OFFICE OF ENVIRONMENTAL HEALTH HAZARD ASSESSMENT

Proposition 65

EVIDENCE ON THE DEVELOPMENTAL AND REPRODUCTIVE TOXICITY OF

Nickel and Nickel Compounds

July 2018

Reproductive and Cancer Hazard Assessment Branch
Office of Environmental Health Hazard Assessment
California Environmental Protection Agency

Preface

Proposition 65[1] requires the publication of a list of chemicals "known to the state" to cause cancer or reproductive toxicity. The Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency maintains this list in its role as lead agency for implementing Proposition 65. The Developmental and Reproductive Toxicant Identification Committee (DARTIC) advises and assists OEHHA in compiling the list of chemicals that cause reproductive toxicity as required by Health and Safety Code section 25249.8. The Committee serves as the state's qualified experts for determining whether a chemical has been clearly shown to cause reproductive toxicity.

After consultation with the DARTIC, OEHHA selected nickel and nickel compounds for consideration for listing by the DARTIC. Upon selection, the public was given the opportunity to submit information relevant to the assessment of the evidence on the reproductive toxicity of nickel and nickel compounds. One comment was received and considered during the development of this document.

On October 11, 2018, the DARTIC is scheduled to deliberate on the reproductive toxicity of nickel and nickel compounds. OEHHA developed this document as part of hazard identification materials that are provided to the DARTIC for the purpose of assisting it in its deliberations on whether or not nickel and nickel compounds should be listed under Proposition 65. To the extent possible, the original papers discussed in the document are also provided to the DARTIC as part of the hazard identification materials. Comments on this hazard identification document received during the public comment period also form part of the hazard identification materials, and are provided to the DARTIC members prior to their formal deliberations.

Nickel carbonyl is already on the Proposition 65 list as causing reproductive toxicity (developmental endpoint), so the committee deliberations and listing decisions will not affect the listing status of nickel carbonyl for this endpoint.

[1] The Safe Drinking Water and Toxic Enforcement Act of 1986 (California Health and Safety Code section 25249.5 *et seq*.)

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Executive Summary

Metallic nickel (Ni) and nickel compounds are used in stainless steel and other nickel alloys, catalysts, batteries, pigments, and ceramics. Because of its corrosion resistance, about 40% of metallic nickel is used in the production of stainless steel, permanent magnets, and other alloys that require resistance to extremes of temperature or stress. Nickel salts are widely used in industry in electroplating baths, batteries, textile dyes, and catalysts or as chemical intermediates to produce other nickel compounds. The most common airborne exposures to nickel compounds are to insoluble nickel compounds such as elemental nickel, nickel sulfide, and nickel oxides present in dusts and fumes associated with welding, electroplating, nickel refineries, and other metallurgical processes. Nickel can be released into the air during nickel mining, combustion of fossil fuels, and waste incineration. Nickel may also be discharged in waste water from facilities engaged in these activities.

Human exposure to Ni or its compounds has the potential to produce a variety of pathological effects, which may include allergic reactions and contact dermatitis (swelling, reddening, and eczema). Nickel and nickel compounds are classified as carcinogens by the National Toxicology Program's Report on Carcinogens and the International Agency for Research on Cancer (IARC), and are listed as known to cause cancer under Proposition 65.

Nickel has been reported as an essential element in several animal species. Signs of Ni deficiency include depressed growth and reduced hematocrit. In the case of human nutrition, the essentiality of Ni has yet to be established.

In human studies of the potential for developmental toxicity of nickel, five cohort studies of air pollution all reported small but statistically significant associations between exposure to Ni particles in ambient air and adverse effects on fetal growth parameters, such as lower birth weight, reduced head circumference, or increased risk of low birth weight (LBW). Two of three case-control studies of air pollution also found higher risk of autism spectrum disorders (ASD) in association with exposure to particulate matter ≤ 2.5 μm ($PM_{2.5}$) Ni in air. The potential for developmental toxicity of nickel compounds has also been investigated in studies using rats or mice. Most studies used the oral route of exposure, with one study conducted by inhalation and other studies employing an injection route of exposure. Regardless of species or route, the most sensitive and commonly reported adverse effects of prenatal exposure to nickel were reductions in viability and reductions in body weights of surviving offspring. Both dose of Ni and timing of exposure were observed to impact the frequency of occurrence and the severity of effects.

Epidemiologic studies of female reproductive effects of Ni examined fecundity and hormone levels and the effects of Ni on milk. One cross-sectional study found Ni exposure to be associated with levels of sex-hormone binding globulin (SHBG) and possibly other hormone levels, and a cohort study reported no effect on fecundity. A

review of the literature in animals found reports of adverse effects of Ni exposure on estrous cyclicity, release of some hormones associated with reproductive function, and alterations to the uterus and ovary. There is also evidence on the effects of Ni on the neuroendocrine control of prolactin in rodents, and negative effects in offspring following changes in milk composition after the dams' exposure to Ni compounds.

Cross-sectional human studies of Ni on male reproductive endpoints found that inhaled Ni was associated with Ni in urine and urinary Ni was in turn associated with lower plasma testosterone. Blood Ni was associated with the following effects on sperm: reduced vitality, slow/nonlinear progressive motility, and tail defects (although other exposures, such as chromium, may at least partly account for this finding). Most studies that measured Ni in semen did not report associations with sperm quality or semen parameters, although one study reported that Ni in seminal plasma was negatively correlated with sperm concentration, volume, and motility. Observations from animal studies include effects of nickel on sperm motility and mortality as well as histopathological effects and biochemical effects on the testis and epididymis. These effects may contribute to serum hormone decreases that are observed in animal studies and are consistent with the findings noted in some studies in humans.

Acronyms and Abbreviations

%ile	percentile
µCi	microcurie
µg	microgram
µm	micrometer
3β-HSD	3β-hydroxysteroid dehydrogenase
8-OHdG	8-hydroxydeoxyguanosine
17β-HSD	17β-hydroxysteroid dehydrogenase
AADT	annual average daily traffic counts
ADDM	Autism and Developmental Disabilities Monitoring Network
Adj.	adjusted
AFP	alpha-fetoprotein
Al	Aluminum
ALH	amplitude of lateral head displacement
AMAD	activity median aerodynamic diameter
ANOVA	analysis of variance
AOR	adjusted odds ratio
As	arsenic
ASD	autism spectrum disorders
AT	apparent temperature
Ba	barium
BCF	beat cross frequency
BGD	N-Benzyl-D-glucaminedithiocarbamate
BMI	body mass index
BMIC	body mass index of child
BTEX	benzene, toluene, ethyl benzenes, xylenes
BW	body weight(s)
Ca	calcium
CADDRE	California Center for Autism and Developmental Disabilities Research and Epidemiology
CARB	California Air Resources Board
CASA	computer-aided sperm analysis
CAT	catalase
CCR	California Cancer Registry
Cd	cadmium
CDTA	trans-1,2-cyclohexanediamine N,N,N',N'-tetraacetic acid
CHO	Chinese hamster ovary (cells)
CI	[95%] confidence interval
CL	corpus luteum (plural: corpora lutea)
cm	centimeters
CO	carbon monoxide
Co	cobalt
Conc.	concentration
Cr	chromium

CT	Connecticut
Cu	copper
CV	coefficient of variation
Cys	cysteine
DCF	dichlorofluorescein
DDS	California Department of Developmental Services
DDTC	diethyldithiocarbamate
DHEAS	dehydroepiandrosterone-sulfate
DHED	dihydroxyethyldithiocarbamate
dL	deciliter
DL	dominant lethal
E_2	estradiol
EC	elemental carbon
EBK	empirical bayesian kriging
ECG	electrocardiography
ELISA	enzyme-linked immunosorbent assay
ERK1/2	extracellular signal-regulated kinases
ERS	endoplasmic reticulum stress
ES	epididymal sperm
ETMs	essential trace metals
EUROCAT	European network of population-based registries for epidemiologic surveillance of congenital anomalies
e-waste	electronic waste
F	Fisher's Distribution
FDR	false discovery rate
Fe	iron
FSH	follicle stimulating hormone
g	gram(s)
Ga	gallium
GAM	generalized additive model
GD	gestation day
GH	growth hormone
G.I.	gastrointestinal
GLUL	glutamate-ammonia ligase
GnRH	gonadotropin releasing hormone
GPS	global positioning system
gsd	geometric standard deviation
GSH	glutathione
GSPE	grape seed proantocyanidin extract
HAP	hazardous air pollutant
His	histidine
hr	hour(s)
hCG	human chorionic gonadotropin
Hg	mercury
HPG	hypothalamic-pituitary-gonadal

H_2O_2	hydrogen peroxide
IARC	International Agency for Research on Cancer
ICD-10	International Statistical Classification of Diseases and Related Health Problems, 10th Revision
ICP-MS	inductively coupled plasma mass spectrometry
im	intramuscular injection
ip	intraperitoneal injection
IQR	interquartile range
iv	intravenous
IUD	intrauterine device
JEM	job exposure matrix
JNK	c-JUN NH2-terminal protein kinase
K	potassium
KBR	Kola Birth Registry
kg	kilogram(s)
km	kilometer
LA	Los Angeles
LBW	low birth weight
LD	lactation day
LDH	lactate dehydrogenase
LH	leutinizing hormone
LIN	linearity
LMP	last menstrual period
LOAEL	lowest observed adverse effect level
LOD	limit of detection
LOQ	limit of quantification
LPO	lipid peroxidation
LUR	land use regression
m	meter
MA	Massachusetts
MAD	mean angular deviation
MAPKs	mitogen-activated protein kinases
MCFA	medium chain fatty acids
MDA	malondialdehyde
Mg	magnesium
mg	milligram(s)
min	minute(s)
MIX	mixed chemicals
Mn	manganese
Mo	molybdenum
MP(s)	microparticle(s)
MTT	3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n	number in study sample
Na	sodium
NAC	N-acetylcysteine

NATA	National Air Toxics Assessment
NC	North Carolina
ng	nanogram
Ni / Ni^{+2}	nickel
$NiCl_2$	nickel chloride
$NiCO_3$	nickelous carbonate
$Ni(CO_4)$	nickel carbonyl
$Ni(NO_3)_2$	nickel nitrate
NiO	nickel monoxide
NiPERA	Nickel Producers Environmental Research Association
NiS	nickel sulfide
NiS(A)	amorphous nickel
Ni_3S_2	nickel subsulfide
$NiSO_4$	nickel sulfate
$NiSO_4•6H_2O$	nickel sulfate hexahydrate
nm	nanometer(s)
NO	nitric oxide
NO_2	nitrogen dioxide
NP(s)	nanoparticle(s)
NS	not statistically significant
NTD	neural tube defect
NTP	National Toxicology Program
O_3	ozone
OR	odds ratio
P	progesterone
Pb	lead
PCOS	polycystic ovary syndrome
PD	pregnancy day
PDD-NOS	pervasive developmental disorder not otherwise specified
PM	particulate matter
$PM_{0.1}$	particulate matter ≤0.1 μm
$PM_{2.5}$	particulate matter ≤2.5 μm
PM_{10}	particulate matter ≤10 μm
PMF	positive matrix factorization
PND	postnatal day
PPD	postpartum day
PRL	prolactin
PUFA	polyunsaturated fatty acids
RACB	reproductive assessment by continuous breeding
RODi	reverse osmosis deionized (water)
ROS	reactive oxygen species
ROS/RNS	reactive oxygen species/reactive nitrogen species
RR	relative risk, risk ratio
RTI	Research Triangle Institute
S	sulfur

SA	spontaneous abortion
Sb	antimony
sc	subcutaneous
SD	standard deviation
SDC	sodium diethyldithiocarbamate
Se	selenium
SE	standard error
SEM	standard error of the mean or median
SES	socioeconomic status
SFA	saturated fatty acid
SGA	small for gestational age
SHBG	sex-hormone binding globulin
Si	silicon
SLI	Springborn Laboratories, Inc.
Sn	tin
SO_2	sulfur dioxide
SOD	superoxide dismutase
Sr	strontium
STR	straightness
T	testosterone
$t_{1/2}$	half-life
TAA	total ascorbic acid
TETD	tetraethylthiuram disulphide
TEMPO	2,2,6,6-tetramethyl-1-piperidinyloxy
TGCT	testicular germ cell tumors
Ti	titanium
TPR	total peripheral resistance
TSH	thyroid-stimulating hormone
TT	total testosterone
TTP	time to pregnancy
UCB	umbilical cord blood
UCD_P	University of California Davis/CIT_Primary chemical transport model
Uf-Ni	ultrafine metallic nickel (20 nm average particle diameter)
V	vanadium
VAP	average path velocity
VCL	curvilinear velocity
VO	vaginal opening
VSL	straight line velocity
W	tungsten
WHO	World Health Organization
WHR	waist-to-hip ratio
WOB	wobble
WV	West Virginia
ZCTA	ZIP code tabulation area
Zn	zinc

A. Introduction

This report presents information relevant to whether nickel and nickel compounds cause developmental and reproductive toxicity (DART). Nickel carbonyl has been listed under Proposition 65 via the authoritative bodies listing mechanism as causing reproductive toxicity (developmental endpoint) since September 1, 1996.

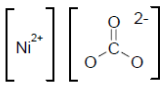
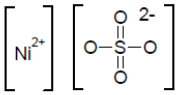
A.1. Compound Identification and Physical Properties

Pure nickel is a hard, silvery-white metal, which has properties that make it very desirable for combining with other metals to form mixtures called alloys. Nickel (Ni) is naturally present in the Earth's crust. It is found in all soil and primarily found combined with oxygen or sulfur as oxides or sulfides.

Nickel has a molecular weight of 58.69. The solubility of Ni in water is 1.13 mg/L at 37°C. While nickel can exist in oxidation states -1, 0, +2, +3, and +4, it commonly exists as Ni^{+2} under normal environmental conditions (US EPA 1986). Nickel is ferromagnetic and a good conductor of both heat and electricity. Elemental nickel is also highly resistant to strong alkali. Nickel dust or powder is flammable. Nickel carbonyl is volatile.

Nickel and some of the Ni compounds frequently tested in animal studies for DART are described below in Table A.1.

Table A.1. Chemical identity of nickel and compounds (Adapted from ATSDR 2005)

Characteristic	Nickel	Nickel carbonate	Nickel chloride	Nickel sulfate
Synonyms	CI 77775; Nickel 200; Nickel 201; Nickel 205; Nickel 270; Alnico[b]; NP 2[b]	CI 77779; Carbonic acid, nickel(2+) salt; nickel (II) carbonate; nickelous carbonate; nickel monocarbonate	Nickel(II) chloride; nickel dichloride; nickelous chloride	Nickel monosulfate; nickelous sulfate; nickel(II) sulfate; sulfuric acid nickel salt[b]
Registered trade name(s)	Monel[b]; Iconel[b]; Icoloy[b]; Raney nickel[c]; Nimonic[d]; Hastelloy[d]; Udimet[d]; Mar M[d]; René 41[d]; Waspaloy[d]	No data	No data	No data
Chemical formula	Ni	$NiCO_3$	$NiCl_2$	$NiSO_4$
Chemical structure	Ni		Cl – Ni – Cl	
Identification numbers:				
CAS registry	7440-02-0	3333-67-3	7718-54-9	7786-81-4

A.2. Use and Exposure Information

Metallic nickel and nickel compounds have many industrial and commercial applications, including use in stainless steel and other nickel alloys, catalysts, batteries, pigments, and ceramics. Because of its corrosion resistance, about 40% of metallic

nickel is used in the production of stainless steel, permanent magnets, and other alloys that require resistance to extremes of temperature or stress (US EPA 1986).

Nickel salts are widely used in industry, and about 20% of nickel is produced as either nickel sulfate or nickel hydroxide for use in electroplating baths, batteries, textile dyes, and catalysts (US EPA 1986; Von Burg 1997). Nickel sulfates are also used as chemical intermediates to produce other nickel compounds, and in nickel flashings on steel to prepare it to be porcelain-enameled. Nickel acetate is used as a catalyst intermediate, as a dye fixative in the textile industry, in electroplating, and as a sealer for anodized aluminum. Nickel chloride is used in nickel catalysts, to absorb ammonia in industrial gas masks, and in electroplating. Nickel carbonate is used to prepare nickel monoxide, nickel powder, nickel catalysts, colored glass, and certain nickel pigments. It also is used in electroplating and as a catalyst to remove organic contaminants from water (HSDB 2009; IARC 1990; NTP 2014).

The most common airborne exposures to nickel compounds are to insoluble nickel compounds such as elemental nickel, nickel sulfide, and nickel oxides present in dusts and fumes associated with welding, electroplating, nickel refineries, and other metallurgical processes. Nickel can be released into the air during nickel mining, combustion of fossil fuels, and waste incineration. Nickel may also be discharged in waste water from facilities engaged in these activities.
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A.3. General Toxicity

Nickel is known as a potentially harmful element for humans. Its concentration in the environment can rise due to industrial activities. Human exposure to Ni or its compounds has the potential to produce a variety of pathological effects, which may include allergic reactions and contact dermatitis (swelling, reddening, and eczema). Nickel and nickel compounds are classified as carcinogens by the National Toxicology Program's Report on Carcinogens (NTP 2014), and the International Agency for Research on Cancer (IARC) (IARC 1990), and are listed as known to cause cancer under Proposition 65.

The various Ni compounds differ in toxicity. The forms of Ni typically used in reproductive toxicity studies are salt forms ($NiCl_2$, $NiSO_4$, $NiCO_3$), and elemental forms (^{63}Ni, Ni microparticles (MP), and Ni nanoparticles(NP)). The chemical form of Ni is one of the determinants of nickel toxicity. Soluble Ni salts found in water (chloride, sulfate) are generally considered to be of low toxicity to humans (Smith et al. 1993). Soluble forms such as nickel chloride seem to be easily excreted through the kidney, and this organ is considered to be the main target organ for Ni; insoluble forms such as nickel subsulfide or nickel oxides are internalized in the cells by phagocytosis and are considered more toxic than soluble forms because of their longer half-life in the body. However, the toxicity is considered to be due to ionic Ni, and the effect of insoluble

forms of Ni would be related to a continuous release of ionic nickel directly inside the cell (Sarkar et al. 1992).

Nickel has been reported as an essential element in several animal species. Signs of Ni deficiency include depressed growth and reduced hematocrit (Nielsen 1996). In the case of human nutrition the essentiality of Ni has yet to be established (IOM 2001). Animal studies associate Ni deprivation with depressed growth, reduced reproductive rates, and alterations of serum lipids and glucose (Barceloux 1999).

A.4. Pharmacokinetics

Much of the following information appears in the OEHHA (2012) document "Nickel Reference Exposure Levels. Nickel and Nickel Compounds. Nickel Oxide. Reference Exposure Levels (REL)". Additional, relevant information, including information that became available after publication of that document, has been added. Further, for this review OEHHA also placed an emphasis on studies reporting on distribution of Ni during gestation and excretion via breast milk.

A.4.1. Absorption

Three routes of absorption: oral, inhalation, and dermal, are discussed below.

A.4.1.1. Oral Route

Ishimatsu et al. (1995) demonstrated that the absorption fraction of orally administered nickel compounds in rats was closely related to their water solubility. They administered eight nickel compounds and nickel metal. The solubilities in saline solution were in the following order: [Nickel nitrate ($Ni(NO_3)_2$)> Nickel chloride ($NiCl_2$)> Nickel sulfate ($NiSO_4$)] >> [Nickel sulfide (NiS) > Nickel subsulfide (Ni_3S_2)] > [Nickel monoxide (NiO) (black, B) > Ni (metal, M) > NiO (green, G)]. The insoluble nickel metal and nickel oxides ranged from 0.01 to 0.09% absorbed. The absorption of the slightly soluble nickel subsulfide and nickel sulfide was 0.47% to 2.12% and the soluble nickel compounds (sulfate, nitrate and chloride) ranged from 9.8 to 33.8 percent. In rats administered $NiCl_2$, $NiSO_4$, and NiS 84-87% of recovered nickel was detected in the kidneys. Lesser kidney ratios were found for Ni_3S_2, $Ni(NO_3)_2$, NiO(B) and Ni(M): 76%, 73%, 62%, and 51%, respectively. However, NiO(G) showed greater recovery from liver than kidney (Ishimatsu et al. 1995).

$^{57}NiCl_2$ was administered orally by gastric intubation (gavage) or by ip injection to groups of mice in doses equivalent to the average human daily dietary nickel intake per mass unit. Orally, the whole body retention was 0.02-0.36% of the administered dose and 1-6% when administered ip There was an adjustment for rapid excretion of Ni and then the intestinal absorption of Ni was estimated to be 1.7-10%. At 8 hours, the tissue concentration was as follows: kidneys>carcass>lungs>testicles>liver> spleen. After 20 hours the Ni concentrations were as follows: kidneys>lungs>liver>carcass. Ni in the

kidneys was rapidly excreted, but excretion was slow in the lungs and liver. When given ip, after 20 hours, Ni was not transported via the portal vein to the liver. (Nielsen et al. 1993).

Nickel is absorbed in the gastrointestinal (G.I.) tract of humans either as free ions or as complexes. The degree of uptake or bioavailability depends on the vehicle (water or food) and has ranged from 1% to 40% in several studies. See Table A.2, from (OEHHA 2012).

Table A.2. Absorption of Ingested Nickel in Humans from Bioavailability Studies (ATSDR 2005; Diamond et al. 1998; OEHHA 2012).

Study	Number of subjects	Vehicle	Duration	Fasting status	Absorption (% of Dose)
Nielsen et al., 1999	8	Water plus scrambled eggs	Acute	Fasted	25.8 to 2.5
Patriarca et al., 1997	4	Water	Acute	Fasted	29-40
Sunderman et al., 1989	8	Water	Acute	Fasted	29.3
Sunderman et al., 1989	8	Food	Acute	Fasted	1.8
Cronin et al. , 1980	5	Capsule plus 100 mL water	Acute	Fasted	12-32
Christensen & Lagassoni, 1981	8	Capsule	Acute	With meal	5.7
Menne et al., 1978	6	Capsule	Acute	Not fasted	2.2 (women)
Menne et al., 1978	7	Capsule	Acute	Not fasted	1.7 (men)
Horak & Sunderman, 1973	10-50	Food	Chronic	Not fasted	1.0
McNeeley et al., 1972	19	Food & water	Chronic	Not fasted	1.6
McNeeley et al., 1972	20	Food	Chronic	Not fasted	1.2

Cronin et al. (1980) reported urinary excretion levels (absorption) after ingestion of a soluble nickel compound during fasting by three groups of 5 female subjects who were given 0.6 mg, 1.25 mg or 2.5 mg of nickel sulphate. Excreted Ni in the urine at 24h after the 2.5 mg dose ranged from 95-206 µg; for the 1.25 mg dose 62-253 µg, and for the

0.6 mg dose, 48-89 µg (Cronin et al. 1980). Sunderman et al. (1989) found that about 40 times more nickel was absorbed from the G.I. tract when nickel sulfate was given to human volunteers in drinking water (27 ± 17 %, mean ± SD) than when it was given in food (0.7 ± 0.4 %). Sunderman et al. (1989) concluded that dietary constituents significantly reduce the bioavailability of Ni^{2+} for alimentary absorption. Twenty-five percent of Ni ingested in drinking water after an overnight fast is absorbed from the intestines and excreted in the urine compared with only 1% of Ni that is ingested in food (Sunderman et al. 1989).

Solomons et al. (1982) and Nielsen et al. (1999) reported similar results. They found that plasma nickel concentrations in fasted human subjects were significantly elevated when they were given nickel sulfate (5 mg Ni) in drinking water with a peak level of about 80 µg Ni/L at three hours after oral administration. When five mg Ni (as nickel sulfate) was administered in whole cow-milk, coffee, tea, orange juice, or Coca Cola®, the rise in plasma Ni was significantly suppressed with all but the Coca Cola®. (Nielsen et al. 1999; Solomons et al. 1982). The elimination half-life for absorbed nickel averaged 28 ± 9 hours (Sunderman et al. 1989).

Nielsen et al. (1999) administered nickel in drinking water (12 µg Ni/kg bw) to eight fasted volunteers at different time intervals, with standardized portions of scrambled eggs. They found that the highest fraction of nickel dose (25.8%) excreted in urine was observed when the scrambled eggs were taken four hours prior to nickel in drinking water. A much lower fraction of nickel dose (2.5%) was excreted when the nickel was mixed into the eggs or when the drinking water was taken together with the eggs (3.4%) (Nielsen et al. 1999).

Patriarca et al. (1997) studied nickel metabolism in humans using the stable isotope ^{62}Ni (98.83%, as metal). Four healthy adult subjects (two women and two men) were fasted overnight and administered 10 µg ^{62}Ni/kg bw in water. Blood samples were drawn in fixed intervals and the total daily output of urine and feces was collected for the first five days after dose ingestion. ^{62}Ni was measured in plasma, urine and feces by isotope dilution using ^{61}Ni and plasma-mass spectrometry. Fecal excretion of ^{62}Ni averaged 66.9 ± 4.9 % of administered dose with an absorbed fraction of 33.1 ± 4.9 %. Urinary excretion over five days ranged from 50.4% to 82% (mean ± SD= 65.2 ± 13.4 %) of absorbed dose. Plasma ^{62}Ni peaked between 1.5 and 2.5 hours after ingestion with concentrations ranging between 269 and 344 nM; ^{62}Ni was rapidly cleared from the plasma but was still detectable at 96 hr post ingestion (< 32 nM). The authors reported no evidence of biliary excretion or enterohepatic circulation of ^{62}Ni as indicated by the appearance of secondary peaks in plasma or urinary nickel concentrations. Also the elimination of ^{62}Ni in feces followed the same pattern as the fecal marker (radio-opaque pellets) indicating that biliary excretion is very low or absent in humans, albeit with a limited number of subjects (Patriarca et al. 1997).

A.4.1.2. Inhalation Route

Deposition, absorption, and retention of nickel particles into regions in the respiratory tract the nasopharyngeal, tracheobronchial, and pulmonary (alveolar) are dependent on lung dynamics and the aerodynamic size and ventilation rate will determine the deposition of nickel particles (NIPERA Inc). Inhaled non-hygroscopic particles > 2 µm deposit in the conducting airways. For particles that are soluble in respiratory tract fluid, systemic uptake may be complete for all deposition patterns. However, particles that are not as soluble that deposit in the conducting airways can be carried out of the respiratory tract to the glottis, where they are then swallowed. Mucociliary transport rates vary along ciliated airways in different individuals. Particles deposited in non-ciliated airways have large surface-to-volume ratios and, if insoluble, are cleared by dissolution or as free particles by passive transport or by phagocytosis. If the particles penetrate the epithelium, they can accumulate in cells or enter the lymphatic circulation. Insoluble particles are cleared from the alveolar region in phases which can last several weeks (Lippmann et al. 1980).

Animal models have been used to estimate the inhalation absorption of water-soluble and water-insoluble nickel compounds. English et al. (1981) administered nickel chloride and nickel oxide intratracheally to rats and reported greater than 50% of the soluble nickel chloride was cleared from the lungs within three days. Most of the nickel was excreted in the urine. In contrast, the water-insoluble nickel oxide persisted in the lung for more than 90 days, and the nickel was excreted equally in urine and feces. Ni in soluble form was rapidly absorbed from the site of deposition following pulmonary exposure, whereas, Ni in its oxide or insoluble form was retained in lungs and related lymphatics for a considerable period (English et al. 1981).

Valentine and Fisher (1984) administered slightly soluble nickel subsulfide intratracheally to mice and observed the pulmonary clearance to have two distinct components with initial and final half-lives of 1.2 and 12.4 days, respectively. The excretion of the chemical (measured as ^{63}Ni) was 60% in the urine and 40% in the feces (Valentine and Fisher 1984). Similar findings were reported by Finch et al. (1987) who observed that the pulmonary clearance of intratracheally administered nickel subsulfide in mice was biphasic with clearance half-lives of two hours and 119 hours for initial and final phases, respectively (Finch et al. 1987).

Tanaka et al. (1985) exposed male Wistar rats to NiO aerosols of mass median aerodynamic diameter (MMAD) and geometric standard deviation (gsd) of 1.2 µm, 2.2 gsd and 4.0 µm, 2.0 gsd. The average exposure concentration was 0.6 mg/m^3 or 70 mg/m^3 and total exposure time was 140 hours. Some rats were sacrificed after exposure while others were kept for 12 and 20 months prior to sacrifice. The biological half-lives of NiO deposited in the lungs based on the assumption of first order clearance kinetics were 11.5 and 21 months for 1.2 and 4.0 µm MMAD aerosols, respectively (Tanaka et al. 1985).

Following a single 70 or 120 minute inhalation exposure of rats to green nickel oxide (^{63}NiO; 9.9 mg Ni/m^3; activity median aerodynamic diameter [AMAD] 1.3 µm, 2.0 gsd), the fraction of the inhaled material deposited in the total respiratory tract was 0.13, with 0.08 deposited in the upper respiratory tract and 0.05 deposited in the lower respiratory tract. Clearance of Ni from the lungs was slow, with approximately 30% of the initially deposited Ni still present 180 days after the exposure. The ^{63}NiO deposited in the lung did not appear to be solubilized; Ni was not detected in any of the extrarespiratory tract tissues examined (Benson et al. 1994).

Tanaka et al. (1988) studied the biological half-life of amorphous NiS(A) aerosols in Wistar rats. The rats were exposed to a NiS aerosol with MMAD (mass median aerodynamic diameter) of 4.0 µm (gsd = 2.0) for either a single four hour exposure at an air concentration of 107 mg/m^3, or repeated exposures to an air concentration of 8.8 mg/m^3 for 7 hr/day, 5 days/week for one month. After exposure, the nickel contents in lung, liver, kidney, spleen, blood and urine were measured. In sharp contrast to the findings with NiO (above), NiS was rapidly cleared from lung tissue following a four-hour exposure with a half-life of 20 hours (f = 0.57) (Figure A.1). Repeated exposures to the lower concentration of NiS showed no accumulation of NiS in the lung and similar clearance kinetics following the final exposure (Tanaka et al. 1988a; Tanaka et al. 1988b).

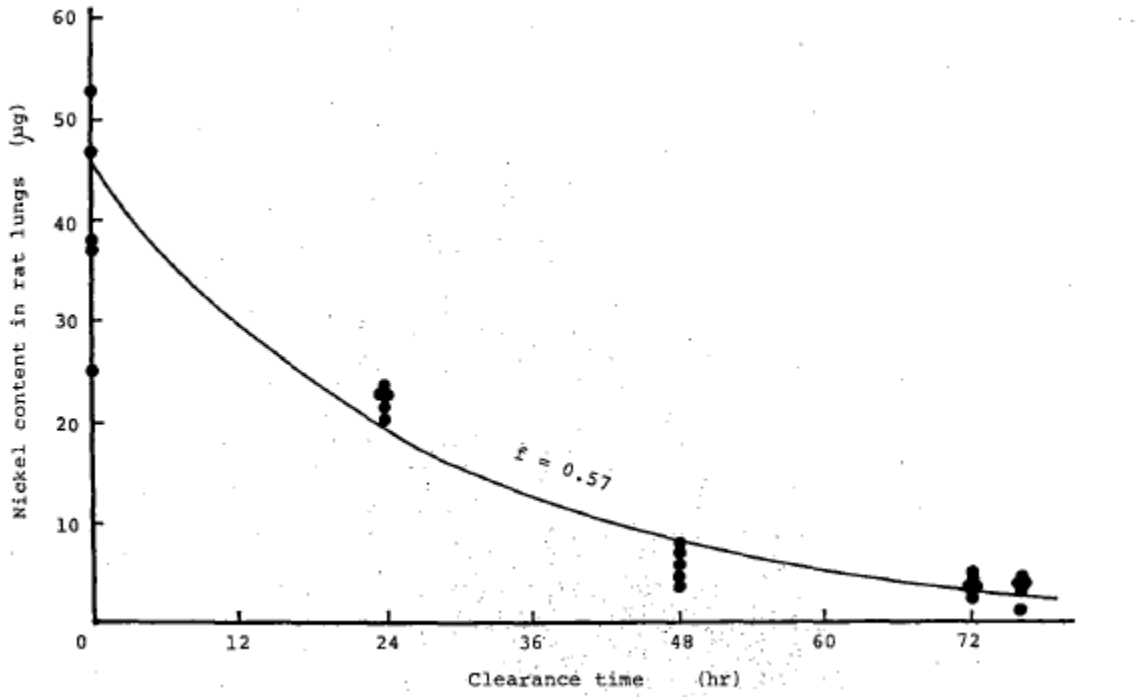

Figure A.1. Nickel content in rat lungs with clearance time after 4 h exposure to NiS(A) (Tanaka et al. 1988a)

Data in rats and mice indicate that a higher percentage of less-soluble nickel compounds was retained in the lungs for a longer time than soluble nickel compounds (Benson et al. 1987; Benson et al. 1988; Dunnick et al. 1989; Tanaka et al. 1985) and that the lung burden of nickel decreased with increasing particle size (≤ 4 μm) (Kodama et al. 1985).

The lung burdens of nickel generally increased with increasing exposure duration and increasing levels of the various nickel compounds (Dunnick et al. 1988; Dunnick et al. 1989). From weeks 9 to 13 of exposure, lung levels of nickel sulfate and nickel subsulfide remained constant while levels of nickel oxide continued to increase (Dunnick et al. 1989). Slow clearance of nickel oxide from the lungs was also observed in hamsters (Wehner and Craig 1972). Approximately 20% of the inhaled concentration of nickel oxide was retained in the lungs at the end of exposure for two days, three weeks, or three months. The retention was not dependent on the duration of exposure or exposure concentration. By 45 days after the last exposure to nickel oxide (two-day exposure), 45% of the initial lung burden was still present in the lungs (Wehner and Craig 1972).

Workers occupationally exposed to nickel have higher lung burdens of nickel than the general population. Dry weight nickel content of the lungs at autopsy was 330 ± 380 μg/g in roasting and smelting workers exposed to less-soluble compounds, 34 ± 48 μg/g in electrolysis workers exposed to soluble nickel compounds, and 0.76 ± 0.39 μg/g in unexposed controls (Andersen and Svenes 1989). In an update of this study, Svenes and Andersen (1998) examined 10 tissue samples taken from different regions of the lungs of 15 deceased nickel refinery workers; the mean nickel concentration was 50 μg/g dry weight (Svenes and Andersen 1998). Nickel levels in the lungs of cancer victims did not differ from those of other nickel workers (Kollmeier et al. 1987; Raithel et al. 1989).

Nickel levels in the nasal mucosa are higher in workers exposed to less-soluble nickel compounds relative to soluble nickel compounds (Torjussen and Andersen 1979). These results indicate that, following inhalation exposure, less-soluble nickel compounds remain deposited in the nasal mucosa. Higher serum nickel levels have been found in occupationally exposed individuals compared to non-exposed controls (Angerer and Lehnert 1990; Elias et al. 1989; Torjussen and Andersen 1979). Serum nickel levels were found to be higher in workers exposed to soluble nickel compounds compared to workers exposed to less-soluble nickel compounds (Torjussen and Andersen 1979). Concentrations of nickel in the plasma, urine, and hair were similar in nickel-sensitive individuals compared to non-sensitive individuals (Spruit and Bongaarts 1977).

Serita et al. (1999) evaluated pulmonary clearance and lesions in rats after a single inhalation of ultrafine metallic nickel (Uf-Ni, 20 nm average particle diameter). Wistar rats (sex unspecified) were exposed to 0.15 (Low), 1.14 (Medium), or 2.54 (High) mg Uf-Ni/m^3 for five hours. Groups of five rats per dose group were sacrificed at 0 hr and 1,

3, 7, 14, and 21 days post exposure. The amount of nickel in the lung accumulated in a dose-dependent manner (1.4, 10.1, 33.5 µg Ni/lung, respectively). The half times for nickel in the lung averaged about 32 days and appeared independent of initial dose (Serita et al. 1999).

Benson et al. (1995) studied the effects of repeated inhalation of relatively insoluble nickel oxide (NiO) and soluble nickel sulfate hexahydrate ($NiSO_4 \cdot 6H_2O$) on lung particle clearance (Benson et al. 1995). They exposed male F344/N rats and B6C3F mice to either NiO or $NiSO_4 \cdot 6H_2O$ 6 hours/day, 5 days/week for up to 6 months. NiO exposure concentrations were 0, 0.62 or 2.5 mg NiO/m^3 for rats and 0, 1.25, or 5.0 mg NiO/m^3 for mice. $NiSO_4 \cdot 6H_2O$ exposure concentrations were 0, 0.12 or 0.5 mg $NiSO_4 \cdot 6H_2O$/m^3 for rats and 0, 0.25 or 1.0 mg $NiSO_4 \cdot 6H_2O$/m^3 for mice. After 2 months of whole-body exposure, rats and mice were acutely exposed for two hours by nose-only to NiO and $NiSO_4 \cdot 6H_2O$ to evaluate lung clearance. To facilitate detection and quantitation of Ni in lungs introduced by acute nose-only inhalation, NiO and $NiSO_4$ were radiolabeled with ^{63}Ni. Then the nose-only exposures were conducted using trace amounts of ^{63}Ni to determine the effect of repeated nickel inhalation on the pulmonary deposition. Animals were returned to the appropriate whole-body inhalation chambers where exposures were resumed until the scheduled euthanization. Animals were euthanized at eight time points (0 to -200 days for NiO-exposed animals; 0 to -30 days for $NiS0_4$-exposed animals) after the acute exposure to the ^{63}Ni-labeled aerosols. After 6 months (26 weeks) of exposure, additional groups of animals from each NiO and $NiS0_4$ exposure level were exposed by inhalation to ^{63}NiO or $^{63}NiSO4$ to further evaluate lung clearance. Repeated inhalation of NiO resulted in accumulation of Ni in lungs of both rats and mice but more so in the lungs of rats. There was some clearance of accumulated Ni from the lungs of both rats and mice exposed to lower NiO concentrations (Benson et al. 1995). In contrast, repeated inhalation of $NiSO_4 \cdot 6H_2O$ did not result in accumulation of Ni in lungs of either rats or mice and did not affect the clearance of radiolabeled Ni inhaled after either 2 or *6* months of $NiS0_4 \cdot 6H_2O$ exposure.

A.4.1.3. Dermal Route

Nickel is absorbed through appendages of the skin (sweat ducts and hair follicles) at a faster rate than through the epidermis. However, the surface area of the appendages is very small; therefore, penetration through the skin is mainly regulated by the diffusion rate through the horny layer. Lipophilic organic nickel compounds pass the dermal barrier quickly and cause systemic toxicity. Nickel penetration of skin is enhanced by many factors including sweat, solvents, detergents, and skin injury (Grandjean et al. 1988).

A.4.2. Distribution

Inhaled particulate nickel compounds are either cleared from the mucous layer of the respiratory tract by mucociliary action, dissolved into Ni^{2+} ions, or taken up by the cells. Phagocytosis of nickel particles, such as Ni_3S_2 or crystalline NiS, results in the formation of a vacuole in which nickel particles are encased and ultimately dissolved.

Extracellular dissolution of soluble nickel compounds results in the release of ionic nickel, which can enter cells via divalent ion transport systems (e.g., magnesium). Both intracellular influx and efflux of nickel ions are described by saturable Michaelis-Menten kinetics. Once in the cytoplasm nickel ions may bind with cytosolic proteins or diffuse through the cytoplasm to the perinuclear cytoplasm. Once there, nickel ions may bind reversibly to perinuclear proteins, enter the nucleus and bind to nuclear proteins (Onkelinx et al. 1973).

Several studies of nickel administered to rodents via the oral route show that nickel was mainly concentrated in the kidneys, liver, and lungs, and the absorbed nickel was excreted primarily in the urine (Borg and Tjalve 1988; Dieter et al. 1988; Jasim and Tjalve 1984, 1986b). Nielsen et al. (1993) showed that retention and distribution of nickel in mice was dependent on the route of administration. As shown in Table A.3, Nielsen at al. (1993) showed that 20 hours after nickel administration, deposition in body tissues resulting from intraperitoneal (ip) injection was much greater than that observed after gavage administration (Nielsen et al. 1993).

Table A.3. Median Nickel Body Burden and Contents of Major Organs in Mice as Percentage of Administered Dose (from Nielsen et al., 1993)*.

Tissue	Gastric Intubation	Intraperitoneal Injection
Liver	0.0439 (0.046)[a]	0.255 (0.044)[b]
Kidneys	0.029 (0.030)	1.772 (0.306)
Lungs	<0.010 (0.010)	0.114 (0.020)
Carcass	0.106 (0.111)	3.164 (0.546)
Stomach	0.014 (0.015)	<0.010 (0.002)
Intestine	0.762 (0.799)	0.490 (0.084)
Total body burden	0.954 (1.0)	5.794 (1.0)

*Note: Measurements made 20 hr after: a) oral dose of 10 µmol Ni/kg bw, or b) intraperitoneal injection of 1.0 µmol Ni/kg bw. Values in parentheses are ratios of relative tissue burden over total body burden.

Ishimatsu et al. (1995) evaluated the distribution of various nickel compounds in rat organs 24 hours after oral administration. Male Wistar rats (10 weeks old, 8/compound) were administered the nickel compounds by gavage as 10 mg of Ni dissolved in a 5% starch saline solution. The animals were sacrificed at 24 hr after dosing and organs and blood taken for Ni determination. Selected results are presented in Table A.4. The kidney stands out as the major site of nickel deposition. This table also demonstrates the high bioavailability of soluble nickel compounds compared to poorly soluble compounds (Ishimatsu et al. 1995).

Obone et al. (1999) measured the accumulation of nickel in tissues of rats exposed to $NiSO_4$ in drinking water for 13 weeks (Table A.5). Accumulation in all organs examined

was observed to increase with increasing dose level. The order of accumulation compared to the control was kidneys > testes > brain > spleen > lung = heart= liver (Obone et al. 1999).

Absorbed nickel is unlikely to exist as free ionic Ni^{2+}, but rather as nickel complexes. Sunderman and Oskarsson (1991) noted that in humans absorbed nickel is transported by binding to a metalloprotein (nickeloplasmin), albumin, and ultra-filterable ligands, such as small polypeptides and L-histidine (Sunderman and Oskarsson 1991). Van Soestbergen and Sunderman (1972) administered nickel chloride (as ^{63}Ni) to rabbits by intravenous injection at 0.24 mg Ni/kg bw. They found that between two and 24 hr after injection, approximately 90% of serum ^{63}Ni was bound to proteins (e.g., albumin) with molecular weights greater than 10,000 and the remaining label was bound to small organic molecules such as short peptides and amino acids (Van Soestbergen and Sunderman 1972).

Onkelinx et al. (1973) conducted a kinetic analysis of $^{63}Ni^{2+}$ clearance in rats and rabbits following a single intravenous injection of $^{63}NiCl_2$ (specific activity 5.9 µCi/µg Ni). In both species $^{63}Ni^{2+}$ was rapidly cleared from plasma or serum during the first two days, and more slowly after two days (Onkelinx et al. 1973).

Table A.4. Mean Nickel Concentrations in Rat Organs 24 Hours after Oral Administration (Adapted from Ishimatsu et al., 1995)*

Ni Compound	Lung µg/g	Liver µg/g	Kidney µg/g	Heart µg/g	Brain µg/g	Blood µg/mL
NiO (Green)	0.04	0.02	0.03	0.03	0.03	0.03
Ni metal	0.18	0.04	0.31	0.04	0.02	0.02
NiO (Black)	0.08	0.04	0.32	0.04	0.02	0.05
Ni_3S_2	0.17	0.07	1.2	0.04	0.02	0.05
NiS	0.34	0.11	6.4	0.60	0.04	0.21
$NiSO_4$	2.50	0.57	25.5	0.47	0.04	0.28
$NiCl_2$	3.70	0.53	28.7	1.20	0.18	0.31
$Ni(NO_3)_2$	6.30	1.10	32.6	2.40	0.15	2.25
Control	0.04	0.03	0.03	0.03	0.02	0.03

* Note: 8 animals/compound; 10 mg Ni oral dose by gavage.

Table A.5. Mean Nickel Concentrations (µg Ni/g tissue) in Rat Organs after 13 Weeks Exposure to $NiSO_4$ in Drinking Water (Adapted from Obone et al., 1999)*

Treatment $NiSO_4$	Liver	Kidney	Spleen	Heart	Lungs	Brain	Testis
0%	1.58	1.39	1.51	1.60	1.22	1.59	1.50
0.02%	1.60	1.88	1.85	1.74	1.60	1.68	1.85
0.05%	1.63	3.45	1.86	1.83	1.95	1.77	2.05
0.1%	2.08	5.48	2.26	2.12	2.11	2.78	2.84

*Note: Values are means of three different experiments. Measurements made 24 hr after termination of exposure.

Chelation of Ni^{2+} by organic compounds has a significant effect on the cellular uptake, absorption, and distribution of Ni^{2+} (Borg and Tjalve 1988; Hopfer et al. 1987; Nieboer et al. 1984; Sarkar 1984). Nierborer et al. (1984) studied cellular uptake of Ni^{2+} in human B-lymphoblasts, human erythrocytes and rabbit alveolar macrophages. They observed that addition of L-histidine or human serum albumin at physiological concentrations to the cell cultures reduced Ni^{2+} uptake by up to 70%. The concentration of Ni^{2+} used in the study was 7×10^{-8} M (4.1 µg/L); it was comparable to serum nickel levels observed in workers occupationally exposed to nickel (Nieboer et al. 1984).

Rezuke et al. (1987) measured nickel concentrations in human postmortem samples in seven to 10 adults. In decreasing order the mean and range in µg Ni/kg dry weight in the tissue specimens were: lung 173 (71-371); thyroid 141 (41-240); adrenal 132 (53-241); kidney 62 (19-171); heart 54 (10-110); liver 50 (11-102); brain 44 (20-65); spleen 37 (9-95); and pancreas 34 (7-71). In five specimens of bile, nickel concentrations averaged 2.3 ± 0.8 µg/L (range 1.5-3.3 µg/L). These values differ markedly from the distribution of Ni in the rat noted in Table A.5 above. The relatively high Ni burden in the human lung and low burden in the human kidney may indicate significantly more inhalation exposure in humans and/or significant differences in the chemical state of nickel absorbed in laboratory rodent versus human environmental exposures (Rezuke et al. 1987).

The distribution of nickel chloride in pregnant and lactating rats following its injection has been studied by a number of authors (Dostal et al. 1989; Mas et al. 1986a; Sunderman et al. 1978). Half-lives of nickel in whole blood following ip treatment of pregnant and non-pregnant rats were similar (3.6–3.8 hours), while the half-life for nickel in fetal blood was 6.3 hours following treatment on gestation day 19 (Mas et al. 1986a). Intramuscular injection of nickel chloride (12 mg Ni/ kg/day) into pregnant and non-pregnant rats resulted in a greater accumulation of nickel in the pituitary of pregnant rats (Sunderman et al. 1978)

In studies on Ni distribution during pregnancy, groups of 12- and 19-day pregnant rats were injected ip with ^{63}Ni (5 µCi/mg Ni; 4 mg Ni/kg BW) (Mas et al. 1985). The animals were killed by decapitation at 0.25, 1, 4 or 24 h after dosing. After ip administration, Ni was cleared rapidly from the blood. The data suggest that the concentrations of Ni in the maternal tissues were maximal at about 1 h after dosing and then decreased progressively. Concentrations of Ni in the placenta and fetuses were maximal at 1 h and 4 h in the pregnant rats (Mas et al. 1985).

Metals, including Ni, are known to be transferred into animal, as well as human, milk. Nickel measurements in human and animal milk are briefly discussed in Section A.4.3. Excretion, and additional information on Ni measurements in milk is presented in Sections C.1. Human Studies of Female Reproductive Toxicity and C.2.6. Milk Composition (Animal Studies of Female Reproductive Toxicity)

Tallkvist et al. (1998) evaluated the olfactory transport and subcellular distribution of $^{63}Ni^{2+}$ solution instilled intra-nasally in rats (4 µg/nostril). Cellular fractionation was conducted at one day, one week and three weeks after exposure. Of the $^{63}Ni^{2+}$ present in the olfactory epithelium, 60% to 70% was present in the supernatant, whereas in the olfactory bulb and the basal hemisphere about 70% - 80% of the nickel was bound to particulate cellular constituents. Gel filtration of the cytosol indicated that the $^{63}Ni^{2+}$ eluted with a molecular weight of about 250, identical to that obtained with histidine. Also, in olfactory tissues $^{63}Ni^{2+}$ was partly present in the cytosol associated with a 25,000 molecular weight component. The authors conclude that: (1) nickel is transported in the primary olfactory neurons via slow axonal transport; (2) the metal is bound to both soluble and particulate cytosolic constituents; and (3) the metal also shows this subcellular distribution in other parts of the olfactory system. The authors also note that neuronal transport of nickel was about 20 times slower than cadmium ($^{109}Cd^{2+}$) or manganese ($^{54}Mn^{2+}$) studied earlier (Tallkvist et al. 1998).

Schwerdtle and Hartwig (2006) evaluated the subcellular distribution of $NiCl_2$ and black NiO in human lung A549 cells exposed for 20 and 24 hr, respectively. Cells treated with $NiCl_2$ at 0, 50, 100, 250, or 500 µM exhibited dose-dependent uptake of Ni into the cytoplasm and nuclei. Intracellular Ni concentrations in cytoplasm were about 10, 20, 50, 275, and 550 µM, respectively. Concentrations in the nuclei were much lower at about 5, 10, 15, 40, and 110 µM, respectively. Cells treated with black NiO at 0, 0.2, 0.5, 1.0, and 2.0 µg NiO/cm^2 showed a similar pattern of intracellular distribution with greater relative concentrations in the nuclei. For cytoplasmic distribution the Ni concentrations were about 5, 110, 150, 240, and 450 µM, respectively. For nuclear distribution the Ni concentrations were about 2, 60, 70, 125, and 230 µM, respectively. The authors concluded that particulate Ni(II) exhibits greater toxicity due to its longer retention times rather than a different MOA which still involves Ni(II) ions as the direct or indirect genotoxicant (Schwerdtle and Hartwig 2006).

A.4.3. Excretion

Nickel burden in humans does not increase with age. A majority of nickel absorbed from environmental media and diet is rapidly excreted via the urine. Solomons et al. (1982) found that nickel in water was quickly absorbed and excreted by humans; they estimated a biological half-life of about eight hours (Solomons et al. 1982). Hogetveit et al. (1978) reported that elevated levels of nickel were detected in urine samples collected from workers exposed to soluble or insoluble nickel through inhalation (Hogetveit et al. 1978).

The kinetics of nickel elimination in humans and animals appear to be similar. Onkelinx et al. (1973) injected nickel chloride iv to rats and rabbits and followed the nickel in plasma over time. Elimination profiles were similar in both species with early and later phases of elimination from plasma exhibiting first-order kinetics with half-lives of 6 and 50 hr for rats and 8 and 83 hr for rabbits, respectively (Onkelinx et al. 1973).

The kidney is the primary route for Ni clearance in both the mother and fetus. Fetuses lack effective means for getting rid of excessive Ni due to their confined environment and relatively weak kidney function. Thus, fetuses are particularly vulnerable to the damaging effects of Ni.

Sweat and milk are also possible excretion routes for absorbed nickel in humans. OEHHA located six studies that analyzed human milk for the presence of Ni. In the UK, Casey and Neville (1987) reported a mean (SD) nickel concentration of 1.16 (0.41) µg/L (range 0.52 – 2.04 µg/L) in 46 milk samples from 13 women during the first five weeks of lactation, resulting in an estimated average daily infant intake of 0.8 µg Ni (Casey and Neville 1987). Aquilio et al. (1996) compared trace element content in multiple samples of milk from six mothers of preterm infants and eight mothers of term infants collected over the first 21 days post-partum in Italy. The infants' umbilical cord blood and peripheral vein blood at 21 days was also analyzed. Mean (SD) Ni concentrations in milk from the first 2-6 days, 12-14 days, and 21 days post-partum are shown in Table A.6. For each period, the mean Ni concentration was statistically significantly lower in the milk for preterm infants (Aquilio et al. 1996).

Table A.6. Ni concentrations in milk from mothers of term and preterm infants (µg/L)

Post-partum period	2-6 days	12-14 days	21 days
Term	11.6 (0.8)	11.2 (0.5)	10.8 (0.7)
Preterm	7.5 (0.2)*	8.1 (0.4)*	7.7 (0.1)*

*p<0.05 compared to term milk

While Ni levels also appeared to be lower in the blood of preterm infants compared to term infants, as shown in Table A.7, the differences were not significant.

Table A.7. Ni concentrations in blood from term and preterm infants (µg/L)

Age	1 day	21 days
Term	3.8 (0.4)	3.8 (0.1)
Preterm	3.2 (0.8)	2.9 (0.5)

Friel et al. (1999) examined milk from 24 mothers of preterm and 19 mothers of full-term infants in Newfoundland, Canada. Milk samples were collected beginning 2-3 days after delivery and then weekly for eight weeks, with a final sample collected at 12 weeks. Weekly median values ranged from 0-28 µg/L, with wide weekly variation within the groups of mothers of preterm and term infants (Friel et al. 1999). Krachler et al. (2000) measured trace elements in 27 human milk samples in Austria and found a median nickel concentration of 0.79 µg/L (range < 0.13-6.35 µg/L) (Krachler et al. 2000). Gurbay et al. (2012) tested milk from 64 mothers 2 – 5 days post-partum in Turkey. Ni was detected in 56 samples, with mean (SD) concentrations of 43.94 (33.82) µg/L (range 8.27 – 148.62 µg/L among those with detected Ni) (Gurbay et al. 2012). Salmani et al. (2016) sampled milk from 150 mothers in Iran three times in the first month after delivery and found mean (SD) Ni concentrations in milk of 47.3 (7.40) µg/L after 3-5 days, 49.9 (8.05) µg/L after 16 days, and 54.8 (7.38) µg/L after 30 days (Salmani et al. 2016).

Hohnadel et al. (1973) observed that, in sauna bathers, the mean concentrations of nickel in the sweat from healthy men and women were significantly higher than the mean concentrations in urine (Hohnadel et al. 1973). Casey and Neville (1987) reported a mean nickel concentration of 1.2 ± 0.4 µg/L in 46 human milk samples from 13 women during the first month of lactation with an average estimated daily infant intake of 0.8 µg (Casey and Neville 1987). Krachler et al. (2000) measured trace elements in 27 human milk samples and found a median nickel concentration of 0.79 µg/L (range < 0.13-6.35 µg/L) (Krachler et al. 2000).

A summary of observations of Ni in human milk is provided in Table A.8.

Table A.8. Overview of studies of Ni in human milk

Study	Location	Ni concentration in milk, µg/L
Casey and Neville (1987)	UK	Mean (SD) 1.16 (0.41) Range 0.52 – 2.04
Aquilio et al. (1996)	Italy	Mothers of term infants, mean (SD) at ages: 2-6 days 11.6 (0.8) 12-14 days 11.2 (0.5) 21 days 10.8 (0.7) Ni concentrations in infants' blood was also reported
Friel et al. (1999)	Canada	Range of weekly medians 0 – 28
Krachler et al. (2000)	Austria	Median 0.79 Range < 0.13 – 6.35
Gurbay et al. (2012)	Turkey	Mean (SD) 43.94 (33.82) Range 8.27 – 148.62 (among those with detected Ni)
Salmani et al. (2016)	Iran	Mean (SD) 3-5 days after delivery 47.3 (7.40) 16 days 49.9 (8.05) 30 days 54.8 (7.38)

Graham et al. (1978) measured the clearance of $NiCl_2$ aerosol in mice exposed to 644 µg Ni/m^3 for two hours. Immediately following exposure and at 24 hr intervals thereafter the mice were sacrificed, their lungs and spleens were removed and weighed, and nickel concentrations were determined by atomic absorption spectroscopy. Clearance of nickel from the lung followed first-order kinetics with a fitted curve of Y = 7.569exp- (-0.291t), where Y is µg Ni/g dry weight lung and t is days post exposure. The spleen did not exhibit a significant uptake of nickel following exposure (Graham et al. 1978).

Koizumi et al. (2004) measured the urinary excretion of nickel nitrate hexahydrate in rats by inductively coupled plasma argon emission spectroscopy (ICPAES). Male Wistar rats received single oral doses of 0, 0.005, 0.01, 0.025, 0.05, 0.075, 0.1, 0.125, 0.25, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 10.0, 20.0, and 50.0 mg $Ni(NO_3)_2 \cdot 6H_2O$/kg bw. Five animals were used for analysis at each dose level. The 24-hr urinary excretion of nickel was observed to fit the relation $Y = 62.68X^{0.8527}$, R = 0.9488, where Y is the excreted Ni in µg and X is the oral dose in mg/kg bw. The proportion of total nickel elimination decreased from 25% at 0.01 mg/kg to about 5% at 0.1 mg/kg and higher doses. Urological analysis of markers of renal toxicity, N-acetyl-β-D-glucosamine (NAG), β_2-microglobulin, urine albumin, and urine protein, showed no indication of toxicity at any dose level used (Koizumi et al. 2004).

Dostal et al. (1989) showed that milk is an excretion pathway of nickel chloride in rodents. Daily subcutaneous injections of lactating rats with 3 or 6 mg Ni/kg bw for four days raised nickel levels in milk from < 2 µg/L to 513 ± 54 and 1030 ± 66 µg/L,

respectively. They also showed that nickel treatment significantly changed the composition of milk by increasing the milk solids (42%) and lipids (110%) and decreasing milk protein (29%) and lactose (61%) (Dostal et al. 1989). Additional studies reporting Ni concentrations in rodent and bovine milk are summarized in Section C.2.6. Milk Composition (Animal Studies of Female Reproductive Toxicity).

Oyabu et al. (2007) studied the biopersistence of inhaled NiO particles in the rat lung. Thirty male Wistar rats were exposed to NiO particles (geometric mean diameter = 139±12 nm, average exposure concentration = 1.0±0.5 x 10^5 particles/m^3) for six hr/day for four weeks. At four days and one and three months after inhalation, a group of 10 rats was sacrificed and the NiO particles deposited in the lung determined by chemical analysis. The retained Ni particle content of the lung decreased exponentially with a calculated half-life of 62 days (Oyabu et al. 2007).

Oliveira et al. (2000) studied urinary nickel excretion in 10 workers from a galvanizing plant using $NiSO_4$, a soluble nickel compound, and 10 control subjects. Personal air monitors were used with 0.8 µm filters (OSHA method). No other particle size information was provided. Nickel airborne levels varied between 2.8 and 116.7 µg/m^3. Pre- and post-shift urinary Ni levels were taken on five consecutive workdays. Post-shift values ranged from 4.5 to 43.2 µg Ni/g creatinine. A significant correlation was observed between urinary and airborne nickel ($r = 0.96$, $P \leq 0.001$) with the relation urinary Ni (µg/g creatinine) = 6.00 + 0.43(airborne Ni, µg/m^3). No differences were observed with respect to different workdays (Oliveira et al. 2000).

Yokota et al. (2007) studied the urinary elimination of nickel and cobalt in relation to airborne exposures in a battery plant where the workers were exposed to nickel hydroxide. No correlation was found between Ni in air and post-shift urine [$Ni\ (\mu g/L)_{urine} = -17.3 + 7.33\ Ni\ (mg/m^3)_{air}$, $r = 0.272$, $P = 0.15$]. The authors note that the workers were using respiratory protection which presumably reduced inhalation exposure to $Ni(OH)_2$. (DFG 2005). The authors argue that $Ni(OH)_2$ should be treated as an insoluble compound with respect to urinary excretion rather than a soluble one (Yokota et al. 2007).

Afridi et al. (2006) measured metal content in biological samples from 56 production workers (PW) and 35 quality control workers (QCW) of a steel mill and 75 unexposed normal controls (all male, age range 25-55 yr). For nickel in scalp hair the PW showed the highest Ni concentration of 13.76 ± 4.48 µg Ni/g with QCW lower at 9.02 ± 2.64 µg Ni/g. These values were significantly higher than the non-occupationally exposed controls at 5.25 ± 1.46 µg Ni/g hair ($P < 0.02$). Surprisingly the mean lead values were quite similar at 16.21, 10.33, and 6.84 µg Pb/g hair, respectively. Urine concentrations were also measured and showed lesser, but also significant, differences i.e. 9.47, 7.62, and 6.31 µg Ni/L urine, respectively (Afridi et al. 2006).

Ohashi et al. (2006) evaluated selected urinary metals in 1000 women in the general Japanese population. The geometric mean concentration for nickel was 2.1 µg Ni/L or

1.8 µg Ni/g creatinine. Unlike copper and manganese both nickel and cobalt showed no substantial age dependency for urinary excretion (Ohashi et al. 2006).

B. Studies of Developmental Toxicity of Nickel and Nickel Compounds

B.1. Human Studies of Developmental Toxicity

The epidemiological studies of the effects of Ni exposure on human developmental outcomes included analyses of spontaneous abortion, fetal growth parameters, congenital malformations, autism spectrum disorders (ASD), neuroblastoma, retinoblastoma, testicular cancer, oxidative damage to DNA, and other adverse pregnancy outcomes. These studies examined exposure to Ni in air pollution, in the workplace, in soil, and through the e-waste recycling industry.

Two of the human developmental studies examined occupational exposures to Ni refinery work as a risk factor for spontaneous abortion in the arctic area of Russia.

Ten studies examined the effects of Ni on fetal growth parameters, including birth weight, body mass index, and small for gestational age birth. Five studies in the US and Europe examined Ni and other air pollutants as risk factors for low birth weight. For some fetal growth studies, Ni concentrations in maternal and cord blood, urine, and placenta were assessed.

Seven studies examined associations between Ni and congenital malformations, including one study each of genital malformations and neural tube defects. Three studies examined effects of Ni refinery work on congenital malformations, and two related studies analyzed metals in soil in a high-risk region as a risk factor. One study of congenital malformations tested newborns' hair to assess metal exposure following military attacks with metal-delivering weapons.

Three studies of ambient air pollutants, including Ni, as risk factors for (ASD) were conducted in the US. Two US case-control studies of air pollution examined risk for neuroblastoma and retinoblastoma. A European cohort study air pollution examined particulate matter in relation to early childhood pneumonia. In one study, the authors examined exposure to metals in an e-waste recycling town as a risk factor for oxidative damage to DNA. Finally, three studies considered other adverse outcomes; one of these examined spontaneous abortion and congenital defects, another examined heavy metals in umbilical cord blood in association with combined adverse pregnancy outcomes, and the last study examined air pollutants in association with pneumonia in early childhood.

Tabulated summaries of the individual studies presented in this section are provided in Appendix 2.

B.1.1. Spontaneous abortion

Chashschin et al. (1994). Congenital defects, abortion and other health effects in nickel refinery workers

The study aims, sampling design, and subject recruitment and selection methods of this cross-sectional study were not described (Chashschin et al. 1994). Study subjects included 758 women who had worked in Ni hydrometallurgy for up to 16 years on the Kola Peninsula in the arctic region of Russia. Analyses of effects on reproduction included 356 mothers, including 232 who worked in Ni hydrolysis and 124 who had worked in purification. Referents were 324 local female construction workers without occupational Ni exposure. Subjects were given a thorough general clinical health examination, including pulmonary X-ray, electrocardiogram(ECG), lung function, routine blood count, and determination of Ni concentrations in 24-h urine samples. Those with work-related symptoms received further examination, including bronchoalveolar lavage, ultrasonic heart investigation, and cutaneous and respiratory provocation tests with Ni.

The authors obtained birth data, including congenital defects, and information about the pregnancies, from the municipal health care registration board. Information on potential confounders, such as smoking, alcohol use, and other diseases, came from a random sample of 60 Ni-exposed mothers of children with malformations and were compared with an unstated number of "appropriate time- and place-matched controls". The authors state "none of these possible confounding factors nor age or number of children (not mentioned) seem to interfere with the conclusions." Cases' and controls' husbands had never been occupationally exposed to Ni.

The exposure assessment methods are not described. The authors report various hazards in Ni hydrometallurgy operations, including sulfuric acid aerosols, temperature, humidity, magnetic field strength, and work energy expenditures. Hazardous amounts of chlorine may be released from the installations for purification of Ni electrolyte, and cause acute intoxication, including deaths. Electrolysis operation requires lifting heavy Ni anodes, repetitive handling operations, and sideways motion. Levels of Ni sulfate aerosols for electrolysis operators (mean 0.201 mg/m^3, range 0.107 – 0.308 mg/m^3) and purification operators (mean 0.136 mg/m^3, range 0.077 – 0.196 mg/m^3), and the Russian safety limit of 0.005 mg/m^3 are shown in Table B.1 below.

Table B.1. Ni sulfate aerosols in Ni hydrometallurgy operations, mg/m^3

Occupation	Mean	Range	Russian safety limit
Electrolysis operator	0.201	0.107 – 0.308	0.005
Purification operator	0.136	0.077 – 0.196	

Ni content in urine was also reported for electrolysis operators (mean 15.6 µg/l, range 5.2 – 22.6 µg/l) and purification operators (mean 10.4 µg/l, range 3.2 – 18.0 µg/l).

Analyses of pregnancy complications included 290 Ni refinery workers and 336 non-Ni workers. The number of pregnancies was not reported. Tables B.2 and B.3 below show pregnancy complications in Ni refinery compared with other workers, and malformations among the specific Ni refinery occupations and non-Ni workers.

Table B.2. Pregnancy complications in Ni and other workers (% of pregnancies)

Pathology	Ni Refinery workers	Non-Ni workers
Normal course of pregnancy	29.0	38.8
Threatened abortion	17.2	7.6
Spontaneous abortion	15.9	8.5
Gestational toxicosis (early)	32.8	27.2
Gestational toxicosis (late)	6.0	8.2
Pregnancy-induced hypertension	6.0	8.2
Anemia	11.2	12.9
Other complications	29.7	20.6

Table B.3. Adverse outcomes in infants by mother's occupation (per 100 live births)

Pathology	Electrolysis operator (n=232)	Purification operator (n=124)	Total in Ni refinery (n=356)	Non-Ni workers
Prematurity	8.6	4.8	7.3	4.1
Hypoxia	7.3	7.3	7.3	9.4
Hypertrophy	15.5	29.0	20.2	15.8
Still birth	0.9	0.8	0.8	1.2
Structural malformations	16.4	17.7	16.9	5.8

Chashschin et al. report that the risk ratio (RR) of spontaneous abortion (SA) was 1.8 in Ni-exposed female workers, and that unspecified complications were also more common in pregnancies of Ni workers. The authors report that the differences between observed and expected values for prematurity and malformations were statistically significant, with RRs of 2.9 for all defects, 6.1 for defects of the cardiovascular system, and 1.9 for musculoskeletal defects (p-values or confidence intervals (CIs) were not reported).

OEHHA comments: The authors acknowledge the possibility of recall bias and expectation bias in their preliminary data and state that the increased risk of pregnancy complications and malformations are the most important results of the investigation. However, the study does not distinguish between exposure to Ni and Ni refinery work (which involves other potentially hazardous exposures).

Vaktskjold et al. (2008a). Spontaneous abortion among nickel-exposed female refinery workers.

Vaktskjold et al. conducted this case-control study between 1996 and 2002 to assess whether women occupationally exposed to elevated levels of water-soluble Ni at the Ni refinery during pregnancy were at higher risk of SA in early pregnancy than other women living in the borough of Monˇegorsk, on the Kola Peninsula in northwest Russia (Vaktskjold et al. 2008a). The Kola Birth Registry (KBR) has included all deliveries in Monˇegorsk since 1973.

In Russia, SA is defined as delivery before 28 weeks or delivery with weight <1,000 g and length <35 cm, where the fetus is dead or survives <168 hours. Pregnancy care was provided without fees and women were advised to visit a gynecologist before 12 weeks of pregnancy. Women in heavy or risk-related work were to be transferred to another task at their workplace as soon as pregnancy was confirmed. Hormone therapy to induce pregnancy was not available in Monˇegorsk until 5-6 years prior to the study and amniocentesis had not been used to identify chromosomal anomalies.

Questionnaire study: All women employed in 14 workplaces in the Ni, cobalt (Co), and copper (Cu) refinery complex in Monˇegorsk (including five production departments), and five workplaces outside the complex, were invited to participate in an interview. Of the 1,638 women available for participation, 1,411 (86.1%) participated. The following were excluded: “young unmarried women who had not been sexually active”, newly-wedded teenage women who had never been pregnant and with no indication of infertility, and women who were currently pregnant for the first time. The women provided information on 2,288 pregnancy outcomes (except induced abortions) in Monˇegorsk. SA was not defined in the interview. The interview included information on employment and workplace during each pregnancy. Interviews for the Ni and Cu refinery workers occurred during 1996, 1999, and 2002. All subjects who worked outside the refinery complex were interviewed in 1996.

The primary inclusion criterion was residence in Monˇegorsk during the onset of pregnancy. Cases were self-recognized pregnancies that terminated spontaneously before 28 weeks. Only a woman’s first SA was included if she had more than one. Controls were at least 37 weeks’ gestation and delivered by a woman who had no history of SA. A woman could have a control infant and a subsequent case infant. Only the first sibling from multiple births was included. Ectopic and molar pregnancies were excluded. The inclusion criteria were met by 1,691 live births and 184 SAs (cases) in

1966 – 2002. The mean (median) year of pregnancy was 1985 (1985) for cases and 1983 (1984) for controls.

Birth Registry Study: Another analysis included SAs noted in the Monče gorsk birth registry during 1973 - 2001. Because only previous SAs each delivering woman experienced were recorded in the registry, a case was only included if it was the woman's first SA and it was both preceded and followed by a live birth that was assigned the same exposure level. Previous births by case mothers and primipara births were not included as controls. The birth registry analysis included 4,571 controls and 474 cases. The median year of pregnancy was 1987 for cases and 1986 for controls.

Each pregnancy was assigned a Ni exposure level according to the woman's occupation at pregnancy onset. Ni exposure levels were derived from air and urine measurements of Ni and other metalloids taken during 1995-2001 with nearly 500 individual workers. These exposure measurements were categorized into *background, low, and high* levels of Ni, based on the water-soluble sub-fraction of the inhalable Ni-aerosol fraction obtained by personal monitoring and urinary Ni concentrations, and knowledge of the refining processes and occupations. The cutoff between low and high exposure categories was 70 µg/L urinary Ni, "corresponding roughly…to 160 µg/m^3 of the water-soluble inhalable sub-fraction." The authors state that quarterly stationary area air measurements since 1980 combined with company records indicate that refinery operations were "materially unchanged from the late 1960s until a few years ago."

The authors used logistic regression analysis to estimate the relative odds of a SA given exposure to water-soluble Ni, adjusted for the following variables, selected *a priori* based on reports of relevant associations, data availability, and consideration of collinearity: maternal age >34 years, previous induced abortions, previous delivery, regular heavy lifting at work, and exposure to paints or solvents (based on occupation). Self-reported smoking was included in a sub-analysis.

Questionnaire study results: Cases and controls represented 1,380 women; of these, 184 (13.3%) had a recognized SA (cases). The highest proportion of women who had a recognized SA was in the matte-separation and roasting, anode casting, and primary Ni reefing units (19.8 ± 10.4%) and in the Cu refinery (19.6 ± 7.4%). The highest proportion of cases to controls was also in the Cu refinery (15.7%). Overall, 38.6% of cases and 31.2% of controls had been exposed. The unadjusted odds ratio for SA was 1.38 (95% CI 1.04 – 1.84) per unit increase in exposure category over the three categories; after adjustment for previous delivery, solvent or paint exposure, heavy lifting, and maternal age >34 years, and maternal smoking, the adjusted OR was 1.14 (0.95 – 1.37). Removing previous induced abortion from the model or adding maternal smoking did not change the result.

Birth registry study results: Inclusion criteria were met by 474 cases and 4,571 controls. 11.4% of cases and 14.2% of controls had been exposed and the unadjusted odds ratio (OR) for SA across the three exposure levels was 0.77 (0.58 – 1.03). After adjusting for previous induced abortion, solvent or paint exposure, and maternal age > 34 years, the OR was 0.87 (0.72 – 1.06). When maternal smoking but not maternal age (which did not appear to change the OR) were included in the model, the adjusted OR was 1.10 (0.82 – 1.47).

OEHHA comments: The authors note that the mean gestational age of cases was highest in the high exposure category (13.6 weeks vs. 11.5 weeks in the background exposure category), although there was no correlation between exposure level and gestational age of cases. They also note that as induced abortions were common, the distribution of cases in the study should be skewed downwards compared to the true risk. The study design did not allow authors to address risk of SA in the first few weeks of gestation, and therefore missed such cases. Vaktskjold et al. (2008a) acknowledge, "…it might have been more appropriate to aim to study the specific risks related to the different stages of pregnancy and limit the inclusion of cases accordingly." The authors add that risks associated with Ni exposure may even appear to be protective due to increased risk of fetal loss before a pregnancy is verified.

The pregnancies included in the questionnaire study date back as far as 1966, although the study commenced among women in the workplace 30 years later. Thus, if pregnancy outcomes influenced women's choice of work within the refinery or to leave the refinery, the study results could be biased. The authors also acknowledge that the inclusion criteria favor the most fertile women. About 39% of women who had experienced SA were registered more than once; the remainder had either changed jobs (and exposure) between the births before and after the SA, or had already had one or more SAs before the first registered birth. For this case selection method to be non-differentially biased, the exposure must not influence job changes differentially for the case mothers vs. control mothers.

B.1.2. Fetal growth

Odland et al. (1999). Urinary nickel concentrations and selected pregnancy outcomes in delivering women and their newborns among arctic populations of Norway and Russia.

With this cross-sectional study, Odland et al. aimed to compare urine nickel excretion in pregnant women and their newborns in three communities in Russia with that of their counterparts living in three cities in Norway, and to assess the influence of urine Ni concentrations and other variables on pregnancy outcomes (birth weight and infant body mass index (BMI)) (Odland et al. 1999).

The Russian communities were Nikel and Monchegorsk in Murmansk County, both with Ni refinery operations, and Arkhangelsk in Arkhangelsk County, which is near five large pulp paper plants. The reference cities in Norway were Kirkenes (near the Russian border and 50 kilometers [km] from Nikel), Hammerfest, and Bergen. Registration and sampling were performed in Arkhangelsk in April – May 1993, Nikel and Monchegorsk in March – June 1994, Kirkenes in November 1993 – January 1994, Hammerfest in December 1993 – January 1994, and Bergen in June 1994.

In each location, at least 50 consecutive women presenting to the hospital delivery department provided urine samples and information in a midwife- or gynecologist-administered questionnaire that addressed age, parity, height, weight, ethnic background, residence, education, occupation, smoking, alcohol consumption, medication, serious diseases, and dietary habits. The Russian questionnaire responses lacked information on years in school, and many Russian respondents were reluctant to answer questions about alcohol use, so analyses did not include these data.

The authors obtained the following information from delivery department medical records: maternal age, height, and weight; Naegele term; infant length, weight, date of birth, Apgar score, congenital malformations, and gestational age; placenta weight; and comments by the doctor or midwife.

Women provided urine and blood samples at 1-2 days post-partum. Women delivering in Nikel and Kirkenes also provided urine samples at week 20 of pregnancy. During the study period, maternity leave in Russia began 56 days before the anticipated delivery date; consequently, the urine samples collected shortly after birth did not represent recent occupational exposures for women in Monchegorsk. Although no women refused to participate, urine samples could not be obtained from some of the infants if the sampling procedure caused skin irritation. First-voided urine was obtained from 265 newborns (137 in Russia and 128 in Norway; in Bergen, samples were not obtained from 19 infants). Statistical analyses excluded women and infants for whom neonatal urine samples were unavailable. Urine Ni concentrations below the limit of detection (LOD) of 10 nmol/L were assigned the value of (1/2)LOD=5 nmol/L.

Ten to twelve tap water samples were collected from randomly selected homes in Nikel, Monchegorsk, Arkhangelsk, Zapolyarniy, and Umba, and tested for Ni. Water in communities with Ni refineries had higher levels of Ni in drinking water compared to the Russian and Norwegian communities without Ni point sources.

The authors used univariate analysis, analysis of variance, and multiple linear regression analysis. On average, the Russian mothers were statistically significantly younger, shorter, much less likely to smoke, and more likely to experience complications related to pre-eclamptic conditions; and had fewer previous deliveries. Mean maternal weight at term and BMI were not significantly different for the two populations.

Russian infants were significantly lighter (mean birth weight: 3,195 vs. 3,590 g) and longer (mean length: 51.8 vs. 50.7 cm), had lower BMI (11.9 vs. 13.9), and were earlier (mean gestational age 38.7 vs. 39.8 wks). Mean head circumference was identical for the two groups. Fifteen women in the Russian group had worked (until maternity leave) in the Ni industry: six who apparently worked in departments with low Ni exposure in Nikel, and nine who were exposed to Ni in electrorefining in Monchegorsk.

Urine Ni concentrations in mothers and infants from each of the three Russian sites were higher than in their Norwegian counterparts: term median urine Ni concentration was 85 nmol/L for Russian mothers vs. 5 nmol/L for Norwegian mothers, and median first-voided urinary Ni concentration was 34 nmol/L for Russian infants vs. 5 nmol/L for Norwegian infants. The median urine Ni concentration in the nine Monchegorsk Ni refinery workers was somewhat lower (66 nmol/L) compared to the total Russian sample (85 nmol/L). After adjustment for creatinine, the urine Ni concentration was lower in the mothers and infants from Arkhangelsk, which does not have a Ni refinery (6 nmol/L and 5 nmol/L), than in the whole Russian group (9 nmol/L and 11 nmol/L). In the Nikel and Kirkenes samples, the maternal urine Ni concentrations did not differ between the 20th week and at term.

For the Russian group, no variables were significantly associated with birth weight or maternal BMI in univariate analyses. For the Norwegian group, maternal weight, height, BMI, smoking, and local food consumption were associated with birth weight; maternal weight, BMI, and smoking were associated with infant BMI. In multivariate analyses, maternal urine Ni was not a significant predictor of birth weight (birth weight change associated with 1 nmol/L increase in maternal urine Ni: -0.1 95% C.I. (-0.5, 0.3) g). Using infant's BMI as the outcome, only maternal BMI and country were significantly associated with infant's BMI; maternal urine Ni was not associated. Categorizing urine Ni levels as below or above 34 nmol/L did not result in an association of Ni with birth weight. Log- or square- transformation of the Ni concentrations and grouping birth weight by 500 g increments added no information.

The authors state that birth-associated trauma may have contributed to slightly higher Ni concentrations in pregnant women than for other residents of Ni (90 nmol/L vs. 58 nmol/L). The authors further speculated that dietary sources and leaching of Ni into drinking water from pipes or cooking utensils, as well as oral prostheses, might explain the higher concentrations of Ni in Russian women.

The absence of data on alcohol use in analyses due to 36% of Russian respondents' refusal to answer questions is a cause for concern. Furthermore, education information was missing from the Russian data. Information about local food consumption was not collected in Monchegorsk. The authors also mention inconsistency between interviewers as a cause for concern, and the possibility that the translation of the questionnaire from Norwegian to Russian may have had a "steering effect" on some of the questions. The authors note that selection bias may have resulted from exclusion of mothers and neonates due to the absence of neonatal urine samples; however,

inclusion of the neonates without urine samples did not affect the difference between the Russian and Norwegian birth weights.

The authors conclude that urinary Ni excretion and thus Ni exposure are unimportant explanatory variables for birth weight, and that the Ni refineries as local point sources only minimally affect the body burden of Ni. However, they caution that this conclusion should not be applied to occupational exposure because Ni is transferred across the placenta and *in utero* Ni exposure cannot be dismissed.

Odland et al. (2004). Elements in Placenta and Pregnancy Outcome in Arctic and Subarctic Areas

The objective of this cross-sectional study was to assess concentrations of essential and toxic elements in maternal and neonatal body fluids and the placenta as predictors of birth weight and newborn body mass index (BMIC) in Norway and Russia (Odland et al. 2004).

In most communities sampled, lifestyle information, placentas, cord blood, maternal serum, and urine specimens were collected from 50 consecutive mother-infant pairs from hospital delivery departments in each of three Norwegian and three Russian communities (Odland et al. 1999; Odland et al. 2004). Availability of maternal and cord blood Pb concentrations determined the eligibility for this study. Registration and sampling took place between May 1993 and June 1994. Women were interviewed for personal and morphometric information, and data on the birth, gestational age, placenta weight, and length of the baby were taken from the delivery records.

Women provided urine and blood samples at 1-2 days post-partum. First-voided urine was obtained from infants. The authors tested for concentrations of Ni, cadmium (Cd), lead (Pb), selenium (Se), Cu, zinc (Zn), iron (Fe), and in a limited sample, mercury (Hg). Concentrations <10 nmol/L (limit of detection; LOD) were assigned the value of (1/2)(LOD)=5 nmol/L (Odland, et al., 1999). Ni concentrations were much lower in Norwegian than in Russian samples, as shown in Table B.4:

Table B.4. Ni in maternal and neonatal urine and placenta in Russian and Norwegian samples

Media	Total group median (range)	Russia median (range)	Norway median (range)	N
Maternal urine (nmol/L)	47.0 (4.3-2108)	84.2 (4.3-2108)	13.6 (4.3-96.9)	252
Neonatal urine (nmol/L)	18.7 (4.3-561)	34.0 (4.3-561)	4.3 (4.3-37.0)	227
Placenta (µg/g)	0.017 (0.005-0.377)	0.023 (0.005-0.119)	0.012 (0.005-0.377)	220

Similarly, Pb, Cu, and Fe concentrations were significantly different in Norwegian vs. Russian samples. There were also large differences in the characteristics of the Norwegian vs. Russian women and their birth outcomes.

In univariate analyses, the only element with an apparent significant negative effect on birth weight was placental Ni: weight change (CI) -2526 (-4659, -394) g/µg/g (Table

B.5). This association loses significance with adjustment for gestational age: -1510 (-3191, 170) g/µg/g. The negative association between placental Ni and BMIC was close to significance, and adjustment for gestational age weakened the association.

Table B.5. Analyses of placental Ni and birth weight and BMIC*

Outcome	β (95% CI) per µg/g*	β (95% CI) per µg/g**
Birth weight, g	-2526 (-4659, -394)	-1510 (-3191, 170)
BMIC	-4.90 (-10.27, 0.47)	-2.73 (-7.49, 2.02)

*Adjusted for country
**Adjusted for country and gestational age

The authors used multivariate linear regression analysis with stepwise variable selection to examine the association between element concentrations and birth weight and BMIC. Multivariate models that included Ni also considered maternal age, smoking frequency, BMI, height, number of deliveries, urinary creatinine; gestational age; country; placental Pb, Ni, and Cu; maternal blood and cord blood Pb. There were no significant associations between placental element concentrations and BMIC. Although placental Ni was considered in multivariate analyses of birth weight and BMIC, it was dropped from final models.

OEHHA comment: The authors did not discuss adjustment for country. If country is a proxy for some mechanism in the causal pathway between exposure and fetal growth, this adjustment may not be appropriate.

Vaktskjold et al. (2007). Small-for-gestational-age newborns of female refinery workers exposed to nickel

The aim of this study was to assess whether women occupationally exposed during pregnancy to elevated levels of water-soluble Ni at the Monˇegorsk Ni refinery were at higher risk of delivering an small for gestational age (SGA) newborn than women not so exposed, in Monˇegorsk, Russia (Vaktskjold et al. 2007). The study used data from the Kola Birth Registry (KBR), combined with questionnaire information on smoking and workplace.

The source population was 25,245 registered singleton births with specified sex and birth weight. Births were included if they met the following inclusion criteria, defined *a priori*: 1) born in 1973-2001; 2) mother was a resident of Monˇegorsk at start of pregnancy; 3) registered gestational age 28-42 weeks; 4) mother's workplace and job were registered; 5) no diagnosis of chromosomal aberrations (trisomies 13,18, and 21, and Turner's syndrome). The study population comprised 22,836 newborns.

SGA was defined as birth weight below the 10th percentile for gestational age, based on the distribution amongst all singleton deliveries in 1973-2003 in Monˇegorsk for each

integer week of gestation from 28 to 40 weeks. Infants born at 41 and 42 weeks were included in the 40-week group in the distribution analysis. The 10th percentile was determined sex-specifically only for infants of at least 37 weeks gestation.

Exposure was characterized using quantitative analyses of air and urine measurements of Ni and other metalloids during 1995-2001. Each occupation in the KBR was assigned a categorical exposure rating of *background* (observed urinary Ni concentration <10 µg/L), *low*, or *high* (urinary Ni concentration ≥70 µg/L, roughly corresponding to ≥ 160 µg/m^3 of the water-soluble inhalable sub-fraction) Ni exposure. *Background* exposure included refinery occupations with exposure levels estimated to be comparable to that of the general population. A comparison of current and past quarterly in-plant stationary (area) air measurements, combined with knowledge of the refining processes, assured the authors that the exposure assessments also reflected past exposures.

Multiple logistic regression was conducted to analyze the relative odds of SGA associated with exposure to Ni. Regression models included adjustment for the following factors, selected *a priori* based on data availability and reports of relevant associations in the literature: first delivery, regular maternal exposure to solvents at work, maternal age >34 years, maternal height, smoking (yes/no), pervious induced abortion (yes/no), and obvious signs of alcohol abuse in pregnancy (yes/no). The authors also conducted several sub-analyses which: 1) excluded smoking from the model; 2) adjusted for genital defects (boys only); 3) included only preterm newborns (with sex as a covariate); 4) included Ni refinery workers only, and 5) included employed women only.

Of the 22,836 newborns, 2,096 (9.2%) were SGA. Mothers of 10.6% of SGA infants and 13.0% of the reference infants were employed at jobs with Ni exposure above the background level. The unadjusted OR for SGA per unit increase in exposure category was 0.79 (0.68 – 0.91) and the adjusted OR was 0.84 (0.75 – 0.93). Excluding maternal height and previous induced abortions from the model resulted in a similar OR of 0.81 (0.73 – 0.90). If only preterm births are included, the OR for SGA was 0.61 (0.31 – 1.20). When newborns of unemployed/housekeeping mothers were excluded, the OR was 0.83.

OEHHA comments: The authors state that although the KBR contains information about occupation, they had very limited insight into what exposures occupations outside the Ni refinery could involve, especially 20 – 30 years ago. Non-Ni workplace exposures associated with SGA could influence the result away from finding an effect of Ni exposure. The authors further note that Russian women had the right to be transferred from potentially hazardous jobs when verified as pregnant, regardless of where they worked. Ten to twenty refinery workers were advised to change jobs to less hazardous or physically demanding jobs for health reasons each year, but the authors state that such voluntary job changes were unlikely for Ni production workers, as they would decrease salaries.

Another possible explanation the authors suggest for the low OR is the "healthy worker effect" – they note that the highest proportion of SGA births was observed among women who were unemployed or homemakers (11.8%). Although they conclude that differences due to workplace regulations and employment status are not likely, bias related to employment status and the type of employment might still be possible.

Bell et al. (2010). Prenatal Exposure to Fine Particulate Matter and Birth Weight: Variations by Particulate Constituents and Sources

The aim of this study was to identify which particulate matter ≤ 2.5 μm ($PM_{2.5}$) constituents or sources are most strongly associated with birth weight (Bell et al. 2010). The authors identified which constituents were most associated with specific sources to assist interpretation of estimates for constituents, and investigated whether observed effect estimates differ by race.

The authors used two exposure metrics for $PM_{2.5}$: 1) elemental constituent (e.g., Ni) concentrations, and 2) source contributions (e.g., traffic). They obtained Teflon filters from $PM_{2.5}$ regulatory monitors for August 2000 – February 2004 from the Connecticut (CT) and Massachusetts (MA) Departments of Environmental Protection to measure levels of $PM_{2.5}$ constituents in Hartford, New Haven, Bridgeport, and Danbury, CT, and Springfield, MA. $PM_{2.5}$ filter samples were available for 92%, 90%, 32%, 30%, and 57% of days for these cities, respectively. Each location had a primary monitoring site; when data were missing from the primary site, filters from other sites were used.

Positive matrix factorization (PMF) uses an algorithm to estimate the contribution of $PM_{2.5}$ sources to constituent levels, based on measured constituent data. The authors applied PMF to data from each monitor separately to convert levels of elemental constituents into daily estimated profiles and source contributions for motor vehicles, road dust/crustal sources, oil combustion, salt, and other regional sources, resulting in a time-series of estimated $PM_{2.5}$ levels from each source. The daily profiles were then combined to estimate county-average exposures over each trimester and the entire pregnancy for each study subject. Distances from the monitors to the centroid of each of the 209 census tracts were calculated, and the population of each tract was assigned to the closest monitor. The distance from a census tract to a monitor in the same county did not exceed 45.0 km.

Constituents contributing the most to each source factor were identified for each county, based on percentage mass. To identify constituents that are closely linked to particular sources, the distribution of constituents in each source was calculated, and each constituent was assigned to an individual source to which it contributed the highest percentage. The ratio of highest percentage contribution to any source to the second highest contribution was calculated to identify constituents that were more likely to originate from a specific source.

Birth weight, demographic, and other data were obtained from birth certificate data. Gestational periods were calculated from the date of birth and last menstrual period (LMP), corrected for two weeks between LMP and conception. Of 232,347 births in the four counties during 1999-2000, the final dataset included 76,788 births after exclusions for missing LMP or covariate data, infeasible delivery dates or birth weight-and-gestation combinations, inconsistent county of residence and delivery (adjacent counties were included), multiple birth, gestation length <37 weeks or >44 weeks, and birth weight < 1,000 g or > 5,500 g. Because previous research linked temperature with birth weight, apparent temperature was calculated for each trimester and the gestational period and included in models.

For each study subject, weekly averages for weather and $PM_{2.5}$ total mass, constituents, and sources were calculated, then combined for the pregnancy and trimesters (weeks 1-13, 14-26, and 27-birth). Linear regression analysis of effects on birth weight of $PM_{2.5}$ total mass, constituents, and sources was conducted in separate models, each of which included *a single constituent, a single source, or $PM_{2.5}$ total mass*. Logistic regression was used for models of small-at-term (LBW; term birth < 2500 g) compared with non-LBW (term birth ≥ 2500 g) birth. Models adjusted for:

- apparent temperature by trimester
- infant's sex
- parity
- nature of delivery (vaginal, primary cesarean, repeat cesarean)
- trimester of prenatal care initiation
- gestation length (weeks)
- year of birth (indicator variables)
- mother's age (grouped)
- marital status
- education
- tobacco use during pregnancy
- alcohol use during pregnancy
- race (white, African-American, other).

The mean (SD) gestational exposure to $PM_{2.5}$ Ni was 0.0031 (0.0015) μg/m^3. Using PMF analysis, the authors identified source tracer constituents that most likely originated from a given source and then used these constituents to function as approximate indicators of source categories. For each constituent, the ratio of the highest to the second highest percentage source contributions was calculated to identify constituents that are more closely linked to a particular source. Ni was closely linked to oil combustion: the source of 66.6% of Ni was oil combustion, and 26.0% was motor vehicle, for a ratio of 66.6/26.0=2.6, indicating that Ni is 2.6 times more likely to be in the oil combustion than the motor vehicle factor. Overall, Ni constituted 0.02% of $PM_{2.5}$ mass.

Regression models were adjusted for the covariates listed above, but not other pollutants. An interquartile range (IQR) increase in exposure to Ni $PM_{2.5}$ was associated with a change in birth weight of −7 (95% CI: −12 to −3) g and change in risk of low birth weight of 11 (95% CI: 3 to 19) percent. In analyses of trimester exposures, an IQR change in third trimester $PM_{2.5}$ Ni exposure was associated with a change in birth weight of −9 (95% CI: −15 to −2) g. First and second trimester $PM_{2.5}$ Ni exposures were not associated with changes in birth weight. Models with adjustment for correlation among trimester exposures were consistent with the trimester-specific results.

The authors also used an interaction model with terms for pollutant level over the gestation period and indicator variables for mother's race to evaluate whether associations differed by race. $PM_{2.5}$ Ni exposure was associated with a change in birth weight of −6 (95% CI: −12 to −1) g in infants of white mothers, and −12 (95% CI: −24 to 0) g in infants of African-American mothers.

The authors noted that due to spatial heterogeneity of $PM_{2.5}$, use of county-wide exposure estimates could have introduced exposure misclassification, and this could vary by constituent and source. Moreover, the monitors do not capture differences in personal exposures that may reflect individual activity patterns, such as time spent outdoors, or occupational exposure. Also, estimates were based on residence at time of delivery. The authors note that research suggests that pregnant women tend to move short distances and stay within the same community, and gestational exposures to air pollutants based on address at delivery are similar to those based on address during pregnancy.

Ebisu and Bell (2012). Airborne $PM_{2.5}$ Chemical Components and Low Birth Weight in the Northeastern and Mid-Atlantic Regions of the United States

This study aimed to investigate associations between birth weight and gestational exposure to particulate matter ≤2.5 μm ($PM_{2.5}$) total mass, $PM_{2.5}$ chemical components, particulate matter ≤10 μm (PM_{10}), and gaseous pollutants carbon monoxide (CO), nitrous oxide (NO_2), ozone (O_3), and sulfur dioxide (SO_2) for the Northeastern and Mid-Atlantic regions of the US (Ebisu and Bell 2012).

The authors obtained birth certificate data for CT, Delaware, Maryland, MA, New Hampshire, New Jersey, Rhode Island, Vermont, Virginia, Washington, DC, and West Virginia from 1 January 2000 through 31 December 2007. Data included county of residence and birth, birth order, trimester of first prenatal care, date of last menstrual period (LMP), gestational age, infant's sex and birth weight, maternal and paternal ages and races, maternal education, marital status, alcohol use, and smoking during pregnancy. Births were excluded for the following: unspecified county of residence or birth, different counties of residence and delivery, plural deliveries, gestation >44 weeks

or <37 weeks, birth weight <1,000 g or > 5,500 g, impossible gestational age and weight combinations, and missing or problematic LMP.

The authors identified the following $PM_{2.5}$ chemical components as having potential links to health and/or as contributing substantially to $PM_{2.5}$ total mass: aluminum (Al), ammonium ion, arsenic (As), Cd, calcium (Ca), chlorine (Cl), elemental carbon (EC), Pb, Hg, Ni, nitrate, organic carbon matter, silicon (Si), sodium ion, sulfur (S), titanium (Ti), vanadium (V), and Zn. Data on $PM_{2.5}$ chemical components were obtained from the US Environmental Protection Agency (US EPA) Air Explorer and PM_{10}, $PM_{2.5}$ total mass, CO, NO_2, O_3, and SO_2 data were obtained from the US EPA Air Quality System for 1999-2007. PM measurements were taken every 3-6 days and gaseous pollutants were measured daily, although O_3 was measured mainly during the warm season. Apparent temperature (AT) was calculated based on daily temperature and dew point temperature data from the National Climatic Data Center. For each birth, the authors calculated the average level of each pollutant during gestation and for each trimester, and average AT for each trimester.

Date of delivery was estimated based on self-reported LMP and gestation length. Trimesters were defined as 1-13 weeks, 14-26 weeks, and 27 weeks to delivery. Exposures were estimated based on county of residence. Many counties had only one monitor; the average number of monitors per county was 1.08 (range, 1-2) for $PM_{2.5}$ chemical components, and 1.57 (range, 1-9) for PM_{10}, $PM_{2.5}$, and gaseous pollutants. The mean (± SD) area of the 49 counties is 540.5 (± 395.3) mi^2 (median = 528.6 mi^2). The average urbanicity rate based on 2000 Census values was 81.7%. Measurements from multiple monitors in the same county were averaged to estimate daily pollutant levels and combined with AT to estimate weekly exposures, which were then averaged to estimate gestational and trimester exposures. Births missing exposure estimates for more than 25% of the weeks for any trimester for a pollutant were excluded from analyses of that pollutant.

The authors used logistic regression analysis to estimate associations between LBW (birth weight <2,500 g) and gestational exposure to each pollutant, adjusted for maternal race, marital status, tobacco and alcohol use during pregnancy, education level, age, gestational age, infant's sex, birth order (first, other), trimester of first prenatal care, delivery method (vaginal, cesarean section, unknown), average AT for each semester, season of birth, year of birth, and regional indicators. Pollutants that showed associations with LBW in single pollutant models were tested in two-pollutant models with pairs of pollutants whose correlation was <0.5. Also, for pollutants associated in single-pollutant models, the authors assessed effects by trimester in models with trimesters' exposures included simultaneously.

Of the 7,098,417 births in the 419 counties in the study area, 2,476,383 lived in the 50 counties with monitors for $PM_{2.5}$ chemical components, and 1,385,466 in 49 counties had exposure estimates for all pollutants for ≥75% of the weeks in all three trimesters. After exclusions, 1,207,800 infants from 49 counties remained in the study.

The mean (SD) gestational exposure to $PM_{2.5}$ Ni was 0.006 (0.006) $\mu g/m^3$ and IQR was 0.0071. The mean (SD) gestational exposure to $PM_{2.5}$ total mass was 13.41 (2.05) $\mu g/m^3$ and IQR was 2.71; mean (SD) gestational exposure to PM_{10} total mass was 22.34 (4.31) $\mu g/m^3$ and IQR was 4.93 $\mu g/m^3$.

The authors examined the births that would have been eligible if not for a lack of air monitors and found them to be similar to included births, except their mothers were more likely to be white (vs. African-American) and married. Births excluded for other reasons were similar to included births with respect to mother's age, race, marital status, and education, but differed with regard to exclusion criteria, e.g., gestational week, birth weight.

Ni was highly correlated ($r > 0.5$) with V (0.64), Zn (0.63), NO_2 (0.72), O_3 (-0.68), and SO_2 (0.61). Associations between LBW and only Ni, elemental carbon, Al, and Ti were robust to adjustment for copollutants with correlation < 0.5 in 2-pollutant models. The odds of LBW was 5.7% (95% CI 2.7, 8.8) higher per IQR increase in $PM_{2.5}$ Ni, adjusted for confounders (not including co-pollutants). This increase in relative odds remained when the analysis was restricted to first births (6.5%, 95% CI 1.6, 11.5). The authors also report that third trimester exposure to $PM_{2.5}$ Ni was associated with LBW.

The OR for LBW associated with $PM_{2.5}$ Ni was 10.2% (95% CI 7.9, 12.4) lower among African American than white mothers, and 4.6% (95% CI 2.2, 7.1) lower for females than for males.

The authors note that among hypotheses concerning biological mechanisms by which air pollution may affect birth outcomes, including direct toxic effects, that fetal growth may be retarded by direct toxic effects of air pollution, possibly by transfer of toxic components to the fetus from PM accumulated in the mother's lungs. They further state that $PM_{2.5}$ metal-related components may increase oxidative stress burdens, leading to adverse health outcomes. Among limitations of the study, the authors mention use of birth certificate data, especially for alcohol and tobacco use, prenatal care, pregnancy complications, and labor. In the present study, unknown smoking status was a risk factor for LBW (though its magnitude was less than half that among known tobacco users). The authors also state that exposure misclassification may have resulted from the possibility that some chemical exposures, such as As, may have been below the detection limit. Use of residence at time of delivery, instead of residence during the pregnancy may also have caused misclassification, though the authors cite studies showing that relocations during pregnancy tend to be within a short distance. Spatial heterogeneity of pollutants within a county, especially for large counties, may have caused misclassification, especially for those living far from monitors. The maximum distance to a monitor from the border of a county was 75.6 km.

Basu et al. (2014). Effects of fine particulate matter and its constituents on low birth weight among full-term infants in California

This retrospective cohort study examined exposures to $PM_{2.5}$ and $PM_{2.5}$ constituents on LBW among term infants in California, with stratification by season and exploration of possible effect modification by maternal characteristics (Basu et al. 2014). The study population comprised infants born between 2000 and 2006 to mothers residing in Los Angeles, Riverside, El Cajon, San Jose, Simi Valley, Bakersfield, Sacramento, and Fresno. The authors accessed birth records from the California Department of Public Health Natality Database. Singleton, live births with gestational age 37 through 44 weeks, with data available on the following covariates were included: infant birth date, weight, gestational age, sex and maternal race/ethnicity, education, age, and zip code.

The authors obtained data on ambient $PM_{2.5}$ mass and concentrations of $PM_{2.5}$ constituents from US EPA monitors in eight sites in California for 2000 through 2006. Start dates varied by site, there were some gaps in operation, and monitoring frequency was every three or six days. Constituents were included in the analysis if monitored continuously throughout the study period and detection levels were above the LOD at least 30% of sampling days. The distance between the monitor and the population-weighted centroid of the 2000 US Census zip code tabulation area (ZCTA) for the maternal zip code was calculated. Births to mothers with zip codes within 20 km of a monitor were included; others were excluded. Births were assigned to the nearest monitor if more than one monitor was within 20 km.

The authors calculated exposure to $PM_{2.5}$ constituents for the full gestational period, estimated as beginning two weeks after the last menstrual period (LMP), which was calculated using the date of birth and total gestation days. The first trimester was estimated as the third through 13th gestation week after the LMP, the second trimester was the 14th through 26th weeks, and the third trimester was the 27th week through birth. Trimester exposures were the average weekly mean for all weeks in the trimester if mean data were available for at least 75% of the weeks in that trimester. Weekly exposures were calculated if there was at least one reading for a given constituent that week, and weekly means for partial weeks at the end of pregnancy were included in the third trimester if the partial week was at least 4 days. Full pregnancy exposure was calculated as the mean of all three trimesters; births missing exposure estimates for one or more trimesters were excluded from the analysis. Average apparent temperatures for each trimester and full gestational period were calculated using temperature data from temperature monitors and humidity data, and techniques similar to those used for $PM_{2.5}$. The authors state that using weekly means reduces bias that may result from variation in sampling frequency across monitors.

The final study population included 646,296 infants with full gestational exposure information available for total $PM_{2.5}$ mass and 23 $PM_{2.5}$ constituents.

The authors used linear regression to analyze associations between birth weight and continuous measures of exposures to total $PM_{2.5}$ and $PM_{2.5}$ constituents, with separate models for each exposure variable, all adjusted for the maternal- and birth-related covariates and apparent temperature. The authors also conducted analyses stratified by season (warm, May-October and cool, November-April) and maternal age, education, and race/ethnicity to assess for effect modification. The authors evaluated the data for confounding by region (Northern California, Southern California, and Central Valley). The authors used groupings based on percentages of employment, non-White race, and home ownership by ZIP code tabulation area (ZCTA) data from the 2000 U.S. Census to adjust for community-level race and socioeconomic status (SES). Because some correlations among pollutants were relatively high, the authors did not examine multi-pollutant models in this study. To analyze the effects of $PM_{2.5}$ on LBW, the authors conducted logistic regression analyses.

For the whole cohort, the mean (SD) gestational exposure to total $PM_{2.5}$ mass was 18.7 (5.0) $\mu g/m^3$, mean (SD) gestational exposure to $PM_{2.5}$ Ni was 0.0033 (0.0040) $\mu g/m^3$, and IQR of $PM_{2.5}$ Ni exposure was 0.001 $\mu g/m^3$. The lowest mean gestational exposure to $PM_{2.5}$ Ni was in Bakersfield (0.0010 $\mu g/m^3$) and the highest was in San Jose (0.0076 $\mu g/m^3$). Among the constituents included in this study, Cl and sodium (Na) were most highly correlated with $PM_{2.5}$ Ni, with correlations of 0.39 and 0.38, respectively. Most other correlations with Ni were much lower. The changes in birth weight and risk of LBW associated with an IQR increase in $PM_{2.5}$ Ni, adjusted for individual demographic and birth characteristics, and adjusted additionally for ZCTA community-level characteristics, are shown in Table B.6.

Table B.6. Adjusted effect estimates (95% CI) for $PM_{2.5}$ Ni on birth weight and risk of LBW, per IQR increase in $PM_{2.5}$ Ni exposure

Outcome	Adjusted*	With additional ZCTA adjustment
Birth weight (g)	-1 (-2 to -1)	-1 (-2 to -1)
LBW (% change in odds)	1 (0 to 1)	1 (0 to 1)

*Adjusted for maternal race/ethnicity, education, and age; infant month of birth, year of birth, gestational age, and sex; region (north, south); and apparent temperature.

For $PM_{2.5}$ Ni, the reduction in birth weight appeared to be smaller for 3rd trimester exposure (Basu 2014, Figure 2). The authors report that nearly all interactions between $PM_{2.5}$ constituents and maternal age, race/ethnicity, and education were significant, though this did not appear to be true for $PM_{2.5}$ Ni and maternal age and race/ethnicity ((Basu et al. 2014), Figures 3 and 4; data for education were not shown).

Basu et al. state their results suggest an association between $PM_{2.5}$ mass and several $PM_{2.5}$ constituent exposures and lower birth weight among term infants born in California. The authors note that many of the constituents had stronger effects on birth weight than $PM_{2.5}$ total mass, indicating that the observed associations were not merely a result of correlations with $PM_{2.5}$ (though $PM_{2.5}$ Ni was not among those most strongly associated with reductions in birth weight). The authors note that although they lacked

information on maternal and passive smoking, both of which are associated with LBW, previous research has not found smoking to confound the relationships between $PM_{2.5}$ and LBW in California. Maternal alcohol consumption, parity, and delivery method were also not available, but are not suspected to be associated with $PM_{2.5}$. The authors also acknowledge the fact that maternal zip code was for the time of delivery, not the pregnancy, and note that exposure classification often does not change significantly following a move. ZCTAs and zip codes are not identical, which may have resulted in some exposure misclassification. The 20 km radius might be too large for $PM_{2.5}$ constituents that have more spatial heterogeneity than total $PM_{2.5}$; for these constituents the associations might be underestimated due to increased misclassification.

McDermott et al. (2014). Does the metal content in soil around a pregnant woman's home increase the risk of low birth weight for her infant?

In this retrospective cohort study, the investigators examined the associations between low birth weight (LBW; <2,500 g) and concentrations of eight metals (As, barium (Ba), chromium (Cr), Cu, Pb, Manganese (Mn), Ni, and Hg) in the soil near the subjects' homes (McDermott et al. 2014). The study included all 9,920 singleton live births to mothers residing in one of ten residential study areas from 1996 through 2002 who were insured by South Carolina Medicaid and therefore living below 185% of the federal poverty level. The authors identified the mothers through Medicaid billing records and followed the pregnancy and delivery using merged reimbursement files and birth certificates. Multiple births and children with birth defects and known causes of disability were excluded. The study areas were in rural, suburban, and urban neighborhoods, and were selected based on having at least 800 births within a 100 km^2 area in the study period.

Covariates were obtained from the birth certificate and Medicaid billing file, and included infant sex and gestational age (>36 weeks, 28-36 weeks, <28 weeks), maternal age (3 categories), maternal race, parity, and tobacco and alcohol use. Density per square mile and median age of housing in the census block group where each mother resided were also obtained.

Addresses for the sixth month of pregnancy (when enrollment was highest) were obtained from a Medicaid eligibility file and geocoded. To maintain confidentiality, a grid was laid over the study area and soil sampling locations were selected as the intersections of grid lines. Samples were taken after the pregnancy at 5 centimeters (cm) depth and 1.0 – 3.0 km apart. In order to estimate soil metal concentrations at the known residential addresses, the authors used data from approximately 100 nodes per area to do Bayesian kriging and statistical methods including variable transformation.

The authors used the multivariable generalized additive model (GAM) with a semi-parametric logistic function to explore the association between soil metal concentrations and LBW. GAM allowed the use of variables that do not have a linear relationship

between metal concentration and LBW. LBW was assumed to follow a Bernoulli distribution. Covariates included characteristics of the mother, child, and neighborhood, and the kriged concentrations for soil metals.

Kriged estimates for mean Ni concentrations were nearly identical for soil near LBW and normal weight infants' homes: 4.58 mg/kg for LBW infants, 4.57 mg/kg for normal weight births; IQR 43.21 mg/kg (whether this IQR represents cases and control combined is not stated). The unadjusted OR (CI) for risk of LBW associated with an IQR increase in Ni was 1.00 (0.98, 1.02). An IQR increase in As was associated with a 4% increase in the odds of LBW, and Hg was associated with a non-significant decrease in odds of LBW. Unadjusted ORs for other metals and LBW were all 1.00.

The authors note that people track soil and dust from outside their homes, the soil and dust accumulate on the floors, and studies confirm that metals in soils are bioavailable to humans.

OEHHA comments: The authors acknowledge that they did not directly measure soil around the mothers' homes and that soil sampling occurred after the pregnancies. However, due to long soil half-lives for inorganic chemicals, these estimates should be good estimates for the concentration during pregnancy. The study was conducted among low-income families, so it may not be generalizable.

Laurent et al. (2014). Sources and contents of air pollution affecting term low birth weight in Los Angeles County, California, 2001-2008.

Laurent et al. studied the relationships between air pollution and term LBW in infants born in Los Angeles (LA) County (Laurent et al. 2014). By studying particulate matter ≤0.1 μm ($PM_{0.1}$), using spatiotemporal chemical transport modeling of primary particles by source and composition and using more common air pollution metrics such as interpolated measurement data, traffic indices, and proximity to roads, this study also enables comparison of results of complementary exposure metrics for traffic-related air pollution.

The authors obtained birth certificate records for all 1,203,782 births to women residing in LA County from January 1, 2001 to December 31, 2008. Records were excluded for multiple births, infants with recorded birth defects or unknown birth defect status, missing information for gestational age, implausible birth weight and gestational age combinations, and estimated gestational age <260 or >308 days. Maternal residence addresses were geocoded at the centroid of tax parcels when feasible; 53% of addresses were geocoded to a specific parcel, 42.5% were geocoded using address range interpolations, 4.5% at the Zip code centroid level, and 0.05% at the city centroid level.

The authors relied on the University of California Davis/CIT_Primary (UCD_P) chemical transport model, which was developed to track PM emitted directly from sources (primary PM) through a simulation of emission, advection, diffusion, and deposition. The UCD_P model utilized size and composition data derived from measured primary particle source profiles, raw emissions inventory data provided by the California Air Resources Board (CARB), meteorological inputs prepared using the Weather Research and Forecast model, and separate model simulations for every individual source in the emissions database. UCD_P did not account for the formation of secondary PM by chemical reactions in the atmosphere.

UCD_P estimates concentrations of primary ground-level PM elements from approximately 900 sources across densely populated areas in California at a 4 km x 4 km grid resolution. The authors defined seven broad source categories: gasoline, diesel, shipping, high sulfur combustion sources (including aircraft, electricity generation, petroleum refining, and other industries), commercial meat cooking, wood burning, and other sources. For this study, concentrations for $PM_{2.5}$ and $PM_{0.1}$ were estimated. Exposure estimates were based on the 448 model grid cells covering LA County. Women were assigned daily concentrations based on the grid cell in which they lived at the time of delivery; these concentrations were then averaged over each trimester and the entire pregnancy.

Measurements from government monitoring stations were obtained from CARB for total $PM_{2.5}$, NO_2, and O_3 for 2000-2008. For $PM_{2.5}$, only filter-based measurements, generally conducted every 3 or 6 days, were included. Hourly NO_2 and O_3 measurements were converted to daily means; for O_3, only data from 10 AM-6 PM were used. Monthly averages were then calculated for stations with more than 75% days of valid data in a month. The authors then used an empirical Bayesian kriging (EBK) model to spatially interpolate and predict pollutant concentrations between stations using 200 m x 200 m grids for all of California. In the study region, there were 5 to 11 monitors providing measurements for $PM_{2.5}$, 11 to 16 monitors for NO_2, and 12 to 16 monitors for O_3. Final pollutant concentration estimates were calculated for each mother by weighing monthly average concentrations by the number of days of each month during pregnancy or specific trimesters. The authors decided *a priori* to include only PM components for which measured $PM_{2.5}$ values and monthly averaged predictions had correlations >0.6 in LA. Correlations for measured and monthly predicted Ni and most other $PM_{2.5}$ species were >0.8.

Traffic densities within radii of 50 meters (m), 150 m, 250 m, and 350 m of mother's homes were calculated based on 2002 annual average daily traffic counts (AADT) data from the California Department of Transportation. AADT on each road segment was weighted by the length of the road segment within the radius and then scaled to other years based on temporal trends in total vehicle miles traveled in LA County.

To study the relationships between air pollution metrics and term LBW, the authors used generalized additive models with a logistic link function and a quasi-binomial

distribution (accounting for observed under-dispersion of the data) for entire pregnancy exposures and for each trimester. Regressions involving EBK interpolation were weighted by the inverse variance of pollutant predictions. Covariates included in the main models were maternal race/ethnicity, education level, parity, trimester prenatal care began, and infant's gender as categorical variables. The authors adjusted for maternal age, gestation length, and median household income by Census block group using smoothing splines due to their J-shape relationship with LBW.

The authors conducted sensitivity analyses to evaluate further adjustment for population density, pre-pregnancy and gestational diabetes, chronic hypertension, and pre-eclampsia. For 2007-08, the authors examined the effects of adjusting for maternal height, BMI at the beginning of pregnancy, and weight gain during pregnancy. The authors also examined separately the births geocoded at the exact parcel centroid. In addition, the authors evaluated effect modification by maternal race/ethnicity, education, median block group income, hypertension, diabetes, preeclampsia, and BMI at the beginning of pregnancy.

Using EBK-interpolated measurements, the OR (95% CI) for LBW associated with total $PM_{2.5}$ was 1.020 (1.010, 1.031) for exposure over the entire pregnancy.

Chemical species associated with increased risk of LBW in this study were elemental and organic carbon, potassium (K), Fe, Mn, and Ni in both particle fractions, and with Ti and Cu in $PM_{2.5}$, and Cr in $PM_{0.1}$. For the entire pregnancy, an IQR increase in primary $PM_{2.5}$ and $PM_{0.1}$ for all sources summed was associated with a 2.5% increase in risk of term LBW. Associations with UCD_P predictions were stronger for third trimester exposures.

The UCD_P predictions for mean (SD) concentration were 0.0030 (0.0026) $\mu g/m^3$ for $PM_{2.5}$ Ni and 0.0004 (0.0004) for $PM_{0.1}$ Ni. IQR increases in $PM_{2.5}$ Ni and $PM_{0.1}$ Ni were associated with LBW, as shown in Table B.7:

Table B.7. ORs for LBW and $PM_{2.5}$ Ni and $PM_{0.1}$ Ni exposures by trimester

	First trimester exposure		Second trimester exposure		Third trimester exposure		Pregnancy-long exposure	
Ni particle size	IQR in exposure ($\mu g/m^3$)	OR (95% CI) per IQR	IQR in exposure ($\mu g/m^3$)	OR (95% CI) per IQR	IQR in exposure ($\mu g/m^3$)	OR (95% CI) per IQR	IQR in exposure ($\mu g/m^3$)	OR (95% CI) per IQR
$PM_{2.5}$	0.0022	1.010 (1.003, 1.016)	0.0022	1.008 (1.002, 1.014)	0.0023	1.010 (1.004, 1.016)	0.0021	1.009 (1.003, 1.015)
$PM_{0.1}$	0.0003	1.009 (1.003, 1.014)	0.0003	1.008 (1.003, 1.014)	0.0003	1.010 (1.005, 1.015)	0.0003	1.009 (1.004, 1.014)

Adjustment for population density, diabetes, chronic hypertension, preeclampsia, maternal height, pre-pregnancy BMI or weight gain during pregnancy had almost no effect on ORs. Adjustment for time of conception also had little effect on most ORs.

Births that were geocoded to the exact parcel were similar to others in terms of maternal characteristics and ORs for most sources and road characteristics.

Effects of $PM_{2.5}$ Ni and $PM_{0.1}$ Ni varied somewhat by maternal race/ethnicity, as shown in Table B.8.

Table B.8. Effects of $PM_{2.5}$ Ni and $PM_{0.1}$ Ni by maternal race/ethnicity

UCD_P primary particles	Asian	African American	Hispanic	White non-Hispanic	Other and multiple
$PM_{2.5}$ Ni	1.067 (1.038, 1.097)	1.008 (0.983, 1.033)	1.006 (0.999, 1.013)	0.973 (0.951, 0.994)	1.084, (1.036, 1.134)
$PM_{0.1}$ Ni	1.054 (1.031, 1.077)	0.997 (0.975, 1.020)	1.007 (1.002, 1.013)	0.978 (0.961, 0.996)	1.064 (1.026, 1.103)

Effects of $PM_{2.5}$ Ni and $PM_{0.1}$ Ni were not statistically significantly different by maternal education level, as shown in Table B.9.

Table B.9. Effects of $PM_{2.5}$ Ni and $PM_{0.1}$ Ni by maternal education level

UCD_P primary particles	<8th grade	9th grade to high school	Some college
$PM_{2.5}$ Ni	0.999 (0.985, 1.014)	1.014 (1.007, 1.022)	1.001 (0.989, 1.014)
$PM_{0.1}$ Ni	1.001 (0.989, 1.014)	1.015 (1.008, 1.021)	0.997 (0.987, 1.008)

Although relatively few women had chronic hypertension (1,549 of 704,143 [0.2%]), the data suggested that ORs for LBW and IQR increases in $PM_{2.5}$ Ni or $PM_{0.1}$ Ni might be somewhat higher among hypertensive women, as shown in Table B.10.

Table B.10. ORs for LBW and IQR increases in $PM_{2.5}$ Ni or $PM_{0.1}$ Ni and hypertension

UCD_P primary particles	Hypertension	No hypertension
$PM_{2.5}$ Ni	1.046 (0.940, 1.164)	1.009 (1.003, 1.015)
$PM_{0.1}$ Ni	1.034 (0.947, 1.128)	1.008 (1.003, 1.013)

ORs for LBW and an IQR increase in $PM_{0.1}$ Ni, and possibly for $PM_{2.5}$ Ni, were slightly higher among women with preeclampsia, as shown in Table B.11.

Table B.11. ORs for LBW and IQR increases in $PM_{2.5}$ Ni and $PM_{0.1}$ Ni and preeclampsia

UCD_P primary particles	Preeclampsia	No preeclampsia
$PM_{2.5}$ Ni	1.045 (0.997, 1.095)	1.011 (1.005, 1.017)
$PM_{0.1}$ Ni	1.054 (1.012, 1.097)	1.010 (1.005, 1.015)

ORs for LBW and an IQR increase in $PM_{2.5}$ Ni might be slightly higher among women with diabetes, as shown in Table B.12.

Table B.12. ORs for LBW and IQR increases in $PM_{2.5}$ Ni and $PM_{0.1}$ Ni and diabetes

UCD_P primary particles	Diabetes	No diabetes
$PM_{2.5}$ Ni	1.039 (1.001, 1.080)	1.008 (1.001, 1.014)
$PM_{0.1}$ Ni	1.018 (0.985, 1.052)	1.009 (1.003, 1.014)

The authors state that their results show modest increases in risk of LBW associated with primary $PM_{0.1}$ and $PM_{2.5}$ from nearly all sources and several chemical species, including Ni, and with total $PM_{2.5}$ interpolated by EBK, NO_2 in the last trimester, and with local traffic indicators. Infants of mothers with lower education, Hispanic ethnicity, diabetes, chronic hypertension, and high BMI appeared to be more susceptible.

OEHHA comments: Air pollution metrics relied on maternal address at time of delivery. The authors did not have data on changes in residential history or time-activity information. As pollution levels can vary substantially among micro-environments, the actual exposure of mothers could not be measured. The authors note that these sources of exposure measurement error contributed random error and might also generate bias.

Data on maternal smoking during pregnancy were available for 2007 and 2008 but were not used because of potentially serious underreporting; the authors state this is "probably the major limitation of this study". They note that Hispanic women, who have the lowest prevalence of smoking during pregnancy in California, were also the group with the highest ORs and represent >60% of the study population, and cannot exclude the possible influence of smoking on their results.

The authors state that the completeness of maternal disease reporting is uncertain. Although the higher ORs among infants of mothers with chronic hypertension could be of great public health interest, potential under-reporting of maternal diseases on birth certificates makes the extent to which these data represent women with chronic diseases questionable.

Hu et al. (2015). Distributions of Heavy Metals in Maternal and Cord Blood and the Association with Infant Birth Weight in China.

The objective of this pilot study was to measure serum levels of heavy metals in pairs of pregnant women and their newborns, and to evaluate associations with birth weight (Hu et al. 2015). From June to August 2011, 81 pairs of mothers and newborns were enrolled from four hospitals in Beijing, Xiamen, Lanzhou, and Taiyuan. The authors did not describe the sampling design and subject selection criteria or methods. Mothers were interviewed at the time of delivery to collect demographic and lifestyle information and medical history. Information on the newborn, including birth date, gestation week, gender, placental weight, and birth weight; mother's previous pregnancy outcomes; and mother's weight at delivery were obtained from the medical delivery records.

Of the 81 infants, 30 (37.0%) were male and 51 (63.0%) were female. "Eleven (13.6%) were small for gestational age infants (gestational week <37 weeks), while the remaining 70 newborns (86.4%) were born following a normal number of weeks of pregnancy (37-41 weeks)." Eight (9.9%) infants were <2,500 g and three (3.7%) were >4,000 g at birth.

The authors used inductively coupled plasma mass spectrometry to measure heavy metals, including Pb, thallium (Tl), Cd, Se, As, Ni, V, Co, and Hg, in maternal and cord blood. The limit of detection (LOD) for Ni was 0.5 ng/g. The authors did not state how they treated concentrations below the LOD in statistical analyses. Ni was detected in 65.4% of maternal blood samples and 51.9% of cord blood samples, and the difference in detection rates was statistically significant (p=0.028). Table B.13 below shows Ni concentrations in maternal and cord blood. The difference between maternal and newborn Ni concentrations was not statistically significant.

Table B.13. Ni in maternal and cord blood

	Detection rate*	25th percentile	Median	75th percentile
Maternal blood	65.4%	< LOD	1.4 ng/g	2.1 ng/g
Cord blood	51.9%	< LOD	0.9 ng/g	2.4 ng/g

*LOD = 0.5 ng/g

The authors conducted multiple regression to examine the association between infant birth weight and exposure to metals that were detected in at least half of the sample. Ni in maternal blood was positively but not significantly associated with birth weight, as shown in Table B.14 below.

Table B.14. Betas for maternal and cord blood and birth weight

	Beta (unadjusted)	95% C.I.	Beta (adjusted)*	95% C.I.
Maternal blood	40.1	-40.5 – 120.7	45.6	-17.2 – 108.4
Cord blood	1.0	-65.9 – 64.0	32.2	-19.8 – 84.1

*Adjusted for infant gender, maternal age, gestational week, and maternal BMI

Correlation among the metals was not addressed, and the regression analyses did not include more than one metal at a time.

Pedersen et al. (2016). Elemental Constituents of Particulate Matter and Newborn's Size in Eight European Cohorts

This study combined eight mother-child cohorts previously formed for studies of PM and nitrogen oxides in Sweden, Denmark, Lithuania, Netherlands (two cohorts), Germany, Italy, and Spain as part of the European Study of Cohorts for Air Pollution Effects project (Pedersen et al. 2016). The aims of this study were to examine effects of constituents of ambient PM on birth weight and head circumference. The study population included 34,923 mother-child pairs with singleton deliveries between 1994

and 2008. Detail on individual characteristics was obtained through self-administered questionnaires and/or interviews (in-person or telephone) of the mothers, depending on the cohort. The timing of the interviews and questionnaire administration varied from early in pregnancy to at least three months after delivery, depending on the cohort. Inclusion criteria also varied across cohorts. Gestational age, birth weight, birth head circumference, sex, and mode of delivery were obtained from birth records or, for one cohort, parental reports. LBW was defined as birth weight < 2,500 g in an infant born after 37 weeks of gestation. Gestational age was defined using the interval between the start of the last menstrual period (LMP) and delivery for 53% of births; ultrasound-based estimation for 10% where LMP was unavailable, or estimates by the obstetrician (usually based on ultrasound) for the remaining 37%.

$PM_{2.5}$ and PM_{10} concentrations in outdoor air were measured between 2008 and 2011, during three 2-week periods in summer, winter, and an intermediate season within one year at 20-40 sites within each study area. Sites were selected to represent spatial variation in air pollution in the areas where participants lived. For each area, $PM_{2.5}$ and PM_{10} filters were analyzed for elemental composition using X-ray fluorescence, and the three measurements were averaged, adjusting for temporal trends using data from a reference site that was monitored continuously for 2-week periods during the year (de Hoogh, 2013). Annual mean concentrations of ambient elemental concentrations, $PM_{2.5}$, and PM_{10} were estimated at the addresses of the mothers during pregnancy using area-specific land use regression (LUR) models. The authors did not estimate cohort exposures if no significant predictors could be included in a LUR model, because the center-specific estimates would be the same for all members of the cohort from the area. For this reason, no Ni $PM_{2.5}$ exposures were estimated for the Lithuanian and Danish cohorts.

Although the pregnancies preceded the air sampling for every cohort, data from routine monitoring stations were used to adjust the annual $PM_{2.5}$ and PM_{10} LUR estimates to the periods corresponding to each individual pregnancy. The authors accounted for changes of address during pregnancy when the date of the move was known and $PM_{2.5}$ and PM_{10} estimates were available at the new address.

The authors conducted pooled analyses using mixed models including a random effect for center. Logistic regression was used to estimate odds ratios for fixed increments of exposure to air pollution and term LBW, and linear regression was used to estimate associations for birth weight and birth head circumference. The authors selected the following adjustment variables *a priori*: gestational age (continuous and quadratic terms); sex; parity; season of conception; and maternal age, height, prepregnancy weight (broken stick model with a knot at 60 kg), education level (cohort-specific definition of low, medium, high), and number of cigarettes/day smoked during second trimester. Each exposure variable was entered in the models separately as a continuous variable. The authors also performed analyses of two-pollutant models for each element adjusted for particle mass ($PM_{2.5}$ or PM_{10}; because some elemental constituents are correlated with particle mass), other elements, and traffic density on the

nearest street, separately. Variance inflation factors were used to assess collinearity problems. The authors also conducted a wide variety of sensitivity analyses. The effect of weather (temperature, humidity atmospheric pressure) was also evaluated to determine whether adjustment was appropriate.

Air pollution exposure levels were on average 17.0 µg/m^3 for $PM_{2.5}$ and 26.9 µg/m^3 for PM_{10}. The mean (SD) Ni $PM_{2.5}$ concentration was 1.6 (0.8) ng/m^3 and Ni PM_{10} was 1.8 (1.2) ng/m^3. The eight selected elements represented 6% and 7% of total mass of $PM_{2.5}$ and PM_{10}, respectively.

Increased odds of term LBW was associated with particle mass, S and Ni in the $PM_{2.5}$ fraction and S and particle mass in the PM_{10} fractions. Adjusted ORs for LBW were 1.14 (1.00, 1.29) for Ni $PM_{2.5}$ and 1.29 (0.96, 1.75) for Ni PM_{10} in single pollutant models, adjusted for gestational age; sex; parity; season of conception; and maternal age, height, prepregnancy weight, education level, and smoking during second trimester.

The authors report that because of correlations between some elemental constituents and particle mass ($PM_{2.5}$ or PM_{10}), associations with elements may reflect relationships with particle mass. In models that additionally included particle mass in 1 ng/m^3 increments, the adjusted ORs for LBW and Ni were 1.11 (0.94, 1.31) for Ni $PM_{2.5}$ and 1.14 (0.90, 1.43) for Ni PM_{10}, as shown in Table B.15 below.

Table B.15. Adjusted* Odds Ratios (95% CI) for term LBW and Ni $PM_{2.5}$ and Ni PM_{10}

Model	N	n (LBW cases)	Ni $PM_{2.5}$ (1 ng/m^3 increments)	Ni PM_{10} (2 ng/m^3 increments)
Ni PM only	27,339	351	1.14 (1.00, 1.29)	1.29 (0.96, 1.75)
With particle mass	27,337	350	1.11 (0.94, 1.31)	1.14 (0.90, 1.43)
With S	27,339	351	1.10 (0.91, 1.33)	1.07 (0.85, 1.35)

* Adjusted for gestational age; sex; parity; season of conception; and maternal age, height, prepregnancy weight, education level, and smoking during second trimester. Adjustment for $PM_{2.5}$ mass was in 5 µg/m^3 increments and for PM_{10} was in 10 µg/m^3 increments. Adjustment for S in both $PM_{2.5}$ and PM_{10} was in 200 ng/m^3 increments.

In analyses of birth weight at term (continuous outcome), $PM_{2.5}$ Ni and PM_{10} Ni were not significant, as shown in Table B.16 below. Only $PM_{2.5}$ S and $PM_{2.5}$ mass were significantly associated with reduced birth weight.

Table B.16. Adjusted* associations (β, 95% CI) between mean birth weight (g) in term births and Ni

Model	Ni $PM_{2.5}$ (1 ng/m^3 increments)	Ni PM_{10} (2 ng/m^3 increments)
Ni PM only	4 (-15, 22)	1 (-22, 24)
With particle mass*	7 (-13, 26)	-6 (-33, 20)
With S	7 (-50, 16)	7 (-26, 39)

* Adjusted for gestational age; sex; parity; season of conception; and maternal age, height, prepregnancy weight, education level, and smoking during second trimester. Adjustment for $PM_{2.5}$ mass was in 5 µg/m^3

increments and for PM_{10} was in 10 µg/m^3 increments. Adjustment for S in both $PM_{2.5}$ and PM_{10} was in 200 ng/m^3 increments.

Nearly all pollutants in both $PM_{2.5}$ and PM_{10} were associated with significant reductions in head circumference at birth. Associations between head circumference and Ni $PM_{2.5}$ and S in both $PM_{2.5}$ and PM_{10} were stronger than those with PM mass concentration. Table B.17 shows the OR for Ni $PM_{2.5}$ remained significant after adjustment for S $PM_{2.5}$, but the OR for Ni PM_{10} was null after adjustment for S PM_{10}. ORs for S in both $PM_{2.5}$ and PM_{10} remained after adjustment for Ni. Head circumference data were not available for three cohorts.

Table B.17. Adjusted* associations (β, 95% CI) between mean head circumference (cm) at birth and Ni

Model	Ni $PM_{2.5}$ (1 ng/m^3 increments)	Ni PM_{10} (2 ng/m^3 increments)
Ni PM only	-0.60 (-0.71, -0.49)	-0.46 (-0.57, -0.36)
With particle mass	-0.49 (-0.61, -0.36)	-0.34 (-0.45, -0.22)
With S	-0.31 (-0.44, -0.19)	-0.05 (-0.20, 0.09)

* Adjusted for gestational age; sex; parity; season of conception; and maternal age, height, prepregnancy weight, education level, and smoking during second trimester.

After application of a false discovery rate correction, the association between LBW and Ni $PM_{2.5}$ was no longer statistically significant (p=0.35 after correction). Effects for head circumference remained significant after correction.

The authors acknowledge that their model did not capture temporal trends. Although exposure assessments did not precede the pregnancies and births, information on individual maternal characteristics such as stature, education, and active and passive smoking during pregnancy was collected prospectively. Exposure assessments were limited to home address, and exposure assessment for other locations was not possible. The authors also note that their approach to false discovery rate correction assumes independence among covariates.

B.1.3. Congenital malformations

Chashschin et al. (1994). Congenital defects, abortion and other health effects in nickel refinery workers. This study is summarized under "Spontaneous Abortion", above (Chashschin et al. 1994).

Friel et al. (2005). Possible altered mineral metabolism in human anencephalic fetuses.

Friel et al. conducted this cross-sectional study to analyze the mineral content selected tissues from autopsied anencephalic and control fetuses (Friel et al. 2005). The authors hypothesized that tissues from anencephalic fetuses would differ from control fetuses in Zn and Cd contents. The study population comprised 33 anencephalic and 22 control fetuses ("term and preterm infants whose tissues had undergone minimal autolytic

changes") from six hospitals in three regions (Newfoundland, Nova Scotia, and Ontario) of Canada. No other description of the sample selection method or criteria is given. Two control fetuses and all but two of the anencephalic fetuses were stillborn.

Samples of liver, kidney, diaphragmatic muscle, sciatic nerve, and pancreas were collected at autopsy from the right side of the fetus. The whole organ was collected when available after necessary tissue was removed for histopathological examination (approximately half of fetuses). Tissue samples were prepared and frozen, thawed, freeze-dried, reweighed, digested, diluted, and then submitted, along with blanks, for analysis by inductively coupled plasma mass spectrometry for trace elements Zn, Cu, Mn, Co, Ni, molybdenum (Mo), and Cd.

The mean concentrations of elements Zn and Cd were different for the two groups. No differences in Ni or other elements between the two groups were observed and concentrations were reported only for the two groups combined.

Vaktskjold et al. (2006). Genital malformations in newborns of female nickel-refinery workers.

The aim of this registry-based cohort study was to investigate whether pregnant women employed in Ni-exposed work areas at a Ni refinery in Monchegorsk were at higher risk of delivering a newborn with a genital malformation (Vaktskjold et al. 2006).

The Kola Birth Registry (KBR) includes registration of up to five diagnoses of congenital malformations and deformations ("malformations") per newborn, with both International Classification of Diseases, 10th Revision (ICD-10) codes and the diagnosis spelled out in words in adjacent fields, as well as an indicator for whether the diagnosis was certain. The woman's employer and job function at the onset of pregnancy were also recorded using both numerical codes and word descriptors, and exposure category assigned accordingly.

The source population was 24,534 registered deliveries. Inclusion criteria, defined *a priori*, were: 1) mother was a resident of Monchegorsk at start of pregnancy; 2) gestational age ≥28 weeks or birth weight ≥ 1,000 g (if gestational age was missing); 3) mother's workplace and job were registered; 4) newborn record with information about malformations was available for registration; and 5) only one record was included if a delivery had more than one baby [selection criteria were not mentioned]. The study population comprised 23,141 live- or stillbirths delivered by 17,301 women. Cases were defined as newborns with an ICD-10 code denoting a malformation of the genital organs (ICD-10 Q50-Q56).

Close to 500 workers participated in surveys of air and urine measurements of Ni and other metalloids during 1996-2001. Each occupation in the KBR was assigned a categorical exposure rating of *background* (observed urinary Ni concentration <10

µg/L), *low*, or *high* (urinary Ni concentration ≥70 µg/L, roughly corresponding to ≥ 160 µg/m^3 of the water-soluble inhalable sub-fraction) Ni exposure. *Background* exposure included refinery occupations with exposure levels estimated to be comparable to that of the general population. A comparison of current and past quarterly in-plant stationary (area) air measurements, combined with knowledge of the refining processes, assured the authors that the exposure assessments also reflected past exposures. Of the births included in the cohort, 87.3% were classified as having *background* exposure, 7.4% *low* exposure, and 5.3% *high* exposure.

Multiple logistic regression analysis was conducted with the presence/absence of one or more malformations of the genital organs as the dichotomous outcome. Sub-analyses with undescended testes and hypospadias as outcomes were also conducted. Regression models included adjustment for the following factors, selected *a priori* based on reports of relevant associations in the literature: parity, maternal malformation, exposure to solvents at work, and infectious diseases in the period from 2-3 months before pregnancy to as weeks of gestation, or influenza in the first 12 weeks of pregnancy. The sub-analysis for undescended testes was also adjusted for gestational age.

There were 103 newborns with one or more malformations of the genital organs (8 girls, 94 boys, 1 indeterminate sex) for a prevalence of 44.5/10,000 births and 82.0/10,000 for boys and 7.9/10,000 for girls. Four of the cases were stillborn and of these, three had multiple malformations. The adjusted OR for a newborn with a genital malformation in nickel-exposed areas was 0.81 (0.52 – 1.26) [This OR is footnoted as “Test for trend across three exposure categories”, p. 46]. Compared to *background* exposure, the OR for *low* exposure was 0.71 (0.31 – 1.64) and for *high* exposure 0.72 (0.26 – 1.95). The adjusted OR for a newborn with undescended testes was 0.76 (0.40 – 1.47). The authors were unable to fit a model for hypospadias.

OEHHA comments: The authors state the observed prevalence rates for undescended testes (23/10,000) and hypospadias (16/10,000) were comparable to those in Norway.

Malformations in the registry were identified in the maternity ward in the first week after delivery; milder hypospadias might not have been detected in that time. Early detections of undescended testes might also be a limitation since the testicle will descend naturally during infancy for a large proportion. However, the authors note that the associations found in population-based studies have been comparable regardless of whether the case group was formed at birth or at time of operation. The authors also acknowledge that an assessment of incidence of malformations would require inclusion of all conceptuses and thus consideration of fetal loss, and that exposure may appear to be protective due to an increased risk of fetal loss.

The authors note that an advantage of this study is that the entire study population resided in the same city environment and therefore shared similar background exposures, and is socioeconomically and ethnically homogeneous.

Misclassification could have occurred if the cut-off levels for categorizing exposure were inappropriate, and could bias the results toward the null. The authors also state that any potential effect of paternal exposures on genital malformations would blur the findings of this study.

The authors did not consider exposure to As, Cd, and Pb to be potential confounders because the exposure levels were "generally at levels well below current occupational exposure levels."

Many malformations of female reproductive organs would likely not be captured using this study design. The potential contribution of fathers, many of whom worked at the Ni refinery, to the occurrence of genital malformations was not analyzed.

Vaktskjold et al. (2008b). Maternal Nickel Exposure and Congenital Musculoskeletal Defects.

Vaktskjold et al. conducted this case-control study to assess whether women occupationally exposed to elevated levels of water-soluble Ni at a Ni refinery during pregnancy were at higher risk of delivering a newborn with a musculoskeletal malformation or deformation than women living in Monˇcegorsk without occupational Ni exposure (Vaktskjold et al. 2008b).

The Ni refinery employed 42.5% of the delivering women in Monˇcegorsk in 1973 – 1997. The Kola Birth Registry (KBR) included data from clinical records of all births of at least 28 weeks' gestation in Monˇcegorsk in the period 1973 – 2001. Births were registered retrospectively beginning in 1997. KBR records include information about the mother's employment at the onset of pregnancy. Data about workplace and smoking were supplemented with information from questionnaires.

The source population was all 24,087 registered singleton births of at least 28 weeks gestation or, in the absence of gestational age information, birth weight ≥1,000 g, delivered in 1973-2001. The inclusion criteria were: 1) resident of Monˇcegorsk at beginning of pregnancy; 2) delivery record was available for registry; 3) mother's workplace and job was registered; 4) newborn had no diagnosis of trisomies 13, 18, or 21, or Turner's syndrome, at birth. The study population comprised 22,965 births to 17,139 women; 5,172 (22.5%) of the births were to women employed within the Ni refinery complex.

Air and urine measurements of Ni and other metalloids were taken during 1995-2001, based on the water-soluble sub-fraction of the inhalable Ni-aerosol fraction and urinary Ni concentrations. A comparison or current and past quarterly in-plant stationary (area) air measurements, combined with knowledge of the refining processes and that processes had not changed substantially since 1973, assured the authors that the exposure assessments also reflected past exposures. Exposure levels were

categorized as *background* (observed urinary Ni concentration <10 µg/L), *low*, or *high* (urinary Ni concentration ≥70 µg/L, roughly corresponding to ≥ 160 µg/m^3 of the water-soluble inhalable sub-fraction) Ni exposure. *Background* exposure included refinery occupations with exposure levels estimated to be comparable to that of the general population. The occupation at the start of pregnancy recorded on each birth record was used to classify each case or control's exposure level. Among the 22,965 births, 87.2% were classified as background exposure, 7.35% low exposure, and 5.4% high exposure [the text ascribes the low and high exposure percentages to women; however, Table I in the paper ascribes the numbers to newborns].

Logistic regression analysis was used to estimate the relative odds of the presence of one or more musculoskeletal defects. The authors conducted two sub-analyses: one in which newborns with associated congenital defects were excluded, and another in which the main musculoskeletal defects were analyzed separately. Analyses adjusted for the following variables, selected *a priori* based on reports of relevant associations and data availability in the KBR: first delivery, tobacco smoking in pregnancy, regular exposure to solvents at work, alcohol abuse, and maternal age >34 years or < 18 years.

Musculoskeletal defects were diagnosed in 341 infants (14.8/1,000 births; 169 girls and 172 boys); of these, 37 also had additional malformations and 21 of those remaining had multiple musculoskeletal defects. The most frequent defect was deformities of the feet (48% of those with only musculoskeletal defects), followed by polydactyly (12%). The highest incidence of musculoskeletal defects was among those with low Ni exposure and those working in the Cu department. The adjusted OR for the association between Ni exposure and musculoskeletal defect was 0.96 (95% CI 0.76 – 1.21) per unit increase in exposure category over the three categories. Excluding the 37 infants with associated non-musculoskeletal defects did not change the estimate. The OR for feet deformity was 1.08 (0.78 – 1.48). The observed incidence of musculoskeletal defects among newborns with no other malformation was 13.3/1,000 births, which is more than double the EUROCAT (European network of population-based registries of congenital anomalies) 2007 figure for all member registries. The incidence of polydactyly (1.6/1,000) was also relatively high.

OEHHA comments: The authors hypothesize that the high incidence of musculoskeletal defects might be explained in part by high proportions of primiparous deliveries (51%) and teenage women (2%) in the study population. The adjusted OR associated with nulliparity was 1.69 (1.34 – 2.14). However, the proportion of teenage mothers seems an unlikely explanation, given the small number in the study.

The authors acknowledge that this study does not address the risk of fetal malformations, which would increase the risk of fetal loss and stillbirth. They note that previous investigations did not reveal an increased risk of fetal loss or SGA newborn.

Most of the studied defects are diagnosable prenatally, and could lead to interruption of pregnancy. However, the authors state that ultrasound was not available in the study area before the early 1990s and was likely non-differential in terms of Ni exposure.

The authors state that a large proportion of the women delivering in Monˇcegorsk were impregnated by men working at the Ni refinery complex. Thus, any potential effect of paternal exposure on risk of a musculoskeletal defect in the offspring would blur the findings in the present study. Data on paternal occupation and workplace was missing too frequently to include in the analyses.

As the KBR covers Monˇcegorsk's whole population, and relatively few records were missing data about occupation, selection bias was unlikely. Russian law required all workers in the Ni refinery to be medically examined each year, and workers who have chronic diseases, injuries, or poor health would likely be transferred from physically demanding or hazardous occupations such as those in Ni production. Occupational health workers recommend that 10-20% of the Monˇcegorsk Ni refinery workers change jobs for health reasons each year.

In a related study, background urinary Ni concentrations in residents of selected Russian and Norwegian cities were measured and found to be substantially higher in the Russian cities. In the two Norwegian cities, the median urinary Ni concentrations measured were 0.6 and 1.2, and highest concentrations were 11.0 and 6.0 µg/L. In these cities, 5.9% and 8.9% of the samples had urinary Ni concentrations >2.5 µg/L. For the four Russian cities in the study, including Nikel (which has a Ni refinery), between 38.6% and 66.0% of the samples had urinary Ni concentrations >2.5 µg/L (Smith-Sivertsen et al. 1998). These data suggest that even the "background" exposure levels in the current study are quite high.

Huang et al. (2011). Effect of Exposure to Trace Elements in the Soil on the Prevalence of Neural Tube Defects in a High-Risk Area of China

The objective of this exploratory ecologic study was to build a mathematical model to explain the association between 12 trace elements (tin (Sn), Pb, Ni, Fe, Cu, Al, As, Se, Zn, strontium (Sr), V, Mo) in cultivated soil and prevalence of neural tube defects (NTDs) (Huang et al. 2011). "Birth defect cases included all live births and still births" in 2002-2004 in the Lvliang Region, Shanxi Province, China. Births in the hospital and at home to residents of the study area were included, as were therapeutic abortions if the estimated date of delivery was in the study period. All NTDs were verified by doctors in the hospital. Data on all therapeutic abortions, still births, and live births with NTDs were collected from hospital records to generate a registry. Each woman on the registry was interviewed for basic information about her pregnancy history and the newborn. The researchers also sent doctors to makeshift clinics in villages, and conducted home visits to reach those who had not been seen in the clinics, to examine all infants for NTDs and other diseases and to interview mothers to ensure all pregnancies were covered (Gu et al., 2007, referenced for data collection and quality control). The

number of cases, subjects, or pregnancies is not reported. The authors state the study area has the highest prevalence of NTDs in China (Gu et al. 2007).

Soil samples were collected from a depth between 2 cm and 20 cm, of at least two patches of cultivated land in each village, in December 2004. All soil samples from a village were mixed thoroughly to represent the village and analyzed using inductively coupled plasma mass spectroscopy to measure levels of the trace elements of interest. All NTD cases were matched by geo-code of the villages' names and locations. Records of both birth defects and soil samples were available from 112 villages.

The authors assumed three possible types of relationship between trace element content in soil and human health: 1) either a positive or negative effect on health; 2) a dose effect; and 3) no relationship between the trace element and the risk of birth defects. The authors built a model that could reflect the association between the trace element levels and the occurrence of NTDs, hypothesizing a Poisson distribution for the NTDs. The authors reported that Sn, Pb, Ni, Fe, Cu, and Al had "layered level effects" on the occurrence of NTDs, while no effects were observed for As, Se, Zn, Sr, and V, and a "threshold value" was observed for the effect of Mo (soil content below or above 8.51 was associated with defects). The level of Ni in soils was as follows: "average" 29.9 µg/ g, mean 41.38 µg/g, standard deviation 6.39, and variance 40.77. The Ni levels and corresponding prevalence of NTDs were 30-34 µg/g (low prevalence), >34 µg/g, (medium prevalence), and <30 µg/g (high prevalence). Definitions of low, medium, and high prevalence were not reported. The authors state that their results show that "both deficiencies in and excessive amounts of nickel cause an increased risk of neural tube defects."

OEHHA comments: The authors did not report important information, such as sample size and results of statistical tests aside from fitness of the model, nor did they address the relevance of a village's average element content in soil to each individual's exposure, other potential risk factors for NTDs, possible interactions among elements and other exposures, and confounding.

Zheng et al. (2012). Contents of heavy metals in arable soils and birth defect risks in Shanxi, China: a small area level geographic study.

Zheng et al. conducted this ecologic case-control study in Shanxi province, a coal-mining region which has one of the highest incidences of birth defects in the world, to examine the effect of heavy metal soil components on risk of birth defects (Zheng et al. 2012).

All live and still births in hospitals and homes in the Lvliang mountain area between January 1, 2002 and December 31, 2004 were included. Study personnel visited households in 144 villages in the study area and took soil samples, and used global positioning system (GPS) data collected during soil sampling to geocode birth and soil heavy metal content data. Of the 144 villages, 97 had complete birth and birth defect

records and were included in the analysis. The authors identified 379 cases of birth defects among 4,736 births, for a rate of approximately 800 per 10,000 births, which is higher than previously observed rates of about 190 per 10,000 in Shanxi province. The exact sources of case and control data were not described in detail.

The researchers collected soil samples from soil used for food cultivation in at least four sampling points in different locations in each village within one week of field surveys. An inductively coupled mass spectrometer was used to measure the components of the multiple samples, and the measurements were averaged to determine each village's soil content. The authors focused primarily on heavy metals As, Cu, Pb, Sn, Sr, V, Zn, Mo, and Ni. All of the metal concentrations for the study area exceeded those in Shanxi, and for As, Pb, Zn, and Ni, the concentrations in the study area exceeded national standards. The mean (SD) concentration of Ni was 41.73 (6.67) mg/kg in the study area.

The authors used Poisson log-linear regression, conditional on intensity parameters representing the expected birth defect case count in each village when covariates are taken into account, to model the relationship between birth defects and heavy metals in soil. To account for potential spatial autocorrelation, the authors state they also specified a conditional spatial autoregressive model to account for the spatial distribution of villages through a contiguity matrix, where distances between villages defined the matrix. Villages were considered adjacent if < 8 km apart.

In the initial Poisson log-linear model with all heavy metal soil components of interest and accounting for spatial autocorrelation, As and Pb were significantly associated with increased risk of birth defect, while Ni was negatively correlated with birth defects. Other elements approached significance when adjusting for covariates. When As, Pb, and Ni were categorized into quartiles and non-significant covariates were excluded, Ni was associated with monotonically decreasing risk of birth defects, significant for each quartile compared to the lowest, as shown in Table B.18.

Table B.18. Soil Ni levels and Adjusted Relative Risk of Birth Defects

Soil Ni level (mg/kg)	Estimate	Adjusted relative risk*	Pr(>|z|)
<37.54	Referent	referent	
37.54 – 41.04	-0.3932	0.67	0.0265
41.04 – 44.86	-0.6216	0.54	0.0034
>44.86	-0.8322	0.44	0.0003

*Calculated by OEHHA

The authors comment that the negative correlations of Ni with birth defects may be attributable to the antagonistic effect of alkaline soil on other poisonous heavy metals such as Hg, which they did not measure, and the form of Ni in the soil was not identified.

OEHHA comments: The authors provide little information about the subjects and how subject data were collected. The extent to which Ni in nearby soil predicts individual

exposure is unclear; the authors did not discuss the relationship between metal content in soils and human exposure. The authors did not report correlations among metals; if correlations among metals were high, then including them together in models may not be appropriate. Combining all birth defects together also may contribute to mixing of effects of different exposures.

Manduca et al. (2014). Specific Association of Teratogen and Toxicant Metals in Hair of Newborns with Congenital Birth Defects or Developmentally Premature Birth in a Cohort of Couples with Documented Parental Exposure to Military Attacks: Observational Study at Al Shifa Hospital, Gaza, Palestine

This case-control study aimed to investigate whether there is a higher metal load in infants born prematurely or with defects than in normal babies due to in utero metal contamination, and to assess the possible impact of metals in affecting epigenetic changes on embryonic and fetal life. The main source of the metal exposure of concern was a 2009 military attack on Gaza known as Operation Cast Lead, in which phosphorus ammunitions were utilized. Manduca et al. state that newborn hair reflects the total accumulation in the period of hair growth in utero, from approximately 22 weeks of gestation, excluding direct contamination from the external environment (Manduca et al. 2014).

Cases with birth defects were those born in succession from May – October 2011 with sufficient hair for testing (87.3%, 48/55) at a hospital in Gaza. Hair was collected from the nape of the neck, when possible, or from the occipital area, within minutes of birth and without any previous treatment except drying with a clean cotton cloth. Hair from all 48 of these birth defect cases was analyzed. Seventy-seven infants born in the study period were defined as premature (<2.0 kg, <37 weeks gestation, without birth defects) and had enough hair for testing; of these, nine children's hair was analyzed. There were 3,892 "normal children", defined as "at term and equal, or above 2.5 kg at birth". Among normal children with no birth defects among siblings and parents' collaterals (i.e., relatives who are not ancestors, such as cousins), 12 were randomly chosen for hair analysis.

Parents were interviewed for birth registration and for a previous study in the delivery room. Questions included parental residence and occupation, weeks at delivery, other children's health status, neighborhood birth defects, reproductive history, exposure to white phosphorus ammunition attacks, among others (Naim et al. 2012). The authors state that the residence of all parental couples had been stable since 2008, and no exposure to environmental accidents, work exposures, or maternal smoking were reported. The father of one infant with a birth defect had been wounded during Operation Cast Lead. Parents of normal and preterm babies declared no direct exposures to white phosphorus ammunition attacks and were not asked about exposure to bombing.
Blinded analyses of the hair samples were conducted using DRC-ICP-MS (dynamic reaction cell inductively coupled mass spectrometry), with analysis for 25 metals,

including Ni, and Se, with various limits of detection. Differences in metal concentrations between groups were assessed by Wilcoxon-Mann-Whitney test. The authors report results for elements with "relevant differences in amounts between the phenotype groups…. All elements analyzed but not shown had no differences for the three groups." Results for Ni were not reported.

OEHHA comments: The study is not clearly described.

B.1.4. Autism Spectrum Disorders

Windham et al. (2006). Autism Spectrum Disorders in Relation to Distribution of Hazardous Air Pollutants in the San Francisco Bay Area.

This exploratory, "semiecologic" case-control study was conducted to explore possible associations between autism spectrum disorders (ASD) and environmental pollutants (Windham et al. 2006). Subjects were children born in 1994 to mothers residing in one of six San Francisco Bay Area counties at delivery, representing approximately 80,000 births. Children with ASD (cases) were identified by the California Center for Autism and Developmental Disabilities Research and Epidemiology (CADDRE), and represented an approximate population-based series of affected children who could be identified from existing records. The sources for case ascertainment were the California Department of Developmental Services (DDS) and the Kaiser Permanente Medical Care Program. DDS is thought to serve 75-80% of children with ASD, "or those on the more severe end of the autism spectrum" and Kaiser members are "generally representative of the general population, except for the extreme ends of socioeconomic status." CADDRE staff used the DDS electronic database to identify children who were diagnosed before the ninth birthday with autism, mental retardation, epilepsy, or other developmental disorder with no known cause. The authors selected children with an ASD diagnosis before their ninth birthday from Kaiser electronic files. The DDS and Kaiser cases were linked to birth certificate data to identify cases born in the study area and duplicates. About 21% of cases were identified only in Kaiser records, and 25% in both systems. Final case status was determined by computer algorithm and several levels of expert review by a CADDRE investigator and/or a child psychiatrist with expertise in ASDs, and the following criteria:

- Diagnosis of ASD from a qualified medical professional,
- Qualification for special education under an autism exceptionality, or
- Autistic behaviors that appear to meet the Diagnostic and Statistical Manual of Mental Disorders, 4th edition criteria for a diagnosis of autistic disorder, Asperger's, or Pervasive Developmental Disorder not otherwise specified, per expert review.

Of the initial cases, 284 (83.3%) met the stricter surveillance definition of ASD. Controls were randomly selected from the California 1994 linked birth-infant death certificate file4with maternal residence at delivery in the study area and matched to the original

cases by sex and month of birth at a ratio of two to one (n=682 for original cases). Birth addresses, demographic data, and infant characteristics were obtained from birth certificates. Birth addresses were geocoded to 1990 census tracts. After exclusions for missing data, there were 284 cases and 657 controls.

As expected, cases were more likely to be male (84.9%). Compared to controls, cases were somewhat more likely to be white and less likely to be Hispanic, and were born to mothers and fathers who were somewhat older and likely to have finished high school and attended college but not graduated from college.

US EPA estimates hazardous air pollutant (HAP) concentrations using a Gaussian air dispersion model that combines emissions inventories from mobile (e.g., motor vehicles, airplanes), point (large industrial manufacturing facilities), and area sources (e.g., dry cleaners, gas stations, residential product use) with data on local meteorology, chemical decay rates, secondary formation, and deposition. Estimated concentrations are summed across the sources and background levels from "clean air locations" are added. The authors used the estimated annual average HAPs concentrations at the US Census tract level for 1996 (the next most recent estimates available were for 1990).

From the 33 chemicals in the 1996 HAPs database, the authors selected 25 compounds that were recognized developmental toxicants, suspected neurologic toxicants, endocrine disruptors, or chemicals that had been identified as being of concern in an autism cluster investigation, and diesel particulates. Six of these compounds had very little geographic variability in the study area and were therefore excluded from further analyses.

As some chemicals were highly correlated (11 had Spearman correlation coefficient ≥ 0.85 with more than one other compound), the authors combined them into structural and mechanistic groups. The structural groups were mutually exclusive and comprised metals (n = 7, including Ni), aromatic solvents (n = 5), and chlorinated solvents (n = 4). The mechanistic groups were developmental toxicants (n = 7) and endocrine disruptors (n = 10), and shared some compounds, but did not include Ni.

Due to large variation in the mean concentrations of compounds within a group, the authors calculated an index score for each group by assigning a level of one to four based on the quartile of exposure of each compound across the census tracts in which controls were born, then summed across the compounds included in each group to obtain an overall score for that chemical group, in each census tract. This group score was assigned to all cases and controls born in that tract. Scores were then categorized into quartiles. Individual compound exposure estimates were also categorized into quartiles.
Metals, vinyl chloride, and hydrazine had the widest range of concentrations among the controls. The mean ± SD exposure to Ni among controls was 0.0037 ± 0.0038 μg/m^3 and among cases was 0.0043 ± 0.0059 μg/m^3.

Maternal age, race, education, and parity; paternal race and age; low birth weight; preterm delivery, and child race were included in statistical models if associated with chemical exposure and case status, and not highly redundant. The final logistic regression models included child race, maternal age, and maternal education.

The authors used logistic regression to estimate odds ratios for ASD in children associated with ambient chemical exposures during gestation or early life. The authors did not use conditional logistic regression because they did not maintain a strict case-control match, though they did use it to check on findings from logistic regression models. To increase power and because there were generally no effects at the second quartile of exposure, the lower two quartiles of exposure were combined as the referent group. The third and fourth exposure quartiles were coded as dummy variables.

Adjusted odds ratios (AORs) (95% CI) for autism and exposure to metals were 1.68 (1.17, 2.41) for third quartile exposure and 1.50 (1.05, 2.12) for fourth quartile exposure, not adjusting for other chemical groups. Exposures to endocrine disruptors and developmental toxicants groups were associated with non significant (NS) increased odds of ASD.

In models that included all three structural groups, AORs were higher for the metals group and lower for the chlorinated solvents group, compared with models that included only one group at a time. The authors noted that the reduced AORs for chlorinated solvents in models with solvents and metals may reflect some overadjustment. In these multi-group models, the AORs (95% CI) for autism and exposure to metals were 1.68 (1.17, 2.41) for third quartile exposure and 1.50 (1.05, 2.12) for fourth quartile exposure.

The AORs (95% CI) for autism and Ni exposure in single chemical models were 1.11 (0.77, 1.59) for the third quartile and 1.46 (1.04, 2.06) for the fourth quartile. Ni was correlated with As (r = 0.86), Cd (r = 0.77), and trichloroethylene ($r \geq 0.77$). Table B.19 shows the AORs for ASD with Ni and the other highly correlated pollutants.

Table B.19. AORs* (95% CI) for ASD with Ni and highly correlated chemicals

Chemical	Third quartile	Fourth quartile
Ni	1.11 (0.77 – 1.59)	1.46 (1.04 – 2.06)
As	1.07 (0.75 – 1.53)	1.28 (0.90 – 1.81)
Cd	1.43 (1.01 – 2.04)	1.54 (1.08 – 2.20)
Trichloroethylene	1.37 (0.96 – 1.95)	1.47 (1.03 – 2.08)

*Adjusted for maternal age, education, and child race, in separate models for each chemical.

OEHHA comments: Due to correlations among chemicals, untangling the effects of specific chemicals was difficult. Actual personal exposures were not estimated; instead, the authors modeled concentrations of chemicals in outdoor air based on emissions in a geographic area. These estimates do not take into account mothers' specific activities, or mobility, although the authors state that US EPA in 1990 found that modeled HAPs concentrations were reasonable surrogates for personal exposure. The exposure estimates were for 1996, whereas the subjects were born in 1994, although the authors

state that available air monitoring data suggest that it is unlikely that the relative concentrations varied greatly in the two years between the estimates and the actual exposures. The authors note that the first trimester of pregnancy might be of the most concern etiologically. Other sources of chemical exposures, such as occupation, diet, and smoking, were not estimated. The aforementioned limitations are sources of exposure misclassification that is not likely to vary by case status. Consequently, the authors state that effect estimates likely shifted toward the null.

Kalkbrenner et al. (2010). Perinatal Exposure to Hazardous Air Pollutants and Autism Spectrum Disorders at Age 8

This prevalent case-control study was designed to examine associations between ASD and perinatal exposure to 35 HAPs for further investigation in ASD etiology (Kalkbrenner et al. 2010). ASD cases and controls were identified from the Autism and Developmental Disabilities Monitoring Network (ADDM) in North Carolina (NC) and West Virginia (WV). The ADDM screens developmental records of children in health and educational settings through the eighth year of life, when most affected children have been identified. The NC site evaluated children in selected counties in 2002 and 2004 (born in 1994 and 1996), and the WV site evaluated children living in the entire state in 2000 and 2002 (born in 1992 and 1994). Cases were all children in the surveillance system with developmental records documenting characteristics and behaviors meeting a definition of ASD based on the Diagnostic and Statistical Manual of the American Psychiatric Association, regardless of whether a previous diagnosis of ASD had been documented.

In NC, 311 children with ASD were identified, of whom 220 had matching NC birth certificates, and 206 were born in a study county. In WV, 257 children with ASD were identified, 189 had matching NC birth certificates, and census tract was identified for 177.

Controls were children in the surveillance system in NC and WV with a school designation of speech and language impairment and no documentation of other serious developmental problems. Kalkbrenner et al. selected this group because the children have equivalent access to developmental evaluations, and they could confirm residency at age 8. In WV, one-third of eligible controls were randomly selected for each study year. The authors linked each child to birth certificate data for residential address in early life and demographic characteristics. In NC, 2,584 children with speech and language impairment were identified, of whom 1,947 had matching NC birth certificates, and 1,733 were born in a study county and whose census tract was identified. In WV, 4,287 children with speech and language impairment were identified, of whom 1,420 were randomly selected for birth certificate matching, 1,146 were found, and a census tract was determined for 1,096. The numbers of cases and controls reported in Table 3 of the article are slightly smaller than those reported in the text of the article and repeated here.

The authors used HAP assessments from the 1996 National Air Toxics Assessment (NATA) program because the 1996 year had improved data input over previous models and was closer to their subjects than the 1999 assessments. Individual exposures were assigned using modeled concentrations for the census tract of the residential address on the birth certificate. Estimated ambient concentrations of Ni compounds for controls are shown in Table B.20.

Table B.20. Concentrations of Ni compounds: Geometric mean (geometric SD), ng/m^3, by state and urbanicity for children with speech and language impairment

North Carolina			West Virginia		
Urban (n=613)	Not urban (n=1120)	All NC (n=1733)	Urban (n=132)	Not Urban (n=964	All WV (n=1096)
1.9 (1.6)	0.8 (1.9)	1.1 (2.0)	1.5 (7.4)	0.1 (4.9)	0.2 (6.3)

The authors estimated prevalence ORs for each air pollutant and ASD using logistic regression. Pollutant concentrations were log-transformed because the resulting ORs were similar to those from models with categorical coding of pollutant concentrations, but more precise. A directed acyclic graph was used to evaluate covariates for confounding. Adjusted models included maternal age, smoking in pregnancy, marital status, maternal education, race, census tract median household income, and urbanicity. Most air pollutants were correlated, with Spearman rank correlations of at least 0.5; pollutants from mobile sources were especially correlated, with many correlations >0.8. To accurately estimate ORs for given pollutants, given their correlation with each another and the multiple comparisons, all pollutants were included in a semi-Bayes hierarchical model, "which improves the plausibility and stability of estimates" by adjusting the beta coefficients for each HAP toward the mean of its exchangeability group.

ORs for Ni were elevated prior to adjustment, but were attenuated upon adjustment. The AOR for ASD and exposure to Ni compounds at the 80th percentile (1.7 ng/m^3) vs. 20th percentile (0.1 ng/m^3) was 1.1 (0.6, 1.9), as shown in Table B.21. For children in 100% urban communities, the AOR was 1.8 (0.6, 4.9); other AORs reported (by state, 100% rural, and mixed urban/rural) were close to the overall AOR.

Table B.21. AORs for ASD and Ni compounds from Hierarchical Semi-Bayes Models*

	Urban/Rural			State		All
	100% Rural	Mixed	100% Urban	NC	WV	
No. cases/all	89/881	177/1446	108/850	201/1931	173/1246	374/3177
OR (CI) for 80th vs. 20th percentile**	1.2 (0.4, 3.4)	0.9 (0.4, 2.0)	1.8 (0.6, 4.9)	0.8 (0.2, 3.3)	0.9 (0.4, 1.8)	1.1 (0.6, 1.9)

*Models include all HAPs (except coke oven emissions) and race, maternal education, maternal age, smoking in pregnancy, marital status, census tract median household income, urbanicity, and a second-stage model with a pre-specified residual in variance estimates of 0.209 and exchangeability predictors of known human developmental neurotoxicant, placental transfer, confidence level in HAP model estimates, and similarity between outdoor and indoor concentrations.
**1.7 vs. 0.1 ng/m^3 Ni

OEHHA comments: There are many sources of non-differential misclassification in this study, probably biasing the results generally toward the null. The authors cite the use of NATA to assign exposure as the largest limitation, acknowledging that they relied on spatial contrasts more than temporal contrasts, that they lacked information on pollutant exposure in early pregnancy, and that they assumed that the rank order of air pollutants remained stable from 1992 through 1996. However, they note that NC and WV ranked as having the highest level of participation in reporting to the National Toxics Inventory, and that inputs for 1996 NATA are more complete than for previous years. The usual concerns with using ambient air pollution data for exposure assessment, such as lack of knowledge about actual individual exposures due to daily activity patterns, relocations, or spatial variation within a census tract, apply to this study.

Kalkbrenner et al. examined the possibility of greater misclassification of exposures for children born before NATA-1996 by performing analyses separately for the NC children born in 1994 and 1996, but reported that the resulting ORs were very unstable.

The choice of control group may have biased the study. Use of speech and language-impaired children as controls was based on the assumption that these impairments are not caused by air pollutants, an assumption for which the authors acknowledge limited support. This study reported negative associations for ASD with Pb and toluene, possibly due to stronger, positive associations with speech and language impairment. ORs for any pollutant associated with speech and language impairment would likely be biased.

Roberts et al. (2013). Perinatal Air Pollutant Exposures and Autism Spectrum Disorder in the Children of Nurses' Health Study II Participants

This case-control study aimed to examine whether perinatal exposure to hazardous air pollutants (HAPs) increases risk of ASD by estimating associations between ASD and air pollutants that were previously associated with ASD or known mutagens, and to examine possible differences by sex (Roberts et al. 2013).

Study subjects were drawn from the Nurses' Health Study II cohort of 116,430 female nurses that was established in 1989 and has been followed with biennial questionnaires. Although the nurses were recruited from 14 US states, they have moved throughout the US, and children in this study were born in all 50 states. In the 2005 questionnaire, respondents were asked if any of their children had been diagnosed with autism, Asperger's syndrome, or other ASD. In 2007-2008, the 756 women who had responded affirmatively were sent a questionnaire about the affected child's sex; birth date; the specific ASD diagnosis, with autism, Asperger's syndrome, and pervasive developmental disorder not otherwise specified (PDD-NOS) as possible answers; and whether the child was adopted. After exclusions for not meeting the case definition, refusal, unreported birth year, specified genetic diseases, and insufficient

address information, 325 cases of ASD remained. The authors validated the ASD diagnosis in a sub-sample of 50 randomly selected cases.

Controls were children born in 1987-2002 to mothers who indicated they had never had a child with ASD in the 2005 questionnaire and had responded to a supplemental 2001-2002 questionnaire that asked about the year and sex of each of their live births and smoking during pregnancy. One child was randomly selected per mother. There were 22,098 controls with adequate address information.

Mailing addresses were updated for the mothers every two years and births were assigned to the mother's address for the year of or year before the child's birth, except that children born in 1987-1988 were assigned the mother's address in 1989. The mailing address was geocoded to obtain a census tract identifier for the child.
HAP concentrations were obtained from US EPA National Air Toxics Assessments, which use an inventory of outdoor point and mobile air pollution sources to estimate average ambient air concentrations of pollutants for each child's residential census tract. HAP assessments were available for 1990, 1996, 1999, and 2002, and used to assign exposures to children born in as shown in Table B.22. The following HAPs were examined: Ni, Sb, As, Cd, Cr, Pb, Mn, Hg, diesel particulate matter, methylene chloride, quinoline, styrene, trichloroethylene, and vinyl chloride. Pollutant concentrations were categorized by quintiles from the entire population.

Table B.22. HAP assessment year and births assigned exposures from each HAP year

Year of HAP Assessment	Birth years assigned HAP concentrations
1990	1987-1993
1996	1994-1997
1999	1998-2000
2002	2001-2002

Roberts et al. examined family and community SES indicators that may be associated with ascertainment of ASD. Community circumstances around the time of likely ASD diagnosis were characterized using census tract median income and percent of residents with a college education six years after the birth. The maximum of the mother's parents' education during her infancy was used as a proxy for maternal childhood SES. Current family income was based on the family income reported in 2001. Educational attainment of the mother's partner or spouse was reported in 1999. The authors also examined factors that could be associated with ASD and air pollutant exposure: smoking (assessed in 2001), year of birth, maternal age at birth, and HAP year.

The authors fit logistic regression models of ASD case status and quintiles of each air pollutant separately, both adjusted for child's sex and stratified by sex, and adjusted for maternal age at birth, year of birth, maternal parents' education, census tract median income, census tract % college educated, and HAP model year to calculate AORs for ASD and the highest vs. the lowest quintiles of HAPs. Ni exposure was statistically

significantly associated with increased odds of ASD; the association was stronger for boys. There was a linear dose-response relationship between Ni exposure and ASD for boys, with a significant test for trend (p=0.004). Table B.23 shows the AORs for quintiles of Ni exposure and ASD.

Table B.23. Quintiles of Ni concentrations and AORs* (C.I.) for ASD, by sex

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5
Median Ni conc., μg/m^3	0.0004	0.0012	0.0024	0.0045	0.0159
Both sexes	1.0 (Ref)	1.3 (0.9, 1.9)	1.6 (1.1, 2.2)	1.5 (1.0, 2.2)	1.7 (1.1, 2.5)
Boys	1.0 (Ref)	1.4 (0.9, 2.0)	1.6 (1.1, 2.4)	1.7 (1.1, 2.6)	1.9 (1.2, 2.9)
Girls	1.0 (Ref)	1.1 (0.5, 2.5)	1.2 (0.5, 3.0)	0.7 (0.3, 2.1)	0.7 (0.2, 2.2)

*Adjusted for maternal age at birth, year of birth, maternal parents' education, census tract median income, census tract % college educated, HAP model year, and sex (if both sexes are included)

Other metals were also associated with ASD, especially in boys, and were correlated. Pearson correlation coefficients for Ni with other HAPs were: Sb 0.66, As 0.41, Cd 0.24, Cr 0.22, diesel 0.28, Pb 0.17, Mn 0.38, Hg 0.32, methylene chloride 0.12, quinoline -0.1, trichloroethylene 0.37, and vinyl chloride 0.15. Because metal concentrations were correlated, the authors calculated two overall measures of association with metals by: 1) pooling ORs for individual metals using a random effects meta-analysis, and 2) summing each subject's quintile category scores for each metal. The results of both of these methods were significant AORs for both sexes combined and for boys only, but not for girls only. The AORs were similar to those for Ni. The authors made false discovery rate adjustments because of the many tests; no linear trend tests remained significant for both sexes, but all tests for boys only remained significant.

The pollutants most strongly associated with ASD were Pb, Mn, Cd, Ni, and methylene chloride. When the authors fit a model including all of these pollutants, all ORs were attenuated compared with single pollutant models. The boys-only AORs (CI) for Pb and Ni were the largest: Pb 1.6 (0.9, 2.9) and Ni 1.5 (0.8, 2.7).

OEHHA comments: The authors refer to the air pollutant exposures in this study as "perinatal", but some of the exposure data actually represent years after some of the births. The authors did not address the stability of the HAP concentrations. This is a source of exposure misclassification, likely biasing the results toward the null. Roberts et al. note other possible sources of exposure misclassification, including the possibility that the mother relocated around the time of pregnancy, and the available address was not the residential address during and immediately following the pregnancy. HAP assessments provide only estimates of air exposures, and individuals' actual exposures, including indoor exposures and exposures when moving between areas, were not measured.

B.1.5. Transplacental Carcinogenicity

Heck et al. (2015). Retinoblastoma and ambient exposure to air toxics in the perinatal period.

Heck et al. (2013). An exploratory study of ambient air toxics exposure in pregnancy and the risk of neuroblastoma in offspring.

These studies, part of a larger case-control study of traffic-related and industrial air pollution effects, aimed to investigate the influence of specific air toxics on risk of retinoblastoma (Heck et al. 2015) and neuroblastoma (Heck et al. 2013). Cases of retinoblastoma and neuroblastoma were ascertained from the California Cancer Registry (CCR) records of diagnoses of children younger than age six from 1990 – 2007. The authors matched 89% of cases to California birth records using names, date of birth, and when available, Social Security number. Controls were selected from California birth records for the same period and frequency matched to all childhood cancer cases by birth year, had no cancer diagnosis in the CCR before age 6, and were not linked to California death records before age 6 (Heck et al. 2013; Heck et al. 2015).

Retinoblastoma study subjects comprised 103 cases and 30,601 controls who were living within five miles of a monitor and had sufficient data for at least one pollutant; among these, there were sufficient Ni data for 62 cases and 18,351 controls. The authors combined unilateral and bilateral retinoblastoma because of sample size constraints and because their previous work had shown both were associated with traffic-related pollution (Heck et al. 2015).

For the neuroblastoma study, Heck et al. reported sufficient Ni exposure data for analyses for births within a 5 km radius (52 cases, 9,730 controls) and a 2.5 km radius (16 cases, 2,944 controls) (Heck et al. 2013).

Maternal race/ethnicity and nativity, paternal age, year of birth, and private health insurance vs. Medi-Cal/other government-sponsored health insurance/ self-pay (as an indicator of socioeconomic status and proxy for family income), which were considered as potential confounders, were obtained from the birth certificates. Analyses examined associations across regions of California and different time periods (1990-1997 vs. 1998-2007) for use of the zip code centroid vs. home address to assign exposure estimates. For children born in 1998 and later, exposure to air pollution was assessed based on the home address listed on the birth certificate. Because address was not available in electronic birth certificate records for births before 1998, exposures were assigned based on the population-weighted centroid of residence zip code for these children. Air toxics data from across California were provided by the California Air Resources Board (CARB). CARB collects 24h samples every 12 days from each monitor, and the information collected from the air monitors varies over time and location. The authors determined the distance from each monitor to each family's home, and used the nearest monitor to assign pollutant values. The authors also tried

kriging to assign pollutant values, but did not observe meaningful differences. The mean distance between retinoblastoma subjects' homes and the nearest monitor was 4.9 km (SD 2.0). The changes in effect estimates associated with use of 3 km and 5 km radii were also evaluated, but point estimates were similar for most pollutants.
For each child, the following time-specific exposure averages were calculated based on the gestational age and date of birth reported on the birth certificate: 3 months preconception, each trimester, entire pregnancy, and first year of life. For each pollutant, children were included in the analysis if there was at least one reading for each full month within the time period of interest, as well as one reading in the last 30 days of pregnancy. Findings are only reported for the entire pregnancy because little to no differences were observed across trimesters.

Because many air toxics share the same source and are consequently highly correlated, the authors applied factor analysis using principal components extraction with varimax rotation. Only two factors had eigen values >1, and neither factor included Ni. For each period of interest, the risk of disease associated with one IQR increase in pollutant exposure was estimated through logistic regression for each pollutant separately.

In the retinoblastoma study, the mean (SD) concentration of Ni was 5.08 (2.27) ng/m^3 (among controls only) and the interquartile range (IQR) was 3.18 ng/m^3. After adjustment for paternal age, maternal race and birthplace, birth year, and method of payment for prenatal care, the OR (CI) associated with retinoblastoma and an IQR increase in average Ni exposure over the pregnancy was 1.48 (1.08, 2.01).

In the neuroblastoma study, the mean (SD) concentration of Ni was 4.851 (2.187) ng/m^3 and the interquartile range (IQR) was 3.193 ng/m^3. After adjustment for maternal age and race/ethnicity, birth year, and method of payment for prenatal care as a socioeconomic indicator, the AOR (CI) associated with neuroblastoma and an IQR increase in average Ni exposure over the pregnancy for births within 5 km was 1.08 (0.71, 1.66), and for births within 2.5 km was 0.67 (0.29, 1.56).

Pollutants in the first factor (benzene, toluene, ethyl benzenes, xylenes [collectively "BTEX"], 1,3-butadiene, perchlorethylene, styrene, and lead) were associated with increased risk of retinoblastoma, though some ORs were not statistically significant. The second factor consisted of polycyclic aromatic hydrocarbons, and was associated with non-significant increases in risk of neuroblastoma.

The authors note that as Ni is quite stable in the atmosphere, risk estimates based on residence within 5 miles of a monitor might be more accurate for Ni than for less stable agents. Further, Ni causes chromosomal aberrations and excesses of lung and nasal cancers in exposed workers and retinoblastoma when injected into rats, and transplacental exposures are associated with renal and pituitary tumors in offspring. Parental employment in metal industries has been associated with retinoblastoma, and attributed to either hexavalent chromium or Ni. In this study, the adjusted OR for

retinoblastoma and hexavalent chromium was 1.22 (0.97, 1.54). Retinoblastoma was also associated with ortho-dichlorobenzene (OR 1.46 (0.90, 2.37)), chloroform (OR 1.35 (1.07, 1.70)), para-dichlorobenzene (1.24 (1.04, 1.60)), and total Cr (OR 1.29 (1.04, 1.60)). The authors state that Cr emissions in California arise in part from gasoline combustion, which may explain correlations with BTEX and the observed elevated risk of retinoblastoma.

OEHHA comments: Exposure misclassification probably resulted from families moving during pregnancy. The authors state that 9-32% of US families move during pregnancy, mostly locally, which would reduce the error from exposure misclassification. Children who moved out of state or whose name changed before cancer diagnosis would not have been included in the study. Adjustment for maternal smoking was not possible because this information was not available on the birth certificate until 2007 (whether smoking causes retinoblastoma is not clear). The authors also point to the lack of information on whether *RB1* mutations were inherited or *de novo,* and that the ability to stratify by germline or somatic mutations would provide a clearer picture of risks in different groups (Heck et al. 2015). Finally, the studies were limited by multiple comparisons and collinearity among pollutants.

Togawa et al. (2016). Parental occupational exposure to heavy metals and welding fumes and risk of testicular germ cell tumors in offspring: a registry-based case-control study.

This case-control study examined associations between parental exposures to heavy metals and welding fumes during preconception and/or prenatal periods and risk of testicular germ cell tumors (TGCT) in offspring (Togawa et al. 2016). The authors used data from the NORD-TEST Study, which is a registry-based case-control study in Norway, Finland, Sweden, and Denmark that investigates various parental exposures in relation to TGCT in offspring. The current study included Norway, Finland, and Sweden, where occupational information was comparable across the registries.

Cases were all males of age 14 to 49 years newly diagnosed with testicular cancer without a previous primary neoplasm (except non-melanoma skin cancer) during 1988-2012 for Finland, 1978-2010 for Norway, and 1979-2011 for Sweden via national cancer registries. TGCT cases that were not classified as either seminoma or non-seminoma, and those without parental occupation information in or before the year of the case's birth, were excluded. Controls were males without a previous primary neoplasm (except non-melanoma skin cancer) at the time of the case diagnosis and who had parental occupation information. Controls were randomly selected from each country's central population registry, and matched individually to each case by country and year of birth, at a ratio of four controls per case. The final study sample included 8,112 cases and 26,264 matched controls.

The authors linked data across registries, including census, birth, and cancer registries. Occupation data were collected in population censuses every five years in Sweden (1960-1990, except 1965) and Finland (1970-1990), and decennially in Norway (1960-1990). The authors obtained job codes for parental occupations from the census the year of the subject's birth, or the most recent census before the birth, and applied Nordic job exposure matrices (JEMs) to determine exposures to Cr, Fe, Ni, Pb, Cd (Finland only), and welding fumes for each parent. The JEMs provide country-specific data on the proportion of workers exposed (P) and the mean level of exposure (L) estimated for over 300 occupations and four periods covering 1945 to 1994. Exposure indices were calculated as the product of the proportion exposed to a given agent and the mean level of exposure (P×L).

Togawa et al. also collected the following information from the various registries: maternal and paternal ages at birth, age at TGCT diagnosis, family history of testicular cancer, personal history of inguinal hernia, hypospadias, and cryptorchidism. Although some of these covariates were associated with TGCT, addition of covariates did not substantially alter the OR estimates, so the authors report unadjusted ORs.

Togawa et al. fitted conditional logistic regression models to assess bivariate associations of TGCT with the covariates, and then multivariate models of TGCT risk associated with Cr, Fe, Ni, Pb, and welding fumes for each parent. Parental exposures were analyzed in two ways. First, three occupational exposure groupings were based on presence/absence of exposure: i) no exposure to heavy metals or welding fumes (referent); ii) exposure to a specific heavy metal agent, with or without other metals/welding fumes; iii) exposure to heavy metal(s)/welding fumes without the specific heavy metal agent. A sensitivity analysis restricted the sample to subjects with information on parental occupations in the year of or the year before the subject's birth, or when the same occupation was listed for last census before, and the first census after the birth. The results of these models for Ni are shown in Table B.24.

Table B.24. ORs* (CI) for TGCT and parental exposure to Ni with or without other metals/welding fumes - main and sensitivity analyses**

Main analysis	
Paternal Ni	1.07 (1.00, 1.16)
Maternal Ni	1.07 (0.74, 1.51)
Sensitivity analysis	
Paternal Ni	1.10 (0.98-1.22)**
Maternal Ni	1.17 (0.68-2.01)**

*Referent is no exposure to heavy metals or welding fumes
**Sensitivity analyses included only subjects with information on parental occupations in the year of or before the subject's birth, or when the same occupation was listed for the last census before and first census after the birth

Second, each exposure was categorized using the exposure indices (P×L), so the occupations were classified as: i) without exposure to a specific heavy metal agent (referent); ii) lower exposure index of the specific agent; iii) higher exposure index of the heavy metal agent. The cutoff between the low and high exposure categories was based on the 90th percentile of paternal exposure index among the exposed (Ni 3.30 µg/m^3) or the 75th percentile of maternal exposure index among the exposed (Ni 0.99 µg/m^3). Results of these analyses are shown in Table B.25.

Table B.25. ORs (CI) for TGCT and parental Ni exposure based on Exposure Index (P×L)

Exposure index	OR (CI)	OR (CI) trend*
Fathers: non-exposed	1.00	
low	1.08 (1.00, 1.18)	1.00 (0.96, 1.04)
high	1.03 (0.85, 1.24)	
Mothers: non-exposed	1.00	
low	1.00 (0.66, 1.51)	1.09 (0.91, 1.31)
high	1.27 (0.66, 2.44)	

*The categorical exposure index was treated as a continuous variable

The associations did not vary by histologic subtype (seminoma vs non-seminoma). The authors acknowledge that exposure misclassification is likely when applying JEMs, especially for jobs with low prevalence of exposure. In addition, nearly 68% of parental occupational information was based on censuses administered two to nine years before the birth.

B.1.6. Oxidative Damage to DNA

Ni et al. (2014). Associations of the neonatal lead, cadmium, chromium, and nickel co-exposure with DNA oxidative damage in an electronic waste recycling town.

This cross-sectional study was intended to investigate the association between levels of Pb, Cd, Cr, and Ni in umbilical cord blood (UCB) and 8-hydroxydeoxyguanosine (8-OHdG) as a marker of oxidative DNA damage among neonates exposed to electronic

waste (e-waste) recycling (Ni et al. 2014). The authors compared neonates from Guiyu, one of the largest e-waste destinations and recycling areas in the world, with neonates from Jinping District in Shantou City, China, which does not have e-waste recycling.

Pregnant women were recruited from Guiyu and Jinping from March 2012 to January 2013. Eligibility criteria for the mothers were age ≥18 years and absence of diseases that could affect levels of oxidative stress markers (e.g., systemic lupus erythematosus, diabetes, acute hepatitis, stroke, myocardial infarction), and singleton pregnancy. Criteria for the neonates were 37-42 weeks gestation, birth weight >2,500 g, and absence of severe neonatal illnesses. Information for eligibility was abstracted from medical records. Of the 201 participants, 126 were from Guiyu and 75 were from Jinping. The women were interviewed in the hospital after the deliveries to collect socio-demographic information, such as maternal age and education level; infant sex, length, and weight; and lifestyle and diet information, such as alcohol, tea, and milk consumption during pregnancy, and maternal and paternal smoking. The authors also asked about possible sources of exposure to the metals of interest, including non-occupational exposures (distance between home and street, environmental contamination sources around the residence), occupational exposures (residence was an e-waste recycling workshop, maternal and paternal occupation); delivery mode, and medication use during gestation. E-waste recycling was further classified into three types of work: e-waste selection or separation; acid baths or baking circuit, and what is described as 'spitting out tiny pieces of plastic' (Huo et al. 2007).

Immediately after delivery, clinical staff collected two UCB samples from the placenta of every subject. One sample from each birth was used to measure levels of metals, and the other was used to measure 8-OHdG concentration. The latter sample was separated by centrifugation, then supernatant plasma was collected and stored for later analysis of 8-OHdG concentration using a competitive enzyme-linked immunosorbent assay. Metal concentrations were measured using graphite furnace atomic absorption spectroscopy. The LOD for Ni, based on three times the standard deviation of 11 measurements of blank sample solution, was 0.92 µg/L. The authors reported quality control measures and good repeatability of the method.

Because the UCB metals concentrations and 8-OHdG concentrations were right-skewed, the authors used non-parametric tests to compare these concentrations, and logarithmic transformations were used for any statistical tests requiring assumptions of normality. Pb concentrations were dichotomized at 5 µg/L. The authors conducted multivariate linear regression analyses to examine associations between metals and 8-OHdG, adjusting for sampling area, maternal age and education, smoking, alcohol and tea consumption, paternal smoking, and maternal and paternal occupation.

Compared with women from Guiyu, women from Jinping were more educated, more likely to drink milk >3 times/week during pregnancy, and the distance between their home and the street tended to be greater. The groups were similar with respect to age, alcohol, tea, and tobacco use during pregnancy, delivery mode, and paternal smoking.

Among the 126 women from Guiyu, 56 women and 87 husbands were involved in e-waste recycling and dismantling.

UCB Ni levels were similar regardless of residence, occupation, and smoking habits. The median (range) UCB Ni for Guiyu was 8.63 (3.68 – 544.20) ng/mL and for Jinping was 9.09 (4.67 – 152.40) ng/mL.

The overall median 8-OHdG concentration was 162.09 (range 58.38 – 422.39) ng/mL. The median 8-OHdG concentration was slightly but not significantly higher among residents of Guiyu than those of Jinping: Guiyu 162.09 (range 74.00 – 422.39) ng/mL; Jinping 153.69 (range 58.38 – 326.84) ng/mL; p=0.117. However, 8-OHdG concentrations were higher if the mother or (to a lesser extent) father was occupied in e-waste recycling (mothers: e-waste 179.77 ng/mL, vs. other 159.00 ng/mL, p=0.03; fathers: e-waste 168.99, vs. other 158.63, p=0.22). No significant differences in 8-OHdG concentration were observed in relation to other investigated factors. When Ni levels and 8-OHdG concentration were log-transformed, the Pearson correlation coefficient was 0.314 ($p<0.001$), suggesting a positive association.

Multivariate analyses also showed an association between 8-OHdG and Ni concentrations: $\beta = 0.215$ (95% CI 0.113 – 0.317) ng/mL, adjusted for Pb, Cd, Cr, sampling area, maternal age and education, smoking, tea drinking, alcohol drinking, maternal occupation, paternal occupation, and paternal smoking. In adjusted analyses, 8-OHdG was more strongly associated with Ni than with other metals.

OEHHA comments: The possibility of correlations among the metals or evaluation of collinearity among the metals in the regression analyses is not mentioned.

B.1.7. Other Adverse Outcomes

Chashschin et al. (1994). Congenital defects, abortion and other health effects in nickel refinery workers. This study is summarized under “Spontaneous Abortion”, above, and includes results for a variety of adverse pregnancy outcomes for Ni refinery vs. other work (Chashschin et al. 1994).

Zheng et al. (2014). Levels of Heavy Metals and Trace Elements in Umbilical Cord Blood and the Risk of Adverse Pregnancy Outcomes: a Population-Based Study

The authors conducted this cross-sectional study to determine concentrations of heavy metals and trace elements in umbilical cord blood (UCB) and to identify the relationship between UCB levels of heavy metals and trace elements and risk of adverse pregnancy outcomes (Zheng et al. 2014). The authors recruited UCB samples from 1,106 pregnant women at a hospital in Xiamen City in Xiamen, China from April 19, 2010 to December 31, 2010. Among these samples, 73 women had adverse pregnancy outcomes, including fetal distress (n=16), premature birth (< 37 weeks gestation; n=18), macrosomia (birth weight ≥4,000 g; n=8), and others (a composite of any adverse

pregnancy outcomes; n=31). Fetal distress in labor was based on the presence of one of more of the following: abnormal fetal heart rate, neonatal Apgar 1 minute score ≤7, and "II~III degree of amniotic fluid contamination". The authors randomly selected 106 samples from the remaining 1,033 women who did not have adverse pregnancy outcomes for the reference group. The women were asked to complete a detailed questionnaire and were interviewed by trained health care personnel, and the authors state that none had ever been occupationally exposed to heavy metals. Subjects with chronic hypertension, diabetes, or chronic renal or cardiac disease during pregnancy were excluded.

UCB was collected at delivery in the Maternal and Child Health Hospital in Xiamen City. The authors used inductively coupled plasma-mass spectrometry to determine UCB heavy metal and trace element concentrations, with multi-element standard solution for calibration and validation of the standard curves, and rhodium as an internal reference. Laboratory quality control and assurance measures are described.

Median Ti and Sb concentrations were higher among the adverse pregnancy outcomes than reference deliveries, but there were no statistically significant differences in median As, Cd, Co, Cr, Cu, Mn, Ni, Pb, Sr, Ti, V, and Zn concentrations (by Mann-Whitney *U* test). The Ni concentration parameters in µg/L for adverse pregnancy outcomes and reference deliveries are shown in Table B.26 below.

Table B.26. UCB Ni levels (µg/L) among deliveries with adverse outcomes and controls

Group	No.*	Mean	S.D.	Median	IQR	p-value
Adverse pregnancy outcome	58	38.82	92.36	11.63	5.07-41.28	0.732
Control	68	46.32	69.75	12.65	4.53-47.62	

*Number with metal concentrations above the detection limit

Maternal age, height, prepregnancy weight and BMI, education, and income, and newborn sex did not differ significantly between adverse pregnancy outcome and referent deliveries. Mean newborn length and weight was lower among the adverse pregnancy outcomes than among the reference births (Zheng et al. 2014).

OEHHA comments: Important details about the study population, sample selection, and data analysis are not reported. Table 2 in the article excludes subjects whose Ni levels were below the detection limit, which was not reported. Up to 15 cases (20.5%) and 38 (35.8%) controls may have been excluded as a result; this could spuriously cause UCB Ni levels among controls to appear higher in aggregate. The study also had a relatively small sample size. Combining all adverse outcomes with various etiologies could make finding relationships between exposure and adverse effects difficult.

Fuertes et al. (2014). Associations between particulate matter elements and early-life pneumonia in seven birth cohorts: Results from the ESCAPE and TRANSPHORM projects

This study, part of a multi-initiative collaborative effort working to improve knowledge about transport-related particulate matter and its effects on health, aimed to examine perinatal exposure to Ni, Cu, Fe, K, S, Si, V, and Zn PM_{10} and $PM_{2.5}$, as a risk factor for pneumonia in early childhood (Fuertes et al. 2014). Seven European population-based birth cohorts were included. Each cohort included births from a period between 1994 and 2008. The primary outcome of interest was a parental report that a doctor had diagnosed pneumonia between birth and two years of age. After exclusions for lack of information on exposure, outcome, or residence, data on 15,962 children were available for analysis. For Ni $PM_{2.5}$, data from one cohort (n=3,971) could not be used due to lack of significant predictors for the LUR model. The cumulative incidence of pneumonia was 5.6% in the total population, and was higher in cohorts that collected data at both the first and second year, compared to only once or up to 1.5 years.

Questionnaire-based information was collected between six and 36 months of age, with some variation in the wording, doctor requirement, number, and timing of the assessments, depending on the cohort.

Between October 2008 and February 2010, particulate matter measurements were taken at 20 or 40 sites in each of seven study areas for 14 consecutive days in cold, warm, and intermediate seasons as part of the ESCAPE project. The three measurement periods were averaged to calculate site-specific annual averages and adjusted for temporal variation using data from a centrally located background reference site that operated continuously throughout the measurement year. Data on traffic intensity, population/household density, and land use were used as predictor variables to develop area-specific LURs for all elements in both particle matter size fractions. Fuertes et al. used these standardized, site-specific LURs to estimate average annual exposures to each element for each participant's home address at birth. The range of cohort-specific mean (SD) concentrations for Ni PM_{10} was 0.8 (0.2) to 3.7 (0.6) ng/m^3, and for Ni $PM_{2.5}$ was 0.4 (0.1) to 2.6 (0.4) ng/m^3.

The authors used random-effects meta-analysis models to account for potential within- and between-cohort variability in combined estimates of associations between elements and pneumonia. Fuertes et al. decided *a priori* to calculate three types of models: 1) "crude models" would adjust for sex and municipality (a four-level categorical variable used in only one cohort); 2) "minimally adjusted models" would add adjustment for older siblings, breastfeeding, season of birth, atopy of either parent, daycare attendance, maternal smoking during pregnancy, secondhand smoke, parental SES, use of natural gas for cooking, mold/dampness in the home, intervention (vs. observational study); and 3) "main models" would further include PM_{10} mass or $PM_{2.5}$ mass. Results for Ni are per 2 ng/m^3, based on rounding down from the mean range between the 10^{th} and 90^{th} percentile Ni conc. from all ESCAPE study areas. The AOR (CI) for pneumonia and Ni

PM_{10} in the fully adjusted main model was 1.09 (0.83, 1.43) and for Ni $PM_{2.5}$ was 0.84 (0.67, 1.05). Table B.27 shows results for Ni PM_{10} and Ni $PM_{2.5}$ for all three models.

Table B.27. AORs* for pneumonia and Ni PM_{10} and Ni $PM_{2.5}$ by level of adjustment

Model adjustment	Crude	Minimal	Main
Ni PM_{10}	1.22 (0.99, 1.49)	1.31 (1.06, 1.63)	1.09 (0.83, 1.43)
Ni $PM_{2.5}$	1.11 (0.93, 1.32)	1.15 (0.96, 1.39)	0.84 (0.67, 1.05)

*per 2 ng/m^3 change in Ni exposure

OEHHA comments: A previous study cited in the current study reported that the LUR models did not perform well for predicting Ni particulate concentrations (de Hoogh et al. 2013). The authors do not provide reasons for including all of the covariates in the models; the result may be overadjustment.

B.1.8 Related Information

Yan et al. (2016). Association of Essential Trace Metals in Maternal Hair with the Risk of Neural Tube Defects in Offspring

Yan et al. conducted this case-control study to investigate the association between NTD risk and concentrations of essential trace metals (ETMs) in maternal hair growth from one month before conception to two months after conception, and to determine relationships between maternal diet and levels of ETMs in maternal hair (Yan et al. 2017). The study was conducted in four counties and the city of Taiyuan in Shanxi Province, and six counties in Hebei Province from January 2003 to December 2007. Shanxi and Hebei Provinces are in northern China, and have the highest NTD prevalence in China. All NTD subtypes (anencephaly, spina bifida, and encephalocele) were included. The authors recruited women with NTD-affected pregnancies, including live births, still births, and terminated pregnancies, as cases. For each confirmed case, one or two women who delivered a full-term, healthy infant at the same hospital were matched to the case by the county or city of residence and last menstrual period, and selected as controls. From the recruited subjects, the authors selected 452 subjects for hair sampling, including 191 cases (85 anencephaly, 79 spina bifida, 24 encephalocele, and 3 without subtype information) and 261 controls. Case ascertainment and sample selection methods were not reported.

Within a week of delivery or pregnancy termination, trained health workers conducted face-to-face interviews for information including maternal age, occupation, education, gravidity, history of previous defects, folate supplementation, fever or flu in early pregnancy, alcohol consumption, periconceptional active or passive smoking, all of which were considered as potential covariates. The interviews also included questions about frequencies of consumption of meat or fish, fresh green vegetables, fresh fruit, and salted vegetables. Cases were somewhat less educated and more likely to have a

history of previous defect, fever or flu during the pregnancy (OR=6.6), and smoking or passive smoking.

Hair samples were cut from as near as possible to the scalp. The authors report that hair samples of all subjects appeared not to be dyed. The authors estimated ~3 cm of hair grown from one month before conception through two months after conception. Concentrations of the 9 ETMs (Fe, Zn, Cu, Co, Mn, Cr, Ni, Mo, and Sn) in hair were measured using inductively coupled plasma-mass spectrometry. To calculate hair ETM concentrations, the authors subtracted the corresponding mean concentration of the blank controls for each batch. Ni was detected in every sample. Median hair Ni concentrations were lower in cases (all NTD subtypes) than controls, as shown in Table B.28.

Table B.28. Concentrations of Ni in Hair

NTD	N	Ni in hair median (IQR), ng/mg
All NTDs	191	0.145 (0.089 – 0.306)
Anencephaly	85	0.138 (0.081 – 0.344)
Spina bifida	79	0.139 (0.089 – 0.228)
Encephalocele	24	0.152 (0.084 – 0.275)
Controls	261	0.189 (0.120 – 0.375)

To maximize the sample size, the authors separated the matched pairs and used unconditional logistic regression to estimate AORs for ETM concentration (dichotomized with a cutoff at the median of the control group) and NTDs, adjusted for maternal age, education, history of previous defects, folate supplementation, fever or flu in early pregnancy, and periconceptional active or passive smoking. Hair Ni conc. above the median was associated with decreased risk of NTDs, as shown in Table B.29.

Table B.29. AORs* (CI) for Ni in hair and NTDs

NTD	AOR (95% CI)
All NTDs	0.53 (0.34 – 0.81)
Anencephaly	0.50 (0.27 – 0.91)
Spina bifida	0.42 (0.23 – 0.76)
Encephalocele	0.82 (0.32 – 2.11)

*Adjusted for maternal age, education, history of previous defects, folate supplementation, fever or flu in early pregnancy, periconceptional active or passive smoking

When the exposure data were classified into quartiles, Ni concentrations above the 1st quartile were associated with decreased risk of NTDs, as shown in Table B.30.

Table B.30. AORs* (CI) for NTDs by Ni exposure quartile

Quartile	All NTDs (n=191)	Anencephaly (n=85)	Spina bifida (*N* = 79)	Encephalocele (*N* = 24)
1st	1.00	1.00	1.00	1.00
2nd	0.57 (0.32-1.03)	0.31 (0.13-0.73)	0.67 (0.32-1.38)	1.45 (0.41-5.19)
3rd	0.38 (0.21-0.69)	0.25 (0.11-0.58)	0.36 (0.17-0.80)	1.34 (0.37-4.83)
4th	0.38 (0.21-0.69)	0.43 (0.20-0.94)	0.34 (0.15-0.78)	0.67 (0.15-3.05)

*Adjusted for maternal age, education, history of previous defects, folate supplementation, fever or flu in early pregnancy, periconceptional active or passive smoking

The authors report that Ni levels in this study were lower than levels in populations that are not near major sources of pollution such as electronic waste recycling, metallurgy, or a mining tailing zone, and interpret the results as indicating "deficiencies of …both Mo and Ni were associated with an increased NTD risk." Ni and Zn were both modestly correlated with consumption of fresh fruits and fresh green vegetables. The only other correlation between diet and an ETM was between Sn and salted vegetables.

B.2. Animal Studies of Developmental Toxicity

Eight studies of the developmental toxicity of nickel were conducted in rats by the oral route (Adjroud 2013; Ambrose et al. 1976; Kakela et al. 1999; Price et al. 1988; Schroeder and Mitchener 1971; Siglin 2000a, 2000b; Smith et al. 1993; US EPA 1991a) and RTI, 1987 as cited by US EPA, 1991a. An additional seven studies were conducted in mice by the oral route (Berman and Rehnberg 1983; Chernoff and Kavlock 1982; Gray and Kavlock 1984; Saini et al. 2013; Saini et al. 2014a; Saini et al. 2014b; Seidenberg et al. 1986). Only one study was identified that used inhalation exposure of rats to nickel (Weischer et al. 1980). Additional studies were conducted by various types of injection protocols (Lu et al. 1979; Mas et al. 1985; Storeng and Jonsen 1981; Sunderman et al. 1978; Sunderman et al. 1983).

In attempting to be as comprehensive as possible, this review includes both well-conducted and reported studies, as well as other studies having limitations in scope, reporting, and/or study design. The former have been summarized and discussed in some detail, while the latter are described as briefly as appropriate.

Many of the studies summarized in this section include specific outcomes such as implantation frequency or fetal viability that might ultimately result from adverse effects directly on the developing offspring or on the maternal reproductive system (or both). A potential for overlap between developmental and male reproductive effects is also a possibility. Therefore, the same studies and even the same adverse effects may be discussed in the developmental and female and/or male reproductive sections of this document.

B.2.1. Studies Conducted in Rats by the Oral Route

Adjroud (2013) The toxic effects of nickel chloride on liver, erythropoiesis, and development in Wistar albino preimplanted rats can be reversed with selenium pretreatment.

Timed-pregnant Wistar albino rats were randomly assigned to seven groups of six animals each (Adjroud 2013). Animals were given nickel in the form of $NiCl_2 \cdot 6H_2O$ (purity not stated) by either the oral route or subcutaneous injection (s.c). Oral dosing was provided in drinking water at a concentration of 0 or 20 mg $NiCl_2$/L for 16 days beginning on gestation day (GD) 3. All maternal animals were sacrificed on GD 20, and uteri excised for fetal examination. Fetal viability was assessed and weights taken; there were no morphological evaluations.

Maternal findings
No maternal mortality was observed in any of the control or treated groups. Maternal body weights at term were significantly reduced by nickel exposure under either of the treatment regimens.

Drinking water exposure to 20 mg $NiCl_2$/L resulted in significantly reduced maternal body weights on GD 5 ($p < 0.001$) and GD 20 ($p < 0.05$).

Fetal findings
Fetal viability and weights on GD 20 were not significantly affected by gestational exposure to 20 mg $NiCl_2$/L drinking water.

Siglin (2000a) A one-generation reproduction range-finding study in rats with nickel sulfate hexahydrate.

The study cited as Siglin 2000a was conducted by Springborn Laboratories, Inc. under contract to the Nickel Producers Environmental Research Association. The same study is often cited as Springborn Laboratory, 2000a; it is sometimes cited as NiPERA, 2000a. (Siglin 2000a)

Animals were assigned to dose groups by a computerized randomization program using a stratified block design that accounted for initial body weight. To avoid sibling matings, rats were ordered from different production areas of Charles River Laboratories. Other components of the study design are summarized in the table of protocol elements below.

Protocol Elements:

Test compound (purity, if reported)	$NiSO_4 \cdot 6H_2O$ (99%)
Route	Oral, gavage
Species and strain	Sprague-Dawley rats; males and females
Doses of $NiSO_4.6H_2O$	0, 10, 20, 30, 50, and 75 mg $NiSO_4.6H_2O$/kg-day
N animals/test group	8 males and 8 females
Days of treatment	2 weeks prior to mating of parental (F0) (Siglin 2000a)animals, continuous through gestation and lactation; direct dosing of F1 offspring commenced on postnatal day (PND) 22 and continued 4 weeks

OEHHA (2001) determined a lowest observed adverse effects level (LOAEL) dose from this study, which they cited as Springborn Laboratory, 2000a. The LOAEL of dose of 10 mg nickel sulfate hexahydrate ($NiSO_4.6H_2O$)/kg-day was converted to 2.23 mg Ni/kg-day (OEHHA 2001). Converting other doses of $NiSO_4.6H_2O$/kg-day by a ratio of 10/2.2 gives estimated doses of 0, 2.2, 4.4, 6.7, 11.2, and 16.7 mg Ni/kg-day.

All F0 females survived treatment during gestation. A single dam in each of dose groups 2.2, 6.7, 11.2, and 16.7 mg Ni/kg-day was sacrificed outside of schedule due to failure to deliver, or loss of complete litter during early lactation. There were no significant changes in body weights during gestation, or gestational weight gain with nickel exposure of F0 dams.

Table B.31. Viability Data for F1 Litters

$NiSO_4.6H_2O$ mg/kg-day	0	10	20	30	50	75
Ni mg/kg-day	0	2.23	4.46	6.70	11.16	16.74
N litters	8	8 or 7	8	7	7	8
Post implantation loss	0.4 ± 0.7	2.6 ± 5.4	1.5 ± 1.6	2.3* ± 2.0	2.7** ± 2.0	4.8** ± 2.3
Total number dead pups	1	12**	10**	10**	4	23**
Live litter size[a] PND 0 (no SD)	16.0	14.3	13.3	13.1	12.2	11.4**

*p < 0.05; **p < 0.01

[a]live litter size on PND 0 includes only females with live pups on that day

Data for post implantation loss and mean live litter size are shown in Table B.31 above. Post implantation loss increased with increasing dose. There appears to have been a general trend for decreasing mean live litter size with increasing dose, although measures of within-group variation were not provided in the report. The total number of pups found dead on PND 0 was significantly greater than controls at all doses excepting

11.2 mg Ni/kg-day. Because the values for dead pups are not presented on a per litter basis, excess death affecting one or a few litters has an outsize impact.

Pup body weights per litter on PND 1 did not differ among groups.

Siglin (2000b) An oral (gavage) two-generation reproduction toxicity study in Sprague-Dawley rats with nickel sulfate hexahydrate.

As for Siglin 2000a, described above, the study cited here as Siglin 2000b was conducted by Springborn Laboratories, Inc. under contract to the Nickel Producers Environmental Research Association. The same study is often cited as Springborn Laboratory, 2000b; it is sometimes cited as NiPERA, 2000b. (Siglin 2000a).

Protocol Elements:

Test compound (purity, if reported)	$NiSO_4.6H_2O$ (99%)
Route	Oral, gavage
Species and strain	Sprague-Dawley rats; males and females
Doses of $NiSO_4.6H_2O$	0, 1, 2.5, 5, and 10 mg $NiSO_4.6H_2O$/kg-day
N animals/test group	28 males and 28 females
Days of treatment	10 weeks prior to mating of parental (F0) animals, continuous through gestation and lactation; direct dosing of F1 offspring commenced on PND 22 and continued through mating, gestation, and lactation of the F1 as they produced the F2.

The study methods used in Siglin (2000b) were very similar to those of Siglin (2000a), but extended into a second generation (Siglin 2000b). The 2000b study also used an adjusted range of doses, and a greater number of animals per dose group. A dose of 10 mg $NiSO_4.6H_2O$/kg-day, which had been the lowest dose used in Siglin 2000a, served as the highest dose in Siglin 2000b.

As described for Siglin 2000a, above, OEHHA (2001) determined a LOAEL dose from the one-generation study, which they cited as Springborn Laboratory, 2000a. The LOAEL of dose of 10 mg nickel sulfate hexahydrate ($NiSO_4.6H_2O$)/kg-day was converted to 2.23 mg Ni/kg-day. Applying the ratio of 10/2.2 to doses used in the two-generation study (Siglin, 2000b) gives estimated doses of 0, 0.2, 0.6, 1.1 and 2.2 mg Ni/kg-day.

F0 maternal body weights and weight gain did not differ among dose groups. Over the course of the study, 0/28 females died in the 0.2 and 0.6 mg Ni/kg-day dose groups. In each of the control, 1.1, and 2.2 mg Ni/kg-day groups, 1/28 females died or were sacrificed moribund.

F1 maternal animals did not show evidence of treatment-related effects on mortality or body weight. Gestational weight gain did not vary among groups.

Among litters of the F1 and F2 generations, no adverse effects of treatment were reported for:

- Mean number of implantation sites per litter
- Mean live litter size
- Mean post-implantation loss per litter
- Total number dead pups per group
- Total number live pups per group
- Number litters with live offspring
- Sex ratio
- Pup viability on Lactation Day 1

The authors concluded that a dose of 10 mg $NiSO_4.6H_2O$/kg-day (2.2 mg Ni/kg-day) was the NOAEL for this compound in their two-generation reproductive toxicity study.

Kakela et al. (1999) Effects of nickel chloride on reproduction of the rat and possible antagonistic role of selenium.

Mature Wistar rats were given nickel in the form of $NiCl_2 \cdot 6\ H_20$ (concentrations expressed as ppm Ni^{2+}; purity not noted) in drinking water (Kakela et al. 1999). Six female rats per group were exposed to 0, 10, 30, or 100 ppm Ni^{2+} from the premating period, continuously throughout cohabitation, gestation, and lactation. For purposes of comparison to other oral studies, these concentrations can be converted to estimated doses of 0, 1.5, 4.4, and 15 mg Ni/kg-day.

Additional groups involved treating of both males and females, or males only. In one female-only group and one male-only group, the drinking water was also supplemented with 0.3 ppm selenium (added as Na_2SeO_3).

For most groups, the premating exposure was two weeks; one group had an extended premating exposure of 100 days. Data collected at birth consisted of number “born-dead pups” and gestation index ((N females delivering live young/N pregnant females) x 100).

Females exposed to 100 ppm Ni^{2+} for two weeks prior to mating continued through lactation were reported as having three pups born dead; it is not clear how many litters had dead pups. Three dead pups were all reported for controls, while no other female-only-exposed groups (including 100 ppm Ni^{2+} and Se) had any born-dead pups. While litter size was significantly lower than controls for the 100 ppm group on PND 21 ($p < 0.05$), there was no effect on gestation index and therefore no clear effect of gestational exposure.

A significant effect on gestation index ($p < 0.01$) was seen with male-only exposure to 30 ppm Ni^{2+} for 28 days prior to the five-day cohabitation period for mating. This finding was in the context of a fertility index of only 50%, as opposed to 100% for controls, as well as no pups born dead. Co-exposure to Se was associated with some protection for gestation index and mean live litter size. It is not clear if there might have been some type of paternally-mediated adverse effect of nickel on embryo-fetal viability beyond decreased fertility alone.

Smith et al. (1993). Perinatal toxicity associated with nickel chloride exposure*.*

Protocol Elements:

Test compound (purity, if reported)	$NiCl_2.6H_2O$
Route	Oral, drinking water
Species and strain	Long-Evans rats; only females treated
Doses of Ni	0, 10, 50, or 250 ppm nickel (0, 1.3, 6.8, or 31.6 mg Ni/kg-day; overall averages calculated by authors as total cumulative water consumption divided by study average body weight)
N animals/test group	34 females/test group; randomized group assignments stratified by body weight
Days of treatment	11 weeks prior to mating, and continuously through two sequential gestation (G1, G2) and lactation (L1, L2) periods (including a two-week "rest period" between breeding cycles).

No overt clinical signs of toxicity were reported for dams in any of the groups (Smith et al. 1993). Maternal weight gain was expressed as percent change over respective gestation periods. Weight change during the G1 period showed significant decrements at the high ($p \leq 0.05$) and mid ($p \leq 0.03$) doses. No statistically significant alterations in percent maternal gestational weight change were seen during the G2 period.

Outcomes reflective of reproductive performance (sperm positive females, number viable litters, and the number of live plus dead pups per litter) were unaltered by nickel exposure in either G1 or G2. Neonatal mortality was expressed as both the total number of dead pups (all litters combined) and the percentage of dead pups per litter. Among G1 litters at the high dose of 31.6 mg Ni/kg-day, both measures were significantly larger than controls ($p \leq 0.01$, and $p \leq 0.03$, respectively).

More severe effects on pup mortality were observed in G2 litters. The number of viable litters with at least one dead pup at birth increased from 2/23 in controls to 10/25 at the high dose of 31.6 mg Ni/kg-day ($p \leq 0.03$). Numbers of total dead pups on PND 1 were significantly greater than controls at all doses ($p \leq 0.03$, $p \leq 0.05$, $p \leq 0.01$), as were the

frequencies of dead pups per litter, although significance at the mid dose of 6.8 mg Ni/kg-day was marginal ($p \leq 0.10$).

Pup birth weights were generally unaffected by nickel treatment for either G1 of G2 litters. Female pups from G1 litters exposed to 1.3 mg Ni/kg-bw showed a slight decrease in birth weight compared to controls. Significance was only marginal ($p \leq 0.10$).

The authors concluded that the lowest dose of 1.3 mg Ni/kg-day represented the LOAEL for the study. Adverse effects on offspring viability appear to have been strongest during the first day of postnatal life. The authors did not propose any specific mechanism for the increase in perinatal mortality, but noted that "nickel disturbs the normal physiological progression of events during late gestation, birth, and the early postnatal period."

Price et al. (1988) Fertility and reproductive performance of the F1 generation; Final study report (III of III). Two-generation reproduction and fertility study of nickel chloride administered to CD rats in the drinking water.

Research Triangle Institute conducted a two-generation reproduction study of nickel chloride under contract to US EPA. The docment is cited here as Price et al. (1988), but is usually cited as RTI, 1988 (Price et al. 1988).

Protocol Elements:

Test compound (purity, if reported)	$NiCl_2$
Route	Oral, drinking water
Species and strain	CD rats
Concentration (dose) of Ni	0, 50, 250, or 500 ppm
N animals	30 breeding pairs, except: 19 pairs of F1 to produce F2a, and 18 pairs of F1 to produce F2b
Days of treatment	F1 animals exposed continuously from prenatal development through weaning and maturation, and on through production of F2 offspring

Ingested doses of nickel were calculated by the authors from concentration in drinking water, water consumption, and body weight. Calculated values varied among generations, parity, and gestational days under consideration. Examples are provided in Table B.32 below.

Table B.32. Calculated doses of Ni^{2+}

Generation, pregnancy number, days of data	0	50 ppm	250 ppm	500 ppm
F1b dams, first pregnancy, GD 6-13	0	6.26 mg/kg-day	24.69 mg/kg-day	45.42 mg/kg-day
F1b dams, second pregnancy, GD 6-13	0	5.67 mg/kg-day	22.24 mg/kg-day	41.82 mg/kg-day

- No maternal mortality during gestation of F2b litters.

- Decreased maternal body weights for F1b dams on GD 20 during production of F2a ($p < 0.01$) and F2b ($p < 0.05$) litters.

- Maternal weight gain, corrected for weight of gravid uterus, was significantly reduced for dams of the 250 ($p < 0.05$) and 500 ($p < 0.01$) ppm groups, during gestation of the F2b offspring. Uncorrected weight gain was significant only at the higher concentration ($p < 0.01$).

- No significant differences among groups for % live litters among treatment groups in the F2a or F2b generations.

- Significantly decreased live litter size on PND 1 for F2a litters of the 500 ppm group. This group also had significantly reduced mean pup weight per litter on PND 1 ($p < 0.01$).

- Live litter size and fetal body weights were not affected by treatment for F2b litters.

- For F2b litters, no effects were seen on many litter outcomes, including: percent preimplantation loss, resorptions per litter, and dead pups per litter.

- No apparent associations between treatment and overall frequencies of malformations or anomalies (gross, visceral, or skeletal) were reported for F2b litters. A significant increase in the frequency of “short ribs” was reported at the 50 ppm level, but not at either of the two higher concentrations.

Results for the earlier part of the study, covering P0 production of the F1a and F1b litters, were reported in a different document (part II of III) that was not available to OEHHA. Information from that part of the study could only be obtained from secondary sources. In those sources, the study is cited as RTI, 1987, rather than by the author’s name, as we have done for part III of III of the same study discussed above (Price et al., 1988).

From the US EPA RfD (1991a):

> In the RTI (1987) F1a generation (postnatal days 1-4) at the 500 ppm dose level the number of live pups/litter was significantly decreased, pup mortality was significantly increased, and average pup body weight was significantly decreased in comparison with controls. Similar effects were seen with F1b litters of P0 dams exposed to 500 ppm nickel. In the 50 and 250 ppm dose groups increased pup mortality and decreased live litter size was observed in the F1b litters (US EPA 1991a).

Reported effects on F1b litters, specifically those seen at 50 and 250 ppm, were called into question "because the room temperature tended to be 10 degrees F higher than normal at certain times (gestation-postnatal days) along with much lower levels of humidity."

The opinion was based on a review of the adverse developmental effects of hyperthermia (Edwards 1986). Specific whole-animal data for pregnant rats exposed to elevated temperatures are shown in Table B.33 below. Two other studies mentioned in the text of the Edwards paper reported no developmental effects with whole-body heating of pregnant rats, but no details of exposure or consequent rectal temperatures were provided. Other studies summarized by Edwards (1986) involved exposing the exteriorized uterus, or in vitro culture of intact rat embryos to temperatures higher than 37 – 38°C.

Table B.33. Temperature and developmental effects data from intact pregnant rats (as presented and reviewed by Edwards, 1986)

Reference as cited by Edwards, 1986	Experiment	External Temp	Rectal Temp*	Effects
Kreshover and Clough, 1953	Pregnant rats exposed for 12, 24, or 48 hrs	37.2 – 39.8°C	38.9 – 40.6°C	Severe tooth defects with exposures after GD 9
Edwards, 1968	Pregnant rats exposed 2X on one day for 40 – 60 minutes	43°C	Not reported	GD 9 – 13: ↑ growth retardation and resorption GD 9: microphthalmia GD 10: anencephaly GD 11 – 14: ↑ limb, toe, and tail defects, hydrocephaly, and cleft palate
Hoffman et al., 1968	"Short wave diathermy applied to [pregnant] abdomen"	Not relevant	Elevated by 1 – 4°C	GD 9 – 10: head defects GD 13 – 14: tail and limb defects
Arora et al., 1979	No details provided	Not reported	Not reported	GD 4, 6, or 8: edema, microencephaly, and microphthalmia GD 10: skeletal defects After GD 14: no effects

*Normal body temperature for rats range from 35.9 – 37.5°C (http://web.jhu.edu/animalcare/procedures/rat.html)

For comparison, typical room temperatures preferred by people indoors range from about 20 – 25.5^{o}C. Without more detailed information on gestation day at time of exposure and internal temperatures of the exposed animals, it is difficult to know how likely animal room temperature alone was responsible for adverse effects reported among F1b litters at nickel concentrations of 50 and 250 ppm.

Ambrose et al. (1976). Long term toxicologic assessment of nickel in rats and dogs.

The Ambrose (1976) study reports on several experiments, including a three-generation feeding study of $NiSO_4.6H_2O$ in "Wistar-derived" rats (Ambrose et al. 1976). This test compound was determined to consist of 22.3% Ni, and was added to feed to provide Ni concentrations of 0, 250, 500, and 1000 ppm.

Protocol Elements:

Test compound (purity, if reported)	$NiSO_4.6H_2O$
Route	Oral, feed
Species and strain	Wistar-derived rats
Doses of Ni	0, 250, 500, and 1000 ppm
N animals/test group	20 females and 20 males
Days of treatment	11 weeks prior to mating of F0, through production of F1a, F1b, F2a, F2b, F3a, and F3b

Additional details of methodology are provided below:

- F0 generation was selected from littermates, "within but not between sexes," separated into four groups of 30 rats/sex, and started on test diets.
- After 11 weeks on the test diet, 20 females per group were chosen for mating with males in the same diet group; the selection process was not explained.
- Males were rotated between female's cages for three successive seven-day mating periods.
- F1a pups were sacrificed at weaning, and the F0 bred again to produce F1b litters.
- F1b offspring were retained to breed the F2 generation (F2a and F2b litters); 30 animals/sex/group started on test diets, reduced to 20 after 11 weeks.
- F2b offspring retained as above to breed F3a and F3b litters.
- For each generation starting with 20 animals/sex, the actual number of litters was inevitably lower; the number of animals giving birth in each group ranged between 11 and 20, with some litters consisting solely of dead pups.

- Data on offspring were presented as total pups, rather than litter means.
- Food consumption data were not provided, hampering determination of ingested doses.

Body weight data for F0 animals were not presented in tabular or graphical form. The text states that at the end of the premating period, female rats in the 1000 ppm group had an average weight decrease not exceeding 8%.

The paper specifically noted the absence of adverse effects of treatment on pup viability. At the same time, an increase in the total numbers of pups born dead was reported for F1a and F1b treated groups. The apparent effect was not reported for F2 or F3 litters. Tabulated data are presented on a total group basis, and without statistical analysis.

The text specifies that there were no malformations observed in any litter at any concentration of Ni. Only gross, external observations of surviving pups could have been made, with no evaluation for internal or skeletal malformations.

Schroeder and Mitchener (1971) Toxic effects of trace elements on the reproduction of mice and rats.

Schroeder and Mitchener (1971) tested the effects of long-term exposure to various trace elements on breeding mice and rats (Schroeder and Mitchener 1971). Nickel was tested only in rats.

Protocol Elements:

Test compound (purity, if reported)	"soluble salt"
Route	Oral, drinking water
Species and strain	Long-Evans BLU:(LE) rats
Doses of Ni	0, 5 ppm
N animals/test group	5 females and 5 males
Days of treatment	F0 treated throughout 9 months of continuous breeding; selected F1 pairs continued through production of F2; F2 continued through production of F3

The study was initiated with five pairs of breeding animals per group, with subsequent generations bred from pairs randomly selected from A, B, and C litters within each filial generation. The numbers of litters produced are stated to have been 11 for the F1 generation, 15 for the F2, and 10 for the F3.

One maternal death was reported for F1 nickel-exposed dams, with no maternal deaths reported for control or treated animals from other generations.

Data for mean litter size and M:F ratio are presented without statistical analysis or measures of variation. Average litter size does not appear to have been affected by nickel exposure; the largest difference reported was a mean litter size of 8.1 for treated and 11.0 for control litters of the F3 generation. M:F ratio for nickel-exposed animals in the F3 generation was 0.44.

The frequencies of "young deaths" were significantly increased in nickel-exposed groups for all three generations ($p < 0.005$ for the F1, and $p < 0.025$ for the F2 and F3, Table B.34). The term "young deaths" was not precisely defined in the paper, but appears to refer to perinatal mortality. The frequency of runting was significantly increased in exposed offspring of the F1 and F3 generations ($p < 0.025$ and $p < 0.0001$, respectively). Runts were defined in the paper as "animals with large heads and small bodies."

Table B.34. Deaths and Abnormalities in Rats Bred While Exposed to Nickel

	Control	**5ppm Nickel**
F1:		
Maternal Deaths	0	1
Dead Litters	0	0
Young Deaths	0	11**
Failures to Breed	0	0
Runts	0	37 ***
Number of rats (offspring)	114	121
F2:		
Maternal Deaths	0	0
Dead Litters	0	0
Young Deaths	0	16 ***
Failures to Breed	0	2
Runts	1	8
Number of rats (offspring)	113	157
F3:		
Maternal Deaths	0	0
Dead Litters	0	0
Young Deaths	1	17 ***
Failures to Breed	0	0
Runts	0	5*
Number of rats (offspring)	121	81

Differs from controls by Chi^2 analysis; *$p < 0.025$; **$p < 0.005$; ***$p < 0.0001$.

As US EPA (1991a) has pointed out, "The major weakness of this study, however, is that the end result is based on a total of five matings." The US EPA (1991a) document also expressed concern about composition of the lab-formulated diet having restricted other trace metals to an extent that might have influenced observed nickel toxicity (US EPA 1991a).

B.2.2. Studies Conducted in Mice by the Oral Route

Saini et al. (2014a) Prenatal exposure to nickel on pregnant Swiss albino mice and fetal development.

The aim of the study was to investigate the effects of nickel exposure specifically during the pre-implantation period of gestation (Saini et al. 2014a). Timed mated female Swiss albino mice were divided into four groups of 10. Gestation day (GD) 0 was the day a vaginal plug was detected. Dosing was by the oral route, but the paper does not clearly state whether treatment was by gavage or via drinking water. Additional protocol details can be found in the table below.

Protocol elements:

Species and Strain	Swiss albino mice
Test compound (purity)	$NiCl_2.6H_2O$ (97%)
Route	Oral (method not stated)
Doses of Ni	0, 46, 92, 185 mg Ni/kg bw-day*
N animals/test group	10
Days of treatment (developmental stage)	GD 0-5 (preimplantation)

*As reported in paper

Daily records were kept of body weight, as well as feed and water intake. Pregnant animals were sacrificed on GD 18 for examination of uterine contents. Each implantation site was classified as being alive, resorbed, or dead. Live fetuses were sexed, weighed, and examined for malformations. Skeletal anomalies were assessed following fixation and double staining for cartilage and bone.

No maternal mortality occurred during the study, and an absence of clinical signs of toxicity was noted. Mean feed and water consumption were significantly ($p < 0.05$) lower than controls at the high dose of 185 mg Ni/kg-day. Final maternal body weights were significantly reduced ($p < 0.05$) at the high and mid dose of Ni (185 and 92 mg/kg-day respectively). It is not clear from the paper whether final maternal body weights included gravid uteri.

Table B.35. Maternal and fetal outcomes

Dose (mg Ni/ kg-day)	Mean Maternal Weight (g)	Mean N implants/dam	Mean Live Litter Size	Mean Fetal Weight (g)	% Fetuses Having Skeletal Anomalies
0	19.0	8.40 ± 0.60	8.40 ± 0.60	1.45 ± 0.01	0
46	18.7	6.33 ± 0.33*	6.00 ± 0.00*	1.40 ± 0.004	11.7
92	16.0*	5.33 ± 0.33*	4.25 ± 0.47*	1.30 ± 0.014*	21.4
185	13.2*	4.80 ± 1.24*	3.00 ± 0.77*	1.05 ± 0.043*	31.8

*Differs from controls, $p < 0.05$

Notable outcomes include dose-related decreases in implantation sites per dam, mean litter size, and mean fetal weights (see Table B.35 above). Implantations and litter size differed significantly from controls ($p < 0.05$) for all three doses of Ni, while fetal weights were significantly reduced at the mid and high doses. Mean placental weights were also significantly lower than controls at the high dose of 185 mg Ni/kg-day.

Skeletal anomalies were reported only as percent fetuses affected, and not on a per litter basis. No statistical analyses of these data were reported. No anomalies were reported among controls, while the frequencies of fetuses with total skeletal anomalies, or anomalies of the skull, sternum, or distal limb elements appeared to increase with increasing dose of Ni.

A particularly interesting feature of this study is the restriction of treatment to the preimplantation period, rather than the more usual organogenesis period or all of gestation. Without data on corpora lutea, implantation frequency could not be calculated, but the average number of implant sites per dam decreased with increasing dose of Ni. This reduction is reflected in decreasing numbers of live fetuses per litter, although resorptions and fetal death also appear to have contributed. Fetal weights were also reduced with increasing dose, despite the smaller litter sizes that are often associated with higher weights for survivors.

The decreases in maternal body weights at the mid and high doses of Ni may reflect the smaller litter sizes and decreased fetal weights also seen in these groups – rather than indicating a causative role for maternal toxicity. Additionally, significant effects on implant sites and live litter size were seen at the lowest dose tested, below the dose level at which maternal effects were described.

Saini et al. (2014b) Effects of gestational administration of nickel on postnatal development in Swiss albino mice.

This study looked at postnatal effects on offspring following prenatal exposure to nickel during the pre-implantation, organogenesis, or fetal period (Saini et al. 2014b). Timed-pregnant Swiss albino mice were assigned to one of three dose groups plus vehicle controls; each dose group was further divided into one of three treatment periods (as shown in Table B.36 below).

Protocol elements:

Species and Strain	Swiss albino mice
Test compound (purity)	$NiCl_2.6H_2O$ (97%)
Route	Oral, gavage
Doses of Ni	0, 46.125, 92.25, 184.5 mg Ni/kg bw-day*
N animals/test group	15 controls and15/dose group; 5/combination of dose and treatment period
Days of treatment (developmental stage)	GD 0-5 (preimplantation), GD 6-13 (organogenesis), GD 14-18 (fetal)

*For purposes of this document, doses are rounded to the nearest whole mg in the text

The dams delivered normally and raised their litters. Pups were non-invasively examined for morphological anomalies, and regularly assessed for body weight and attainment of developmental landmarks. Indices of survival were determined at birth, and on PNDs 4, 7, 14, and 21 (weaning).

No effects of nickel exposure were observed on sex ratio, or on days at which developmental landmarks (pinna detachment, hair appearance, eye opening, vaginal opening or testes descent) were attained. Litter size at birth was significantly lower ($p < 0.05$) than controls for offspring exposed to 92 mg Ni/kg-day during the preimplantation period, as well as for those exposed to 185 mg Ni/kg-day during any of the three treatment periods.

Table B.36. Mean Litter Size in Mice Exposed to Ni During Different Stages of Gestation

Groups	Period of Gestation	Mean Litter Size
Control	Preimplantation Organogenetic Fetal	6.33 ± 0.33 7.00 ± 0.00 8.25 ± 0.47
46 mg/kg-day	Preimplantation Organogenetic Fetal	6.33 ± 0.88 6.66 ± 0.33 7.33 ± 0.33
92 mg/kg-day	Preimplantation Organogenetic Fetal	5.33 ± 0.33[a] 6.00 ± 0.57 7.00 ± 1.15
185 mg/kg-day	Preimplantation Organogenetic Fetal	4.66 ± 0.66[a] 5.33 ± 0.33[a] 6.00 ± 0.00[a]

[a] $p < 0.05$

No abnormalities were described for controls or animals exposed to 46 mg Ni/kg-day during organogenesis (GD 6-13). Offspring exposed to higher doses of nickel during this period showed increased incidences of specific morphological abnormalities (see Table

B.37 below). As reported, “total limb anomalies” were a combination of “absence of limbs” and “absence of phalanges.” Similarly, “total tail anomalies” were a combination of “absence of tail” and “short tail.”

Table B.37. Morphological anomalies in mouse offspring exposed to nickel during organogenesis (no affected animals indicated by -)

Groups	Microphthalmia	Total limb anomalies	Total tail anomalies
Control	-	-	-
46 mg/kg-day	-	-	-
92 mg/kg-day	5.55%	-	-
185 mg/kg-day	6.25%	18.75%	25.00%

Several points should be noted about the reporting of the malformation data:

- The percentages in the table refer to numbers of total pups affected, rather than the numbers of litters having at least one affected pup.
- Actual numbers of affected animals and group sizes are not provided in the paper.
- At the high dose of 185 mg/kg-day with treatment during organogenesis, five litters per dose, each with an average litter size of 5.33, would mean an estimated total of 27 pups in that group. Approximately five of those 27 pups would have had a limb anomaly (for example).
- Although no statistical analysis is reported, the defects described and shown in photographs in the paper are unusual and present at frequencies high enough to be concerning.

All indices of viability, from gestation through weaning, remained at 100% for control pups, and hence control data are not represented in Table B.38 below.
The various viability indices were calculated as follows:

- Gestation index = (N live litters/N successful matings) X 100
- Live birth index = (N live born pups/N offspring delivered) X 100
- Viability index = (N live pups on PND 4, 7, or 14/N offspring delivered) X 100
- Weaning index = (N live pups on PND 21/N live born pups) X 100

Table B.38. Offspring viability shown by dose and period of exposure*

Dose	Exposure period	Gestation index	Live birth index	Viability on PND 4 or 7**	Viability on PND 14 or 21**
46 mg/kg-day	Preimplantation	**75%**	100%	100%	100%
-	Organogenesis	100%	100%	100%	100%
-	Fetal	100%	100%	100%	100%
92 mg/kg-day	Preimplantation	**75%**	100%	100%	100%
-	Organogenesis	100%	100%	**94%**	**89%**
-	Fetal	100%	100%	**91%**	**91%**
185 mg/kg-day	Preimplantation	**75%**	**88%**	**88%**	**88%**
-	Organogenesis	100%	**94%**	**88%**	**81%**
-	Fetal	100%	100%	**89%;83%**	**83%; 78%**

*All control viability indices were 100%, and are not shown in the table.
**Except where two values are noted in a field, indices on PND 4 and PND 7 were identical, as were values on PND 14 and PND 21.

Points to note about viability data include the following:

- Preimplantation exposure to any of the test doses of nickel (46, 92, or 186 mg Ni/kg-day) was associated with a reduction in gestation index to 75%. The identical extent of reduction was seen at each dose.
- The live birth index was impacted only at the high dose of 186 mg/kg-day, and only with treatment during the preimplantation or organogenesis periods of gestation; high dose exposure during the fetal period had no effect on the gestation or live birth indices.
- With preimplantation exposure to 185 mg nickel/kg-day, postnatal viability indices showed no further decrease from the live birth index.
- Excepting for exposure to 185 mg nickel/kg-day, viability indices for PNDs 4 and 7 were identical with no further reductions between those days. The same pattern was seen for PND 14 and 21.
- At the mid-dose of 92 mg/kg-day, a further decrease in viability from PND 7 to PND 14 was seen with exposure during the organogenesis period, but not the fetal period.

Body weight data from birth through the sixth week of postnatal life are presented in the three figures below (Figures B.1, B.2, and B.3). These figures were prepared from data presented in “Table 4” of Saini et al. (2014b), to clarify the relationships between dose and time of treatment on birth and postnatal weights of gestationally-exposed offspring.

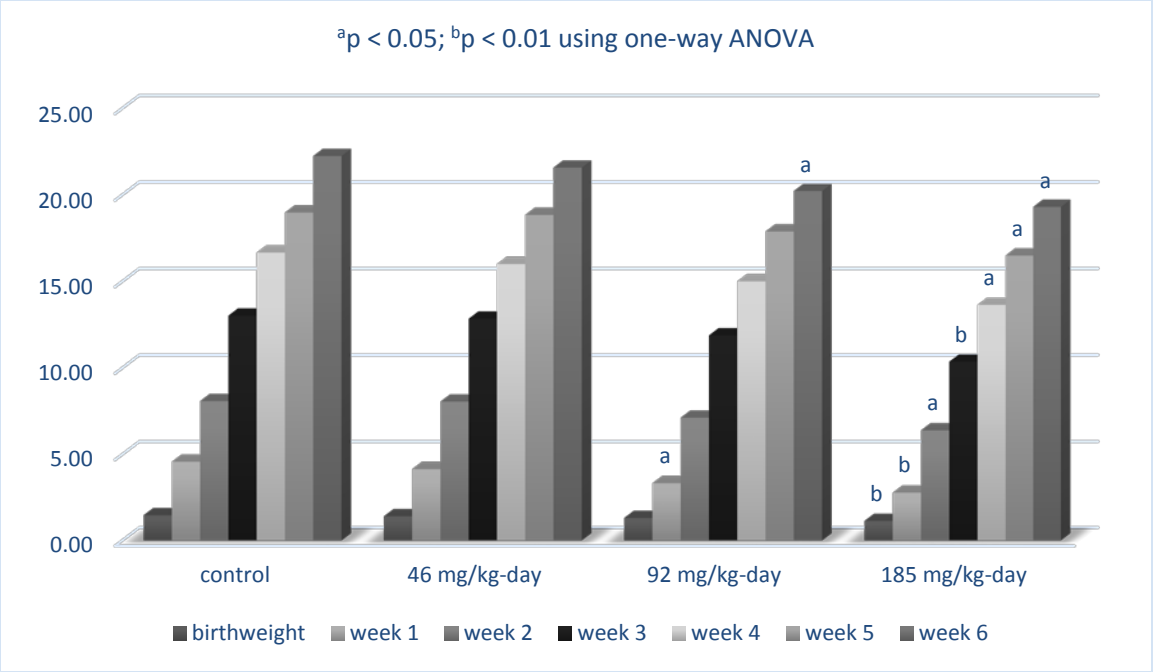

Figure B.1. Body weight (g) of offspring exposed to nickel during the preimplantation period

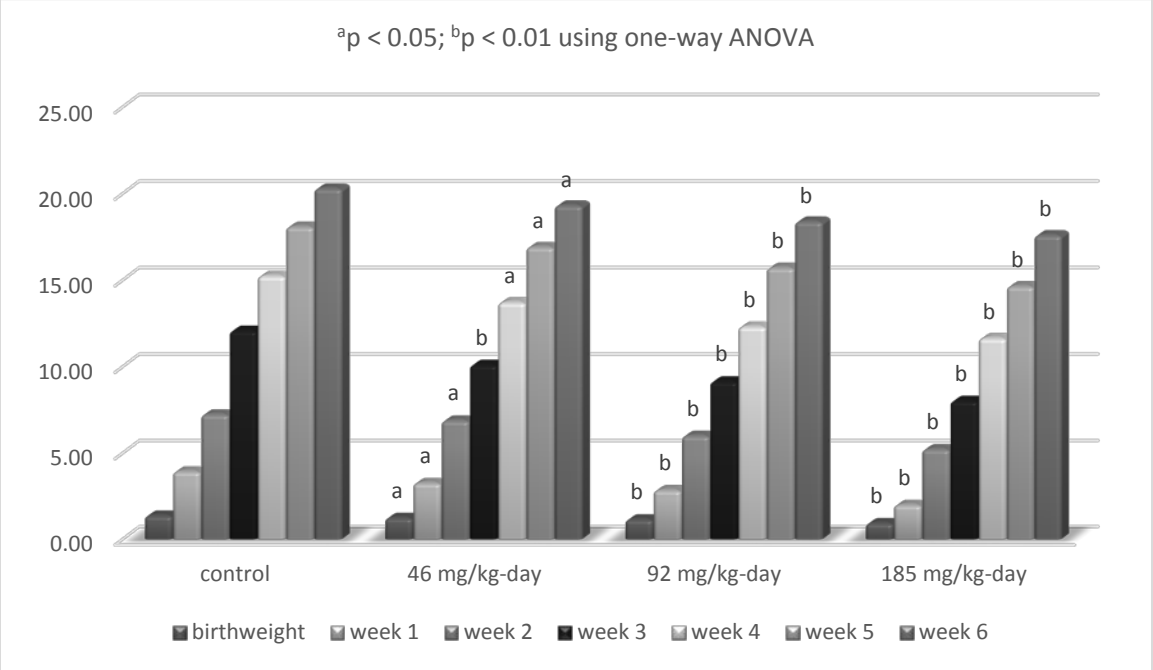

Figure B.2. Body weight (g) of offspring exposed to nickel during the organogenesis period

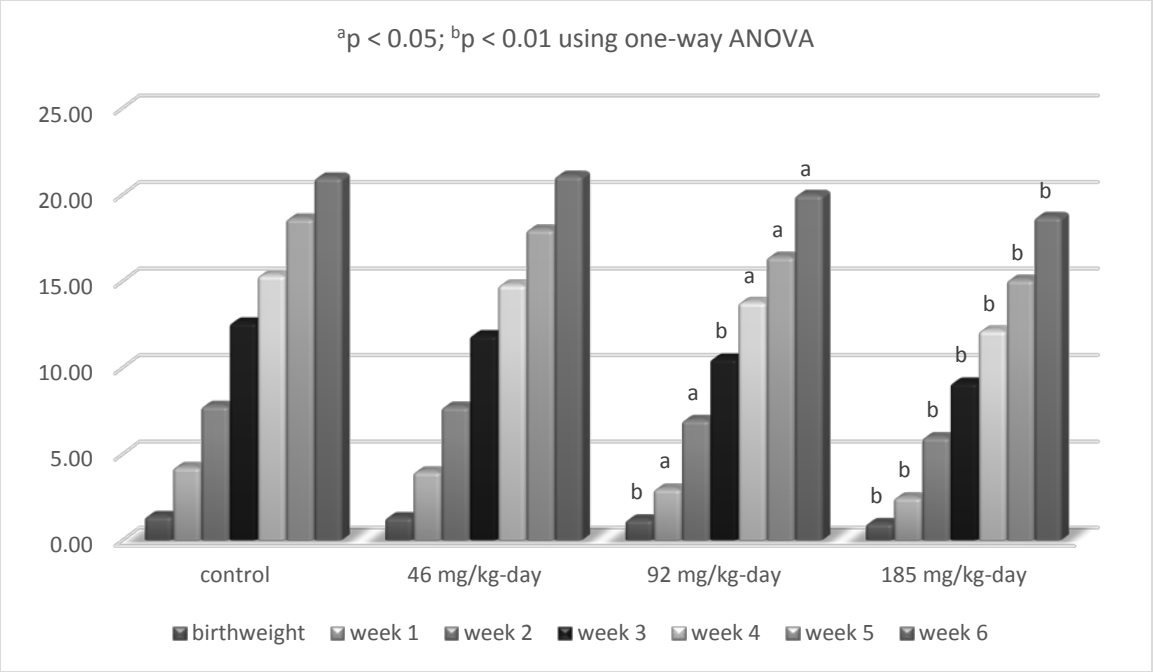

Figure B.3. Body weight (g) of offspring exposed to nickel during the fetal period

Points to note about offspring weight data include the following:

- Both the nickel dose and gestational stage at time of treatment appear to have affected the degree of impact on postnatal offspring growth. These factors were subjected to separate statistical analyses, rather than for combined contributions.
- The greatest impact on postnatal growth was associated with treatment during the organogenesis stage of gestation.
- The impact of treatment during the fetal period was greater than that of treatment during the preimplantation period.
- Data appear to have been analyzed without consideration of possible litter effects.
- The numbers of offspring in various dose/treatment-period groups were not provided, though we know that sizes of some groups would have been affected by reduced viability.

Overall, prenatal exposure to nickel was associated with gestational effects on viability, birth weight, and malformation frequency. Postnatal viability and growth were also affected by nickel exposures restricted to specific stages of gestational development. It would have been interesting to evaluate postnatal effects of prenatal exposure in offspring cross-fostered to untreated dams (and vice versa). Although not critical for the purposes of Proposition 65, it would be interesting to see cross-fostering data to investigate whether postnatal decrements were due to lasting effects of gestational exposure, or to lasting changes in the dams' physical or behavioral ability to care for their pups. While nickel is expected to have a relatively short half-life in the body, at

least for adult animals (OEHHA, 2001), a possible role for some form of continued exposure has not been eliminated by the study design employed by Saini et al. (2014b).

Saini et al. (2013) Embryotoxic and teratogenic effects of nickel in swiss albino mice during organogenetic period.

This study was conducted to evaluate the developmental effects of oral exposure to nickel during the organogenesis stage of gestation in Swiss albino mice (Saini et al. 2013). Doses were determined as fractions of the LD_{50} (369 mg Ni/kg). Treatment was given on each of GD6 – GD13, with examination of fetuses on GD18. Standard teratological evaluations were performed on uterine contents. Details of the protocol can be found in the table below.

Protocol elements:

Species and Strain	Swiss albino mice
Test compound (purity)	$NiCl_2.6H_2O$ (97%)
Route	Oral (method not stated)
Doses of Ni	0, 46.125, 92.25, 184.5 mg Ni/kg bw-day*
N pregnant animals/test group	10
Days of treatment (developmental stage)	GD 6-13 (organogenesis)

*For purposes of this document, doses are rounded to nearest whole mg in the text.

No maternal mortality was observed in any of the test groups. Compared to controls, maternal feed intake, water intake, and body weight were all significantly reduced ($p < 0.05$ or $p < 0.01$) at the two higher doses of 92 and 185 mg Ni/kg-day. At the high dose, mean maternal body weight was reduced to 58% of controls. No effects on any maternal measures were seen in the low dose group of 46 mg/kg-day. See Figure B.4 below for a graphical representation of these data.

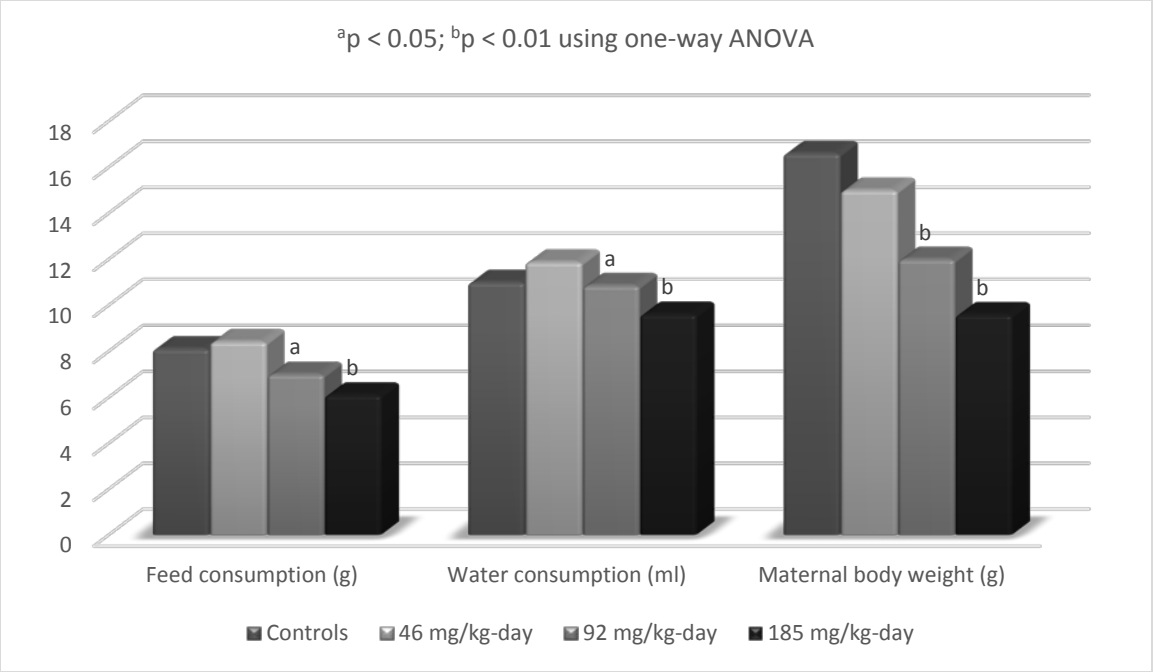

Figure B.4. Maternal Swiss albino mice treated with $NiCl_2 \cdot 6H_20$ on GD6-13

The paper is unclear on whether final maternal weights were taken with or without prior removal of gravid uteri. If the latter, reduced litter size could explain some or all of the difference between groups in maternal body weight.

Uterine contents were evaluated for the numbers of implantation sites, live fetuses, dead fetuses, and resorbed fetuses. Live fetuses were sexed, weighed, and examined for external evidence of altered morphology. Three quarters of the live fetuses were randomly selected for skeletal evaluations; the remainder were fixed for brain studies. Mean live litter size and mean fetal weights were significantly reduced ($p < 0.05$ or 0.01) in a dose-dependent manner with increasing doses of nickel (see Table B.39 below). Mean implantation sites per dam, sex ratio, and placental weights were among the litter outcomes not showing statistically significant differences between dose groups.

Table B.39. Litter outcomes

Dose (mg Ni/ kg-day)	Mean Implants/dam[1]	Mean Live Litter Size[1]	Mean Fetal Weight[2] (g)
0	8.00 ± 0.57	8.00 ± 0.57	1.35 ± 0.00
46	8.00 ± 0.57	7.66 ± 0.66	1.29 ± 0.018*
92	7.33 ± 0.33	6.66 ± 0.33	1.18 ± 0.022**
185	6.80 ± 1.49	4.40 ± 0.87**	0.82 ± 0.063**

*p < 0.05; **p < 0.01 by [1]Mann Whitney U-test or [2]one-way ANOVA

Points to note about litter outcome data include the following:

- As nickel exposure did not begin until GD6, treatment is unlikely to have an effect on implantation frequency.

- The frequencies of resorbed fetuses, dead fetuses, macerated fetuses, and post implantation death (not shown in Table B.39 above) were all reported as percentages of control values without statistical analysis or consideration of possible litter effects. Although not presented in a directly comparable manner, dose-related increases in the frequency of post implantation death, particularly resorption frequency, appear to generally correspond with decreases in mean live litter size.
- Mean live litter sizes were decreased in a dose-related manner, which reached statistical significance ($p < 0.01$) at the high dose of 185 mg Ni/kg-day.
- Mean fetal weights were significantly reduced ($p < 0.05$ or 0.01) relative to controls at all test doses, and in a dose-related manner. The fetal weight data do not appear to have been analyzed on a per litter basis.
- The lowest test dose of 46 mg/kg-day was associated with a significant reduction in mean fetal weight ($p < 0.05$) in the absence of a concurrent decrease in maternal body weight.

Data on external anomalies are presented as percentages of affected fetuses, without total numbers of fetuses examined (see Table B.40 below). The numbers of litters having affected fetuses are not presented, nor is it clear if individual fetuses had multiple malformations. Hence the reported anomalies may represent a very few litters with several malformed fetuses.

Table B.40. Fetuses with different anomalies after maternal exposure to nickel.

Anomaly (%)	46 mg Ni/kg-day[1]	92 mg Ni/kg-bw[1]	185 mg Ni/kg-bw[1]
Hydrocephaly	0%	5%	12.5%
Microcephaly	0%	5%	0%
Open eyelids	0%	10%	12.5%
Microphthalmia	5%	5%	6.25%
Exophthalmia	0%	5%	0%
Club foot	0%	0%	6.25%
Umbilical hernia	0%	5%	6.25%

[1]Control data have been omitted as no control fetuses were affected.

Data for skeletal anomalies were presented in the same manner as morphological anomalies. The frequencies of total skeletal anomalies for the groups given 0, 46, 92, or 185 mg Ni/kg-day were 0%, 22.7%, 35%, and 50%, respectively. Results of the “brain studies” mentioned in the paper’s methods are not discussed in any detail. One photograph shows a sagittal section of a hydrocephalic fetal brain; a second shows a transverse section through the orbits of a fetus with unilateral microphthalmia.

Berman and Rehnberg (1983) Fetotoxic effects of nickel in drinking water in mice.

The document is an unpublished study report from US EPA’s Health Effects Research Laboratory (Berman and Rehnberg 1983).

Protocol Elements:

Test compound (purity, if reported)	$NiCl_2$
Route	Oral, drinking water
Species and strain	Timed pregnant female CD-1 mice
Concentration (dose) of Ni	0, 500, or 1000 ppm (0, 80, or 160 mg/kg-day, estimated by authors)
N animals (N pregnant/pregnant + bred but not pregnant)	51/75 controls, 26/40 low concentration group, 7/34 high concentration group
Days of treatment	GD 2-17

Maternal toxicity was not evaluated directly, but was presumed for the 160 mg/kg-day group based on "the loss of mass in non-pregnant bred mice from the same high-dose group."

Pregnancy rates at sacrifice on GD 18 were reported as 68% for controls, 65% at the low dose of 80 mg/kg-day, and 21% at the high dose of 160 mg/kg-day. The decrease in pregnancy rate observed at the high dose was interpreted by the authors as "increased spontaneous abortions."

Uterine contents from pregnant animals were examined for numbers of live and dead or resorbed fetuses, and weights of individual fetuses. No statistically significant differences between treated and control groups were reported for the numbers live, dead, or total fetuses per litter. Fetal "mass" or weight per litter was reported as significantly decreased in litters exposed to 1000 ppm Ni ($p = 0.007$).

Seidenberg et al. (1986) Validation of an in vivo developmental toxicity screen in the mouse.

Nickel chloride was one of 55 compounds tested in a proposed screen for developmental toxicity (Seidenberg et al. 1986). A single dose level of 200 mg $NiCl_2$/kg-day was selected as producing minimal, but detectable, adult toxicity in range-finding studies of non-pregnant female mice.

Of 28 females treated by gavage daily on GD 8 – 12, one died during the course of the study. Surviving females gained an average of 3.2 g during treatment, which was significantly less than weight gain by control dams ($p < 0.01$). No significant differences from controls were noted in the frequencies of liveborn or fully resorbed litters. No significant differences between treated and control litters were found for mean litter size, numbers of dead pups, or mean pup weights on PND 1.

Gray and Kavlock (1984) An extended evaluation of an in vivo teratology screen utilizing postnatal growth and viability in the mouse.

Chernoff and Kavlock (1982) An in vivo teratology screen utilizing pregnant mice.

A large, *in vivo* screening study of multiple compounds included $NiCl_2$ ($\geq$ 97% pure). Initially reported in 1982 by Chernoff and Kavlock (Chernoff and Kavlock 1982), an extended report was later published by Gray and Kavlock (1984) (Gray and Kavlock 1984). Timed-pregnant CD-1 mice were treated with $NiCl_2$ on each of GDs 8-12, at an oral gavage dose of 100 mg/kg-day for comparison to untreated controls. Dams delivered normally, and pups were examined for viability and growth (body weight). On PND 6, four to 12 dams were randomly selected from the pool of treated animals, and given six pups (three females and three males) randomly selected from the pool of gestationally-exposed pups.

In the oral experiment, $NiCl_2$ exposure was not associated with any statistically-significant changes in pup viability or body weight on PND 3, 22, 30, or 57. At necropsy of male offspring on PND 250, body weight, and weights of liver, testes, seminal vesicles, and kidneys were unaffected by treatment.

B.2.3. Studies Conducted by the Inhalation Route of Exposure

Weischer et al., (1980) Effects of $NiCl_2$ and NiO in Wistar rats after oral uptake and inhalation exposure respectively

The study included an experiment on pregnant Wistar rats exposed to NiO aerosols at concentrations of 0, 0.8, 1.6, or 3.2 mg/m^3 (Weischer et al. 1980). Sperm-positive females were continuously exposed to treatment conditions throughout gestation until sacrifice for evaluation on GD 21. Each treated group consisted of 10 dams, with 13 air-exposed controls.

Gestational weight gain of maternal animals was significantly decreased at all three exposure levels of NiO ($p < 0.05$ at the lowest concentration). Fetal weights were significantly decreased at the two higher concentrations of NiO: 1.6 and 3.2 mg/m^3 ($p < 0.01$ in both cases). Fetal weight data do not appear to have been analyzed on a per litter basis.

Data were not provided on live litter size, or other objective measures of fetal viability. The text states:

> "Number of fetuses, and of placentas, wet weights of placentas, hemoglobin, hematocrit, erythrocytes, MCV, alkaline phosphatase in serum did not significantly differ from controls."

Based on a comment by Sunderman et al. (1968), that metabolism is similar for nickel entering the body by either the inhalation or parenteral route (Sunderman and Selin 1968), the authors suggest that their results for NiO by inhalation might be justifiably compared with other studies conducted by the parenteral route .

B.2.4. Studies Conducted by Injection Routes of Exposure

Intraperitoneal (ip) injection

Mas et al. (1985) The acute toxicity and teratogenicity of nickel in pregnant rats.

Timed-pregnant Wistar rats were given nickel chloride by ip injection. Doses of 1, 2, or 4 mg Ni/kg body weight were given on one of gestation days 8, 12, or 16. Results for N =12 control dams were compared to test groups of one to six litters (Mas et al. 1985).

Average fetal weights were found to be significantly ($p < 0.05$) reduced from control values for offspring of dams treated on GD12 with doses of 2 or 4 mg Ni/kg body weight. However, there were only three litters in each test group, and it is not clear if statistical analysis was conducted on a litter-mean basis.

Viability and malformation data are presented for individual fetuses, with no litter-based information. It is not clear if statistical analyses were performed. Cases of hydrocephalus, hydronephrosis, and hemorrhage appear to increase with dose following treatment on GD8. Small numbers of litters and the lack of litter-based statistics undermine any clear attribution of effects to treatment.

Chernoff and Kavlock (1982) An in vivo teratology screen utilizing pregnant mice

In addition to the oral exposure study described above, thirty additional pregnant females were given 30 mg/kg $NiCl_2$ by the ip route on GD 8. In the ip experiment, none of the 30 exposed maternal animals died during the course of the study, but only 12 animals were pregnant at term. Gestational weight change was significantly decreased in treated dams ($p < 0.05$). The number of live pups per litter was significantly reduced on PND 1 ($p < 0.05$), but pup weights were unchanged. On PND 3, neither mean live pups per litter nor mean pup weight differed significantly from controls.

Storeng, R. and J. Jonsen (1981) Nickel toxicity in early embryogenesis in mice.

Timed pregnant NMRI/Bom mice were given a single ip injection of $NiCl_2 \cdot 6H_2O$ on one of GDs 1, 2, 3, 4, 5, or 6 (Storeng and Jonsen 1981). The dose provided was 20 mg Ni_{2+}/kg body weight. Each day had its own treated and sham-injected control groups, with group sizes ranging from 23-29 pregnant females. Uterine contents were evaluated on GD 19.

The average number of fetuses/litter classified as “normal” was lower in treated than control groups for all test days; statistical significance was reported following treatment on GD 1, 3, and 5 ($p < 0.05$ or 0.01). Morphological anomalies were reported in all

treated, and some control, groups. Specific observations consisted of hematoma, exencephaly, and anemic appearance with “hypodevelopment.”

The mean number of implantation sites/dam was lower than controls ($p < 0.1$) for dams treated on GD 1. The highest overall frequency of abnormal plus “stillborn” (dead) fetuses was 4.3%, following treatment on GD 1. Total resorption frequency (early plus late resorptions) was significantly increased over controls following treatment on any of GDs 1-5 ($p < 0.005$), or on GD 6 ($p < 0.010$).

Mean fetal body weights appear to have been analyzed on a per group basis, rather than per litter. Average fetal weight per group was significantly reduced following treatment on any one of GD 1-4 or GD 6 ($p < 0.001$), but not GD 5.
The highest overall frequency of abnormal plus stillborn fetuses was 4.3%, following treatment on GD 1.

Lu et al. (1979) Teratogenic effects of nickel chloride and its transfer to embryonic mice.

Timed pregnant ICR mice were given a solution of $NiCl_2$ as an ip injection (Lu et al. 1979). Seven to 10 pregnant dams were give a single dose of 0, 1.2, 2.3, 3.5, 4.6, 5.7, or 6.9 mg Ni/kg on one of GDs 7 – 11. Pregnant dams were sacrificed on GD 18 for fetal evaluations.

Maternal and fetal effects were dependent upon both dose and day of treatment, with the greatest toxicity evidenced with exposure to higher doses at later gestation days. For example, 3/7 dams and no fetuses survived exposure to 6.9 mg Ni/kg given on GD 11. Increasing dose was associated with decreased fetal and placental weights, as well as increased frequencies of fetal death and skeletal anomalies.

Fetal data were not evaluated on a per litter basis, but for each entire treatment day/dose group. The frequency of fetal death reached 100% following exposure to 6.9 mg Ni/kg on any one of GDs 9, 10, or 11. Fetal death was also 100% at the lower dose of 5.7 mg Ni/kg on either of GDs 10 or 11. Significant reductions in fetal weight were seen at some doses after treatment on any of the treatment days; the lowest effective dose/day was 1.2 mg Ni/kg given on GD 10 ($p < 0.05$).

Intramuscular (im) injection

Sunderman et al. (1978) Embryotoxicity and fetal toxicity of nickel in rats.

Seven timed-pregnant Fischer rats were given a 16 mg Ni/kg bw as 0.4 ml $NiCl_2$ by im injection on GD 8 (Sunderman et al. 1978). Eight control dams were given 0.4 ml $NaCl_2$. At birth, the mean number of live pups per dam was reduced from 11.4 in controls to 7.9 for treated animals ($p < 0.001$). Body weights of prenatally-exposed male and female

pups were significantly lower than controls at 4, 6, and 8 weeks of postnatal life ($p < 0.01$ or 0.001).

In a second experiment, pregnant rats were again given $NiCl_2$ on GD 8. This time a range of doses were used, and fetuses were examined on GD 20. Results are summarized in Table B.41 below:

Table B.41. Effects Ni on Fetal Viability and Weight

	Control	**8 mg Ni/kg bw**	**12 mg Ni/kg bw**	**16 mg Ni/kg bw**
N	13	12	11	12
Live litter size	9.7 ± 1.1	8.9 ± 2.0	7.7 ± 1.9*	7.0 ± 3.9**
Fetal wt (g)	3.45 ± 0.26	3.43 ± 0.36	3.19 ± 0.43	3.03 ± 0.32*

*p < 0.01; **p < 0.05

It is worth noting that both litter size and mean fetal weight decrease with increasing dose, as there is typically an inverse relationship between these outcomes (US EPA 1991b).

In a third experiment, pregnant rats were given two daily i.m. injections of $NiCl_2$ on each of GDs 6, 7, 8, 9, and 10. Each injection contained 1.5 mg Ni/kg bw, for a total to each animal of 15 mg Ni/kg over the dosing period. An additional dose level of 2.0 mg Ni/kg/injection provided a total of 20 mg Ni/kg bw over the treatment period. The lower dose regimen was not associated with significant changes in fetal viability or weight. The higher dose resulted in decreased live litter size ($p < 0.05$), and no change in mean fetal weight.

In a fourth experiment, Ni_3S_2 was suspended in a penicillin solution and given to pregnant rats by i.m. injection on GD 6. Dosed animals were given 80 mg Ni/kg bw, while controls were give penicillin only. Dams were sacrificed for examination of uterine contents on GD 20. As in the previous (third) experiment, live litter size was significantly decreased in the Ni-exposed litters ($p < 0.01$), while fetal weights were not significantly affected.

In a fifth experiment, $NiCl_2$ to doses of 0, 6, 8, or 16 mg Ni/kg bw were given by i.m. injection on GD 18. Dams were sacrificed for evaluation on GD 19. No significant effects were seen on live litter size at any dose, although the ratio of dead fetuses to total conceptuses was significantly increased at the high dose ($p < 0.001$). According to the text of the paper, approximately half of treated dams died within 24 hours of injection with the high dose of 16 mg Ni/kg bw, and their litters were excluded from analysis.

Overall, the experiments demonstrated adverse effects on fetal viability and (in some cases) fetal weight, at doses of Ni not associated with maternal mortality.

Subcutaneous (sc) injection

Adjroud (2013) The toxic effects of nickel chloride on liver, erythropoiesis, and development in Wistar albino preimplanted rats can be reversed with selenium pretreatment.

In addition to the oral exposure study described above, timed-pregnant Wistar albino rats were randomly assigned to seven groups of six animals each (Adjroud 2013). Animals were given nickel in the form of $NiCl_2 \cdot 6H_2O$ (purity not stated) by either the oral route or sc injection. Doses of 0, 25, 50, or 100 mg $NiCl_2$/kg sc were given only on GD 3. The seventh group was given 0.3 mg/kg selenium sc concurrently with 100 mg/kg $NiCl_2$; this last experimental group will not be discussed further here. All maternal animals were sacrificed on GD 20, and uteri excised for fetal examination. Fetal viability was assessed and weights taken; there were no morphological evaluations.

Maternal findings
No maternal mortality was observed in any of the control or treated groups. Maternal body weights at term were significantly reduced by nickel exposure under either of the treatment regimens. As compared to controls, all doses of $NiCl_2$ given subcutaneously resulted in significant reductions in maternal weight on GD 5 and GD 20 ($p < 0.05$ or 0.01 in all cases).

Fetal findings
Subcutaneous $NiCl_2$ on GD 3 was not associated with changes in fetal weight on GD 20. Exposure to the highest subcutaneous dose of 100 mg/kg resulted in a mean live litter size of 3.2 $\pm$ 1.6 fetuses, reduced from 8.0 $\pm$ 1.8 in controls ($p < 0.01$). Mean fetal loss for the same group was 7.4 $\pm$ 1.7, compared to 2.2 $\pm$ 0.8 for controls ($p < 0.01$).

Intra-renal injection

Sunderman et al. (1983) Embryotoxicity and teratogenicity of nickel compounds.

The paper reports on three experiments conducted by the injection or inhalation routes of exposure (Sunderman et al. 1983). Two of these experiments used nickel carbonyl ($Ni(CO)_4$). As nickel carbonyl is already listed under Proposition 65 on the basis of developmental toxicity, those experiments will not be discussed further here, but are included under “Related Information” below.

In the third experiment, Ni_3S_2 was given to female rats by intrarenal injection at a dose of 30 mg Ni/kg bw (seven treated, and seven control) one week prior to breeding. This treatment of pre-mating female rats resulted in “intense” erythrocytosis.

Their pups were found to have reduced hematocrits at two-weeks postnatal age ($p < 0.001$). Hematocrit values normalized as older pups began to eat rat chow. Offspring of

treated dams had significantly reduced body weights at two ($p < 0.001$ for male and female pups) and four weeks ($p < 0.001$ for male pups, and $p < 0.01$ for female pups) postnatal age. These findings could indicate a postnatal developmental effect on offspring following pre-mating maternal exposure to a nickel compound.

Intravenous (iv) injection

Ferm (1972). The teratogenic effects of metals on mammalian embryos.

The review of metal teratogenicity by Ferm (1972) contains some original data on nickel, which do not appear to have been published elsewhere (Ferm 1972). Reporting of methods and results is brief and incomplete, but included here for the sake of completeness.

Pregnant golden hamsters were given “nickelous acetate” by iv injection on GD 8. There does not appear to have been a control group, but doses of 2, 5, 10, 20, 25, or 30 mg/kg test compound were given to groups of 2-6 pregnant hamsters. The day of evaluation was not specified.

No mention was made of maternal effects. Results were reported by group, not per litter, and no statistical analysis was performed. No embryos survived the top dose of 30 mg/kg. Nine out of 68 total embryos survived 25 mg/kg, compared to 24/24 survivors at 2 mg/kg.

B.2.5. Related Information

Nickel carbonyl

Nickel carbonyl ($Ni(CO_4)$) was added to the Proposition 65 list for developmental toxicity on September 1, 1996. Among other adverse effects, prenatal exposure to $Ni(CO_4)$ has been associated with ocular malformations that appear specific to this nickel compound (Sunderman et al., 1983; Sunderman et al., 1980; Sunderman et al., 1979).

Unlike other nickel compounds, $Ni(CO_4)$ has only poor solubility in water, and is relatively unstable and highly reactive – as well as highly toxic (ATSDR, 2005; OEHHA, 2012). Because of differences from other nickel compounds, including breakdown products of carbon monoxide (CO) as well as Ni, $Ni(CO_4)$ is often evaluated separately from other nickel compounds (US EPA, 1991a; ATSDR, 2005; OEHHA, 2001; OEHHA, 2012).

Sunderman et al. (1983) Embryotoxicity and teratogenicity of nickel compounds.

The paper reports on three experiments, two of which used $Ni(CO)_4$ administered by the injection or inhalation routes of exposure:

1. $Ni(CO)_4$ was given iv to pregnant Fischer rats (eight treated, and five control) on GD 7 to a dose of 11 mg Ni/kg bw.

2. Dominant lethal tests were performed on male rats given $Ni(CO)_4$ by inhalation (10 treated, and 10 control) at 0.05 mg Ni/L/15 min or by iv injection at 22 mg Ni/kg (eight treated, and 12 control).

The third experiment used Ni_3S_2, and has been described and discussed above.

The first experiment resulted in increased frequencies of dead fetuses/implants ($p < 0.01$), although the numbers of live fetuses/litter and dead fetuses/implants/litter were unaffected. Mean fetal weights/litter were significantly decreased ($p < 0.05$). The frequency of litters with malformed fetuses, and the overall frequency of malformed/live fetuses were both statistically significant ($p < 0.02$ and $p < 0.01$, respectively). Specific malformations included anopthalmia, micropthalmia, cystic lungs, and hydronephrosis.

Inhalation exposure of males at two to six weeks prior to breeding had no effect on fertility or offspring viability. Intravenous exposure on the same schedule resulted in reduced live litter size for litters sired during the fifth week following exposure ($p < 0.001$). This result is consistent with a dominant lethal effect: chromosomal damage occurring during the meiotic stage of spermatogenesis. Such an effect can be considered a manifestation of paternally mediated developmental toxicity.

Sunderman et al. (1980) Teratogenicity and embryotoxicity of nickel carbonyl in Syrian hamsters

Timed-pregnant Golden Syrian hamsters were exposed to vapors of $Ni(CO)_4$ (nickel carbonyl) at an atmospheric concentration of 8.6 ppm for a single 15-minute exposure on GD 5. Treated and control groups consisted of 14 pregnant dams. Litters were delivered normally and followed for viability at intervals until PND 65. Pup body weights were taken on PNDs 31 and 65. Statistically significant changes in treated versus control animals were:

- Decreased frequency of dams surviving until GD 16 ($p < 0.005$)
- Decreased live pups/litter on PND 4 ($p < 0.01$), 31, and 65 ($p < 0.05$ for both). No change in live pups/litter on the day of delivery.
- Increased mean pup body weights (not expressed per litter) for male and female pups on PND 31 ($p < 0.05$ for both sexes). No effect on pup weight on PND 65.

In another experiment, test groups of pregnant hamsters were exposed to the same dosing regimen as above, but restricted to one of GDs 4, 5, 6, 7, or 8. Controls were

sham-exposed on GD 6. Nine dams were assigned to the control group, with 12-33 dams in each of the treatment groups. All dams were sacrificed for evaluation on GD 15.

- Maternal survival to GD 15 was decreased for dams exposed on GDs 5, 6, 7, or 8 ($p < 0.01$), but not significantly affected by treatment on GD 4.
- Live litter size and mean fetal weights per litter did no show significant changes for any treatment day.
- The frequency of litters with malformed fetuses was significantly increased with treatment on GD 4 or 5 ($p < 0.05$ for both days), but not GD 6, 7, or 8. Specific malformations included exencephaly, cystic lung, anopthalmia, cleft palate, and fused rib.

Sunderman et al. (1979) Eye malformations in rats: induction by prenatal exposure to nickel carbonyl

In one experiment, timed-pregnant rats were exposed to 0.03 mg $Ni(CO_4)$/L/15 minutes on GD 7. Litters were carried to term, delivered, and nursed by their dams. Control and treated groups consisted of 8-9 litters.

Following treatment on GD 7:

- Live litter size (at birth) was significantly decreased in exposed group ($p < 0.001$).
- Body weights of male and female pups were significantly decreased at four and 16 weeks postnatal age ($p < 0.001$ in all cases for treated groups).
- The frequency of litters with live, malformed pups (at birth) was significantly increased with treatment ($p < 0.01$).
- Twenty-eight percent of treated offspring had ocular malformations. Bilateral or unilateral micropthalmia and anopthalmia were frequently observed, with some pups having both conditions.

In a second experiment, groups of 12-22 dams were exposed to 0.16 or 0.30 mg $Ni(CO_4)$/L/15 minutes on GD 7; to 0.08 or 0.16 mg $Ni(CO_4)$/L/15 minutes on GD 8; or to 0.16 mg $Ni(CO_4)$/L/15 minutes on GD 9. Control dams were either sham treated, or exposed to 0.5 percent carbon monoxide. All animals were sacrificed for evaluation on GD 20.

- Live litter size did not significantly differ between controls and any of the treated groups.

- Mean fetal weights of all fetuses (not on a per litter basis) were significantly reduced ($p < 0.01$) in all $Ni(CO_4)$-exposed groups, excepting no effect was found with 0.08 mg/L on GD 8.
- Exposure to 0.16 or 0.30 mg $Ni(CO_4)$/L/15 minutes on GD 7 was associated with significantly increased total frequency of malformations ($p < 0.001$). Bilateral or unilateral micropthalmia and anopthalmia were frequently observed.
- Increased total malformations ($p < 0.001$), primarily ocular, were also observed with 0.16 mg $Ni(CO_4)$/L/15 exposure on GD 8.

The authors concluded that the findings of ocular defects are specific to $Ni(CO_4)$, rather than an effect of nickel compounds in general.

Mixtures including nickel

George et al. (1990). Mixed chemicals (MIX): reproduction and fertility assessment in Swiss (CD-1) mice when administered in the drinking water: final study report, volume I of II.

A reproductive assessment by continuous breeding (RACB) study conducted by the National Toxicology Program (NTP) was published in 1990 (George et al. 1990). This study discussed in further detail in section C.2.1. in *Related Study* was conducted in CD-1 Swiss mice with a complex drinking water mixture (MIX) containing 25 chemicals including Ni which was designed to simulate groundwater near hazardous waste dumps. The low dose (1%) mixture contained 0.68 ppm Ni, the medium dose (5%) contained 3.4 ppm Ni, and the high dose (10%) mixture contained 6.8 ppm Ni. The concentrations of Ni given by the study author were as a percentage of a technically achievable stock solution. Minimal effects on developmental endpoints were shown as a result of treating mice. A dose-dependent decrease in the number of female pups per litter and adjusted live pup weight at 10% MIX indicated developmental effects at that dose. Also, a small but significant decrease in adjusted live pup weight at the high dose indicated developmental toxicity in the F1 generation.

Specific conclusions on the developmental effects of Ni from the RACB MIX study are difficult to discern due to the complex treatment mixture.

B.3. Integrative Evaluation of Developmental Toxicity

B.3.1. Developmental Toxicity in Humans

Spontaneous Abortion

Two studies reported analyses of effects of occupational Ni exposure on spontaneous abortion (SA) in Russia. Chashschin et al. (1994) report that the RR of SA was 1.8 in Ni-exposed female workers, but the study does not distinguish between exposure to Ni and other potentially hazardous exposures involved in Ni refinery work. Vaktskjold et al. (2008) report on a larger birth registry and questionnaire-based occupational study that used Ni exposure categories based on historical occupational exposure assessment, and found modest, non-significant increased odds of SA associated with Ni exposure for both data sources. Other exposures (including metals, e.g., in the Cu refinery) were not included in analyses.

Fetal growth

Table B.42 provides a broad overview of the ten studies that examined the effects of exposure to Ni on fetal growth parameters, including birth weight, risk or odds of LBW, BMIC, head circumference, and SGA. Authors studied exposures via air and soil and by occupation, and measured Ni in blood, urine, and placenta. Some studies were focused on Ni specifically, while others analyzed effects of numerous chemicals. Five studies that investigated the association between inhalation exposure to Ni and low birth weight are presented in Figure B.5.

Three fetal growth studies examined associations with personal biologic measurements. The studies by Odland et al. (1999) and Hu et al. (2015) had small sample sizes and although Hu et al. (2015) did report positive associations between Ni in maternal and cord blood and birth weight, both studies found no statistically significant associations between birth weight and Ni exposure. The study by Odland et al. (2004) had a larger sample and found that of the various elements examined, only Ni in placenta was associated with reduced birth weight, though this was not significant after adjustment for gestational age. Placental Ni was also weakly associated with lower BMIC; this association was not robust to adjustment for potential confounders.

The four studies of air pollution all examined multiple pollutants, including Ni, and were conducted in the past decade. All of the air studies analyzed Ni as $PM_{2.5}$, and Ni $PM_{0.1}$ and Ni PM_{10} were also examined in some studies; outcomes were birth weight, LBW, and head circumference. None of these studies could separate the potential effects of Ni from those of other pollutants due to correlation among pollutants. However, the studies were large and all reported small associations between Ni particles in air and lower birth weight, reduced head circumference, or increased risk of LBW.

One study, by McDermott et al. (2014), found no association between LBW and Ni concentrations in soil.

Table B.42. Overview of studies of Ni and fetal growth

Study	Exposure Assessment	Ni Levels	Result
Odland et al. (1999)	Maternal and newborn urine	Mothers Russia: 85 nmol/L, Norway: 5 nmol/L Infants Rus:34 nmol/L, Nor: 5 nmol/L	No associations Small sample
Odland et al. (2004)	Placenta, maternal and neonatal urine	Ni levels, Median (range), including Russian and Norwegian groups: Maternal urine 47.0 (4.3-2108) nmol/L Neonatal urine 18.7 (4.3-561) nmol/L Placenta Ni 0.017 (0.005-0.377) µg/g	Estimates are adjusted for country Placental Ni was associated with weight change (CI) -2526 (-4659, -394) g/µg/g; adjusted for gestational age: -1510 (-3191, 170) g/µg/g. Association between placental Ni and BMIC was -4.90 (-10.27, 0.47) per µg/g; adjusted for gestational age: -2.73 (-7.49, 2.02)
Vaktskjold et al. (2007)	Occupation category (based on historical urine and air measurements)		Unadjusted OR for SGA per unit increase in exposure category was 0.79 (0.68 – 0.91); AOR was 0.84 (0.75 – 0.93).
Bell et al. (2010)	Air ($PM_{2.5}$)	$PM_{2.5}$ Ni gestational mean (SD): 0.0031 (0.0015) µg/m^3	IQR increase in Ni $PM_{2.5}$ was associated with: Δ BW -7 (-12 – -3) g, and Δ risk of LBW 11 (3 – 19)%
Ebisu and Bell (2012)	Air ($PM_{2.5}$)	$PM_{2.5}$ Ni mean (SD) 0.006 (0.006) µg/m^3, IQR= 0.0071.	Ni was highly correlated (r > 0.5) w/ V, Zn, NO_2, O_3, SO_2. Adjusted odds of LBW was 5.7% (2.7 – 8.8%) higher per IQR increase in $PM_{2.5}$ Ni (not adjusted for co-pollutants).

Basu et al. (2014)	Air ($PM_{2.5}$)	$PM_{2.5}$ Ni mean (SD) 0.0033 (0.0040) $\mu g/m^3$, IQR=0.001 $\mu g/m^3$	IQR increase in $PM_{2.5}$ Ni was associated with Δ -1g BW, Δ 1% odds of LBW
McDermott et al. (2014)	Soil	Kriged Ni conc. in soil: 4.58 mg/kg for LBW, 4.57 mg/kg for normal weight births; IQR 43.21 mg/kg	Unadjusted OR (CI) for LBW and an IQR increase in Ni was 1.00 (0.98, 1.02).
Laurent et al. (2014)	Air (UCD_P predictions for $PM_{2.5}$ and $PM_{0.1}$)	$PM_{2.5}$ Ni mean (SD) 0.0030 (0.0026) $\mu g/m^3$ $PM_{0.1}$ Ni mean (SD) 0.0004 (0.0004) $\mu g/m^3$	ORs for LBW and IQR increase in exposure over the entire pregnancy $PM_{2.5}$ Ni 1.009 (1.003, 1.015) $PM_{0.1}$ Ni 1.009 (1.004, 1.014)
Hu et al. (2015)	UCB and maternal blood	UCB median 0.9 ng/g Maternal blood median 1.4 ng/g	Maternal blood Ni and BW Beta (CI)=45.6 (-17.2 – 108.4) UCB Ni and BW Beta (CI)=32.2 (-19.8 – 84.1)
Pedersen et al. (2016)	Air ($PM_{2.5}$ and PM_{10})	$PM_{2.5}$ Ni mean (SD) 1.6 (0.8) ng/m^3 PM_{10} Ni mean (SD) 1.8 (1.2) ng/m^3	AORs for LBW and Ni $PM_{2.5}$: 1.14 (1.00, 1.29) Ni PM_{10}: 1.29 (0.96, 1.75) in single pollutant models; adjustment for S and particle mass attenuated these effects. Adjusted associations (β, 95% CI) between head circumference (cm) and Ni $PM_{2.5}$: -0.60 (-0.71, -0.49) Ni PM_{10}: -0.46 (-0.57, -0.36); adjustment for S and particle mass attenuated these effects.

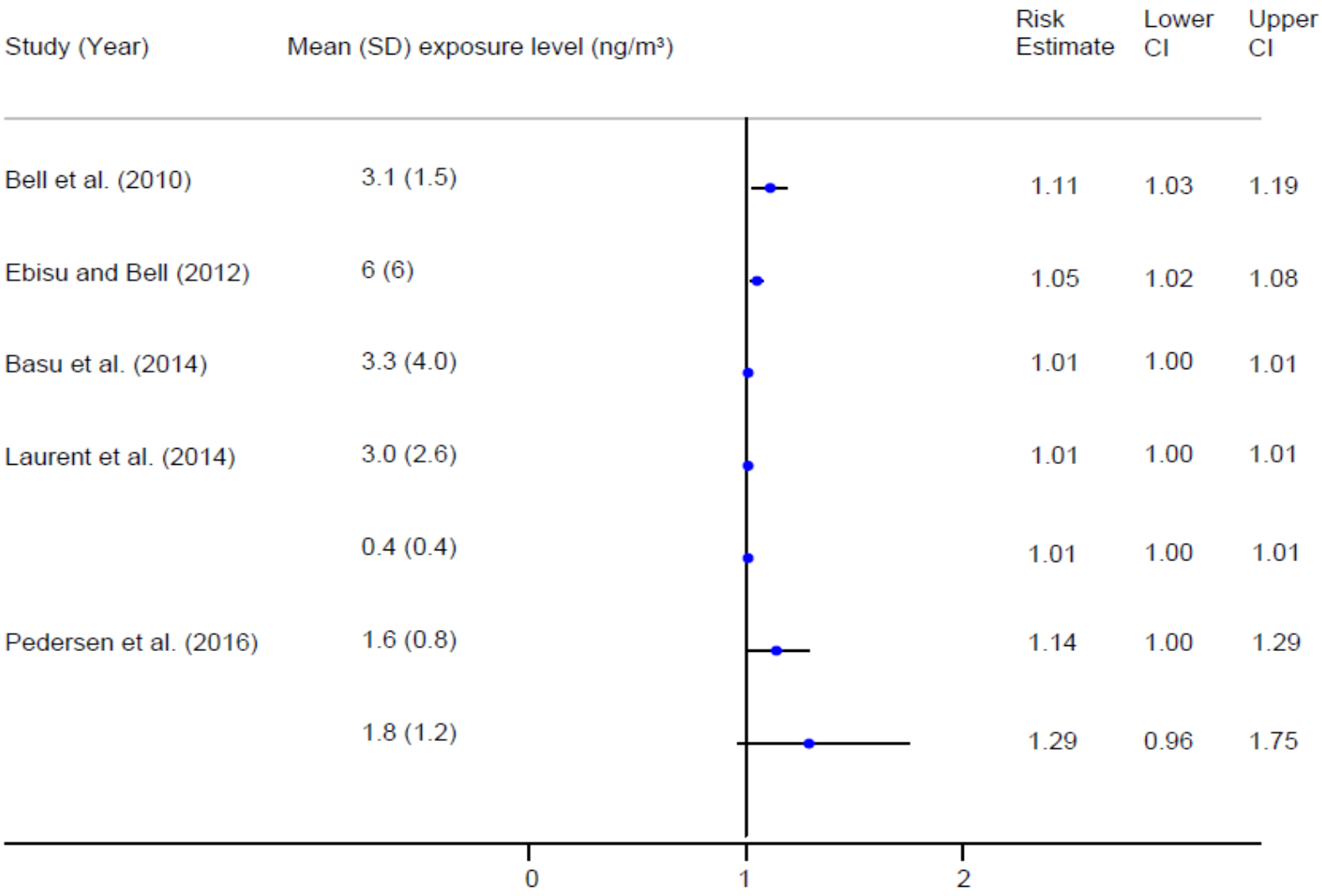

Figure B.5. Forest plot of the association between Ni exposure and Low Birth Weight. Confidence intervals (95%) are denoted by "CI".

Congenital malformations

As shown in Table B.43, seven studies examined associations between exposure to Ni and birth defects, including an autopsy study of fetuses that found no association between anencephaly and Ni in fetal tissues. There were three occupational studies among Russian Ni workers; one reported increased risk of structural malformations among offspring of Ni hydrometallurgy workers, and others reported non-significant reductions in risk of genital malformations or no difference for musculoskeletal defects associated with Ni work. The authors acknowledge that assessing incidence of malformations would require inclusion of all conceptuses and thus consideration of fetal loss, and that Ni exposure may appear protective due to increased risk of fetal loss. In addition, “background” Ni levels in this area may be so high that the “unexposed” are actually highly exposed to Ni relative to other locations.

Two studies examined Ni in soil as a risk factor in Shanxi province, a coal-mining region which the authors report has one of the highest incidences of birth defects in the world, and the highest prevalence of NTDs in China. Zheng et al. (2012) examined heavy metal levels in samples from soil used for food cultivation in each village in the province, and report that higher levels of soil Ni were associated with statistically significant decreased risk of birth defects in this study. Huang et al. (2011) modeled the association between risk of NTDs and 12 trace elements in cultivated soil, and report that Ni had “layered level effects” on the occurrence of NTDs, showing that “both deficiencies in and excessive amounts of nickel cause an increased risk of neural tube defects.”

Finally, Manduca et al. (2014) examined metal concentrations in hair of newborns whose parents might have been exposed to white phosphorus ammunition in military attacks. No differences in Ni levels were reported for normal children compared with those born with defects.

Table B.43. Overview of studies of Ni and congenital malformations

Study	Exposure Assessment	Ni Levels	Result
Chashschin et al. (1994)	Air (Ni sulfate aerosols) in Ni hydrometallurgy work Urine	Urine Ni, mean (range), µg/l Electrolysis operators: 15.6 (5.2 – 22.6) Purification operators: 10.4 (3.2 – 18.0)	RRs for Ni hydrometallurgy vs. other work: All structural malformations* 2.9 Cardiovascular defects 6.1 Musculoskeletal defects 1.9 *Reportedly significant
Friel et al. (2005)	Fetal liver, kidney, sciatic nerve, pancreas, muscle	Range: 1.6 (liver) – 36 (sciatic nerve) ppm	No differences between anencephalic and control fetuses
Vaktskjold et al. (2006) Vaktskjold et al. (2008b)	No measurement of individual subjects' exposure. Categorical exposure was based on occupation.	Categories of urine Ni, µg/L Background <10 Low 10 to <~70 High ≥ ~70	ORs (CI) for genital malformations Low (vs. background) exposure 0.71 (0.31 – 1.64) High exposure 0.72 (0.26 – 1.95). OR (CI) for undescended testes 0.76 (0.40 – 1.47). OR (CI) for musculoskeletal defect and unit increase in Ni exposure category 0.96 (0.76 – 1.21)
Huang et al. (2011)	Soil samples from each village	Soil Ni, µg/g Mean (SD) 41.38 (6.39)	Authors report "layered level effects" of Ni levels on prevalence of NTDs: <30 µg/g Ni (high NTD prevalence) 30-34 µg/g Ni (low prevalence) >34 µg/g (medium prevalence) Statistical significance not reported
Zheng et al. (2012)	Samples of soil used for food cultivation in each village	Soil Ni, mg/kg Mean (SD) 41.73 (6.67)	Higher concentrations of Ni in soil were associated with lower risk of birth defects.
Manduca et al. (2014)	Newborn hair	Not reported	No differences between birth defects cases and normal births

ASD

Three US case-control studies examined associations between ASD and air pollutants, including Ni. All of these studies used US EPA NATA estimates for exposure data, and were subject to many of the same exposure misclassification concerns: ambient air data do not necessarily reflect actual personal exposures, women may have relocated shortly before the birth, mismatch between NATA year and gestation or birth year. Each study examined multiple pollutants, many of which are highly correlated. False positives with multiple measurements is also a concern, although Kalkbrenner et al. in particular tried to address this. Table B.44 provides an overview of some of the distinctions among the ASD studies. The studies are also presented graphically in

Figure B.6. Roberts et al. (2013) and Windham et al. (2006) compared ASD cases with general population controls, and Kalkbrenner et al. (2010) selected controls with speech and language impairment. The Ni concentrations appear to be lower in the study by Kalkbrenner et al.

Table B.44. Overview of case-control studies of ASD and prenatal Ni ambient air levels

Study	Study sample	Ni concentration, ng/m^3	Result
Windham et al. (2006)	ASD diagnosed by age 9, n=284 Controls from general population, n=657	Mean (SD) Cases: 4.3 (5.9) Controls: 3.7 (3.8)	AOR (95% CI) for autism and Ni exposure in single chemical models, compared to 1st quartile Ni conc.: 3rd quartile 1.11 (0.77, 1.59) 4th quartile 1.46 (1.04, 2.06)
Kalkbrenner et al. (2010)	ASD identified at 8 yrs, n=374 Controls from same registry had speech and language impairment, n=2,829	Geometric mean (geo. SD) NC All: 1.1 (2.0) Urban: 1.9 (1.6) Not urban: 0.8 (1.9) WV All: 0.2 (6.3) Urban: 1.5 (7.4) Not urban: 0.1 (4.9)	OR (CI) for 1.7 vs. 0.1 ng/m^3 (80th vs. 20th percentile Ni conc.): All 1.1 (0.6, 1.9) 100% rural 1.2 (0.4, 3.4) Mixed 0.9 (0.4, 2.0) 100% urban 1.8 (0.6, 4.9) NC 0.8 (0.2, 3.3) WV 0.9 (0.4, 1.8)
Roberts et al. (2013)	ASD diagnosed by 2005 and birth after 1987, n=325 Controls from general population, born 1987-2002, n=22,098	Quintiles 1st 0.4 2nd 1.2 3rd 2.4 4th 4.5 5th 15.9	AORs (CI) for ASD by quintiles of Ni conc. All / Boys only 1 (Ref) / (Ref) 2 1.3 (0.9, 1.9) / 1.4 (0.9, 2.0) 3 1.6 (1.1, 2.2) / 1.6 (1.1, 2.2) 4 1.5 (1.0, 2.2) / 1.7 (1.1, 2.6) 5 1.7 (1.1, 2.5) / 1.9 (1.2, 2.9)

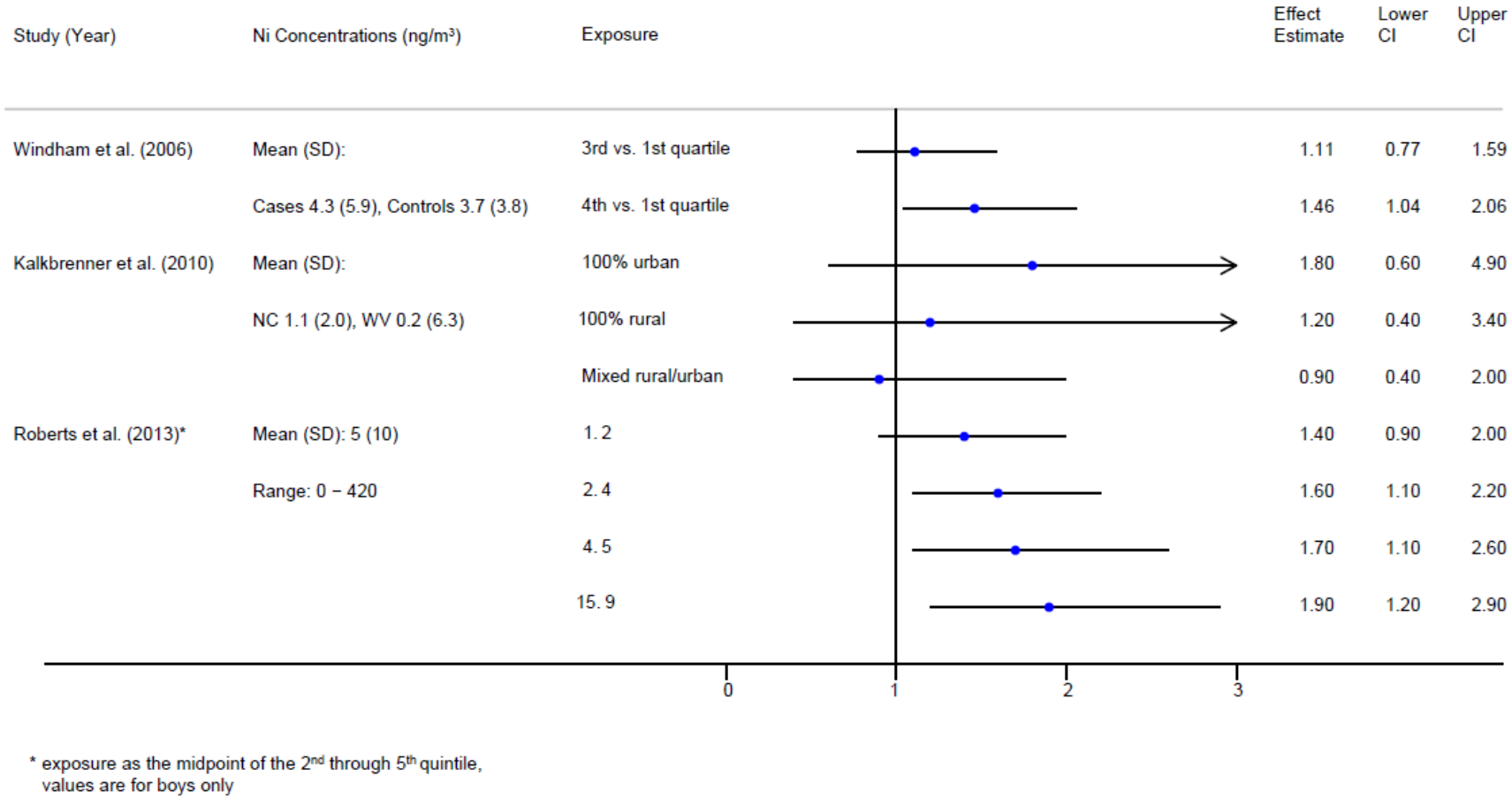

Figure B.6. Forest plot of the association between Ni exposure (ambient air concentration) and autism spectrum disorders. Confidence intervals (95%) are denoted by "CI". Exposures are to Ni $PM_{2.5}$ except: Laurent et al. (2014) - the 2nd OR and CI are for Ni $PM_{0.1}$; and Pedersen et al. (2016) - the 2nd OR and CI are for Ni PM_{10}

Other Developmental Outcomes

Three studies examined exposure to Ni in association with preterm birth, among other outcomes. The number of preterm births in each of these studies was small. Zheng et al. (2014) examined preterm birth, fetal distress, macrosomia, and a group of adverse outcomes in a study where metals were measured in UCB and each outcome was represented by few deliveries (e.g., n=18 premature infants), and reported no associations for Ni. Manduca et al. (2014) reported no differences in Ni levels for 12 normal children compared with nine premature children. Chashschin et al. (1994) included prematurity among the pregnancy complications they examined in association with Ni refinery work, and report that differences between observed and expected values for prematurity were statistically significant, though neither p-values nor confidence intervals were provided.

Other adverse developmental outcomes, including neuroblastoma, retinoblastoma, and oxidative damage to DNA were the subject of one study each. Heck et al. (2013) examined air pollution in the neuroblastoma study, and found no evidence of elevated risk for neuroblastoma associated with Ni exposure (Heck et al. 2013). Heck et al. (2015) also examined air pollution in the retinoblastoma study, and found that perinatal exposure to Ni was associated with increased risk of later retinoblastoma diagnosis (Heck et al. 2013).

Ni et al. (2014) compared levels of heavy metals in UCB of infants born in areas with vs. without e-waste recycling in China in their study examining 8-OHdG concentration as a marker of oxidative damage to DNA. Although UCB Ni levels were higher in the area without e-waste recycling, Ni was associated with 8-OHdG concentration, adjusted for other heavy metals.

B.3.2. Developmental Toxicity in Animals

To provide an overview of the available studies on developmental toxicity in experimental animals, summary tables are provided below. These tables are focused on the most sensitive outcomes seen across studies, but do not necessarily include all outcomes reported at effective doses.

Most of the studies employed an oral route of exposure, and were conducted in either rats or mice (Table B.46 and B.47). The oral route of exposure is generally preferred for developmental toxicity studies (US EPA, 1991b and 1998a), because it is a relevant route for human exposure. While injection is not a preferred route, data from various types of injection studies in rats and mice are presented in Table B.48.

Only one study, conducted in rats, reported on the effects of Ni exposure by inhalation (Weischer et al. 1980). Statistically significant adverse effects were found for maternal gestational weight gain and fetal weights. Data were not presented for any objective

measure of fetal viability. Because there was only a single relevant inhalation study, no summary table has been prepared.

To facilitate comparisons across studies in the summary tables, doses have been expressed as mg Ni/kg-bw (Table B.45). Many of the studies cited reported this information, as well as the identity of the specific nickel compound used. For some other studies, doses had to be calculated based on data provided in the study; for still other studies, it was necessary to estimate doses according to standardized assumptions.

Table B.45. Nickel Content of Nickel Compounds Used in Animal Studies[1]

	Nickel	**Nickel sulfate**	**Nickel sulfate hexahydrate**	**Nickel chloride**
Formula	Ni	$NiSO_4$	$NiSO_4 \cdot 6H_2O$	$NiCl_2$
Mw	58.69	154.75	262.85	129.6
% Ni	100%	38%	22.3%	45%

[1]Presents Ni content only for compounds used in studies that required dose conversion.

Where data on body weights and water or feed consumption were not available from the study report we used standardized factors from US EPA (1998b)

Reference Body Weights (kg):

- For mature, female Sprague-Dawley rats – 0.35 kg
- For mature, female Wistar rats – 0.32 kg

Reference Water Consumption (L/day) for use with Reference Body Weights:

- For mature, female Sprague-Dawley rats – 0.046 L/day
- For mature, female Wistar rats – 0.043 L/day

Reference Feed Consumption (kg/day):

- For mature, female Sprague-Dawley rats – 0.028 kg/day
- For mature, female Wistar rats – 0.026 kg/day

A few studies that were summarized in the body of this document were omitted from summary tables of results, specifically:

- RTI 1987, as described by US EPA, 1991, is not in the summary tables because we do not have the original study report.
- Ambrose (1976) was omitted from the summary tables due to the study's poor reporting of methods and results, such as lack of feed consumption and birthweight data, and presenting pup viability data on a total group, rather than per litter basis.
- Schroeder and Mitchener (1971) was omitted from the summary tables because the nickel compound used in the study was not identified.

- Ferm (1972) was omitted from the summary tables because of the study's poor reporting of methods and results.

Table B.46. Summary of Data on Oral Exposure to Nickel Compounds in Rats

Reference	Compound (method of admin)	Dose: mg Ni/kg-day (N)	Treatment day(s)	Evaluation day	Maternal Systemic effects*	Offspring effects*
Adjroud, 2013	$NiCl_2 \cdot 6H_2O$ (drinking water)	0 (6) 1.3 (6)	GD 3-19	GD 20	↓ bw on GD 20	None reported (only evaluated for viability and bw)
(Siglin 2000a)	$NiSO_4 \cdot 6H_2O$ (gavage)	0 (8) 2.2 (8) 4.5 (8) 6.7 (8) 11.2 (8) 16.7 (8)	Pre-mating through gestation and lactation	PND 0	No effect on bw or wt gain	↑ number total dead pups at all doses except 11.16 mg/kg-day ↓ mean live litter size at 16.74 mg/kg-day No effects on PND 1 pup weight
(Siglin 2000b)	$NiSO_4 \cdot 6H_2O$ (gavage)	0 (28) 0.2 (28) 0.6 (28) 1.1 (28) 2.2 (28)	Pre-mating of F0 through production of F1 and F2 generations	PND 0	None reported	None reported
Kakela et al., 1999	$NiCl_2 \cdot 6H_2O$ (drinking water)	0 (6) 1.46 (6) 4.38 (6) 15 (6)	Premating through gestation and lactation	PND 0	Not noted	No effect on gestation index at birth Birthweight not reported
Smith et al., 1993	$NiCl_2 \cdot 6H_2O$ (drinking water)	0 (34) 1.3 (34) 6.8 (34) 31.6 (34)	Pre-mating through gestation and lactation for 2 sequential litters	PND 1	↓ wt gain at 6.8 and 31.6 mg/kg-day during G1 (but not G2)	↑ % dead pups/litter at all doses (marginal significance at 6.8 mg/kg-day) in G2 litters; only at 31.6 mg/kg-day for G1 litters ↓ female pup birth weight at 1.3 mg/kg-day in G1 litter (marginal significance)

Price et al., 1988	$NiCl_2$ (drinking water)	0 (21) 6.26 (28) 24.69 (27) 45.42 (16)	F1b dams, preconception through production of F2a litters	PND 1	↓ maternal wt on GD 20 at 45.42 mg/kg-day	↓ live pups/litter and mean pup wt/litter at 45.42 mg/kg-day
		0 (18) 5.67 (22) 22.24 (22) 41.82 (16)	F1b dams, preconception through production of F2b litters	GD 20	↓ maternal wt gain at 22.24 and 41.82 mg/kg-day	No effect on live litter size or mean fetal wt/litter

* Reported outcomes significantly differ from controls with p value < 0.05.

Table B.47. Summary of Data on Oral Exposure to Nickel Compounds in Mice

Reference	Compound (method of admin)	Dose: mg Ni/kg-day (N)	Treatment day(s)	Evaluation day	Maternal Systemic effects*	Offspring effects*
Saini et al., 2014a	$NiCl_2 \cdot 6H_20$ (not stated)	0 (10) 46 (10) 92 (10) 185 (10)	GD 0-5	GD 18	↓ bw at 92, 185 mg/kg-day	↓ live litter size at all doses ↓ fetal wt at 92, 185 mg/kg-day
Saini et al., 2014b	$NiCl_2 \cdot 6H_20$ (gavage)	0 (15) 46 (5) 92 (5) 185 (5)	GD 0-5	PND 0	Not noted	↓ live litter size at 92, 185 mg/kg-day ↓ pup wt at 185 mg/kg-day
		0 (15) 46 (5) 92 (5) 185 (5)	GD 6-13	PND 0	Not noted	↓ pup wt at all doses ↓ live litter size at 185 mg/kg-day
		0 (15) 46 (5) 92 (5) 185 (5)	GD 14-18	PND 0	Not noted	↓ pup wt at 92, 185 mg/kg-day ↓ live litter size at 185 mg/kg-day
Saini et al., 2013	$NiCl_2 \cdot 6H_20$ (not stated)	0 (10) 46 (10) 92 (10) 185 (10)	GD 6-13	GD-18	↓ bw at 92 and 185 mg/kg-day	↓ fetal wt at all doses ↓ live litter size at 185 mg/kg-day
Berman & Rehnberg, 1983	$NiCl_2$ (drinking water)	0 (51) 80 (26) 160 (7)	GD 2-17	GD 18	Not evaluated for pregnant dams	21% pregnancy rate at 160 mg/kg-day ↓ "fetal mass" at 160 mg/kg-day
Seidenberg et al., 1986	$NiCl_2$ (gavage)	0 (28) 200 (28)	GD 8-12	PND 1	1/28 died ↓ wt gain over treatment period	None noted
Gray & Kavlock, 1984; Chernoff & Kavlock, 1982	$NiCl_2$ (gavage)	0 (23-40) 100 (23-40)	GD 8-12	PND 3	Not noted	No effects on pup viability or bw

* Reported outcomes significantly differ from controls with p value < 0.05.

Table B.48. Summary of Data on Injection Exposure to Nickel Compounds in Mice and Rats

Reference (species)	Compound (method of admin)	Dose: mg Ni/kg-day (N)	Treatment day(s)	Evaluation day	Maternal Systemic effects*	Offspring effects*
Mas et al., 1985 (rats)	$NiCl_2$ (ip)	0 (12) 1 (1-6) 2 (1-6) 4 (1-6)	One of GDs 8, 12, or 16	GD 20	Not noted	↓ fetal wt at 2 or 4 mg/kg given on GD 12 No clear evidence of effects on fetal viability
Storeng and Jonsen, 1981 (mice)	$NiCl_2{\cdot}6H_2O$ (ip)	0 (23-29) 20 (23-29)	One of GDs 1, 2, 3, 4, 5, or 6	GD 19	Not noted	↑ frequency of resorptions following treatment on any of GDs 1-6 ↓ fetal wt with treatment on any of GDs 1-4 or 6
Lu et al., 1979 (mice)	$NiCl_2$ (i.m.)	0 (7-10) 1.2 (7-10) 2.3 (7-10) 3.5 (7-10) 4.6 (7-10) 5.7 (7-10) 6.9 (7-10)	One of GDs 7, 8, 9, 10, or 11	GD 18	Some maternal deaths, most notably 4/7 dams following 6.9 mg/kg on GD 11	↑ frequency of fetal death*; 100% with 6.9 mg/kg on GD 9, 10, or 11, and 5.7 mg/kg on GD 10 or 11 ↓ fetal wt** seen at higher doses on all days, lowest effective dose/day 1.2 mg/kg on GD 10
Sunderman et al., 1978 (rats)	$NiCl_2$ (i.m.)	0 (8) 16 (7)	GD 8	PND 0	Not noted	↓ Live litter size ↓ postnatal growth
		0 (13) 8 (12) 12 (11) 16 (12)	GD 8	GD 20	Not noted	↓ Live litter size at 12 and 16 mg/kg ↓ Fetal wt at 16 mg/kg

		0 (12) 3 (12) 4 (12)	GD 6-10	GD 20	Not noted	↓ live litter size No effect on fetal wt
	Ni_3S_2 (i.m.)	0 (14) 80 (12)	GD 6	GD 20	None noted	↓ live litter size No effect on fetal wt
	$NiCl_2$	0 (12) 6 (10) 8 (12) 16 (15)	GD 18	GD 19	Death in 50% of dams given 16 mg/kg	No effect on live litter size ↑ ratio of dead fetuses to total conceptuses No effect on fetal wt
Sunderman et al., 1983 (rats)	Ni_3S_2 (intra-renal)	0 (7) 30 (7)	1 week prior to breeding	2 and 4 weeks postnatal	"intense" erythrocytosis	↓ hematocrit at 2 weeks postnatal age ↓ pup weights at 2 and 4 weeks postnatal age

* Reported outcomes significantly differ from controls with p value < 0.05.
**Evaluated on total group basis, not per litter.

Regardless of species or route, the most sensitive and commonly reported adverse effects of prenatal exposure to nickel were reductions in viability and offspring weight. Both dose of Ni and timing of exposure impacted occurrence and magnitude of effects. For example, Saini et al. (2014b) tested the same range of doses during the pre-implantation, organogenesis, and fetal stages of development in mice. The live birth index was affected only at the highest dose, and only following treatment during the preimplantation and organogenesis periods. Birthweights were observed to have been adversely affected at all doses with exposure during organogenesis, only at the two higher doses with exposure during the fetal period, and only at the highest dose with preimplantation exposure.

B.3.3. Integrative Evaluation of Human and Animal Developmental Toxicity

Most of the animal studies on the developmental toxicity of nickel compounds were performed by the oral route of exposure in rats or mice (Adjroud 2013; Ambrose et al. 1976; Berman and Rehnberg 1983; Chernoff and Kavlock 1982; Gray and Kavlock 1984; Kakela et al. 1999; Price et al. 1988; Saini et al. 2013; Saini et al. 2014a; Saini et al. 2014b; Schroeder and Mitchener 1971; Seidenberg et al. 1986; Siglin 2000a, 2000b; Smith et al. 1993; US EPA 1991a) RTI, 1987 as cited by US EPA, 1991a. Only one study exposed rats by inhalation (Weischer et al., 1980), while other studies employed

an injection route in treating rats or mice (Lu et al. 1979; Mas et al. 1985; Storeng and Jonsen 1981; Sunderman et al. 1978; Sunderman et al. 1983).

Regardless of species or route, the most sensitive and commonly reported adverse effects of prenatal exposure to nickel were reductions in viability and reductions in body weights of surviving offspring. Graphical summaries of these effects are provided in Figures B.7-B.12. Both dose of Ni and timing of exposure were observed to impact the frequency of occurrence and the severity of effects.

In integrating the data from animal developmental toxicity studies with findings from human epidemiology studies, it must be remembered that while adverse effects in animals are generally predictive of risk to human development, "…the types of developmental effects seen in animal studies are not necessarily the same as those that may be produced in humans" (US EPA, 1991b). However, five cohort studies of air pollution all reported small but statistically significant associations between exposure to Ni particles in ambient air and adverse effects on fetal growth parameters, such as lower birth weight, reduced head circumference, or increased risk of LBW. Two of three air pollution case-control studies also found higher risk of ASD in association with exposure to $PM_{2.5}$ Ni in air. The observations from studies of human populations for effects of Ni on spontaneous abortion, congenital defects, and preterm birth were inconsistent.

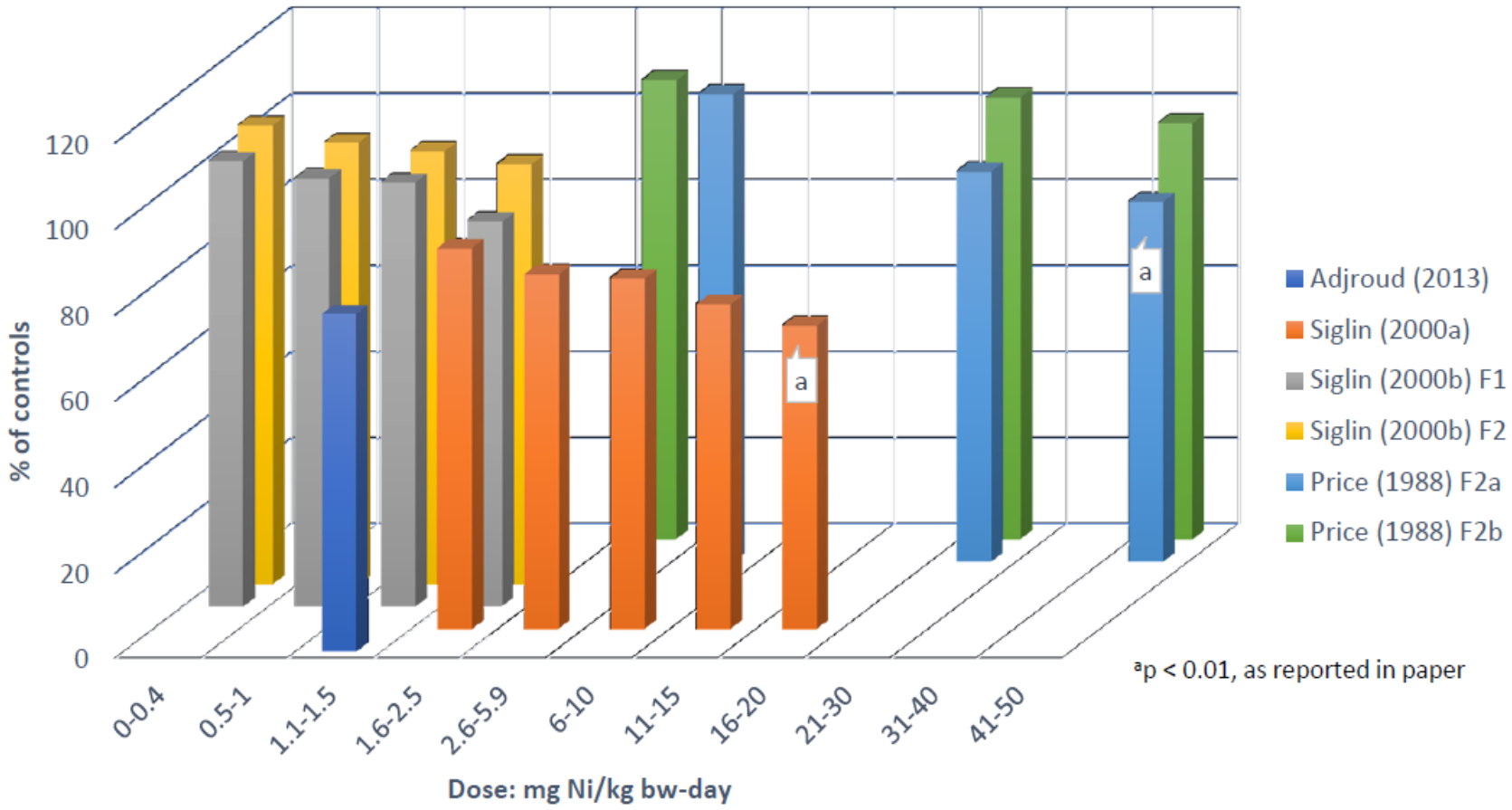

Figure B.7. Oral Exposure In Rats: Live Litter Size as % of Controls

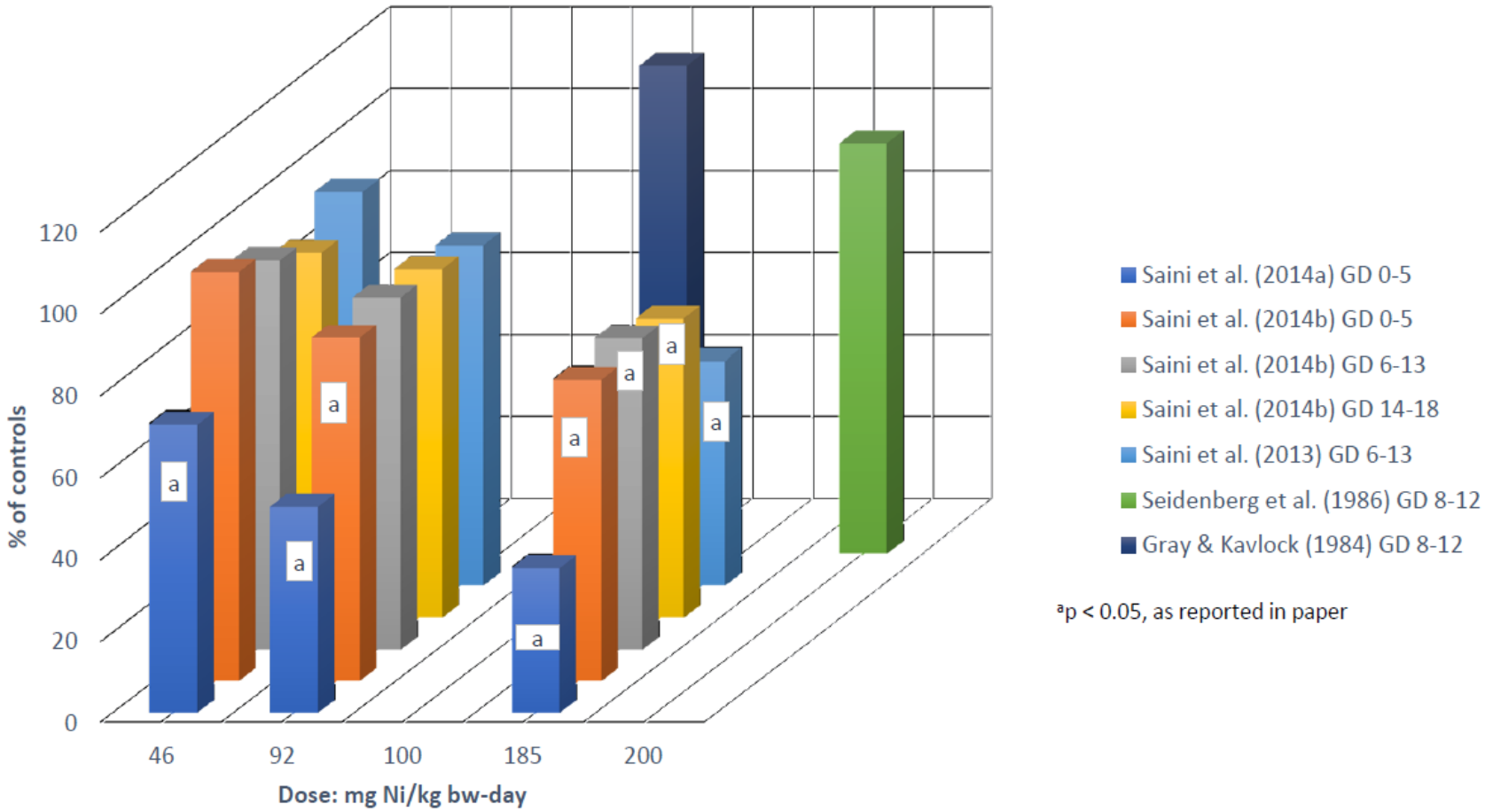

Figure B.8. Oral Exposure In Mice: Live Litter Size as % of Controls

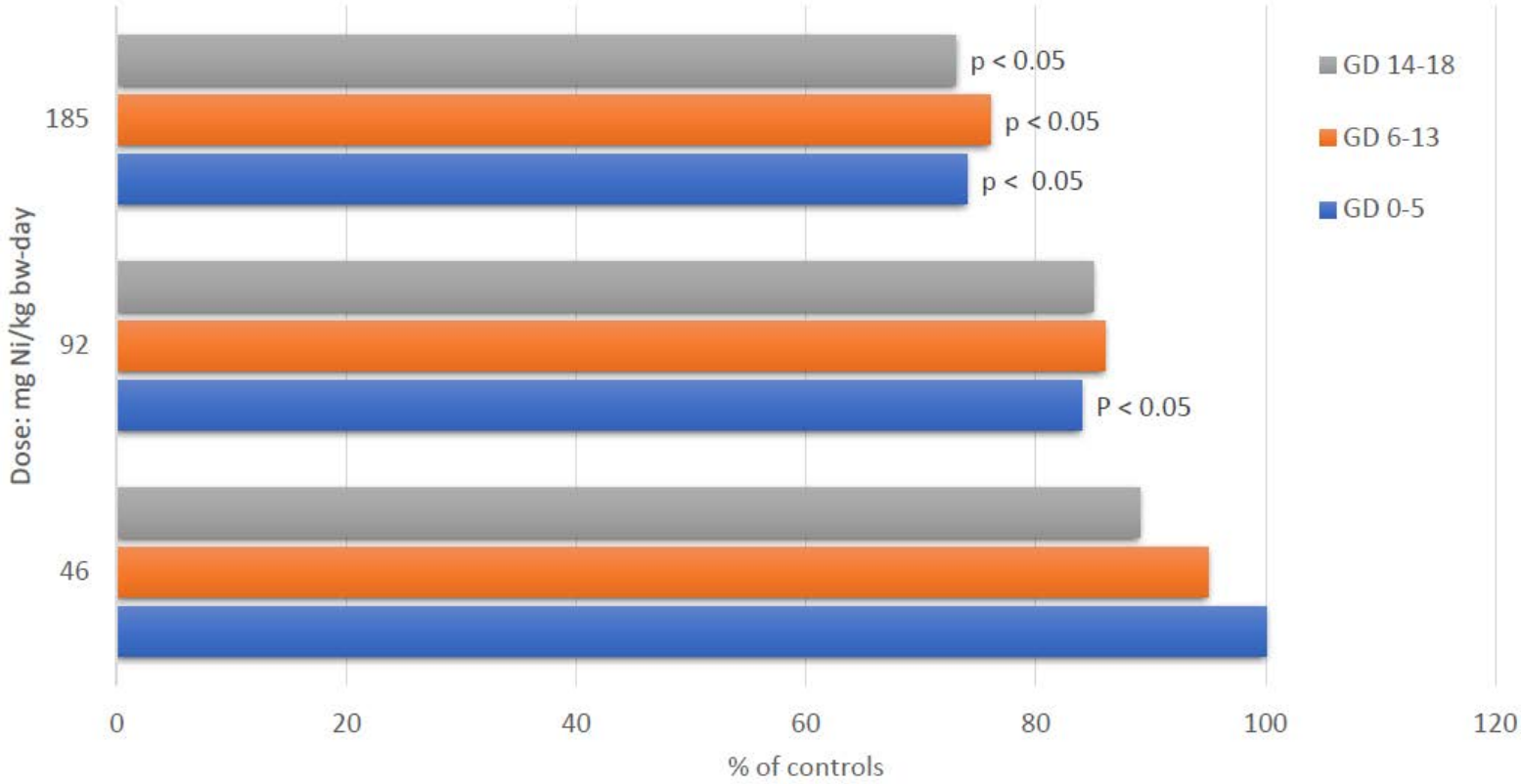

Figure B.9. Oral Exposure In Mice: Live Litter Size as % of Controls.
Saini et al (2014b) Exposure on: GD 0-5, 6-13, or 14-18

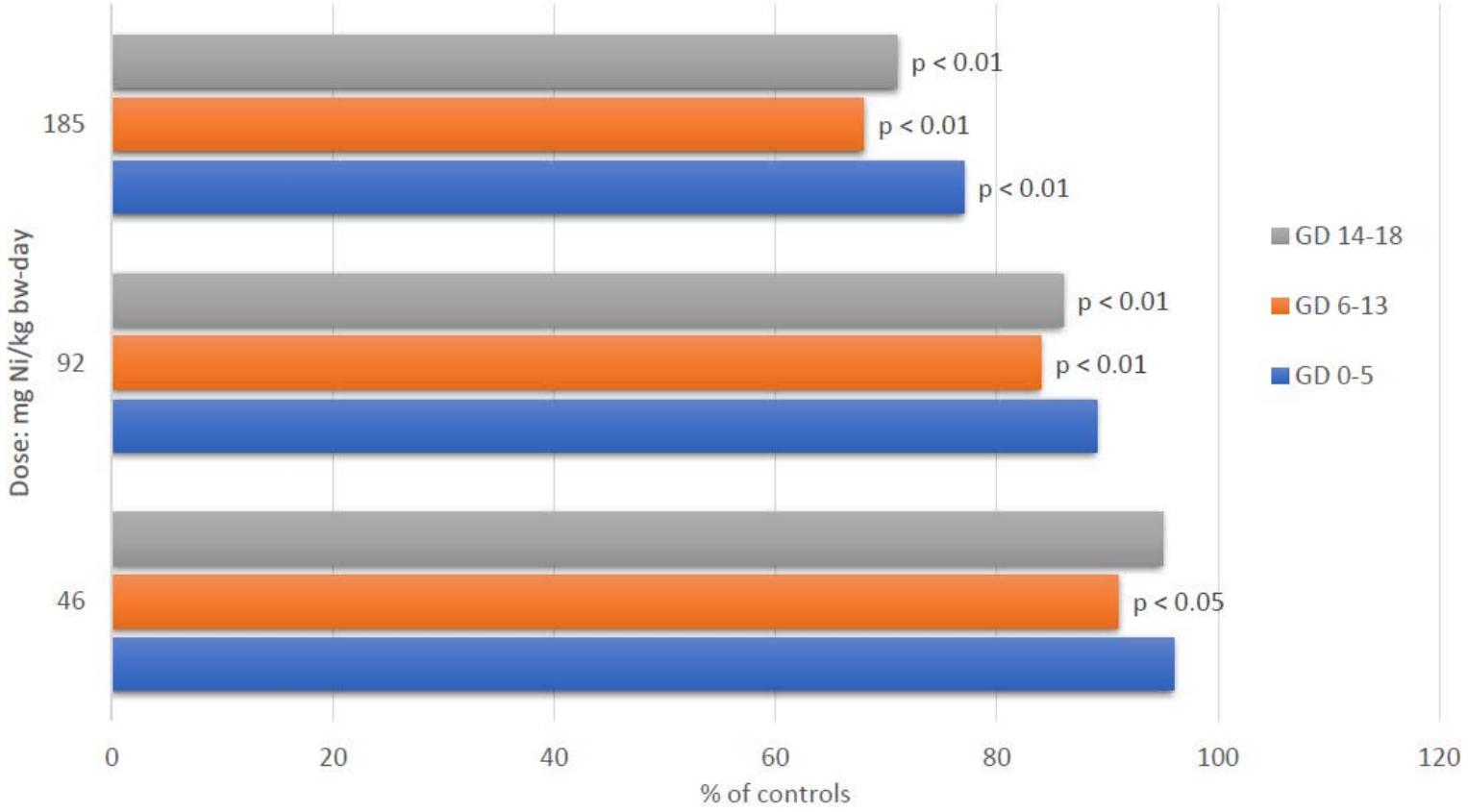

Figure B.10. Oral Exposure In Mice: Fetal Weight as % of Controls.
Saini et al (2014b) Exposure on: GD 0-5, 6-13, or 14-18

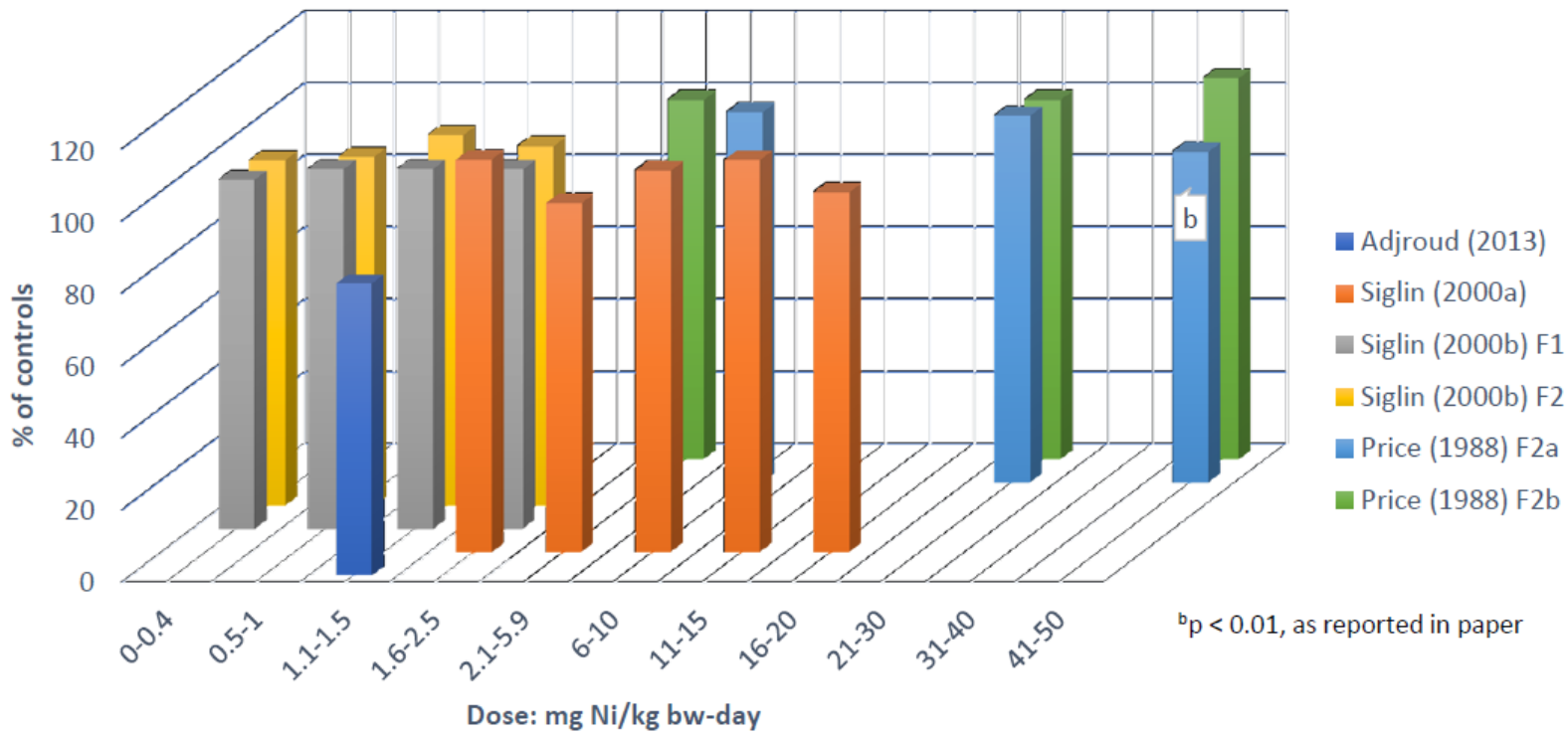

Figure B.11. Oral Exposure In Rats: Fetal/Birth Weight as % of Controls

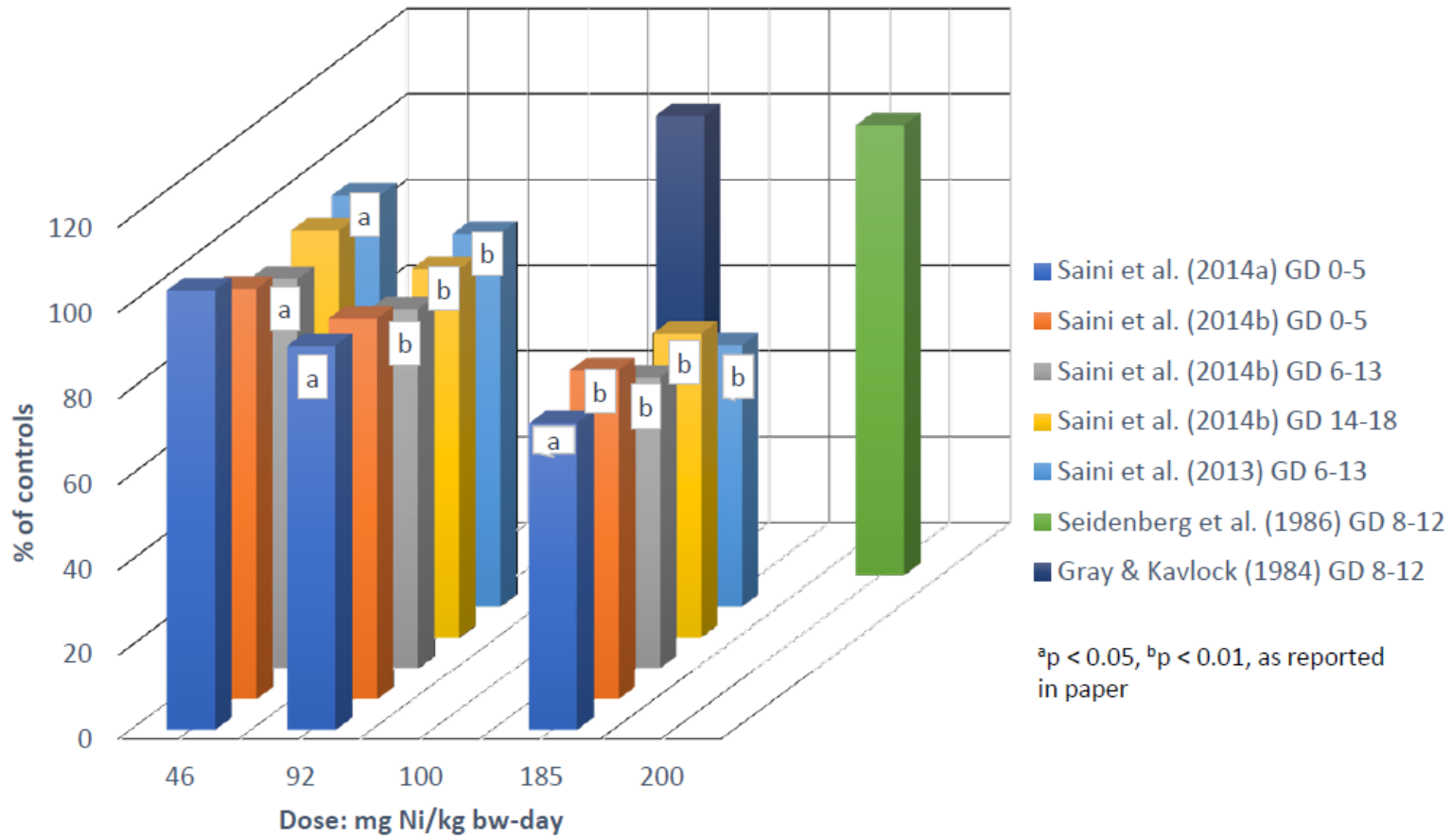

Figure B.12. Oral Exposure In Mice: Fetal/Birth Weight as % of Controls

C. Studies of Female Reproductive Toxicity of Nickel and Nickel Compounds

C.1. Human Studies of Female Reproductive Toxicity

Epidemiologic studies of female reproductive effects of Ni examined fecundity, hormone levels, and pre-eclampsia. In addition, some researchers studied the presence of Ni in milk; these studies are briefly summarized in Section A.4.3 above.

Tabulated summaries of the individual studies presented in this section are provided in Appendix 2.

Bloom et al. (2011). Associations between blood metals and fecundity among women residing in New York State.

Bloom et al. drew a convenience sample from a cohort of anglers who had participated in a fish consumption survey in New York State in 1991-92. In 1996-1997, the authors contacted from the original cohort 244 women age 18-34 years who had not had children. Of these, 113 women were not pregnant at the beginning of the current study and were discontinuing contraception to become pregnant. Eighty women completed the study and provided sufficient blood samples for metals testing (Bloom et al. 2011).

A research nurse administered an in-person interview in the participant's home and participants completed daily diaries to record menstruation, sexual intercourse, and use of cigarettes and caffeinated and alcoholic beverages. Prior to the study, the nurse instructed the participants on the 'fertile window' and use of home pregnancy kits. Kits were capable of detecting 50 milli-international units/mL of human chorionic gonadotropin (one IU is equal to approximately 2.35×10^{-12} moles or 6×10^{-8} grams) with sensitivity and specificity >99%, corresponding to levels expected on the first anticipated day of menstruation following conception. Upon completion of the interview and instructions, the nurse collected a 25 mL non-fasting blood specimen for the metals analyses. Blood samples were also taken at the first prenatal visit or after pregnancy loss, four to six weeks postpartum, upon initiation and cessation of breastfeeding, and after 12 unsuccessful months of attempting pregnancy (Buck Louis et al. 2009).

An 'at-risk' cycle was defined as a cycle with at least one act of sexual intercourse in the 'fertile window' (estimated as 5 days before, through 2 days after ovulation, with ovulation assumed to occur 14 days before the end of the cycle, based on the Ogino-Knaus method (Cooney et al. 2009)).

Whole blood specimens were pooled by subject and analyzed by inductively coupled plasma mass spectrometry (ICP-MS) in a modular class 1000 clean laboratory for concentrations of ^{75}As, ^{111}Cd, ^{114}Cd, ^{206}Pb, ^{207}Pb, ^{208}Pb, ^{25}Mg, ^{60}Ni, ^{62}Ni, ^{77}Se, ^{82}Se, ^{63}Zn, and ^{65}Zn. Time to pregnancy (TTP) was estimated using Cox proportional hazards models for discrete time. Models adjusted for age, parity (0, 1+), cigarette smoking, alcohol use, caffeinated beverage use, groupings of PCB congeners

(estrogenic, anti-estrogenic, other), serum lipids (mg/dL), and frequency of intercourse during the fertile window.

Ni levels were slightly higher among women who had a positive pregnancy test than those who did not, as shown in Table C.1 below, though the difference was not statistically significant (NS). The LOD for Ni was 0.003 µg/L whole blood.

Table C.1. Blood Ni concentrations (µg/L) among women with and without a positive pregnancy test

Positive pregnancy test?	Mean (SD)	Minimum	Maximum
Yes	6.94 (1.47)	4.00	16.00
No	6.81 (1.44)	4.09	14.00

In the multivariable model, β for Ni = -0.176 (β < 0 suggests longer TTP or lower fecundability); however, p=0.79, adjusted for age, parity (0, 1+), cigarette smoking, alcohol use, PCBs, serum lipids, frequency of intercourse during fertile window, and other metals. An interquartile range (IQR) increase in blood Ni was associated with a NS 8.6% decrease in the conditional probability of a pregnancy in an 'at-risk' cycle.

OEHHA comments: Ni (and other metal) levels were within population reference levels, with relatively low variability. The authors note that a single preconception measure of blood metals may have introduced exposure misclassification of nonpersistent metals. The sample may not be representative of the general population.

Zheng et al. (2015). Association of serum heavy metals and trace element concentrations with reproductive hormone levels and polycystic ovary syndrome in a Chinese population.

Zheng et al. conducted this cross-sectional study among women attending the Reproductive Medicine Center of an army hospital in Xiamen, China. The objectives were to determine the serum concentrations of 11 heavy metals and trace elements in women with polycystic ovary syndrome (PCOS) and women without PCOS, and to identify and evaluate the possible relationship between PCOS and these concentrations (Zheng et al. 2015).

From March through August 2013, 369 women gave blood samples. Of these women, 96 were diagnosed with PCOS, where PCOS was defined as the presence of at least two of the following: oligo-/amenorrhea (<8 menstrual cycles in the presenting year), hyperandrogenism (and/or hirsutism), and polycystic ovaries. From the remaining 273 samples, the authors randomly selected 105 samples from women who had no symptoms of hyperandrogenism, history of menstrual dysfunction, infertility, sonographic signs of PCOS, or history of occupational exposure to heavy metals or trace elements. Women were excluded if they had a history of hypertension, diabetes, or cardiovascular events, or had been treated for at least six months with oral

contraceptives, antiandrogens, insulin sensitizers, or drugs that might interfere with clinical and/or biochemical variables. All were non-pregnant non-smokers and were studied within 10 days after onset of menstruation in the case of mild oligomenorrhea, or at random for those with severe oligo-/amenorrhea. The authors mention questionnaire data but do not explain how the questionnaires were administered or what information was collected.

Blood samples were collected at day two or three of the menstrual cycle (except as noted above for women with severe oligo-/amenorrhea) and were immediately centrifuged to separate serum, then stored at -80°C. Heavy metal and trace element concentrations (Ba, Cd, Pb, As, Cr, gallium (Ga), Ni, Sr, V, Cu, Zn) were determined by inductively coupled plasma-mass spectrometry. The researchers used a variety of methods to test for levels of hormones and other clinical characteristics, including serum follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E_2), prolactin, total testosterone (TT), progesterone, serum thyroid-stimulating hormone (TSH), sex hormone binding globulin (SHBG) dehydroepiandrosterone-sulfate (DHEAS), fasting glucose, fasting insulin, cholesterol, low and high density lipoprotein-C, body mass index (BMI), and waist-to-hip (WHR) ratio.

Differences in clinical characteristics and heavy metal and trace element levels for the PCOS and comparison groups were compared using Mann-Whitney U-tests. Associations between metal levels and natural log-transformed hormone levels were examined using linear regression.

Ni concentrations differed in the PCOS (median 1.52; mean 1.96 µg/L) vs. comparison group (median 1.11; mean 1.23 µg/L; P=0.000). Concentrations of Cu and Zn also differed across the two groups, while other metals and trace elements did not.

As shown in Table C.2 below, in unadjusted analyses, a 1-µg/L increase in Ni was associated with small increases in fasting insulin and fasting glucose and a small decrease in SHBG; adjustment for age, BMI, and WHR attenuated these differences and only the decrease in SHBG remained statistically significant. Ni was also marginally associated with DHEAS after adjustment, but the percentage change was small.

Table C.2. Selected results for % difference in serum hormone levels or metabolic profile per 1-µg/L increase in serum Ni levels

Hormone or metabolic parameter	Unadjusted % difference	95% CI	P	Adjusted* % difference	95% CI	P
DHEAS (µg/L)	2.885	-0.651, 6.421	0.109	3.234	-0.452, 6.919	0.085
SHBG (nmol/L)	-13.190	-24.220, -2.160	0.019	-12.602	-24.083, -1.122	0.032
Fasting insulin (µU/mL)	8.201	2.110, 14.291	0.009	2.655	-2.866, 8.177	0.344
Fasting glucose (mg/dL)	1.353	-0.034, 2.739	0.056	0.978	-0.437, 2.393	0.175

* Adjusted for age, BMI, WHR. Results were selected for statistical significance or marginal significance.

OEHHA comments: If age, BMI, and/or WHR are causally associated with Ni, DHEAS, SHBG, insulin, or glucose concentrations, adjustment for these covariates may be inappropriate and considered overadjustment. However, the authors note that previous research has shown higher serum Ni concentrations in subjects with diabetes than healthy subjects, and state that the pathogenesis of PCOS has been linked to the development of insulin resistance and hyperinsulinemia, which can progress to type 2 diabetes mellitus. The authors state that hyperinsulinism may cause increased androgen production and greater serum levels of free androgens. Increases in free androgens may also arise due to reduced hepatic synthesis of SHBG. In this study, increased Ni exposure was associated with decreased SHBG levels.

In addition to the studies described above, some epidemiologic studies summarized in the developmental toxicity section of this document address outcomes such as spontaneous abortion and preterm delivery, which may be manifestations of direct toxicity to the conceptus, but may also be mediated wholly or partly through toxicity to the reproductive system of the mother. Thus, the studies in the developmental toxicity section can also be considered in the context of identifying female reproductive toxicity.

Maduray et al. (2017). Elemental analysis of serum and hair from pre-eclamptic South African women

This study compared concentrations of elements in hair and serum samples from pre-eclamptic women with samples from normotensive women in a large urban regional hospital in South Africa (Maduray et al. 2017). The sampling design, subject identification and recruitment methods, and inclusion and exclusion criteria were not reported. The study population consisted of 43 pre-eclamptic women and 23 normotensive women.

A research nurse collected clinical and demographic data. Pubic hair samples and venous blood samples were collected from the same women for each study group. Three replicates for serum samples and two replicates for hair samples were prepared, and digests were analyzed in triplicate. The authors analyzed the digested samples for

As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, Pb, Se, and Zn using inductively coupled plasma-optical emission spectrometry.

The concentrations of Ni in hair and serum for the two groups were not significantly different, as shown in Table C.3.

Table C.3. Median ± SEM Ni concentration in hair and serum of normotensive and pre-eclamptic women, µg/g

Sample medium	Normotensive (n=23)	Pre-eclamptic (n=43)	P-value
Hair	8.40 ± 1.31	6.86 ± 0.81	0.85
Serum	0.14 ± 0.0	0.02 ± 0.0	0.16

Pearson correlations between hair Ni and maternal and fetal parameters were low in both groups of women. Serum Ni was also not correlated with maternal parameters; however, in the pre-eclamptic group, serum Ni was correlated with maternal age (r = 0.45), gestational age (r = –0.5), diastolic blood pressure (r=0.3), and infant weight (r= –0.35). Hair concentrations of Co, Cr, and Zn, and serum concentrations of Ca, Co, Cu, Mg, Mn, Se, and Zn were significantly different for the two groups (Maduray et al. 2017).

C.2. Animal Studies of Female Reproductive Toxicity

Relevant animal studies pertaining to the potential for nickel and nickel compounds to cause female reproductive toxicity are summarized below, and in some cases, excerpts of text taken directly from the original publications and study reports are incorporated into the summaries. In some cases, topics discussed in the developmental toxicity section are also discussed in the female reproductive toxicity section because it is not always possible to determine if an observed developmental outcome is the result of a direct effect on the conceptus, an effect on the female reproductive system, or a combination of such effects.

C.2.1. Multi-generation Reproductive Toxicity Studies

Two multi-generation reproductive toxicity studies of Ni^{+2} were identified in the literature search.

Price et al. (1988) Fertility and reproductive performance of the F1 generation. Final study report (III of III). Two-generation reproduction and fertility study of nickel chloride administered to CD rats in the drinking water.

The developmental toxicity endpoints from a two-generation reproduction and fertility study of nickel chloride ($NiCl_2$) hexahydrate in drinking water were previously discussed (Price et al. 1988). Here, the fertility and reproductive performance of the F1 generation

of those rats will be discussed. The experimental animals were viral antibody free Crl: CD BR VAF/Plus outbred albino rats supplied by the Charles River Laboratories. Males and females were 27-32 days old at the time of arrival at Research Triangle Institute (RTI) for the P0 generation and the animals for the F1 generation were born at RTI. Nickel chloride hexahydrate contains 24.70% Ni, 29.83% Cl, and 45.47% H_2O. Doses of 0, 50, 250, or 500 ppm $NiCl_2$ were administered in filtered deionized water to some males and females of the F1b generation. F1b males (218) and females (204) were selected as potential F1 breeders and all remaining pups were sacrificed. On PND 42, F1 breeders were selected randomly from the 0, 50, or 250 ppm groups (30 males and 30 females); all surviving pups (22 males and 10 females) from the 500 ppm group were retained as F1 breeders. All remaining F1b animals were sacrificed. Exposure of F1 breeders was initiated indirectly via administration to their parents. Direct exposure from the drinking water began for the F1b pups before weaning and continued during the post-weaning period until scheduled sacrifice (21 to 24 weeks of age for males and 27 to 30 weeks of age for females). Progeny from F1 were used for two purposes; F2a (from the first mating) were used to assess postnatal development, and F2b (from the second mating) were used to assess embryo/fetal development.

No adverse effects of treatment were observed for the mating index (% mated females), fertility index (% fertile matings), gestational index (% live litters), perinatal viability index (% viable litters on PND 4), or the lactational index (%viable litters on PND 21) of F1 animals. During GD 0-6 of the F2a litter, F1 dams consumed 6, 21 and 42 mg Ni^{+2} kg-day in the 50, 250 and 500 ppm groups, respectively. Nickel intake remained stable throughout most of gestation, but noticeably decreased near the time of parturition, especially for 500 ppm animals (5, 16 and 15 mg/kg-day for the 50, 250 and 500 ppm groups between GD 0 and PND 1). Nickel consumption increased in all groups during lactation with average consumption reaching 13, 54 and 89 mg/kg-day, respectively, for the final week (PND 14-21).

During F2a gestation, fluid intake was not affected at 50 ppm (96-100% of control intake), but was significantly reduced at 250 ppm (71-77% of control) and t 500 ppm (58-73% of control). During the F2b gestational period, Ni^{+2} consumption by F1 dams was similar to that observed for P0 dams during gestation of the F1b litter. During F2b gestation, ranges of 5-6, 21-22, and 35-42 mg Ni^{+2}/kg-/day were observed for the low- through high-dose groups during measurement periods of GD 0-6, 6-13 and 13-20. Reduction of fluid intake was most severe between GD 20 and PND 1, showing decreases to 66% and 31% of control intake at 250 ppm and 500 ppm, respectively. During F2a lactation (PND 1-21), fluid intake was not significantly affected at 50 ppm or 250 ppm, but was reduced at 500 ppm (73-82% of control intake). During F2b gestation, fluid intake was reduced at all exposure levels; 88% of control intake at 50 ppm, 65-77% of control at 250 ppm, and 53-72% of control at 500 ppm. At the end of the F2a gestational period, a transient reduction of food intake was observed at 500 ppm (93% of control intake between GD 13-20 and 45% of control between GD 20 and PND 1). F1 female body weight was significantly below controls in the 500 ppm group on GD 20 (87% of control weight), and during lactation (82-92% of control) of the F2a

litters. F1 dam body weight during the F2b gestational period was significantly below controls on GD 20 for the 500 ppm group (91% of control weights).

Four F1 females in the 250 and 500 ppm groups died during delivery of the F2a litter; 3 from the 250 ppm treatment group and 1 from the 500 ppm treatment group. The incidence of pregnancy-related deaths was reported to be not statistically significant. Circumstances were similar to the maternal deaths in the P0 generation; the attribution of pregnancy complications to $NiCl_2$ consumption was compromised by the reduced water intake of treated animals. Based upon the absence of similar complications in pregnant historical control animals, as well as the low incidence of mortality in nickel-exposed adult P0 and P1 females during the non-reproductive portions of this study, nickel-exposed females in both generations showed the greatest probability for compromised status near the time of parturition. The author stated further evaluation was needed to determine the potential contribution of chronically reduced maternal fluid intake to these maternal deaths.

Exposure levels of 50, 250 and 500 ppm Ni^{+2} in drinking water were generally well-tolerated by F1 animals with the following exception, an increased incidence of adverse effects among pregnant females (reduced body weight or mortality) occurred at higher dose levels during late gestation, parturition and lactation. As also noted in the P0 generation, susceptibility of females appeared to be greatest during the perinatal phase of the reproductive process, in association with complications of pregnancy. The attribution of pregnancy complications to $NiCl_2$ consumption was compromised by the reduced water intake of treated animals.

Body weights of timed-mated females exhibited differences among nickel-exposed groups on GD 0 ($p<0.05$), showing 108%, 101% and 98% of the control weight at 50, 250 and 500 ppm, respectively. Gestational body weight for nickel-exposed females (timed-mated with litters) did not differ from controls on GD 0, 6 or 13. On GD 20, the 500 ppm group was significantly below controls (91% of control weight; $p<0.05$). Gestational exposure to Ni (mg/kg-d) was comparable for the F2a and F2b litters (Price et al. 1988). During gestation, maternal body weight was not affected except for a significant ($p<0.05$) reduction to 91% of the average control weight on GD 20 at 500 ppm. Maternal weight gain during gestation was 103% of control gain at 50 ppm (not significant), 92% at 250 ppm (not significant) and 74% at 500 ppm ($p<0.01$). Corrected maternal gain (maternal weight gain during gestation minus gravid uterine weight) exhibited a dose-related decreasing trend ($p<0.001$) across all groups with reduction to 94% of control weight gain at 50 ppm (not significant), 73% at 250 ppm ($p<0 .05$) and 34% at 500 ppm ($p<0.01$)(Price et al. 1988).

Evaluation of the F1 generation suggests that nickel chloride exposure interferes primarily with the normal processes associated with late gestation, parturition, lactation and/or postnatal development, and that the severity of these effects shows considerable variability among individual females and their litters. Further studies are needed to distinguish between the direct effects of nickel chloride upon the physiological/

endocrine processes associated with reproduction as opposed to possible indirect effects associated with decreased fluid intake.

Siglin (2000b) An oral (gavage) two-generation reproduction toxicity study in Sprague-Dawley rats with nickel sulfate hexahydrate.

A study conducted by Springborn Laboratories and authorized by NiPERA, Inc. (Nickel Producers Environmental Research Association, an independently incorporated division of the Nickel Institute) to evaluate the potential effects of nickel sulfate hexahydrate on the integrity and performance of the reproductive system in male and female rats was published in 2000. This study was discussed earlier in the developmental toxicity section, but only the female reproductive toxicity portion of the study will be discussed here. Sprague Dawley rats were ordered from different production areas of Charles River Laboratories (to avoid potential sibling matings). The rats were allowed to acclimate for ten days prior to randomization, and only healthy animals were maintained for possible assignment in the study. The selected rats were treated orally with nickel sulfate hexahydrate for two generations to evaluate parameters of reproductive toxicity (Siglin 2000b).

The study consisted of a vehicle control group, and four treatment groups. Each group contained 28 rats per sex (F0 and F1 generations). Nickel sulfate hexahydrate was dissolved in reverse osmosis deionized water and administered to rats daily once a day by oral gavage at levels of 1.0, 2.5, 5.0 or 10.0 mg/kg-d. Controls only received reverse osmosis deionized water at the equivalent dose volume of 10 ml/kg. Dosage levels for the two-generation study were selected based on the results of a one-generation range-finding study in rats with nickel sulfate hexahydrate. The one-generation range-finding study was conducted using gavage as the route of exposure due to palatability problems with Ni in drinking water and bioavailability problems with Ni in food. The first part of the range-finding study was a dose response probe using small numbers of animals and nickel sulfate hexahydrate exposures of 0, 5, 15, 25, 50, 75, and 150 mg/kg-d (Siglin 2000a). Lethality was seen at 150 mg/kg-d exposure.

The second part of the range-finding study utilized nickel sulfate hexahydrate exposures of 0, 10, 20, 30, 50, and 75 mg/kg-d. There were statistically significant increases in post-implantation lethality among offspring of treated parental rats at the 30 to 75 mg/kg-d exposures and questionable increases at the 10 and 20 mg/kg-d exposures (Siglin 2000a). The mean litter size was significantly decreased at the 75 mg/kg-d level and was lower than historical controls at or above 30 mg/kg-d. Another variable, stillbirth, was significantly increased in all exposure groups except the 50 mg/kg-d group.

Dosing of the F0 began at 10 weeks prior to mating; dosing of the F1 rats began at postpartum day 22. For both generations, daily dosing was continued until the day prior to scheduled euthanasia. Adult females and their pups were euthanized and

necropsied on lactation day 21. Experimental female reproductive toxicity endpoints in the F0 and F1 adults that were assessed include mating and gestation, parturition and lactation, reproductive indices, estrous cyclicity and histopathology of the female reproductive tracts. Throughout lactation, pups were closely evaluated for clinical signs, viability and growth. Pups selected to produce the F2 generation were evaluated for vaginal opening during the growth phase. Developmental toxicity aspects of this study are discussed further in the developmental toxicity section.

Estrous cycle determinations of F0 and F1 females were evaluated by daily vaginal smears for a minimum of three weeks prior to mating and throughout cohabitation until evidence of copulation was observed or until conclusion of the mating period. The vaginal smears were performed at approximately the same time daily. The stage of estrous was also determined for each F1 female on the day of scheduled euthanasia. Females were considered to be cycling if they had at least three estrous cycles during the evaluation period. Cycle length was defined by the number of days between estrous observations (interceded by at least one non-estrous stage) or between estrous and evidence of copulation (copulatory plug or positive smear).

Twenty-eight F0 animals in each group were assessed. There were no statistically significant or toxicologically meaningful differences in F0 copulation and fertility indices; estrous cyclicity; precoital intervals or gestation lengths. No statistically significant or toxicologically meaningful differences were observed among groups with respect to mean implantation scar counts, mean number of live pups on lactation day 0, or mean post-implantation loss (calculated as implantation scar counts minus live pups on lactation day 0). The percentage of F0 females with evidence of estrous cycling (ex: at least three estrous cycles during the evaluation period) ranged from 92.9 to 100%. The mean cycle lengths of F0 females were 4.35, 4.36, 4.17, 4.63, and 4.48 days, respectively. F0 gestation lengths ranged from 21.9 to 22.1 days Table C.4 summarizes some specific data from the author's individual animal data (Adapted from Appendix L (Siglin 2000b)).

Table C.4. Summary of Individual F0 Estrous Cyclicity Data

Group	No. of females not cycling / total females	No. of females with a mean cycle length of > 7 days / total females	No females with one cycle length > 10 days / total females
control	2/28 (7.1%)	N/A	N/A
1.0 mg/kg-d	1/28 (3.6%)	N/A	N/A
2.5 mg/kg-d	2/28 (7.1%)	N/A	N/A
5.0 mg/kg-d	0/28 (0)	2/28 (7.1%)	3/28 (10.7%)
10.0 mg/kg-d	0/28 (0)	N/A	N/A

N/A: not applicable

Authors defined cycle length (days) as the number of days between estrous observations (interceded by at least one non-estrous stage) or between estrous and evidence of copulation (copulatory plug or positive smear).

No significant treatment-related differences were noted in the onset of vaginal opening (VO) in the F1 offspring (Siglin 2000b). All control females (n=27) exhibited VO by day 35 of age, as did all 1.0 mg/kg-d treated F1 females. All 2.5 mg/kg-d treated F1 females exhibited VO by day 38 of age. All 5.0 mg/kg-d treated F1 females (n=27) showed VO by 36 of age. There was no explanation for the later VO of the 2.5 mg/kg-d treatment group. During lactation, mean F1 BWs were comparable among the groups; however, mean weight gain was significantly lower in the 1.0 and 5.0 mg/kg-d treatment groups (groups 2 and 4, respectively) during lactation days 14-21. The mean weight gain differences were not considered toxicologically meaningful since slight weight loss is expected during the final week of lactation and no dose response pattern was observed. No statistically significant differences in F1 copulation and fertility indices; estrous cyclicity; precoital intervals, or gestation lengths were noted.

The percentages of F1 females with evidence of estrous cycling (ex: at least three estrous cycles during the evaluation period) were 66.7, 85.7, 60.7, 59.3, and 60.7% in groups 1-5, respectively. Mean estrous cycle lengths were 5.2, 5.7, 5.2, 5.5, and 6.0 days in groups 1-5, respectively (Table C.5). These parameters were not significantly different among the F1 groups; however, the number of cycling F1 females in each group was lower and mean F1 cycle lengths were longer compared to the F0 generation. The study author's evaluation of the individual F1 animal data suggested this was likely the result of inadvertent induction of pseudopregnancy, as evident by persistent diestrous periods lasting approximately 12-16 days.

Table C.5. Summary of F1 Estrous Cyclicity Data (Adapted from Siglin, 2000b)*

Group	**1**	**2**	**3**	**4**	**5**
Level (mg/kg-d)	**0**	**1.0**	**2.5**	**5.0**	**10.0**
Mean cycle length (days)	5.22	5.66	5.22	5.47	6.04
Standard Deviation	2.019	1.666	1.385	1.674	1.656
# of females evaluated	27	28	28	27	28
# (%) cycling	18 (66.7)	24 (85.7)	17 (60.7)	16 (59.3)	17 (60.7)

*None significantly different from control. Authors defined cycle length (days) as the number of days between estrous observations (interceded by at least one non-estrous stage) or between estrous and evidence of copulation (copulatory plug or positive smear).

Examination of individual animal data of the control F0 females and F1 females revealed very different incidences of naturally occurring estrous cycles between F0 and F1 females. As shown in Table C.6 below, many of the treated F1 females were not cycling, albeit the control F1 females also had a low rate of cycling. The low-dose treatment group (group 2) had the highest percentage of normally cycling females. These individual F1 animal data were made obscure by the author's reporting of the data as group means. The author attributed the low rate of estrous cycling to inadvertent induction of pseudopregnancy.

Table C.6. Summary of Individual F1 Estrous Cyclicity Data

Group #	Level (mg/kg-d)	No. females not cycling / total females	No. females with a mean cycle length > 7 days / total females	No. females with one cycle length > 10 days / total females	No. females with no cycle data / total females
1	Control	9/28 (32.1%)	4/18 (22.2%)	4/18 (22.2%)	1/28 (3.5%)
2	1.0 mg/kg-d	4/28 (14.3%)	8/24 (33.3%)	10/24 (41.7%)	N/A
3	2.5 mg/kg-d	11/28 (39.3%)	4/17 (23.5%)	5/17 (29.4%)	N/A
4	5.0 mg/kg-d	11/28 (39.3%)	5/16 (31.3%)	7/16 (43.8%)	1/28 (3.6%)
5	10.0 mg/kg-d	11/28 (39.3%)	9/17 (52.9%)	9/17 (52.9%)	N/A

N/A: not applicable.
Authors defined cycle length (days) as the number of days between estrous observations (interceded by at least one non-estrous stage) or between estrous and evidence of copulation (copulatory plug or positive smear).

Among F2 pups, no statistically significant or toxicologically meaningful differences were noted in BW during lactation. The most notable gross necropsy observations in F2 pups which were found dead consisted of atelectasis and absence of milk in the stomach. It is not known from the study report, however, if milk quality and/or quantity of F1 mothers played a role in F2 pup BW.

Oral administration of the test article over the course of two generations at dosage levels of up to 10.0 mg/kg-d had no effect on F0 and F1 survival, growth, mating behavior, fertility, gestation, parturition or lactation. No toxicologically meaningful differences were noted among the groups with respect to estrous cycling, fertility indices, gestation lengths, or the onset of sexual maturation in F1 rats. Yet, there was a low incidence of cycling in the F1 controls, which suggests an inherent difference from the F0 controls. However, parameters that were not assessed such as hormone profiles and ovarian follicle counts of the different treatment groups would be informative towards understanding altered cyclicity among the F1 generation. Histopathological evaluations did not reveal any test-article-related changes in reproductive organs.

Related Study

One reproductive assessment by continuous breeding study treated mice with a mixture of 25 chemicals, including Ni, tested at varying concentrations.

George et al. (1990). Mixed chemicals (MIX): reproduction and fertility assessment in Swiss (CD-1) mice when administered in the drinking water: final study report, volume I of II.

In a National Toxicology Program (NTP) Reproductive Assessment by Continuous Breeding (RACB) study, CD-1 outbred albino Swiss mice were administered a mixture of 25 chemicals (MIX), including nickel, formulated to simulate groundwater near hazardous waste dumps (George et al. 1990). Mice were given one of three dose-level mixtures ad libitum, and fresh treatment water was given every 2-3 days because not all compounds were stable in the MIX for 48 hours. The low dose (1%) mixture contained 0.68 ppm Ni, the medium dose (5%) contained 3.4 ppm Ni, and the high dose (10%) mixture contained 6.8 ppm Ni. The concentrations of Ni given by the study author were as a percentage of a technically achievable stock solution.

COBS Crl:CD-1 (ICR)BR VAF/Plus outbred albino Swiss mice were purchased from Charles River Breeding Laboratories for this study. The four tasks in a RACB study were as follows:

- Task 1 -- dose selection, which was not conducted since sufficient data had been generated by other NTP-sponsored studies to allow dosage preselection for conduct of Task 2.
- Task 2 -- the continuous breeding phase, which consisted of a control group (40 breeding pairs) and three dose groups (20 pairs/group). Task 2 dose levels were set so that the highest dose was expected to depress weight gain by approximately 10% and permit >90% survival. The middle dose was selected to produce little or no systemic toxicity, whereas the low dose was designed to be a no-effect level. The animals were housed as breeding pairs for 98 days. Endpoints for Task 2 were clinical signs, parental body weight, fertility (number producing a litter/number of breeding pairs), litters per pair, live pups per litter, proportion of pups born alive, sex of live pups, pup body weights within 24 hours of birth, and feed and water consumption. At the end of the 98 days, the pairs were separated and housed one animal/cage with continued dosing. Any litters born (F1) after the continuous breeding phase were reared by the dam until weaning. Selected weanlings were reared in same sex groups until 74 days of age. Dosed water was provided at the same concentration as during Task 2. These F1 animals were used for assessment of second-generation fertility.
- Because a positive effect on fertility was not detected during Task 2, a one-week crossover mating trial (Task 3) was not performed on the parental animals to determine the affected sex. Low- and mid-dose males were sacrificed after separation of the breeding pairs, with no data collection. Low and mid-dose females were sacrificed after weaning of their litters, with no data collection. After weaning of their litters, vaginal cytology was evaluated for the control and high-dose females for 12 days prior to necropsy. At necropsy, body weight was taken, but no tissues were submitted for histopathological evaluation.
- Task 4 was offspring assessment.

Vaginal cytology data was analyzed using a multivariate analysis of variance to test for the simultaneous equality of measurements across dose levels. An arcsine transformation was applied to vaginal cytology data, representing the proportion of the observation period that an animal was in a given estrous stage, to bring the data closer to conformance with normality assumptions. Cycle length was analyzed using Shirley's or Dunn's test.

Dose of each of the test compounds consumed in the MIX was a function of the concentration of the compound, the amount of the MIX consumed in the drinking water by the animal, and animal body weight. During weeks 1-13 of Task 2, water consumption for males averaged 170.2, 136.4, 114.8 g/kg-d for the 1%, 5%, and 10% dose groups, respectively. Female Task 2 animals averaged 172.6, 144.3, and 120.9 g/kg-d water consumption during weeks 1-13. Exposure of the F0 mice to MIX in the drinking water had no significant effect on fertility, average number of litters per pair, numbers of live male pups per litter, proportion of pups born alive, absolute pup weight (males, females, or sexes combined), or adjusted female live pup weight. Treatment with MIX had no effect on cumulative days to litter, dam body weights at delivery, or F0 male or female body weight throughout Task 2 to week 18. There was no significant effect of MIX treatment at 10% on the stages or length of the estrous cycle in Task 2 females. Results of the fertility trials of F1 animals (Task 4), showed no effect of MIX treatment on the mating or fertility index, the number of live male pups per litter, the total number of live pups per litter, the proportion of pups born alive, the sex ratio of live born pups, absolute pup weight, or average number of days to litter. Water consumption was significantly depressed in the 5% and 10% groups for mating pairs at the beginning of Task 4 and for males and females separately housed at the end of Task 4. At necropsy, there was no significant effect of treatment on female body weight, or absolute weight of liver, uterus, or ovary/oviduct. There was a significant increase in the proportion of days for which females in the 5% and 10% MIX groups exhibited no cycle or an unclear estrous cycle. In addition, females in the 5% group spent less time in metestrus.

Overall, while there were many known reproductive and developmental toxicants in the MIX, it is unclear which chemicals contributed to specific reproductive effects. The RACB study design also makes discerning the reproductive toxicity of a specific chemical difficult to identify as to being male or female-mediated on subsequent generations. For F0 mice, water consumption was depressed in a dose-related manner that was consistently significant in the mid- and high-dose groups. The authors attributed decreased water consumption to palatability problems as there was no evidence of generalized toxicity. Exposure of F0 mice to MIX had no significant effect on fertility, average number of litters per pair, number of live male pups per litter, proportion of pups born alive, absolute live pup weight, or adjusted female live pup weight. Treatment with MIX had no effect on cumulative days to litter, dam body weights at delivery or F0 male or female body weight throughout Task 2 to week 18. After approximately 20 weeks of exposure, there was no effect of high-dose treatment on vaginal cytology in F0 animals. For F1 mice, water consumption was depressed in a

dose-related manner that was consistently significant in the mid- and high-dose groups. In task 4, there was no significant effect of treatment on female body weight, absolute weight of liver, uterus, or ovary/oviduct. However, there was a significant increase in the proportion of days in which females in the 5% and 10% MIX groups exhibited no cycle or an unclear estrous cycle; and, females in the 5% group spent less time in metestrus (Table C.7) (George et al. 1990). The proportion of days that the females exhibited no estrous cycle or an unclear cycle increased in a dose-related manner with the mid- and high-dose groups significantly above the control group. The effects on estrous cycle in this study are consistent with other Ni female reproductive toxicity studies.

Table C.7. Summary of MIX in CD-1 Mice (Adapted from (George et al. 1990))*

	Low (1%)	Medium (5%)	High (10%)
Unclear vaginal cyclicity	–	↑	↑

*(–) no effect
(↑) significant increase in the parameter

Specific conclusions on the female reproductive effects of Ni from the RACB MIX study are difficult to discern due to the dosing of both males and females, and the complex treatment mixture. However, the unclear estrous cycle findings from the RACB MIX study are consistent with the rest of the body of literature on Ni and female reproductive toxicity.

C.2.2. Uterus

The effect of Ni on the uterus is not well studied. Only two references were identified for this endpoint, one of which was an in vitro study.

Chang et al. (1970). Effect of intrauterine copper and other metals on implantation in rats and hamsters.

Inserting Ni and other metals into the uterus is part of the history of intrauterine contraceptive development (Margulies 1975). An intrauterine device (IUD) prevents the lining of the uterus (endometrium) from growing very thick, which makes the lining a poor place for a fertilized egg to implant and grow. In 1970, Chang et al. investigated the effect of intrauterine copper and other metals (including Ni) on implantation in rats and hamsters. Hamsters were used only to examine the effects of copper, and will not be discussed further here. This study, while not well reported, was conducted on adult virgin female rats of the Holtzman strain by inserting 0.005 inch diameter Ni wire into the uterus (other metals tested will not be discussed further here) (Chang et al. 1970). Loops of Ni were inserted on day 3 of pregnancy. In some experiments laparotomies were performed on days 9 and 15 of pregnancy to determine whether implantation or embryonic resorption had occurred. In all experiments, the animals were sacrificed on day 21. Live fetuses, resorbing sites, and corpora lutea in both ovaries were counted.

Chang et al. reported Ni inserted into the lumen near the tubouterine junction was somewhat effective in preventing pregnancy in rats if inserted by day 3 (prior to implantation). The fertility index was calculated by dividing the number of viable implants in the treated horn (x100) by the number of viable implants in the control horn. The fertility after Ni treatment in rats was 7.1 (n=5). The effect of Ni on fetal mortality was calculated for the treated and the control horns by dividing the number of resorbing sites (x100) by the sum of implants and resorbing sites. Fetal mortality after Ni exposure was 60.0 (6.7). Chang et al. found the effect of Ni inserted into the uterus to be localized to the uterus.

Rubanyi and Balogh. (1982). Effect of nickel on uterine contraction and ultrastructure in the rat.

The in vitro effects of $NiCl_2$ on uterine contractile activity and ultrastructure were studied in uterine strips isolated from 20-day-pregnant Wistar rats (Rubanyi and Balogh 1982). Nickel had dual action on uterine spontaneous contractions. In low concentrations (10^{-7} M to 10^{-5} M), $NiCl_2$ increased basal tone significantly, but had no effect on the amplitude or frequency of development of isometric force. High concentrations of $NiCl_2$ (10^{-4} M to 10^{-3} M) inhibited spontaneous contractile activity and decreased basal tone, which was antagonized by elevation of the extracellular concentration of calcium. Electron microscopic localization of Ni by the dimethyl glyoxime cytochemical technique showed that, after incubation of uterine strips in a physiologic medium that contained 10^{-6} M $NiCl_2$, electron-dense Ni-dimethyl glyoxime particles could be observed in the cytoplasm and in the mitochondria of uterine smooth muscle cells. Exposure to Ni caused mitochondrial structural damage and accumulation of glycogen.

C.2.3. Ovary

Several studies demonstrate Ni can affect ovarian function. The effects of Ni vary from histological changes to functional alterations.

Chang et al. (1970). Effect of intrauterine copper and other metals on implantation in rats and hamsters.

In a short study published in 1970, ovarian function was not affected by the presence of Ni in the uterus (Chang et al. 1970). This study did not produce much information in regards to ovarian toxicity of Ni as the purpose of the study was to investigate the effect of introducing various metals in the uterus on fertility in rats and hamsters. (The study design was described in section C.2.2. Uterus.) In all instances, the average number of corpora lutea present in the ovary of the metal-treated uterine horn was about the same as found on the control side and in untreated control rats.

Forgacs et al. (1997). Effects of NiSO4 on the ovarian function in rats.

Forgacs et al. investigated the effects of Ni sulfate treatment on the ovarian cycle and progesterone levels. SPRD rats weighing 240-300 g were treated on the day of estrus on the 4th cycle with 10, 20, or 40 mg/kg $NiSO_4$ ($NiSO_4$ x $7H_2O$) or 0.9 % NaCl solution (1 mL/kg) subcutaneously (Forgacs et al. 1997). This treatment was repeated through 4 cycles. In the majority of animals 10 mg/kg bw $NiSO_4$ disturbed the regular ovarian cycles, while at 40 mg/kg it inhibited ovulation. In the group of $NiSO_4$-treated animals, the number of ova shed remained at the control level. Treatment with $NiSO_4$ did not alter either the number of corpora lutea or the weight of ovaries. In the $NiSO_4$-treated rats the basal progesterone (P) secretion before the human chorionic gonadotropin (hCG) injection characteristic of estrus did not differ from that in the control animals. The injected hCG evoked a rise in P secretion within the first 10 minutes, and a further continuous rise until the 40th minute. The basal ovarian P secretion rates in the treated rats were found not to be significantly lower than those in the controls. The P response to hCG stimulus in the rats treated with 40 mg/kg bw $NiSO_4$ was completely abolished. This decreased capacity of P synthesis and the anovulation found in treated rats indicate a loss in reproductive capability. There were no Ni^{+2}-related changes in blood pressure, ovarian venous blood flow, body weights or in the weights and histology of adrenals, pituitary glands and kidneys.

Rao et al. (2009). Protective role of vitamin E on nickel and/or chromium induced oxidative stress in the mouse ovary.

Rao et al. investigated the protective antioxidant qualities of vitamin E against Ni induced toxicity in the ovary (Rao et al. 2009). Healthy adult female albino mice (Mus musculus) of Swiss strain were used in this study. The animals were divided into 9 groups; some of these groups received potassium dichromate (which will not be discussed further here). Group I served as a control, and received only distilled water. Group II received only vitamin E. Nickel chloride ($NiCl_2$) was diluted in distilled water based on LD_{50} values. Groups III and IV received $NiCl_2$ at doses of 8 and 16 mg/kg body weight. Daily treatments were given orally using a feeding tube for 30 days. At the end of the treatment period, the animals were weighed and sacrificed by cervical dislocation. The ovaries were dissected out carefully, blotted free of blood and weighed up to the nearest milligram and used for the estimation of protein, malondialdehyde levels (MDA), catalase (CAT), glutathione (GSH), total ascorbic acid (TAA), and superoxide dismutase (SOD). Body weights were reduced ($p<0.05$, $p<0.001$) in the high dose $NiCl_2$ and $NiCl_2$ + $K_2Cr_2O_7$ treated groups. Similarly, ovary weights exhibited decreases ($p<0.05$, $p<0.01$) (Table C.8). Ovary protein levels declined significantly following $NiCl_2$ exposure (Table C.9). Likewise, anti-oxidative levels in the ovary were decreased significantly following $NiCl_2$ exposure (Table C.10).

Table C.8. Body and organ weights of control and some experimental groups (truncated, from Rao et al. 2009)*

Parameter	Control	Vitamin E	$NiCl_2$		$NiCl_2 + K_2Cr_2O_7$		$NiCl_2 + K_2Cr_2O_7$ + vit E
			8 mg	16 mg	16 mg	10 mg	
BW (g)	43.00 ± 0.89	43.40 ± 0.78	41.20 ± 0.81	40.00 ± 0.84[a]	37.50 ± 1.04[b]	35.30 ± 0.84[c]	40.60 ± 0.49
Ovary weight (mg)	26.30 ± 0.57	26.80 ± 0.55	25.90 ± 0.31	24.60 ± 0.47[a]	24.80 ± 0.24[a]	24.30± 0.30[b]	25.80 ± 0.35

*Values are mean ± S.E.; [a] p<0.05; [b] p<0.01; [c] p<0.001; n=10

Table C.9. Protein and MDA levels in ovary of control and $NiCl_2$ treated groups (Adapted from Rao et al. 2009)*

Parameter	Control	Vitamin E	$NiCl_2$		$NiCl_2 + K_2Cr_2O_7$		$NiCl_2 + K_2Cr_2O_7$ + vit E
			8 mg	16 mg	16 mg	10 mg	
Protein (mg/100 mg tissue weight)	12.42 ± 0.35	13.00 ± 0.25	11.36 ± 0.36	9.81 ± 0.56[b]	7.12 ± 0.42[c]	5.36 ± 0.22[c]	12.01 ± 0.15
Lipid peroxidation (MDA/100 mg tissue weight	65.23 ± 0.83	65.04 ± 0.55	68.26 ± 1.20	91.85 ± 1.51[c]	121.40 ± 0.91[c]	142.74 ± 1.94[c]	66.83 ± 0.70

*Values are mean ± S.E.; [a] p<0.05; [b] p<0.01; [c] p<0.001; n=10

Table C.10. Antioxidative levels in ovary of control and $NiCl_2$ treated groups (Adapted from Rao et al. 2009)*

Parameter	Control	Vitamin E	$NiCl_2$		$NiCl_2 + K_2Cr_2O_7$		$NiCl_2 + K_2Cr_2O_7$ + vit E
			8 mg	16 mg	16 mg	10 mg	
Glutathione (µg/100 mg tissue weight)	37.06 ± 0.95	37.59 ± 1.27	34.31 ± 0.92	29.81 ± 1.64[b]	22.67 ± 1.04[c]	16.82 ± 0.94[c]	36.30 ± 0.44
Total ascorbic acid (mg/g tissue weight)	3.28 ± 0.07	3.29 ± 0.03	3.12 ± 0.03	2.57 ± 0.16[b]	1.67 ± 0.03[c]	1.08 ± 0.05[c]	3.19 ± 0.02
SOD (units SOD/mg protein)	0.374 ± 0.02	0.382 ± 0.02	0.350 ± 0.01	0.280 ± 0.02[b]	0.192 ±0.01[c]	0.151 ± 0.01[c]	0.360 ± 0.01
Catalase (µmoles of H_2O_2/mg protein)	24.04 ± 0.27	24.45 ± 0.17	22.92 ± 0.46	18.93 ± 0.32[c]	16.88 ± 0.12[c]	13.25 ± 0.23[c]	23.06 ± 0.28

*Values are mean ± S.E.; [a] $p<0.05$; [b] $p<0.01$; [c] $p<0.001$; n=10

Rao et al. suggested that vitamin E exerts its protective effect against Ni (and/or chromium) induced toxicity by preventing lipid peroxidation and protecting the antioxidant defense system in the mouse ovary.

Krockova et al. (2013). Nickel-Induced Structural and Functional Alterations in Porcine Granulosa Cells In Vitro.

Nickel chloride was reported to cause a concentration-dependent depletion of the P release and alter the ultrastructure and apoptosis in porcine granulosa cells (Krockova et al. 2013). Ovaries were obtained from pubertal Slovak white gilts at 7 months of age, immediately prior to the onset of estrous cyclicity. One hour after the animals were killed, the contents of all 1–4-millimeter follicles were aspirated using a 2-mL syringe, and granulosa cells were separated from the follicular fluid by centrifugation (200×g in 4°C for 5 min). Nickel chloride was added to the cells to achieve a Ni^{+2} concentration of 62.5, 125, 250, 500 and 1,000 µmol/L. A control group contained no $NiCl_2$ addition. This experiment was performed with 4–15 replicate samples at each concentration, and repeated seven times (n=7). Following 48 h of granulosa cell culture in the presence of Ni, a concentration-dependent depletion of the P release was observed (Figure C.1).

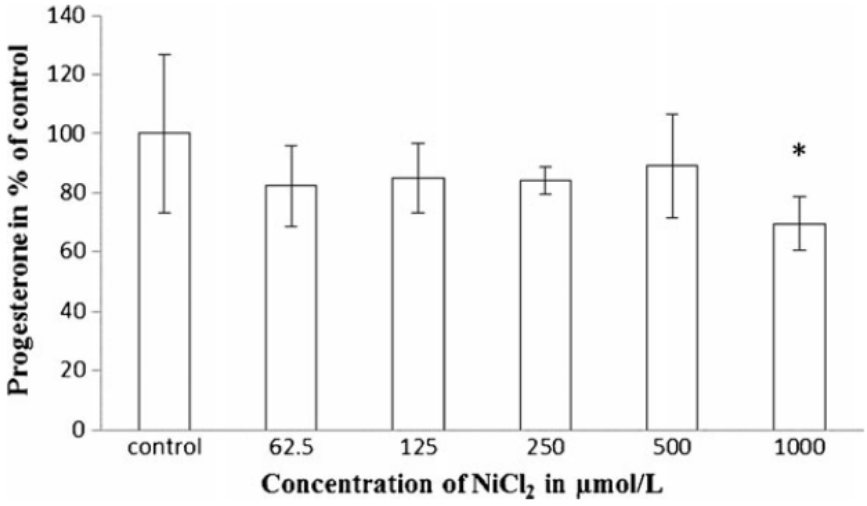

Figure C.1. The effect of $NiCl_2$ on progesterone production (µmol/L), *p<0.05 (Krockova et al. 2013)

The lowest P production was detected after treatment with 1,000 µmol/L $NiCl_2$ (69.47±9.04 %, p<0.05). The percentage of apoptotic cells was increased in each of the experimental groups except for the concentration of 250 µmol/L $NiCl_2$ (41.80 ± 25.11%). The decrease was by 3% in comparison with the control group (44.86 ± 22.97%). The highest percentage of apoptotic cells was found after treatment with 1,000 µmol/L $NiCl_2$ (79.17 ± 36.08%, p<0.05). No significant differences were detected in groups with the addition of 62.5 µmol/L $NiCl_2$ (62.21 ± 20.00%), 125 µmol/L $NiCl_2$ (49.90 ± 26.37%) and 500 µmol/L $NiCl_2$ (51.40 ± 31.13%). The ultrastructure of porcine granulosa cells was also altered; there was a decrease in the volume of smooth endoplasmic reticulum after incubation with the highest nickel concentrations (1,000 µmol/L). The granular endoplasmic reticulum was present in the control group only. After treatment with the highest Ni concentrations (≥500 µmol/L), a lower occurrence of mitochondria was seen compared to the control group. Euchromatin had a more prominent presence in the Ni-exposed cultures. Other observations of the granulosa cell ultrastructure included a greater occurrence of lipid droplets and vacuoles after exposure to all Ni concentrations in comparison with the control group.

Kong et al. (2014). Nickel nanoparticles exposure and reproductive toxicity in healthy adult rats.

In another study, experimental results showed treatment with Ni nanoparticles (NPs) resulted in ovarian lymphocytosis, vascular dilatation and congestion, inflammatory cell infiltration, and an increase in apoptotic cells in ovarian tissues from Ni NP exposure groups (Kong et al. 2014). Fifty male and 100 female Sprague-Dawley rats weighing 80–100 g were divided into five groups including a control group, Ni NPs (90 nanometers, nm) groups (high dose 45, mid dose 15 and low dose 5 mg/kg-day, respectively) and Ni microparticles (MP; 3 micrometers, µm) group (45 mg/kg-day) in such a way as to equalize group means and standard deviations of body weights. Each

group consisted of 10 males and 20 females, as F0 parental rats. Both male and female F0 parental rats were given different doses of Ni NPs, Ni microparticles (MPs) and 0.9% sodium chloride solution (control group) by gavage for 10 weeks before the initiation of the mating period. Dosing of females continued through gestation and lactation.

Kong et al. reported Ni NPs increased the level of serum FSH and LH, and decreased E_2, which was significant and dose-dependent in females. Kong et al. also reported the effects of Ni NPs on the female rat ovarian reserve. There were no adverse histopathological observations in the control group (Figure C.2A). However, pathological results showed vascular dilatation and congestion (Figure C.2B, C.2C), ovarian lymphocytosis (Figure C.2D), luteal cells increasing and becoming cavitated (Figure C.2E), and increased eosinophils and inflammatory cell infiltration (Figure C.2F) in rat ovarian tissue from the Ni NPs group. There were decreased levels of serum E_2 and ovarian hormone secretion following ovarian damage with Ni NPs that increased the level of serum FSH and LH by negative feedback
.

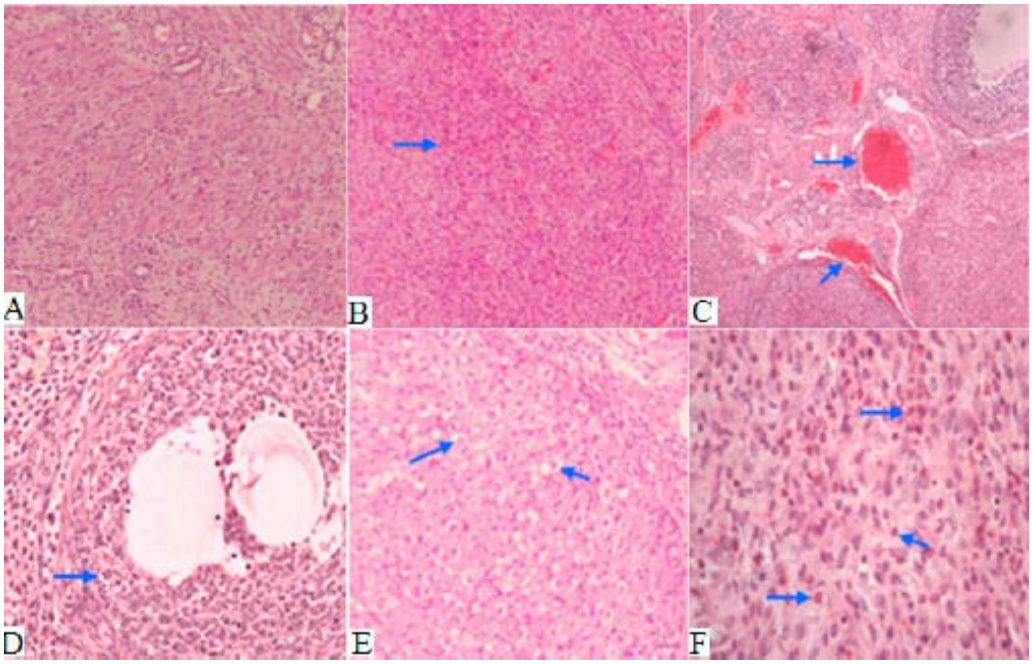

Figure C.2. Effects of Ni NPs on histopathology of ovaries in female rats.
The control group (A); 15 mg/kg BW (B); 45 mg/kg (C–E); Ni MPs group (F). Original magnification was 100×, 200× and 400× (100× refers to picture A, B and C; 200× refers to picture D and E; 400× refers to picture F). The arrows on (B) and (C) point to vascular dilatation and congestion, on (D) the arrow points to lymphocytes, on (E) arrows point to luteal cells, and on (F) arrows point to eosinophils and inflammatory cells (Kong et al. 2014).

Related Study

Costa et al. (1978). Alteration in morphology of chinese hamster ovary cells by Ni3S2 and dibutyryl cAMP.

Costa et al. conducted a study on Chinese hamster ovary cells (CHO), which are an epithelial cell line often used in toxicity screening; albeit this is not a straight-forward reproductive toxicity study. Costa et al. exposed CHO cells to Ni metal compounds to compare and contrast the early morphological and biochemical changes that occur following exposure to potentially carcinogenic metal compounds with those produced by elevation of cellular cAMP concentrations (Costa 1978). Chinese hamster ovary cells were plated at a concentration of 5 x 10^6 cells per plate (150-mm diameter) about 12 hr prior to addition of the compounds. The cultures were treated with the compounds for either 3 or 24 hr. Other treatments (Ni_3S_2, NiS, and $NiCl_2$) were ground to a mean particle size of 2 µm in an agate mortar within an inert atmosphere. The purity of the metallic powder was verified by emission spectroscopy and crystal structures of the compounds were checked by X-ray diffraction. Elongation of CHO cells resulted from exposure to Ni_3S_2. No changes in morphology were observed with $NiCl_2$ or NiS treatments.

C.2.4. Reproductive Index

Some of the studies discussed in this section are also discussed in the developmental toxicity section given the biological overlap of embryotoxicity.

Schroeder and Mitchener. (1971). Toxic effects of trace elements on the reproduction of mice and rats.

Schroeder and Mitchener investigated the toxic effects of trace elements, such as Ni, on the reproduction of mice and rats (Schroeder and Mitchener 1971). This study was complicated by multiple sources of Ni (water and diet) and two species, mice and rats. Drinking water was from a forest spring and double-deionized. Among the elements added to the water was Ni at 5 ppm. The diet contained multiple elements, including 0.31 ppm Ni (wet weight). Five pairs of mice or rats were randomly selected for each element from divided litters at the time of weaning, placed in separate cages and given the element in drinking water continuously. Mice were allowed to breed as often as they would up to 6 months of age and rats up to 9 months of age or longer. Criteria used to estimate innate toxicity were the intervals between litters, the age at which the pair produced its first litter, the ratio of males to females, and the numbers of runts (animals with large heads and small bodies), deaths, stillborn offspring, failures to breed, and congenital abnormalities. The effects of Ni were assessed in breeding rats (as well as lead and titanium). Schroeder et al. found Ni was less toxic than lead with respect to breeding, with 9.1% young deaths, one maternal death, and 30.6% runts in the first generation; 10.2% young deaths and 5.1% runts in the second; and 21.0% (17) young deaths and 6.2% (5) runts in the third (Table C.11).

Table C.11. Deaths and abnormalities in rats bred while exposed to Ni (Adapted from Schroeder and Mitchener 1971)

	Control	Nickel
F1 generation		
Maternal deaths	0	1
Dead litters	0	0
Young deaths	0	11*
Failures to breed	0	0
Runts	0	37 ‡
Number of rats	114	121
F2 generation		
Maternal deaths	0	0
Dead litters	0	0
Young deaths	0	16 ‡
Failures to breed	0	2
Runts	1	8
Number of rats	113	157
F3 generation		
Maternal deaths	0	0
Dead litters	0	0
Young deaths	1	17 ‡
Failures to breed	0	0
Runts	0	5†
Number of rats	121	81
Total number of rats	348	359

* Differs from controls by x^2 analysis; P < 0.005.; † P < 0.0001.; ‡ P < 0.025.

Mizejewski et al. (1990). Effects of heavy metals on alpha-fetoprotein in maternal sera and amniotic fluid of pregnant mice.

Mizejewski et al. conducted a study of the effects of heavy metals on alpha-fetoprotein in maternal sera and amniotic fluid of pregnant mice, in which they also conducted a secondary study of fecundity (Mizejewski et al. 1990). Several of the metal-injected Nya:Nylar outbred female mice gave birth before the scheduled autopsy for the primary assessment of metals on alpha-fetoprotein in maternal sera and amniotic fluid could be performed. The progeny of these injected mice were allowed to attain breeding age and were interbred in each family to determine whether the metals would exert secondary effects on F1 litter size. The fecundity data showed no effect on litter size in the F1 generation and F1 progeny (Tables C.12 and C.13).

Table C.12. Effect of heavy metals on fetal wastage and primary litter size in F1 (Adapted from Mizejewski et al. 1990)

Treatment Group	No. of Litters	Mean Litter Size	No. fetuses resorb. per total litters	No. fetuses dead per total litters	Mean body weight (g) ± SE	Mean gest. age (days)**	Range of gest. age (days)
Distilled water	34	10.82	(14/34) 41%	(1/34) 3%	39.09 ± 7.78	14.90	10.5 - 18
$NiCl_2$	40	10.93	(11/40) 28%	(5/40) 13%*	39.86 ± 4.93	14.80	10.5 - 18
NTC ◊	558	10.25	38%	4%	39.88 ± 3.93	14.00	10 - 20

*Significant at $p < 0.05$, Chi-Square Analysis
**Determined by Crown-rump fetal measurement (pregnancies were untimed; gestational age was determined by fetal-crown rump length on day of autopsy)
◊ Taken from the non-treated (NTC) Nylar mouse colony over a 3-year period

Table C.13. Effect of heavy metals on secondary litter size (Adapted from Mizejewski et al. 1990)

Litter #	Metal	Concentration injected (mg/kg)	F1 litter size (F1 progeny)
6	$NiCl_2$	1.00	9
7	$NiCl_2$	1.00	9

Mean litter size from NTC mice: 10.25 ± 0.98 (SE)
Mean litter size of mice injected with distilled water: 10.82 ± 1.10 (SE)

Smith et al. (1993). Perinatal toxicity associated with nickel chloride exposure.

Female Long-Evans rats were treated with $NiCl_2$ for 11 weeks prior to mating and then during two successive gestation (G1, G2) and lactation (L1, L2) periods (Smith et al. 1993). Four groups of 34 females each were given $NiCl_2$ drinking water solutions at 0, 10, 50, and 250 ppm Ni^{+2} for a period of 11 weeks prior to breeding. During gestation periods, females were weighed on GD 0 and 21. During lactation periods, weights were collected on PND 1, 4, 7, 14, and 21. Dams were rested for 2 weeks after weaning of the first litters before initiating the second breeding. The reproductive performance of females, as measured by mating success, rate of impregnation, pups per litter (total or live), and gestation length, reflected no differences across dose groups in either the first or second gestation period . The only significant reduction in pup growth was seen in L1 male pups in the 50 ppm group . Pups in L2 (including controls) weighed significantly less than those in L1, and this difference persisted until weaning . This study is also briefly discussed in section C.2.6.1 and in the developmental toxicity section.

Kong et al. (2014). Nickel nanoparticles exposure and reproductive toxicity in healthy adult rats.

The effect of Ni nanoparticles (NPs) on reproductive index was examined by Kong et al. in this one-generation reproductive toxicity test as discussed earlier in section C.2.3. Ovary. Briefly, 50 male and 100 female Sprague-Dawley rats were divided into five groups including the control group, Ni NPs (90 nm), groups (high dose: 45 mg/kg-d, medium dose: 15 mg/kg-d, and low dose: 5 mg/kg-d) and Ni microparticles (MPs; 3 µm; 45 mg/kg-d) group. Both male and female F0 parental rats were administered Ni treatment by gavage with different doses of Ni NPs and Ni MPs and 0.9% sodium chloride solution (control group) for 10 weeks before the initiation of the mating period. One male and two females were selected randomly and mated within each dose group for 14 days. All females were allowed to give birth naturally, and rear their own pups until weaning. Dosing of females continued through gestation and lactation. Male rats were killed at the end of the 14-day mating period. Females that delivered were killed on day 22 after parturition. Females that did not produce litters were killed on day 3 after the last expected parturition date. At necropsy, special attention was paid to the reproductive organs (ovary, uterus, testis and epididymis). The authors stated Ni NPs could reduce the chance of mating success and pregnancy rate in rats, but this was not statistically significant ($p > 0.05$) . Birth survival rate in fetal rats to parents with Ni NPs and Ni MPs significantly decreased compared with control, and the birth survival rate of the high dose of Ni NPs significantly decreased compared with Ni MPs (Table C.14). At the same time, the feeding survival rates in the Ni NPs and Ni MPs groups were also lower than the control group. However, the feeding survival rate of the high dose Ni NPs was higher than that of Ni MPs. Out of 104 live born rats 67 survived (64.4%) after 21 days at a high dose of Ni NPs and out of 174 live born rats 75 survived (43.1%) after weaning (21 days) in Ni MPs.

Table C.14. Effects of Ni NPs on rat reproductive index (%) (Kong et al. 2014)

Group	Mating Success Rate	Pregnancy Rate	Live Birth Rate	Birth Survival Rate	Feeding Survival Rate
Control	100 (20/20)	100 (20/20)	100 (20/20)	94 (185/196)	79 (147/185)
NP Low dose: 5 mg/kg-d	90 (18/20)	90 (18/20)	100 (18/18)	86 (171/198) *	73 (125/171)
NP Mid dose: 15 mg/kg-d	80 (16/20)	80 (16/20)	100 (16/16)	75 (142/190) *	65 (93/142) *
NP High dose: 45 mg/kg-d	80 (16/20)	80 (16/20)	100 (16/16)	67 (104/156) *,1	64 (67/104) *,1
Ni MPs	90 (18/20)	90 (18/20)	100 (18/18)	82 (174/211) *	43 (75/174) *

* $p < 0.05$, compared with control group; [1] $p < 0.05$, compared with Ni MPs (45 mg/kg BW).

Saini et al. (2014a). Prenatal exposure to nickel on pregnant Swiss albino mice and fetal development.

Saini et al. assessed the embryotoxic effects due to exposure to Ni as $NiCl_2*6H_2O$ in Swiss albino mice in two separate, but similar studies (Saini et al. 2014a; Saini et al. 2014b). In one study, Ni was administered orally (46, 92, or 185 mg Ni/kg BW) to pregnant females from GD 0-5. Dams were sacrificed by cervical dislocation on GD 18 and uteri examined. Implantation sites and live fetuses per dam were counted and the conceptus at each site was classified as being alive, resorbed, or dead. A significant decrease in maternal and fetal BW was noted at doses of 92 and 185 mg Ni/kg BW (Table C.15; (Saini et al. 2014a)).

Table C.15. Effect of Ni^{+2} ($NiCl_2*6H_2O$) on diet consumption, water intake, and body weight of pregnant mice (from Saini et al. 2014a)

(Mean ± SEM)					
Groups	**Dose**	**Diet Consumption**	**Water Intake**	**Maternal BW**	**Percent Mortality**
	mg Ni/kg BW	**g**	**Ml**	**g**	
I	0	9.12 ± 0.06	12.26 ± 0.09	19.00 ± 0.31	0
II	46	9.11 ± 0.00	12.12 ± 0.01	18.66 ± 0.66	0
III	92	9.00 ± 0.11	11.76 ± 0.00	16.00 ± 1.15 *	0
IV	185	8.12 ± 0.12*	10.94 ± 0.64 *	13.20 ± 0.80 *	0

Note: The data were evaluated by one-way analysis of variance (ANOVA).
* The level of significance from control $p < 0.05$.

Reproductive parameters including implantation sites and live fetuses per dam were significantly reduced in a dose-dependent manner. The authors stated the reduction in maternal body weight might be due to implantation failure and increased number of resorptions . The decrease was significant at all Ni doses. Post-implantation deaths or embryonic resorptions were found to be 5.26%, 12.5%, and 37.5% in mice at 46, 92, and 185 mg Ni/kg BW, respectively. A significant decrease in placental weight was also evident at 185 mg Ni/kg BW (Saini et al. 2014a).Exposure increased skeletal aberrations including absence/reduced ossification of nasal, frontal, parietal, intraparietal, supraoccipital bones of skull, reduced number of ribs, sternebrae and caudal vertebrae, absence/reduced ossified carpals, metacarpals, tarsals, metatarsals, and phalanges.

Saini et al. (2014b). Effects of gestational administration of nickel on postnatal development in Swiss albino mice.

In the other study by Saini et al., the effects of Ni were investigated at different gestational periods (Saini et al. 2014b). Pregnant Swiss albino mice were given $NiCl_2*6H_2O$ by gavage (46.125, 92.25, and 184.5 mg Ni/kg BW) at doses below median lethal dose during GD 0–5 (preimplantation period), GD 6–13 (organogenetic period), and GD 14–18 (fetal period). Each group had 15 mice. Tap water served as the control. The dams were allowed to deliver and raise their pups.

No notable changes in behavior or morphology were evident in experimental dams. The maternal body weight fell significantly at 92 and 185 mg Ni/kg BW compared to control. However, no change in mortality rate was recorded in any of the experimental groups. A significant ($p<0.05$) decrease in litter size was observed after 184.5 mg Ni/kg BW exposure during either of the three gestation periods. The greatest treatment-related effect was associated with exposure during the preimplantation period, followed by the fetal period. The gestation index (number of females delivering live young divided by the number of females with evidence of pregnancy multiplied by 100) from preimplantation period was low at all the doses. The gestation index during the preimplantation period was decreased by 75% at all three dose levels, while no effect was seen with exposure during the organogenetic or fetal periods. Live birth index decreased with exposure to 184.5 mg Ni/kg BW during the preimplantation and organogenetic periods. The viability index and weaning index of pups decreased after 92.25 or 184.5 mg Ni/kg BW doses with exposure during all periods. The viability index was calculated on PND 4, 7, and 14 of age as the number of live offspring on day 4, 7, or 14 divided by number of offspring delivered divided by 100. The authors described the weaning index as a percentage that was equivalent to a lactation index; the number of live offspring at day 21 divided by the number of live offspring born multiplied by 100. A dose-dependent, highly significant ($p<0.01$) decrease in the body weight of offspring from day 0 to 6 weeks of age at all the doses during different gestation periods was observed. Saini et al. concluded that young are vulnerable to Ni exposure throughout gestation and lactation, and that Ni ingested by mothers constitutes a great threat to the progeny.

Related Study

Storeng and Jonsen. (1981). Nickel toxicity in early embryogenesis in mice.

Timed pregnant NMRI/Bom mice were given a single ip injection of $NiCl_2 \cdot 6H_2O$ on one of GDs 1, 2, 3, 4, 5, or 6. The dose provided 20 mg Ni^{2+}/kg body weight. Each day had its own treated and sham-injected control groups, with group sizes ranging from 23-29 pregnant females. Uterine contents were evaluated on GD 19.

The mean number of implantation sites/dam was lower than controls ($p < 0.1$) for dams treated on GD 1. The average number of fetuses/litter classified as “normal” was lower

in treated than control groups for all test days; statistical significance was reported following treatment on GD 1, 3, and 5 ($p < 0.05$ or 0.01). Morphological anomalies were reported in all treated, and some control, groups. Specific observations consisted of hematoma, exencephaly, and anemic appearance with "hypodevelopment." The highest overall frequency of abnormal plus stillborn fetuses was 4.3%, following treatment on GD 1.

C.2.5. Maternal Fetal Distribution of Ni

As noted in Section A.4. Pharmacokinetics, materno-fetal transfer of Ni occurs in mammals via the placenta. The kidney is the primary route for Ni clearance in both the mother and fetus. Fetuses lack effective means for getting rid of excessive Ni due to their confined environment and relatively weak kidney function. Thus, fetuses are particularly vulnerable to the damaging effects of Ni.

Given the biological overlap of information in maternal-fetal distribution of Ni and the earlier pharmacokinetics section, more published studies encompassing these two topics are described in Appendix 1.

Mas et al. (1985). The acute toxicity and teratogenicity of nickel in pregnant rats.

Mas et al. conducted a study of Ni to investigate: (a) Ni distribution during pregnancy, (b) the influence of changes in body weight during pregnancy on the toxicity of Ni, and (c) the effect of a teratogenic dose of Ni on the uptake, incorporation and/or transport of certain essential metabolites in the placenta and fetuses of the pregnant rat (Mas et al. 1985).

For studies on Ni distribution, groups of 12- and 19-day pregnant rats were injected ip with ^{63}Ni (5 µCi/mg Ni; 4 mg Ni/kg BW). Pregnant Wistar Porton rats were from an inbred laboratory colony. The animals were killed by decapitation at 0.25, 1, 4 or 24 h after dosing. After ip administration, Ni was cleared rapidly from the blood of 12- and 19-day pregnant rats. The data for 15 min, 1 hr, 4 hr, and 24 hr suggest that the concentrations of Ni in the maternal tissues of both groups were maximal at about 1 h after dosing and then decreased progressively (Mas et al. 1985). Concentrations of Ni in the placenta and fetuses were maximal at 1 h and 4 h in the 12-day and 19-day pregnant rat.

LD_{50} determinations were made on virgin, 12-day, and 19-day pregnant rats. Four animals per group were given $NiCl_2$ ip in a constant volume of solution (1.0 ml/kg BW) as described in Table C.16.

Table C.16. LD_{50} values for Ni administered ip to non-pregnant and pregnant rats (Adapted from Mas et al. 1985)

Animal group (n=4)	LD_{50} (mg Ni/kg BW)	95% Confidence limits (mg Ni/kg BW)
Virgin	9.33	8.51 – 10.23
12-day pregnant	6.30	5.62 – 7.08
19-day pregnant	5.96	5.45 – 6.50

Teratogenic effects were determined on the survivors from these experiments and also with other groups of animals that were injected on day 8, day 12 or day 16 of pregnancy with 1, 2 or 4 mg Ni/kg BW. Many of the fetuses of dams that survived the administration of Ni at or near the LD_{50} on day 12 of pregnancy, when removed on day 20, were malformed. The survivors of the group treated on day 19 of pregnancy, however, delivered normal young and at 5 days postpartum, the litter sizes and weights were similar to those of control, untreated females. Although treatment of the dam with Ni during organogenesis decreased the incidences of fetal reabsorption and skeletal retardation (which are high in this inbred colony), it induced specific malformations e.g. hydrocephalus, hydronephrosis and heart defects, and other effects e.g. bleeding into the subachranoid spaces and pin-point hemorrhages in various organs, particularly in the lungs. These abnormalities were common to fetuses of dams that were dosed with Ni on either day 8 or day 12 of pregnancy, but fetal weight (at day 20) was reduced significantly only in the latter group. When injected on day 16 of pregnancy, Ni (1–4 mg/kg BW) was neither teratogenic, nor highly fetotoxic (Mas et al. 1985). Mas et al. noted there may be species differences in Ni toxicity, as some of their findings in their inbred rats were different from results in mice and golden hamsters (Ferm 1972; Lu et al. 1979).

C.2.6. Milk Composition

Metals, including Ni, are known to be transferred into animal, as well as human, milk. The following studies discuss the effects of Ni on milk in rats and cows.

O'Dell et al. (1970). Effect of nickel supplementation on production and composition of milk.

O'Dell et al. investigated the effect of Ni on milk production and on Ni, fat, protein, and solids-not-fat in milk when lactating cows were fed Ni in amounts lower than those at which toxicity symptoms have been observed with monogastric animals (O'Dell et al. 1970). Three concentrations of nickel carbonate were fed to 3 groups of 5 lactating dairy cows each. The 15 cows were multiparous, lactating dairy cows with daily production between 16.5 and 30.0 kg. These cows were equalized into groups of 3 based on age, weight, milk production, and stage of lactation. Concentrates are low-fiber, high-energy additives for cattle feed (hay). Nickelous carbonate ($NiCO_3$) was added to the concentrate to provide 0, 50, and 250 ppm of elemental Ni and

concentrate fed at a ratio of 1 kg per 3 kg of milk produced. Average daily consumption of supplemental Ni per cow was 0, 365 or 1,835 mg, respectively. There was no significant effect on milk production, milk composition, animal, health or feed consumption (Table C.17). Fifty milliliter samples of milk, in duplicate, from each cow during both the pretreatment period and at the end of 6 weeks treatment. Daily intakes of supplemental Ni up to 1.8 g exerted no statistically significant influence on concentrate intake, milk production, or milk composition. Within the detectable limits of the analytical procedure, feeding Ni did not increase Ni in milk. None of the milk samples from cows fed 250 ppm Ni contained as much as 0.1 ppm Ni, which was the lower reliability limit of the procedure. Nickel in concentrate at 250 ppm greatly exceeds the amount that a lactating cow would consume in feeds under any conceivable normal circumstances. Less than 0.12% of the supplemental nickel appeared in the milk.

Table C.17. Effect of supplemental Ni in concentration on cow performance[a] (Adapted from O'Dell et al. 1970)

	Supplemental Ni, ppm			
	0	**50**	**250**	**SE[b]**
Concentrate fed (kg/cow-d)	7.76[c]	7.76	7.76	
Concentrate refused (kg/cow-d)	0.45	0.46	0.41	
Concentrate consumed (kg/cow-d)	7.31	7.30	7.35	
Supplemental Ni consumed (mg/cow-d)	0.	365	1,835	
BW change (kg/cow-d	0.67	0.83	0.83	
Milk produced				
Preliminary period (kg/cow-d)	24.2	23.8	23.4	
Treatment period (kg/cow-d)[d]	22.0	22.0	23.1	0.68
Fat in milk				
Preliminary period (kg/cow-d)	3.60	3.36	3.52	
Treatment period (kg/cow-d)[d]	3.80	3.66	3.63	0.09
Protein in milk				
Preliminary period (kg/cow-d)	3.85	3.87	3.75	
Treatment period (kg/cow-d)[d]	3.95	3.90	3.85	0.05
Solids-not-fat in milk				
Preliminary period (kg/cow-d)	8.75	8.70	8.48	
Treatment period (kg/cow-d)[d]	8.63	8.55	8.46	0.10

[a] Measures of milk quality and quantity. Average values for 5 cows per treatment
[b] Standard error of a treatment mean
[c] None of the measures in this table were significant at the 5% level of probability
[d] Adjusted by covariance for differences in milk and milk components during standardization period

Dostal et al. (1989). Effects of nickel chloride on lactating rats and their suckling pups, and the transfer of nickel through rat milk.

Dostal et al. (1989) reported on the effects of nickel chloride hexahydrate on lactating rats and their suckling pups and the transfer of Ni through rat milk (Dostal et al. 1989). Single and multiple dose experiments were conducted to investigate the possible effects of maternal $NiCl_2$ administration on lactating Sprague Dawley rats and their suckling

pups, and to determine if these effects could be correlated with plasma or milk Ni concentrations. Either a single dose (0 [vehicle], 10, 50, or 100 µmol/kg), or four doses (0 [vehicle], 50, or 100 µmol/kg per injection) of $NiCl_2$ were administered by subcutaneous (sc) injection to lactating Sprague Dawley rats. An additional multiple (four) dose experiment (0 [vehicle], 0 [pair-fed, vehicle], 100 µmol/kg per injection) included a pair-fed vehicle control group. Nickel levels were assessed in plasma and milk after single or multiple doses. The composition of the milk was determined after multiple doses, and milk production was assessed by determining the content of RNA and DNA in the mammary gland as indices of milk synthetic activity and cellularity of the gland.

Administration of a single dose of 10, 50, or 100 µmol $NiCl_2$/kg caused dose-related increases in plasma and milk Ni concentrations (correlation coefficient r = 0.98 and 0.92, respectively) 4 hr after dosing to levels of 9740 and 188 µg/liter in plasma and milk, respectively, at the highest dose. The time course of appearance of Ni in rat milk and plasma following a single sc dose of 100 µmol $NiCl_2$/kg showed that peak plasma concentrations of 10.3 mg/liter were reached by 4 hr and decreased to 2.0 mg/liter by 24 hr. If a single half-life ($t_{1/2}$) is calculated for all of the data points from 4 to 24 hr using a one-compartment model, a $t_{1/2}$ of 5.2 hr is obtained. In contrast to plasma, peak milk Ni concentrations of 650 µg/liter were not reached until 12 hr, and were 580 µg/liter 24 hr after injection. After four daily doses of 50 and 100 µmol $NiCl_2$/kg, average milk Ni concentrations were 513 and 1030 µg/liter, respectively, 4 hr after the last dose. However, plasma Ni concentrations were the same as those found after a single dose, giving milk/plasma Ni ratios of 0.10.

In the multiple dose experiments, 50 or 100 µmol $NiCl_2$/kg treatment were observed to significantly decrease total mammary gland weight and the weight relative to body weight. The total amount of DNA in the gland was not reduced compared to ad libitum-fed controls, but the DNA per gram of gland was significantly increased, possibly reflecting a loss of fluid from the tissue. When compared to pair-fed controls, 100 µmol $NiCl_2$/kg treatment was associated with a significant decrease in total mammary gland DNA. The total amount of RNA in the gland was significantly reduced compared to both ad libitum and pair-fed controls, but the RNA per gram of gland was not significantly affected.

The relatively large decreases in RNA with only small changes in DNA led to RNA/DNA ratios which were significantly reduced compared with both ad libitum-fed and pair-fed controls; the authors suggested this indicated reduced protein synthetic activity in the mammary cells.

The composition of the milk was also significantly altered by the $NiCl_2$ treatment, and most of the changes were not due to the decreased food consumption. For example, $NiCl_2$ treatment (100 µmol/kg) was associated with a significant increase in milk solids (42%) and lipid (110%), a decrease in milk lactose (61%), and no change in milk protein compared with ad libitum-fed controls.

Treatment of lactating rats with 50 or 100 µmol $NiCl_2$/kg for 3 days led to plasma Ni concentrations of approximately 24 and 50 µg/liter, respectively, in their suckling pups. Maternal $NiCl_2$ administration at 100 µmol/kg also was associated with a significant decrease in body weight in the female and male pups, and a decrease in the absolute liver weight of the female pups only, but did not affect the relative liver weight.

Kong et al. (2014). Nickel nanoparticles exposure and reproductive toxicity in healthy adult rats.

Kong et al. (2014) examined the relationship between nickel nanoparticle (NP) exposure and reproductive toxicity in Sprague-Dawley rats. Briefly, as this study was described earlier, F0 male and females were gavaged with different doses of Ni NPs, Ni MPs and 0.9% sodium chloride solution (control group) for 10 weeks before the initiation of the mating period. Females continued to receive test samples during gestation and lactation. Nickel NPs were associated with decreased weight gain in the neonatal rats at 4, 7, 14, and 21 days of age (Table C.18). The offspring weights of groups receiving the mid- and high- doses of Ni NPs, and Ni MPs were significantly decreased compared with the control group. However, there was no statistical significance between high dose of Ni NPs and Ni MPs (Kong et al. 2014). The authors stated the decreased weight gain of neonatal rats may be due to alterations in milk quality being less nutritional for pups.

Table C.18. Body Weight changes of pup rats during the experiment (grams) (Kong et al. 2014)

Group	Birthday	The 4th day	The 7th day	The 14th day	The 21st day
Control	7 ± 1	11 ± 2	16 ± 3	31 ± 3	50 ± 5
Low dose	7 ± 1	10 ± 2 *	15 ± 3	27 ± 5 *	48 ± 5
Mid dose	7 ± 0	10 ± 2 *	14 ± 3 *	25 ± 7 *	46 ± 6 *
High dose	7 ± 0	10 ± 2	14 ± 2	24 ± 2 *	42 ± 6 *
Ni MPs	7 ± 0	9 ± 2 *	13 ± 3 *	26 ± 2 *	45 ± 7 *

* $p < 0.05$, compared with control group.

C.2.6.1. Neuroendocrine Control of Prolactin

Metal ions are known to affect the function of tissues, such as the brain and pituitary, which are involved in the maintenance of reproductive function. Prolactin (PRL) has many diverse actions in many species of animals, including osmoregulation, growth and developmental effects, metabolic effects, actions on ectodermal and integumentary structures, and actions related to reproduction. Abnormal female PRL patterns are known to alter latency period for the onset of maternal behavior critical to successful nurturing of young (Bridges et al. 1985). In rats, which is the primary species used in the studies discussed here, PRL is known to feedback negatively on its own secretion (autofeedback) (Clemens and Meites 1968). Prolactin is secreted episodically; its levels

also change with cyclicity and age. For those reasons examination of PRL perturbation with an exogenous substance is complicated in females. Some studies discussed here purposely examined the effects of Ni on PRL in male rats. While PRL has multiple actions related to reproduction in rodents (such as in ovulation in females and development of the reproductive tract in males), its primary action in human reproduction is the preparation of the breast for lactation.

LaBella et al. (1973). Prolactin secretion is specifically inhibited by nickel.

Nickel was reported to localize within the pituitary gland and the hypothalamus of rats (LaBella et al. 1973), and it has been shown to inhibit prolactin secretion both in vitro and in vivo. The study by LaBella et al., while not well reported, demonstrated with bovine pituitary glands that commercial $NiCl_2$ specifically inhibited the basal release of prolactin in vitro. Bovine pituitary glands from animals of unspecified age and sex were obtained 30 to 60 minutes after death. They were maintained at 25ºC at all times before incubation. Pituitary anterior lobes were cut into 1 mm cubes incubated in buffer at 37ºC under 95% O_2/5% CO_2 in the presence or absence of increasing concentrations of Ni, and the medium assayed for prolactin by radioimmunoassay. As the concentration of Ni was increased, prolactin release was almost completely inhibited. In other in vivo experiments with adult male Sprague Dawley rats, 100 µg Ni^{+2} (as $NiCl_2$) resulted in a 40% decrease in serum prolactin at 30 min, and 200 µg Ni^{+2} resulted in significant inhibition even after 60 min.

Clemons and Garcia. (1981). Neuroendocrine effects of acute nickel chloride administration in rats.

Clemons and Garcia investigated the neuroendocrine effects of $NiCl_2$ hexahydrate administration in rats. Subcutaneous injections of 10 or 20 mg $NiCl_2$/kg (42 and 84 µmol/kg, respectively) were administered to young adult male Sprague Dawley rats. Groups of eight animals were killed by decapitation at 3 and 6 hours and 1, 2, 4, and 7 days after the injection. Two groups of control rats received saline and were sacrificed at the beginning and end of the experiment. The results confirmed LaBella's finding that Ni produces an acute fall in PRL in the hours after Ni administration. However, the effects of $NiCl_2$ increase serum PRL in the following days (Table C.19).

Table C.19. Serum level of Prolactin in Ni($^{+2}$)-treated rats (Adapted from Clemons and Garcia 1981)

Treatment Group	Time after $NiCl_2$	Serum Concentration Prolactin (ng/ml)
Control	3 hr	45.7 ± 6.5
	7d	48.9 ± 3.2
$NiCl_2$ 10 mg/kg	3 hr	32.7 ± 7.0
	6 hr	81.0 ± 16.0
	1 day	122.9 ± 17.3 *
	2 days	130.2 ± 16.5 *
	4 days	105.2 ± 11.3 *
	7 days	85.8 ± 15.0 ◊
$NiCl_2$ 20 mg/kg	3 hr	21.8 ± 3.6 ○
	6 hr	32.1 ± 8.2
	1 day	189.9 ± 28.5 *
	2 days	175.1 ± 18.5 *
	4 days	174.5 ± 14.4 *
	7 days	91.7 ± 15.1 ◊

* $p<0.001$, computed by Student's t test
◊ $p<0.005$
○ $p<0.02$

One to 4 days after $NiCl_2$ treatment there is a profound three- to four-fold increase in serum PRL concentration. Nickel chloride promotes high circulating PRL levels lasting 1 to 4 days instead of specifically inhibiting PRL secretion from the pituitary (Clemons and Garcia 1981) as reported in previous short-term studies (LaBella et al. 1973).

Carlson, HE. (1984). Inhibition of prolactin and growth hormone secretion by nickel.

Nickel is a potent inhibitor of PRL secretion from isolated rat pituitary quarters in vitro. Secretion of hormones from rat pituitary tissue was studied in two systems: in static incubations in flasks and a continuous-flow perifusion apparatus (Carlson 1984). Anterior pituitary glands from male Sprague Dawley rats (weighing 200-300 g) were quartered and cultured in incubation buffer. In static incubations, pituitary quarters were pre-incubated in buffer for 2 h at 37^{o}C, rinsed, placed in fresh medium containing NiCl2, then incubated for 1 h; test medium was removed and frozen for later hormone measurements (Carlson 1984). For continuous-flow experiments, pituitary quarters were pre-incubated for 2 h, then 2 ml aliquots of effluent were collected every 4 min and frozen at -20^{o}C for later hormone assay. Nickel suppressed both basal PRL release and the stimulation of PRL secretion due to theophylline and dibutyryl cyclic AMP (Tables C.20 and C.21).

Table C.20. Effect of Nickel on Basal PRL Secretion (Adapted from Carlson 1984)

Agent	Medium PRL (Percent of Control)
Static flask incubations	
Control	100
1 mM $NiCl_2$	46 ± 5[a] (55)
Perifusions	
Control	100
1 mM $NiCl_2$	38 ± 2[a] (8)
0.1 mM $NiCl_2$	86 ± 3[a] (4)
0.01 mM $NiCl_2$	91 ± 4[a] (5)
1 mM $NiCl_2$ + metoclopramide 20 µg/ml	42 ± 3[a] (6)

[a] Significantly different from control, $p<0.05$.
Mean ± SE is shown. Numbers in parentheses indicate number of perifusion experiments or number of replicate flasks in static incubations. In the static incubations, contro] levels of PRL secretion were in the range of 150-450 ng/ml/mg. Perifusion control hormone levels were 20-40 ng/ml for PRL.

Table C.21. Effect of Nickel on Stimulated PRL Secretion (Adapted from Carlson 1984)

Agent	Medium PRL (Percent of Control)
Static flask incubations	
Control	100 (6)
10 ng/ml GHRH	109 ± 19 (5)
10 ng/ml GHRH + 1 mM $NiCl_2$	30 ± 6[a] (5)
1 mM $NiCl_2$	20 ± 4[a] (5)
Perifusions	
Control	100
1 mM DbcAMP	118 ± 4[a] (4)
1 mM DbcAMP + 1 mM $NiCl_2$	43 ± 1[a,b] (4)
5.5 mM theophylline	149 ± 2[a] (10)
5.5 mM theophylline + 1 mM $NiCl_2$	42 ± 6[a,c] (7)

[a] Significantly different from control, $p<0.05$.
[b] Significantly different from DbcAMP alone, $p<0.05$.
[c] Significantly different from theophylline alone, $p<0.05$.
[d] Significantly different from GHRH alone, $p<0.05$.
Mean ± SE is shown. Numbers in parentheses indicate number of perifusion experiments or number of replicate flasks in static incubations. Control hormone levels in the perifusion and flask experiments were in the ranges given in Table C.20.

Stimulation of growth hormone (GH) secretion by synthetic growth hormone releasing hormone (GHRH) is also blunted by Ni^{+2}, although basal GH release and stimulated GH release due to theophylline or dibutyryl cyclic AMP are not suppressed. Nickel antagonizes the stimulation of both PRL and GH secretion by the barium ion, suggesting that the inhibitory effects of Ni^{+2} on hormone release are due to an antagonism of calcium uptake or redistribution.

Cooper et al. (1987). Effects of metal cations on pituitary hormone secretion in vitro.

Cooper et al. also investigated the effects of metals on pituitary hormone release. Anterior pituitary fragments from adult male Long-Evans rats were evaluated using an in vitro perfusion system. In order to establish a baseline secretory rate of PRL, the pituitary fragments were perfused for 2 hr in standard buffer. After the initial period, the tissues were exposed to experimental buffer containing 50 µM Ni. After 1.5-hour exposure to the metal, the experimental buffer was replaced by the standard buffer. The tissue was then perfused for an additional 1.5 hr. Control tissue received only standard buffer during the entire perfusion. Figure C.3 illustrates how Ni reduced PRL release compared with baseline secretion, but the result was not statistically significant (Cooper et al. 1987).

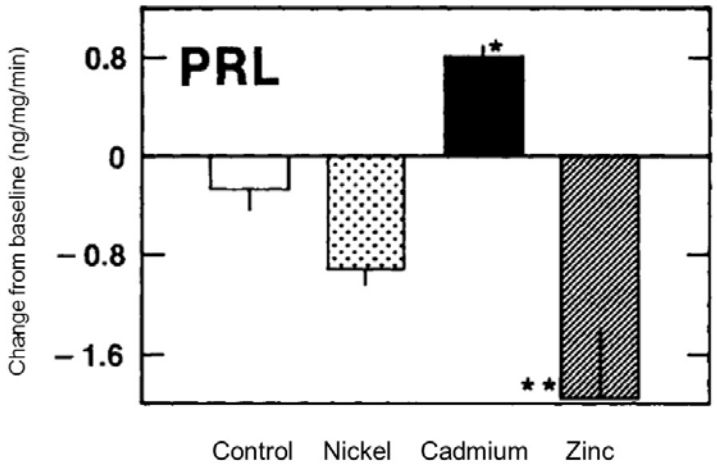

Figure C.3. Spontaneous prolactin release following addition of metal cations
Bar represents the difference in cumulative release (mean ± SE, n=6) of pituitary hormones compared to the 30-minutes prior to exposure.
* P≤ 0.05. ** P≤ 0.01. Adapted from Cooper et al. (1987).

Smith et al. (1993). Perinatal toxicity associated with nickel chloride exposure.

In a study by Smith et al., exposure of dams to $NiCl_2$ significantly reduced PRL in the 250 ppm group. Virgin female Long-Evans rats were given 0, 10, 50, or 250 ppm Ni in $NiCl_2$ drinking water solutions for 11 weeks prior to mating and then for two successive gestation (G1, G2) and lactation (L1, L2) periods. Plasma PRL levels in dams and pups were analyzed using ANOVA. The data distributions were highly skewed and a log transformation of the response values were used prior to analysis. The average daily liquid intake (mg/kg) was comparable across doses during all parts of the study with the exception of those females drinking 250 ppm Ni. The overall average throughout the study was 1.3, 6.8, and 31.6 mg/kg per day at 10, 50 and 250 ppm, respectively (Smith et al. 1993). Prolactin levels in dams were 6.6 ± 2.1 in controls, 5.8 ± 1.8 in 10 ppm

treated dams, 6.3 ± 1.8 in 50 ppm treated dams, and 5.2 ± 1.1 in 250 ppm treated dams ($0.01<p\leq0.03$) (Smith et al. 1993). The significance levels were assessed by pairwise comparison to the controls. Prolactin levels in pups were unchanged by treatment (comparable within generations in pups from either L1 or L2) and were reduced in dams at the high dose. All pups in L2 showed PRL levels less than half of those in L1 (males, $P\leq0.002$; females, $P\leq 0.02$).

C.3. Integrative Evaluation of Female Reproductive Toxicity

C.3.1. Female Reproductive Toxicity in Humans

Ni is associated with levels of SHBG and possibly DHEAS, fasting insulin, and fasting glucose, but no effect on fecundity, as indicated by TTP, was observed in one study of a population with blood Ni levels in the range of 4.00 – 16.00 µg/L but with little variability, as shown in Table C.22. Serum Ni appeared to be lower in a small sample of pre-eclamptic women, though the difference was not statistically significant. Some of the outcomes examined in studies classified as developmental toxicity studies in Section B.1., such as spontaneous abortion and preterm birth, may also be considered female reproductive outcomes, as the effects of Ni may be mediated through the mother's reproductive system.

Table C.22. Overview of human studies of Ni and female reproductive toxicity

Study	Exposure assessment	Ni concentration, µg/L	Results
Bloom et al. (2011)	Whole blood Ni	Mean (SD): Pregnant women 6.94 (1.47) Non-pregnant women 6.81 (1.44) Range 4.00 – 16.00	TTP was not associated with blood Ni
Zheng et al. (2015)	Serum Ni	Median, mean: PCOS cases 1.52, 1.96 Controls 1.11, 1.23	1 µg/L Δ in serum Ni was associated with: 12.6% ↓ SHBG adjusted for age, BMI, WHR (p=0.032)
Maduray et al. (2017)	Ni in hair and blood	Median ± SEM <u>Serum</u> pre-eclamptic 0.02 ± 0.0 normotensive 0.14 ± 0.0 <u>Hair</u> (*µg/g) pre-eclamptic 6.86 ± 0.81 normotensive 8.40 ± 1.31	Ni in serum (p=0.16) and hair (p=0.85) were not significantly correlated with pre-eclampsia

C.3.2. Female Reproductive Toxicity in Animals

A review of the literature on the female reproductive toxicity of nickel in animals report adverse effects on estrous cyclicity, the uterus and ovary. There are also studieson the

maternal fetal distribution of Ni, alteration of milk composition, and effects on the neuroendocrine control of prolactin.

Two multi-generation reproductive toxicity studies of Ni^{+2} report adverse reproductive effects. In the Siglin (2000b) report from Springborn Laboratories Inc., very different incidences of naturally occurring estrous cycles between F0 and F1 females were described. Many of the treated F1 females were reported to not be cycling, although the control F1 females also had a low rate of cycling. There was a lower incidence of F1 control females who displayed normal estrous cyclicity compared with F0 control females. These findings, when assessed together, make it difficult to discern whether there was an effect on cycling that was purely treatment related. The data for individual F1 animals were not reported in the main study; the author reported of the data as group means. Female reproductive parameters such as hormone levels and ovarian follicle counts would have been informative for evaluating the cause of altered cyclicity, which could ultimately affect female reproduction, but were not assessed. As discussed in Price et al. 1988, the RTI study reported that $NiCl_2$ exposure primarily interferes with late gestation, parturition, lactation and/or postnatal development; however, the findings are complicated by the effects associated with decreased fluid intake in the high dose treatment groups.

Some studies showed Ni and Ni compounds altered the reproductive function of the uterus, more specifically alterations in the uterine lining and lumen. Investigating effects on uterine lining, Chang et al. (1970) reported Ni inserted into the lumen near the tubouterine junction was somewhat effective in preventing pregnancy. In vitro, $NiCl_2$ was found to induce alterations in uterine contractile activity in uterine strips isolated from 20-day-pregnant rats. At low concentrations $NiCl_2$ significantly increased basal tone, while at higher concentrations $NiCl_2$ abolished uterine contractions. Treatment-related mitochondrial structural damage and glycogen accumulation were shown in the uterine smooth muscle cells (Rubanyi and Balogh 1982). Observed effects of Ni and Ni compounds on the uterus are summarized below in Table C.23.

Regarding the effects of nickel compounds on the ovary, nickel has been reported to disturb regular ovarian cycles, induce a dose-dependent anovulation, and alter the secretion of several hormones, notably progesterone (Forgacs et al. 1997; Krockova et al. 2013). Ovarian histological alterations were also noted after exposure to Ni (Kong et al. 2014). Other reported ovarian changes as a result of Ni exposure include changes in weight and signs of oxidative stress (Rao et al. 2009). In a related study, nickel subsulfide (Ni_3S_2) was observed to affect the growth and morphology of a hamster ovarian epithelial cell line (Costa 1978). Reported effects on ovarian function are summarized below in Table C.24.

The placenta provides partial protection to the fetus against certain metals, such as Ni, that are toxic to the fetus (Mas et al. 1985). Nickel primarily distributes to the kidney, liver, then placenta in the very short term (Mas and Sarkar 1988). After 24 hours, Ni distributes equally to the maternal kidney and placenta (Mas and Sarkar 1988). Nickel

is actively transferred across the blood-placenta-barrier into the fetus, but to a lesser extent from the fetus to the mother (Hou et al. 2011; Szakmary et al. 1995; Wang et al. 2010). Nickel has been detected in fetal blood and amniotic fluid. Nickel uptake, retention and transport in placenta increase dose- and gestation age-dependently (Lu et al. 1981). The fetus has a lesser ability to excrete Ni compared to the mother.

The kinetics of $NiCl_2$ in fetal tissues showed a different pattern from that in maternal tissues (Lu et al. 1981). Concentration of Ni in blood and placentas were found to be at maximum level (19.8 and 3.9 µg/g) 2 hr after injection, and those in liver, spleens, and kidneys reached maximum levels of 4.9, 1.3 and 56.2 µg/g, respectively, 4 hr after injection (Lu et al. 1981). The relative concentrations of Ni in blood, organs, and tissues of pregnant mice at 24 hr after injection were found in order from highest to lowest concentration: kidneys > blood > placentas > fetuses ~ liver > spleen. In the presence of other chemicals, the absorption and distribution of Ni can be altered. Nickel uptake in fetal tissues and distribution was accentuated with concurrent exposure to other compounds, such as thiuram sulphides and glutathione (Jasim and Tjalve 1984; Jasim and Tjaelve 1986a).

Nickel and Ni compounds might have effects on measures of reproductive index; however, it is difficult to pinpoint as the study designs evaluated are complicated by variables including mating parameters, maternal-fetal interaction, and period of evaluation. Studies reporting reproductive index are summarized below in Table C.25. Measures of reproductive index are more clear when evaluated as a whole with consideration of developmental toxicity endpoints. After ip or im injection of Ni during pregnancy in mice and rats, reductions in number of live pups, body weights of fetuses and offspring, or malformations were observed (Mas et al. 1985; Sunderman et al. 1978). Intraperitoneal injections of $NiCl_2$ in mice embryos on the first day of gestation were reported to have a higher frequency of both early and late resorptions, and a higher frequency of stillborn and abnormal fetuses (Storeng and Jonsen 1981).

Nickel has been reported to affect milk composition in rodents, but not cows. In cows, adding Ni to their diet produced no significant effect on milk production, milk composition, animal health or feed consumption (O'Dell et al. 1970). Rats exposed to high doses of $NiCl_2$, excreted Ni into milk and showed changes in milk quality and production (Dostal et al. 1989). Reductions in liver weight in the suckling pups were also observed, which may have been due to Ni exposure or to changes in milk composition (Dostal et al. 1989). Decreased weight gain was seen in pups after maternal gavage dosing with Ni NPs and Ni MPs during gestation and lactation (Kong et al. 2014). In rodent studies, pups consuming milk from Ni exposed mothers are observed to gain less weight and milk composition is observed altered in dams. Observed effects on milk composition are summarized below in Table C.26. Milk solids, lipid, lactose, and fatty acid ratios are reported to be altered, along with pup weights.

Historically, the role of PRL in mammalian physiology was believed to be limited to lactation but it is now known that PRL is involved in a great variety of mechanisms,

often acting permissively to modify the effects of other hormones (Kaufman 1981). Hormonal effects may play an important role in the reproductive toxicology of nickel both at the neuroendocrine and gonadal levels in the hypothalamic–pituitary–gonadal (HPG) axis (Forgacs et al. 2012). Nickel chloride has effects on the endocrine system that appear mediated through the neuroendocrine system (pituitary and hypothalamus glands); alterations in neurotransmitter release following exposure to metal cations have been described by several investigators (Clemons and Garcia 1981; Cooper et al. 1987; LaBella et al. 1973). Prolactin is the primary regulatory hormone for milk synthesis. Nickel interferes with normal pituitary function and PRL secretion in the dam. Abnormal female PRL patterns are known to alter the onset of maternal behavior needed for successful nurturing of young (Bridges et al. 1985). Given that PRL secretion is reduced by exposure to Ni (Carlson 1984), it is possible that this action contributes to the changes in milk production and milk quality observed in Ni-exposed rodents, as well as to negative consequences on the suckling offspring as suggested by the perinatal mortality seen in some studies.

Table C.23. Studies of Uterine Toxicity

Reference	Compound	Animal Model (Species/ Strain/Sex/Age) N	Exposure (Route/Period/ Frequency) Vehicle	Doses/ Concentrations	Results
Chang et al., 1970	Ni wire, 0.005" diameter	Adult virgin female Holtzman rats n=5	Intrauterine device (IUD) inserted in one uterine horn on pregnancy day (PD) 3, or on subsequent PDs, up to PD 21	Not clearly defined	<u>Insertion on PD 3</u> Ni fertility = 7.1 Fertility = [Number of implants (treated horn) X 100] / Number of implants (control horn)] Ni was somewhat effective for preventing pregnancy in rats <u>Insertion on PD 4 or later</u> no reported effects on fertility
Rubanyi and Balogh, 1982	$NiCl_2$	Pregnant Wistar rats	In vitro GD 20 uterine strips 1 hr incubation	10^{-7} – 10^{-3} M	10^{-7} M to 10^{-5} M $NiCl_2$ increased basal tone significantly 10^{-4} to 10^{-3} M $NiCl_2$ inhibited spontaneous contractile activity and decreased basal tone; dose-related mitochondrial structural damage and glycogen accumulation

Table C.24. Studies of Ovarian Toxicity

Reference	Compound	Animal Model (Species/ Strain/Sex/Age) N	Exposure (Route/Period/ Frequency) Vehicle	Doses/ Concentrations	Results	Comments
Chang et al., 1970	Ni wire, 0.005" diameter	Adult virgin female; Holtzman rats n=5/group	Intrauterine device (IUD) inserted in one uterine horn on pregnancy day (PD) 3, or on subsequent PDs, up to PD 21	Not clearly defined	Average number of corpora lutea (CL) in the ovary of the treated horn was the same as the control horn, and as that of untreated controls	Ten metals assessed; Ni tested only in rats Vague study design
Forgacs et al., 1997	$NiSO_4$ x 7 H_2O	Female SPRD rats 240-300g; regular (4-day) estrus cycles n=13-14/group	sc injection First injection given on the day of estrus of the 4th cycle; treatment was repeated through 4 cycles Vehicle: 9% saline	10, 20 or 40 mg/kg $NiSO_4$ x 7 H_2O; injection volume was 1 ml/kg	10 mg $NiSO_4$/kg disturbed regular ovarian cycles 40 mg $NiSO_4$/kg inhibited ovulation and abolished ovarian P secretion response to hCG stimulus $NiSO_4$ did not alter the number of CL or the weight of ovaries	Saini et al., 2014 noted this study reported decreased ovarian steroidogenesis in rats after the administration of $NiSO_4$
Kong et al., 2014	Ni NPs, average size 90 nm Ni MPs, average size 3μm	Female Sprague-Dawley rats 80–100 g n=20/group	Gavage for 10 weeks before initiation of mating, dosing continued through gestation and lactation (i.e., 8 weeks postpartum) Vehicle: 0.9% NaCl	Ni NPs: 5, 15, 45 mg/kg/day Ni MPs: 45 mg/kg/day Control	Pathology changes observed in Ni NP exposure groups: ovarian lymphocytosis, vascular dilatation and congestion, inflammatory cell infiltration, and an increase in apoptotic cells in ovarian tissues from Ni NP exposure groups, luteal cells increasing and becoming cavitated, increased eosinophils and inflammatory cell infiltration in ovaries	

Table C.24. Studies of Ovarian Toxicity (continued)

Reference	Compound	Animal Model (Species/ Strain/Sex/Age) N	Exposure (Route/Period/ Frequency) Vehicle	Doses/ Concentrations	Results	Comments
Krockova et al., 2013	$NiCl_2$	Female Slovak white glits (7 months old; pubertal) Granulosa cells 4-15 replicate cultures/concentration; the experiment was repeated 7 times.	In vitro cultured granulosa cells harvested immediately prior to onset of estrous cyclicity 48 h incubation	0, 62.5, 125, 250, 500 and 1,000 µmol $NiCl_2$/L	Significant decrease in P release at 1,000 µmol/L $NiCl_2$ Highest percentage of apoptotic cells after treatment with 1,000 µmol/L $NiCl_2$ (79.17±36.08 %, $p<0.05$); no significant differences detected in groups with the addition of 62.5 µmol/L $NiCl_2$ (62.21±20.00 %), 125 µmol/L $NiCl_2$ (49.90±26.37 %) and 500 µmol/L $NiCl_2$ (51.40±31.13 %) Decrease in volume of smooth endoplasmic reticulum of porcine granulosa cell after 1,000 µmol/L $NiCl_2$; lower occurrence of mitochondria after ≥500 mol/L $NiCl_2$	
Rao et al., 2009	nickel chloride ($NiCl_2$)	adult female albino mice (Mus musculus) of Swiss strain, 35 and 40 g	Oral; using a feeding tube attached to a hypodermic syringe 30 d 9 groups –control (distilled H_2O), combinations of vitamin E, chromium, and/or Ni	8 and 16 mg $NiCl_2$/kg BW	Protein levels in the ovary declined significantly ($P<0.01$) following high dose $NiCl_2$ A significant ($P < 0.001$; n=10) elevation was noted in lipid peroxide levels in the ovary after high dose $NiCl_2$ $NiCl_2$ ovarian weight (mg): Control: 26.30 ± 0.57 8 mg $NiCl_2$/kg BW: 25.90 ± 0.31 16 mg $NiCl_2$/kg BW: 24.60 ± 0.47 ($P<0.05$)	Vitamin E is protective against Ni effects

Table C.25. Studies of Reproductive Index

Reference	Compound	Animal Model (Species/ Strain/Sex/Age) N	Study Design Exposure (Route/Period/ Frequency) Vehicle	Doses/ Concentrations	Results	Comments
Mizejewski et al., 1990	$NiCl_2$	Adult Nya:Nylar outbred pregnant mice, 35--40 g n=40 litters n=173 mice total study	ip injection; twice Autopsied 2 d after second injection (injected on Monday and Wednesday, autopsied on Friday) Autopsy usually on GD 17	11.0 mg $NiCl_2$/kg (87.5 mg $NiCl_2$/100ml double-distilled, deionized water)	The natural rise of serum AFP concentrations was not influenced by 1.5 mg $NiCl_2$/kg1.5 mg $NiCl_2$/kg was associated with elevated AFP levels in amniotic fluid which exceeded control levels in later gestation A large increase in fetal death was seen with 1.5 mg $NiCl_2$/kg, while embryo resorption was not affected	Pregnancies were untimed; gestational age was determined by fetal crown-rump length on day of autopsy Copper and lead also examined
Kong et al., 2014	Ni NPs Ni MPs	Female Sprague-Dawley rats of 80–100 g n=100 n=20 females/group 5 groups	Gavage Female F_0 given doses of Ni NPs and Ni MPs and 0.9% NaCl (controls) for 10 weeks before the initiation of the mating period; then continued to receive test samples during gestation and lactation	Ni NPs (90 nm) 45 mg/kg-d, 15 mg/kg-d, or 5 mg/kg-d Ni MPs (3 μm): 45 mg/kg-d 0.9% NaCl (controls)	Birth survival rate in fetal rats of parents with Ni NPs and Ni MPs significantly decreased compared with fetal rats of control parents Birth survival rate of 45 mg/kg-d Ni NPs significantly decreased compared with Ni MPs	

Table C.25. Studies of Reproductive Index (continued)

Reference	Compound	Animal Model (Species/ Strain/Sex/Age) N	Study Design Exposure (Route/Period/ Frequency) Vehicle	Doses/ Concentrations	Results	Comments
Saini et al., 2014a	Nickel chloride hexahydrate ($NiCl_2 \cdot 6H_20$)	Swiss albino mice (7-9 weeks of age, 24 ± 2 g) n=4 groups/10 each	Oral GD 0-5 Dams sacrificed on GD 18	46, 92, or 185 mg $NiCl_2 \cdot 6H_20$/kg BW Control: tap water	Implantation sites and live fetuses per dam were significantly reduced in a dose-dependent manner; decrease was significant at all Ni doses Post-implantation deaths or embryonic resorptions were 5.26, 12.5, or 37.5 in mice at 46, 92, or 185 mg/kg BW, respectively No marked change was observed in the sex ratio (M:F) after administration of $NiCl_2 \cdot 6H_20$ at all dose levels Significant decrease in placental weight after 185 mg Ni/kg BW	
Saini et al., 2014b	Nickel chloride hexahydrate ($NiCl_2 \cdot 6H_20$)	Pregnant Swiss albino mice n=4 groups of 15 mice each	Gavage Pre-implantation period *GD 0-5, Organogenic period *GD 6-13, or Fetal period *GD 14-18 *Authors referred to GD as post-conception day	46.125, 92.25, and 184.5 mg Ni/kg BW Control: tap water	92.25 and 184.5mg/kg BW during GD 0-5: a significant decrease in average litter size per dam compared with control group Percentage of male and female was not altered in the preimplantation, organogenetic, and fetal periods at all dose levels Gestation index (=number of females delivering live young divided by number of females with evidence of pregnancy multiplied by 100) during preimplantation period was affected by 75% at all three dose levels	

Table C.25. Studies of Reproductive Index (continued)

Reference	Compound	Animal Model (Species/ Strain/Sex/Age) N	Study Design Exposure (Route/Period/ Frequency) Vehicle	Doses/ Concentrations	Results	Comments
Schroeder et al., 1971	Ni	Rats of the Long-Evans BLU: (LE) strain 5 pairs of rats	Oral; Ni in drinking water and diet Started at weaning and given continuously up to 9 months of age during continuous breeding	Drinking water from a forest spring was double deionized and element was added as soluble salts (ppm element) Ni 5ppm Diet contained (ppm wet weight) Ni 0.31 ppm	Ni was less toxic than lead with respect to breeding, with 9.1% young deaths, one maternal death, and 30.6% runts in the first generation; 10.2% young deaths and 5.1% runts in the second; generation and 21.0% young deaths and 6.2% runts in the third generation	Unclear study design with 6 trace elements
Smith et al., 1993	Nickel chloride ($NiCl_2$ - $6H_2O$)	Virgin female Long-Evans rats, aged 40-43 days 4 groups of 34 females each Females bred the 12th week	Oral For a period of 11 weeks prior to breeding	0, 10, 50, and 250 ppm $NiCl_2$ - $6H_2O$ in drinking water	Reproductive performance (mating success, rate of impregnation, pups per litter (total or live), and gestation length) reflected no differences across dose groups in either the 1st or 2nd gestation period 50 ppm dose group showed a significant reduction in pup growth in L1 male pups Pups in L2 (including controls) weighed significantly less than those in L1	

Table C.26. Studies of Milk Composition

Reference	Compound	Animal Model (Species/ Strain/Sex/Age N	Study Design Exposure (Route/Period/ Frequency/ Vehicle	Doses/ Concentrations	Results
O'Dell et al., 1970	Nickelous carbonate ($NiCO_3$)	multiparous, lactating dairy cows 3 groups of 5 each (n=15)	Oral; mixed with feed 24-hour sample was taken at the end of a 1-week preliminary, at 2-week intervals for 6 weeks during treatment, and at the end of 1-week post-treatment	$NiCO_3$ was mixed in the concentrate ration at 0, 50 and 250 ppm of elemental nickel;concentrate fed at a ratio of 1 kg per 3kg of milk produced Average daily consumption of nickel per cow was 0, 365 or 1,835 mg, respectively	Milk production, milk fat, solids-not-fat, and protein were unaffected by the treatments Average daily milk production during the treatments with 0, 50 and 250 ppm groups, were 93, 93 and 97% of production during the 1 week one-period before nickel supplementation Average milk fat tests were 0.20, 0.30 and 0.11 % higher for the 0, 50, and 250 ppm nickel groups during the treatments None of the milk samples from cows fed 250 ppm nickel contained as much as 0.1 ppm Ni
Dostal et al., 1989	Nickel chloride hexahydrate ($NiCl_2 \cdot 6H_2O$)	Sprague Dawley (CD) rats n=6-7 rats/group with 10-12 pups per dam	sc injection Single dose: one injection on LD 12 Multiple dose: sc 50 or 100 µmol/kg or vehicle were given once daily on LD 12-15	0, 10, 50, or 100 µmol/kg 0.4 ml/kg (0.4mM) NaCl (control)	Plasma and milk Ni concentrations increased in a dose dependent manner 4 hr after single doses 10, 50, or 100 µmol $NiCl_2$/kg to lactating rats, giving milk/plasma Ni ratios of 0.02 High doses led to the excretion of Ni into rat milk and changes in milk quality and production Treatment resulted in significant decreases in mammary RNA content and the RNA/DNA ratio compared to both ad libitum-fed and pair-fed rats

Table C.26. Studies of Milk Composition (continued)

Reference	Compound	Animal Model (Species/ Strain/Sex/Age N	Study Design Exposure (Route/Period/ Frequency/ Vehicle	Doses/ Concentrations	Results
Kong et al., 2014	Ni NPs Ni MPs	Female Sprague-Dawley rats; 80–100 g n=100 n=20 females/group 5 groups	Gavage Female F_0 given Ni NPs, Ni MPs or 0.9% NaCl (control) for 10 weeks before mating period; then continued to receive treatment during gestation and lactation	Ni NPs (90 nm): 45 mg/kg/day, 15 mg/kg/day, or 5 mg/kg/day Ni MPs (3 μm): 45 mg/kg/day 0.9% NaCl (control)	Feeding survival rates in the Ni NPs and Ni MPs groups were lower than the control group Feeding survival rate of the 45 mg/kg/d Ni NPs was higher than that of Ni MPs 67/104 survived (64.4%) after 21 days at 45 mg/kg/d Ni NPs and out of 174 live born rats 75 survived (43.1%) after weaning (21 days) in Ni MPs

C.3.3. Integrative Evaluation of Human and Animal Female Reproductive Toxicity

Data summarized in this document include reports of adverse effects of nickel on a wide variety of female reproductive endpoints.

Epidemiologic studies of female reproductive effects of Ni examined fecundity, hormone levels, and pre-eclampsia. One study found Ni exposure to be associated with SHBG and possibly other hormone levels, while no significant associations with fecundity or pre-eclampsia were observed in other studies. A review of the literature in animals found reports of adverse effects of Ni exposure on estrous cyclicity, release of some hormones associated with reproductive function, and alterations to the uterus and ovary. There is also evidence on the effects of Ni on the neuroendocrine control of prolactin in rodents, and negative effects in offspring following changes in milk composition after the dams exposure to Ni compounds.

Female reproductive toxicity and developmental toxicity are closely linked biologically due to the maternal-fetal relationship. Some epidemiologic studies summarized in the developmental toxicity section address outcomes such as spontaneous abortion and preterm delivery, which may be mediated through toxicity to the reproductive system of the mother and can also be considered in the context of identifying female reproductive toxicity. Thus, in some cases, topics discussed in the animal developmental toxicity section were also discussed in the female reproductive toxicity section because it is not always possible to determine if an observed developmental outcome is the result of a direct effect on the conceptus, an effect on the female reproductive system, or a combination of such effects.

D. Studies of Male Reproductive Toxicity of Nickel and Nickel Compounds

D.1. Human Studies of Male Reproductive Toxicity

Four cross-sectional studies in humans examined the effects of Ni on male reproductive endpoints, namely sperm and semen parameters and plasma testosterone. Three of these studies were focused on workers or retired workers and were conducted in India, Russia, Italy, and California. Multiple exposures and sample size are important concerns with some of these studies. Additionally, five other recent cross-sectional studies examined the association between exposure to metals and a number of sperm parameters among men attending infertility centers. Four studies are from the same center in China (Wang et al. 2016; Zeng et al. 2013; Zeng et al. 2015; Zhou et al. 2016) and one study (Zafar et al. 2015) was conducted in Pakistan.

Tabulated summaries of the individual studies presented in this section are provided in Appendix 2.

Danadevi et al. (2003). Semen quality of Indian welders occupationally exposed to nickel and chromium

This cross-sectional study was conducted to analyze the semen of welders exposed to Ni and Cr in South India, and to quantify Ni and Cr levels using the ultra mass 700 inductively coupled mass spectrometer (ICP-MS) (Danadevi et al. 2003).
The authors selected 57 workers who had been exposed for 2-21 years to welding fumes at a welding plant and 57 controls who were matched to the exposed workers for age, lifestyle, economic status, and "were not exposed to known harmful chemicals." The sample selection procedure is not described. The welders and unexposed workers were the same age, on average.

Each subject completed a detailed questionnaire that was intended to elicit information on age, smoking habits, duration of exposure, and medicine use. Welders were exposed to welding fumes containing Ni and Cr for a mean (SD) 11.2 (4.5) years. The subjects were given physical examinations, with emphasis on the genitourinary tract. Medical histories, including questions about reproductive history, possibility of exposure to other gonadotoxic agents, and some addictions (e.g., smoking, alcohol abuse), were obtained.

28 welders and 27 unexposed men ("controls") were randomly selected from the subjects for blood sampling for metals. Whole blood was sampled on Thursday morning using ultra mass 700 ICP-MS.

Participants were given instructions on avoiding contamination of semen samples, which were collected for 2 weeks after a 3-day sexual abstinence period into glass test tubes that were warmed to 37°F. Procedures and interpretations were in accordance with World Health Organization criteria. Samples were assessed for volume, liquefaction, and pH, and aliquots of each specimen were evaluated for viscosity, sperm agglutination (motile sperm stuck to each other), nonspecific aggregation (immotile sperm adhering to each other, or motile sperm adhering to other cells or debris) sperm count, percentage with motility grades 1-3, immotile sperm, and concentration of white blood cells. A normal semen profile was defined as $>20 \times 10^6$ spermatozoa/ml or $>40 \times 10^6$ spermatazoa/ejaculate; ≥50% progressive motility (grades 2 and 3), and ≥30% sperm with normal forms. The samples were randomized and coded to blind the scorer.

Motility was assessed at room temperature within 1 hr of ejaculation by counting motile and immotile sperm in 10 randomly selected fields per 10-µl drop in 5 drops analyzed per sample under a light microscope (125x magnification). Motility grades ranged from 3 (very active forward progression) to 0 (no motility).

At least 300 sperm per sample were categorized as normal or abnormal based on the presence of head, mid-piece, and tail defects. One hundred sperm per drop were assessed and expressed as percent of live sperm in the five drops analyzed in each sample.

Semen parameters were log transformed to improve normality. Analyses included the X^2 test, Mann-Whitney *U*-test, and Spearman's correlation analysis, and non-parametric Mann-Whitney test.

Compared to controls, welders had significantly lower sperm counts, a lower percentage of rapidly linear progressive sperm, higher percentage with slow linear progressive motility, lower percentage with normal morphology, higher percentage with head defects, higher percentage with mid-piece defects, lower percentage sperm vitality, and higher percentage with nonspecific aggregation.

For the subsample with blood levels, the mean blood Ni level was 123.3 ± 35.2 µg/l among welders (n=28) and 16.7 ± 5.8 µg/l among controls (n=27) and, and mean blood Cr level was 131.0 ± 52.6 µg/l among welders and 17.4 ± 8.9 µg/l among controls. The authors conducted "simple regression analysis" of semen parameters on blood metal concentration based on years of exposure. Among welders, blood Ni was negatively associated with rapid linear progressive motility (coefficient=-0.381, p=0.045) and vitality (coefficient=-0.420, p=0.026), and positively associated with slow/nonlinear progressive motility (coefficient=0.386, p=0.042) and tail defects (coefficient=0.485, p=0.036). Chromium was more strongly associated with three of the four parameters (all except slow/nonlinear progressive motility).

OEHHA comments: Based on the description of "simple regression," Ni and Cr were probably not included in the same models (and might be so highly correlated that they really could not be validly included in the same models). Observed associations may be attributable, at least in part, to Cr, and possibly to other exposures associated with welding.

Slivkova et al. (2009). Concentration of trace elements in human semen and relation to spermatozoa quality.

The aim of this cross-sectional study was to determine the concentrations of Pb, Cd, Fe, Ni, Cu, and Zn in human semen, the occurrence of pathological spermatozoa, and correlations between the elements and the pathological spermatozoa (Slivkova et al. 2009).

Forty-seven men, age 22-48, who were undergoing semen analysis at a state infertility center provided semen samples on-site. The sample selection method is not described.

Specimens were analyzed for sperm morphology using a modified Papanicolau staining. At least 500 spermatozoa were evaluated for each slide. Slides were analyzed at 500x magnification for the following pathological changes: knob-twisted flagellum, separated flagellum, flagellum torso, broken flagellum, retention of cytoplasmic drop, acrosomal changes, large heads, small heads, flagellum ball, and

other pathological forms (teratoid spermatozoa, spiral twisted flagellum, deformation of mitochondrial segment, etc.).

Semen samples were stored at -20°C and subsequently mineralized by adding 2 mL HNO_3-$HClO_4$ (4:1) mixture, and heated to 120°C for 65 minutes. The resulting solution was diluted to 10 mL with demineralized water. The authors do not state how Ni was subsequently measured. The concentration of Ni in the semen was as follows: mean (SD) 0.40 (0.07); median 0.39; minimum 0.23; and maximum 0.55 mg/kg.

Correlations among metals were not high. Ni was correlated with Fe (r=0.36). Slivkova et al. conducted correlation analyses of semen parameters with metal concentrations and two-way analysis of variance to determine the significance of the relationships between trace elements and the number of pathological spermatozoa. The authors report no findings relating to Ni and sperm morphology from this study.

OEHHA comment: This study is not described in detail.

Zeng et al. (2013). Associations of urinary metal concentrations and circulating testosterone in Chinese men

This cross-sectional study examined associations between urinary concentrations of 13 metals and circulating testosterone (T) in men presenting to a fertility clinic in Wuhan, China (Zeng et al. 2013). Of 148 men who agreed to participate in March-May 2012, 118 men were selected after exclusions due to endocrine disease (e.g., diabetes, thyroid, or adrenal disorders), other medical conditions associated with infertility (azoospermia, orchiditis, epididymitis, vesiculitis, undescended testicle, injury of testis, hernia repair complicated by testicular atrophy). Participants completed a face-to-face questionnaire about demographics, lifestyle habits, occupational exposures, and medical characteristics.

The authors collected a 5-ml morning peripheral blood sample and a single-spot urine sample from each participant. The blood was centrifuged and T concentration in serum measured. The LOD for T in serum was 10 ng/dL. The mean (range) T concentration was 450.53 (114.95 – 941.65) ng/dL.

The urine sample and analyzed using inductively coupled plasma mass spectrometry and the picric assay was used to determine urinary creatinine. The creatinine-adjusted urine Ni concentration (µg/g) was as follows: mean 3.55, range 0.36 – 91.16, 25th percentile 1.11, 75th percentile 2.98.

Potential confounders considered included age, body mass index (BMI), smoking status, alcohol use, education level, and income. The authors entered potential

confounders into the multivariate model if they changed effect estimates for at least one metal exposure by 10% or more. Age and BMI as continuous variables, alcohol use as a dichotomous variable, and smoking status (current and former vs never smoker) and income as dummy variables were included in the models.

The authors considered full linear regression models to simultaneously examine effects of multiple metals on T, using backward elimination with alpha=0.1 to retain variables in the final model. The adjusted associations (β) between serum T and creatinine-adjusted urinary Ni exposure, relative to first quartile Ni exposure, were as follows: second quartile: –0.86 (–81.25, 79.53); third quartile –83.79 (–163.85, –3.74); fourth quartile –36.35 (–116.31, 43.61). When simultaneously considering the effects of multiple metals and potential confounders, Cd, Cr, Mn, Zn, and BMI were retained in the final model.

Sancini et al. (2014). Correlation between urinary nickel and testosterone plasma values in workers occupationally exposed to urban stressors

This cross-sectional study evaluated the correlation between occupational exposure to low levels of air Ni present in urban pollution and alterations of testosterone plasma values in municipal police workers assigned to different types of outdoor tasks in a large Italian city (Sancini et al. 2014). The authors state that in this city, the Ni pollution in the air mainly adheres to respirable dusts.

The authors randomly selected workers who had joined a workplace health promotion program from eight different areas of the city. From most areas, 42 workers were selected (25 traffic police, 10 drivers and/or motorcycle riders, and seven in other outdoors positions), and from the busiest central area, 65 were selected (35 traffic police, 20 drivers/motorcycle riders, and 10 other), for a total of 359 workers. Most of the activities were carried out outdoors or in cars at least 80% of working time (8 hours/ day, 5 days/week), all were without protective equipment against dust and fumes.

Each worker completed a questionnaire, including clinical case history, age, 5-year residence history, “physiological anamnesis” (especially focused on diet, consumption of water from the water supply and /or mineral water, exposure to cigarette smoking), near and remote medical history, fertility (including diagnosed problems, unsuccessfully trying to get pregnant >6 months, fertility investigations), and fertility treatment history. Men who were exposed to solvents, paints, or pesticides in leisure activities; used illicit drugs; habitually drank >2 units of alcohol/day; were >50 yrs; did shift or night shift work; competed in sports; performed other outdoor tasks in the past year; or had urinary Ni below the LOD were excluded. The final sample included 274 men.

For each worker, urinary Ni and plasma testosterone assays were collected at the end of the shift after five continuous working days. Workers were asked to abstain from eating foods containing cocoa, soybeans, oatmeal, almonds, walnuts, and fresh and

dried legumes during the four days prior to the examination. Urinary Ni was determined by complexation with ammonium pyrrolidinedithiocarbamate and atomic absorption analysis in graphite furnace, with LOD 1.0 mg/g of urinary creatinine. For plasma testosterone, 10 mL venous blood samples were analyzed by immunoassay within three days of collection.

Urinary Ni and plasma testosterone were log-transformed for correlation and multiple linear regression analyses with plasma testosterone as a dependent variable, and urinary Ni, age, length of service, and cigarette smoking as independent variables. Covariates were job position (traffic police, drivers, motorcyclists, and outdoor workers with other tasks), age, seniority, and smoking habit.

The mean (SD) for urine Ni was 4.78 (3.68) mg/100mL in smokers and 4.62 (4.22) mg/100mL in nonsmokers. Urine Ni levels did not vary by type of police duty, as shown in Table D.1.

Table D.1. Urine Ni (µg/g creatinine) by police duties

Subjects	Mean (SD)	Geometric mean	Min-max	Median
Traffic control	4.79 (3.68)	3.56	1 – 17.2	3.6
Drivers	4.62 (4.22)	3.26	1-24.9	3.1
Motorcyclists	4.67 (3.77)	3.42	1-19.4	3.55
Other outdoor	4.76 (4.68)	3.23	1-24.9	2.8

The Pearson correlation coefficient for log urinary Ni and log plasma testosterone was significant and negative: R=-0.468 (p=0.000) in the total sample and the association was significant for each smoking status and type of work. In multiple linear regression, log plasma testosterone was negatively associated with log urinary Ni in the entire sample (beta=-8.631, p=0.000) and in each job position, adjusted for age, years of work, and smoking.

Neither urinary Ni nor plasma testosterone level was significantly associated with smoking, age, length of service, or type of task.

The authors performed personal dosimetry for environmental monitoring of Ni on a subsample of eight men in eight different work areas considered to be most representative of the city's air quality, and four police drivers of cars with at least two people for each shift to represent exposure for colleagues in the car with the monitored man. Air, blood, and urine samples were taken on the same day and subjects were asked not to smoke during sampling. Air samples were collected for the entire 7-hour shift using cyclones with a cut-point for 5 micron diameter particles, and each cyclone was attached to a pump for personal sampling, calibrated to a flow rate of 1.7 L/min. Each cyclone was fitted with a cassette holding a membrane filter of 37 mm in polyvinyl chloride that was attached to the worker's collar in the breathing area. Digested

particulate samples were analyzed by atomic absorption spectrometry in graphite furnace. Air Ni levels are shown in Table D.2.

Table D.2. Air Ni (ng/m^3) from personal dosimetry

Subjects (n)	Mean (SD)	Geometric mean	Min-max	Median
Traffic police (8)	178.4 (142.3)	104.52	11.6 – 378.3	96.3
Police drivers (4)	113.2 (123.4)	85.18	30.2 – 538.7	78

Among the 12 subjects who carried out personal air sampling, log Ni in air was significantly associated with log urinary Ni: beta=0.924, p=0.000. In multiple regression, log Ni in air was the only significant predictor of urinary Ni.

OEHHA comment: No other air pollutants were explored.

Skalnaya et al. (2015). Association between semen quality and level of 20 essential and toxic metals in ejaculate

The aim of this cross-sectional study was to estimate the association of ejaculate levels of 20 toxic and essential metals (Al, As, beryllium (Be), bismuth (Bi), Cd, Co, Cr, Cu, Fe, Hg, iodine (I), lithium (Li), Mn, Mo, Ni, Pb, Se, Sn, V, Zn) and sperm quality parameters in 148 adult men. The study was approved by an ethics committee of the Orenburg State University in Russia (Skalnaya et al. 2015).

The study population and sample selection are not described. "[S]emen samples were collected according to current WHO recommendations" and assessed in an independent laboratory for ejaculate volume, absolute and relative sperm count, motility, and vitality "according to the WHO manual". Inductively coupled plasma mass spectrometry was employed to analyze ejaculate for metals.

The authors used the Shapiro-Wilk test to analyze data distribution, nonparametric Mann-Whitney U-tests for paired-group comparisons, and Spearman's coefficient of rank to analyze correlations between semen parameters and semen metal concentrations.

The Mean (SD) Ni concentration in ejaculate was 0.026 (0.022) µg/mL, median was 0.021 µg/mL, minimum was 0.005 µg/mL, and maximum was 0.197 µg/mL. Ejaculate volume <1.5 mL was significantly associated with Ni, Cu, Mn, and Mo, while greater seminal volume was associated with lower Ni, Cu, Mn, and Mo content (Mann-Whitney U-test p=0.015 for Ni). However, the authors report that "normalization" [not explained by the authors] of the ejaculate volume resulted in a 31% decrease in seminal Ni concentration and lower concentrations for the other metals. Absolute sperm count

(<39 x 10^6 vs. ≥39 x 10^6), relative sperm counts (<15 x 10^6/mL vs. ≥15 x10^6/mL), progressive sperm motility (< 32% vs. > 32%), and sperm vitality (< 58% vs. > 58%), were not statistically significantly associated with semen Ni concentrations based on Mann-Whitney U-tests.

Correlations between semen parameters and Ni were as follows: Ejaculate volume -0.123, total sperm count -0.069, relative sperm count 0.005, sperm motility -0.041, sperm vitality -0.049.

OEHHA comment: The study is not described in detail. Analyses are for single metals.

Zeng et al. (2015). Urinary Metal Concentrations in Relation to Semen Quality: A Cross-Sectional Study in China.

The study examined the association between urinary metal concentrations and measures of semen quality in a sample of men attending an infertility clinic, the Reproductive Center of Tongjing Hospital in Wuhan, China in 2011-2012 (Zeng et al. 2015). A total of 2,540 men of infertile couples were invited to participate in the study regardless of prior knowledge of their fertility status. Men were excluded if they were azoospermic (332 men), because the mechanism responsible for azoospermia may be associated with an obstructive mechanism or Y-chromosome deletions, or if they had any of a number of medical conditions that might alter semen quality (118 men) (including orchiditis, epididymitis, vesiculitis, vasectomy, undescended testicle, injury of testis, hernia repair complicated by testicular atrophy, and endocrine disease (e.g., diabetes, thyroid, or adrenal disorders). A total of 394 men were randomly selected.

Urinary concentrations of 13 metals were determined from a single-spot urine sample provided by the participants. Semen samples were analyzed for sperm concentration, motility, count, normal morphology, and abnormal head according to World Health Organization (WHO) guidelines. Study participants were dichotomized as either below or at/above the WHO reference values for sperm concentration (20 million/mL), sperm motility (50% motile), and sperm count (40 million). Those participants for whom all three parameters were at or above the reference values were defined as the comparison group. Multivariable linear and logistic regression models were used to assess the relationship between creatinine-adjusted urinary metals and sperm quality measurements.

Potential confounders considered included age, body mass index (BMI), abstinence time, smoking status, alcohol use, education level, income, and tap water consumption. Age as a continuous variable and both abstinence time (3−5 and >5 vs <3 days) and smoking status (current and former vs never smoker) as dummy variables were included in the models as they had changed the effect estimates by 10% or more.

According to the WHO standard, 46 men (11.7%), 222 men (56.3%), and 38 men (9.6%) had sperm concentration, sperm motility, and sperm count below the reference, respectively. A total of 169 men (42.9%) had the three semen parameters at or above the reference values and were thus defined as the comparison subjects.

All 13 metals (As, Cd, Co, Cr, Cu, Fe, Pb, Mn, Hg, Mo, Ni, Se, Zn) were detected in all of the urine samples at various magnitudes. The creatinine-adjusted concentration of Ni in urine, µg/g creatinine, was as follows: geometric mean 1.46, minimum 0.01, 25th percentile 0.87, median 1.53, 75th percentile 2.47, and maximum 91.16. There were significant associations among the urinary metal concentrations (Spearman's Rho ranged from 0.11 to 0.79), except for the association between molybdenum and manganese and that between selenium and manganese. The correlations for Ni ranged between 0.24 and 0.59.

The median sperm concentration, motility, and count were 56.36 million/mL, 46.08%, and 165.18 million, respectively, while the geometric mean sperm normal morphology and abnormal head were 22.36% and 62.50%, respectively.

Urine Ni concentration was not associated with sperm count or concentration < reference level, or % normal morphology. Although Ni was not significantly associated with % motile sperm, there was an indication of decreasing % motile with increasing Ni concentration.: AORs (CI) for % motile sperm < reference level and quartiles of urine Ni concentration (compared to the first quartile) were as follows: second quartile 0.80 (0.45, 1.44), third quartile 0.77 (0.43, 1.39), and fourth quartile 0.67 (0.37, 1.02). Also, the authors stated that "Ni was significantly associated with an increasing trend for sperm abnormal head": regression coefficients (CI) for % abnormal head and quartiles of urine Ni concentration (compared to the first quartile) were as follows: second quartile -1.65 (-3.9, 0.60), third quartile 0.92 (-1.32, 3.16), fourth quartile 1.67 (-0.57, 3.92); p trend=0.03. When multiple metals were included to predict abnormal head, Ni and Cr remained in the model, and the trend test for the association between % abnormal head and Ni concentration was significant, p=0.01

In addition, a significant dose-response association was observed between chromium and a decline in sperm count when considering the co-exposure to multiple metals.

Strengths of the study include the use of a biomarker for exposure assessment, although the half-life of Ni in urine is short, and control for multiple metals in the regression analyses. However, the use of a single urine sample may result in misclassification of Ni exposure, given the short half-life of Ni in urine.

Wang et al. (2016). Associations of urinary metal levels with serum hormones, spermatozoa apoptosis and sperm DNA damage in a Chinese population.

This study examined the association between the urinary concentration of 18 metals and reproductive hormones, spermatozoa apoptosis and sperm DNA integrity in

participants recruited from a population of male partners of sub-fertile couples without knowledge of their fertility status (Wang et al. 2016). After exclusion criteria were applied (see study description in Zeng et al. (2015) above), 1,052 participants provided a blood sample, a semen sample and two spot urine samples separated by approximately four hours, and answered a questionnaire at the time of their clinic visit. Urine samples were analyzed for 18 metals including Al, As, Cd, Co, Cr, Cu, Fe, Pb, Mn, Mo, Ni, Sb, Se, Sn, Ti, U, W, and Zn. Blood samples were analyzed for reproductive hormones, spermatozoa apoptosis using Annexin V assay, and sperm DNA integrity using the comet assay. Of the 1,052 participants, 511 were measured for reproductive hormones, 460 for spermatozoa apoptosis and 516 for DNA integrity (171 men were measured for the three sets of outcome measures).

The urinary Ni concentrations, µg/L, were as follows: first sample (6.8% < limit of quantification (LOQ)), geometric mean 2.0, median 2.4, IQR 1.3–4.1; second sample (7.1% < LOQ), geometric mean 1.8, median 2.3, IQR 1.2–4.0. The reproducibility, as measured by intraclass correlation coefficient, for repeat samples of creatinine-corrected urinary Ni, as well as for Cr, Cd, and W was ≥0.75, which the authors deemed "excellent". Values < LOQ were assigned LOQ/√2.

Since multiple metals may simultaneously affect these markers of male reproductive health, the authors tested full multivariable linear models that considered all metals and covariates that showed statistically significant or suggestive association estimates together.

A significant inverse dose-response relationship was observed across Ni, Zn, and Mo quartiles with total testosterone/luteinizing hormone (T/LH) ratio, after adjusting for multiple testing (false-discovery rate (FDR)-adjusted p trend <0.05), indicating impaired Leydig cell function at high exposure levels. Men in the highest quartile of Ni had on average 20% (95% CI: −38,−4.2%) lower total T/LH ratios compared with the lowest quartile. This association remained significant when simultaneously considering multiple metals and persisted when modeled as continuous variables in the cubic spline analysis (P-value ≤ 0.01). Similar findings were reported for Zn and Mo. The study also observed an association between manganese exposure and spermatozoa apoptosis. No significant associations were observed between urinary metals and sperm DNA integrity parameters.

The authors note that strengths of this study included the collection and analysis of fresh semen samples to measure sperm DNA integrity and apoptosis parameters without cryopreservation which has been found to cause sperm DNA damage. In addition, a repeat measure of urinary metals was used to improve the accuracy of exposure estimation for each individual. However, the mean interval between the two samples was 4.4 hours (± 3.7 hours; range: 2.0–11 hours).

OEHHA comment: Correlations among metals are not reported and could be an important consideration in analyses that include multiple metals.

Zhou et al. (2016). Evaluation of urinary metal concentrations and sperm DNA damage in infertile men from an infertility clinic.

This study examined the association between urinary concentrations of 13 metals and sperm DNA damage in men attending an infertility clinic in 2012 (Zhou et al. 2016). A total of 252 infertile men agreed to participate; no criteria were presented for the determination of infertility in the participants. After excluding men with azoospermia, medical conditions that might alter semen quality, or endocrine disease,207 men were selected. A single urine sample was used to determine metal concentrations of 13 metals including arsenic, cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, molybdenum, nickel, selenium, and zinc. Sperm DNA damage was measured on a thawed semen sample using the neutral comet assay test. Parameters evaluated included tail length, which indicates single-strand breaks of DNA damage, as well as percent DNA tail, and tail distributed moment, which indicate double-strand breaks of DNA damage.

Age, BMI, race, smoking status, alcohol use, education, income, and total tap water consumption were considered as potential confounders. Based on changing effect estimates with inclusion in models, multivariate models included adjustment for age and BMI as continuous variables, smoking status as dummy variables, and abstinence time as an ordinal variable.

All metals were detected in the urine samples of 100% of the participants. Urinary Ni concentrations, µg/g creatinine, were as follows: mean 2.29, geometric mean 1.03, 25th percentile 0.60, 50th percentile 1.05, 75th percentile 1.98, range 0.01–91.16. Most metals were not statistically significantly associated with any of the comet assay parameters.

However, urinary Ni and Hg concentrations were associated with significant increasing trends for tail length (both p for trend < 0.05); with first quartile values as the reference, men with values in the fourth quartiles of Hg and Ni had significant increases in tail length of 3.14 µm (CI: 0.70, 5.59) and 2.74 µm (CI: 0.36, 5.12), respectively. When multiple metals were included in the model, the effect persisted; the fourth quartiles of Hg and Ni exposure was associated with increases in tail length of 3.72 µm (CI: 1.05, 6.38) and 2.95 µm (CI: 0.34, 5.56), respectively. Urinary Mn was also associated with significant increasing trends for tail length (p for trend = 0.04) as well as with tail distributed moment (p for trend = 0.02). Synergistic or antagonistic effects, or additivity of multiple metals were also examined by including them simultaneously in the models. When considering the effects of multiple metals, these dose-response relationships remained significant. Thus, the authors concluded that environmental exposure to Ni, Hg, and Mn may result in increased sperm DNA damage.

Zafar et al. (2015). Toxic metals signature in the human seminal plasma of Pakistani population and their potential role in male infertility.

This study examined the association between the occurrence of trace metals in seminal plasma and semen quality in male partners of couples undergoing infertility assessment at the National Institute of Health in Pakistan (Zafar et al. 2015). Concentrations of 17 different metals were measured in seminal plasma.

The study design, population, and sample selection methods are not described. Semen samples were obtained after 3-5 days of sexual abstinence. The specimens were assessed for sperm quality parameters such as liquefaction time, volume, pH, viscosity, presence of pus or epithelial cells, semen quantity, sperm motility, sperm count and sperm morphology. Semen and sperm analyses were conducted according to the WHO guidelines (WHO, 1999). Seminal plasma samples from the 75 participants were classified into three groups according to sperm concentrations: normozoospermia (n = 25); oligozoospermia (n = 25); and azoospermia (n = 25). The participants with normozoospermia were used as the control group. Three semen parameters (volume, sperm concentration and motility) showed statistically significant differences ($p<0.05$) among the three groups (normozoospermic, oligozoospermic, azoospermic).

The authors measured metals in semen using inductively coupled plasma mass spectrometry. Statistical analysis of metals in seminal plasma was conducted using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test to evaluate mean significant differences for toxic metals in different semen samples. The concentrations of most metals were higher in azoospermic participants compared with the other two groups. Ni values (mean ±SD), ppb, in the three groups were: normozoospermic group (3.07 ±1.63), oligozoospermic group (1.92 ±0.77), azoospermic group (10.49 ±10.94). The authors reported that Ni and Cd concentrations in seminal plasma showed significant differences in all three groups ($p = 0.01$). Ni concentrations were negatively correlated with sperm concentration ($r = -0.26$), sperm volume ($r = -0.44$) and motility ($r = -0.33$), $p< 0.05$ for each correlation. Ni was also highly correlated ($r > 0.70$) with Cd, Cu, Sn, and V.

The levels of trace metals in this sample were lower and/or comparable to that found in populations of other countries. The authors suggested that exposure of Ni and Cd is mainly related with the consumption of contaminated dietary items, including ghee (cooking oil), flour and other agricultural products, because Ni is used as a catalyst in ghee and flour production for the hydrogenation reaction.

OEHHA comment: Limitations include the limited generalizability of the results due to the nature of the sample population. In addition, although each patient completed a questionnaire concerning occupation, residence, diet, alcohol consumption, water source, smoking habits, height, weight and detailed medical history, there was no further mention of these variables' potential association with metals and sperm quality.

D.2. Animal Studies of Male Reproductive Toxicity

Relevant animal studies pertaining to the potential for nickel and nickel compounds to cause male reproductive toxicity are summarized below, and in some cases, excerpts of text taken directly from the original publications and study reports are incorporated into the summaries.

Twenty-four animal studies examining effects of nickel on the male reproductive system were identified from the peer-reviewed literature. Additionally, three studies conducted by contract research laboratories were available to OEHHA staff and have been summarized here. These include a one-generation and a two-generation study in rodents exposed to nickel via oral gavage. Another two-generation study in CD rats exposed to nickel in the drinking water is also summarized. Also 13 related articles were identified (these include 8 *in vitro* studies and 4 *in vivo* studies, and 1 study which conducted both *in vivo* and *in vitro* studies) which are presented below. Several studies in the peer-reviewed published literature state that nickel appears to be a potential gonadotoxin and has adverse effects on the male reproductive system. Some of the effects include reducing DNA, RNA, and protein concentrations in the testes of rats (Das and Dasgupta 2000). Researchers state that the necessity of monitoring nickel concentrations in animal as well as human semen is confirmed by the many studies that demonstrate and describe the toxic effect of nickel on male reproductive abilities (Lukac et al. 2011). Some of the studies summarized below used nickel to exert toxic effects on male reproduction in order to investigate the mechanisms underlying that toxicity.

The studies are presented in chronological order, with the exception of multiple publications reporting on a common research focus and from the same research group, which are discussed together.

Hoey (1966). The effects of metallic salts on the histology and functioning of the rat testis.

In a series of experiments in male albino rats, various metals (silver, copper, tin, nickel and cobalt) were individually administered subcutaneously as aqueous salts in a single dose or as daily injections for 1 to 30 days (Hoey 1966). The testes then underwent normal histological examination and dark ground (e.g., dark field) microscopy, and radiography following intra-arterial injection of Micropaque in one experiment. Only the results of nickel treatment on the male reproductive system are discussed here. The authors considered silver (silver nitrate) as the standard metal for comparison with other metals.

The authors report the following:

Experiment 1: One rat was injected with nickel sulfate and killed 18 hr after injection and the testes removed and examined. Nickel resembled silver nitrate, with damage to central and peripheral tubules but without the interstitial damage. There was some shrinkage of the epididymal tubules, but they retained their regular shape. The spermatozoa in the epididymis were completely degenerated and dark ground microscopy showed the presence of nickel in the testicular interstitial tissue, the lumina of all the tubules (in the spermatozoa), sometimes in the tissue of the peripheral tubules, in the spermatozoal contents of the epididymis and occasionally in the tunica albugínea.

Experiment 2: Four rats were injected daily and one rat was killed at 2, 10, 21 or 30 days, respectively, after the first injection. Nickel produced much less testicular damage (necrosis and blockage of ductuli efferentes, presence of mitotic figures throughout testis, interstitial damage especially in central area) in the early stages than silver nitrate, and a similar effect on spermatogenesis (fewer spermatozoa in semeniferous tubules and pyknotic sperm heads). Damage to interstitial tissue and the body of the epididymis increased while testicular recovery was occurring, and finally full spermatogenesis resumed while the ductuli remained necrotic.

Experiment 3: Three rats were given a single injection of nickel and one rat was killed at 4, 8 or 12 days after injection, respectively. Nickel closely resembled silver nitrate but spermiogenesis was more severely affected in the early stages, being present in only 16% of tubules, and the condition of the tissues regressed until spermiogenesis virtually ceased (4%). Some recovery occurred but spermiogenesis remained very limited.

Experiment 4: Three rats were given 10 daily injections of nickel and one rat was killed at 1, 4, or 8 days after injection, respectively. This experiment showed the extent of damage and recovery of tissue after a short course of injections. Nickel had a similar effect to silver nitrate but the suppression of spermiogenesis is far less than with other metals. Necrosis of ductuli was progressive, but the testicular tissue and spermatogenesis had returned to virtual normality by 12 days.

Experiment 5: One rat was injected with nickel and 24 hours later the rat was anaesthetized, the thoracic cage opened to expose the thoracic aorta, and 25 ml of Micropaque were injected into the aorta, which was then ligated. The testes were removed and X-rayed. Arteries, veins and lymphatics were all prominent, but haemorrhages were not as marked as with silver nitrate treatment.

According to the authors, all five metals produced acute and chronic changes in the histology of the testis and interfered with spermatogenesis. All tissues showed improvement following the initial injection. The effects and possible modes of action of these metals were discussed and contrasted with cadmium. The authors report that the metals act differently than cadmium, and may limit their own action by precipitating proteins in the membrana propria, and making it impermeable to further metallic ions. The authors also speculate that the action of the metals on the epididymis varies from

that on the testis, and the damage produced in the epididymis and ductuli efferentes shows less tendency to recover.

The authors stated that overall, nickel allowed recovery despite initial damage and while profound changes were obtained between 1 and 8 days in this series of experiments, previous work in rats failed to notice any histological change in testis or epididymis at 2 or 7 days after a single sub cutaneous injection.

Schroeder and Michener (1971). Toxic Effects of Trace Elements on the Reproduction of Mice and Rats.

In this study, male and female Long-Evans rats were exposed to low doses of six trace elements in double deionized drinking water from a forest spring (Schroeder and Mitchener 1971). Five pairs of rats were randomly selected for each element from divided litters at the time of weaning, placed in separate cages and given the element in drinking water continuously. One group included nickel at 5 ppm compared to controls given only doubly deionized water. The diet for all groups contained several elements including nickel at 0.31 ppm. Each group was carried through three generations.

The authors reported that the size of the litters decreased somewhat with each generation, and, with two failures to breed, the number of rats was reduced and few males were born in the third generation. While the authors state that nickel fed for life was not toxic to rats (unpublished data), they comment that these experiments provide a much more sensitive method for detecting toxicity of an element than feeding the element for life and feeding of these trace elements resulted in relative toxicities in the following order: lead > cadmium > selenium > nickel, > titanium > molybdenum > arsenic.

Mathur et al. (1977a). Effect of nickel sulfate on male rats.

This study investigated the effects of dermal application of nickel sulfate ($NiSO_4$) on the liver, kidneys, and testes in male rats (Mathur et al. 1977a).

Thirty-two male adult rats were divided into groups of 8 and the hair on the lateroabdominal area was clipped. Groups 1, 2, and 3 were painted with 40, 60, and 100 mg Ni/kg in the form of $NiSO_4 \bullet 6H_2O$ dissolved in 0.25 mL of normal saline for 30 days. Group 4 served as control. Four animals from each group were sacrificed after 15 and 30 days of Ni application and the skin, liver, kidneys, and testes were removed and fixed, then histopathologically examined.

There were no clinical symptoms of poisoning or mortality after dermal application of nickel sulfate. No gross (macroscopic) changes were noticed in the skin, liver, kidney or testis of rats painted with nickel sulfate. There was no liver enlargement and the weight of liver showed no significant difference from those of controls. The testes also did not

show any marked atrophy or hypertrophy. The color and weight of the testis did not differ significantly from those of controls. Cytopathological and histopathological changes were observed in the skin, liver, kidney and testis in treated rats; effects on the testes are described below.

Effects on the testes: After 15 days, there were no effects, however, by 30 days, groups 2 and 3 (60 and 100 mg/kg) showed tubular damage; the lumen was filled with degenerated sperm and edematous fluid. There was an increase in tubular degeneration and edema. The epithelium of the seminiferous tubules was distorted, and the testes of one rat had necrotic tubules carrying giant cells (Figures D.1 and D.2 below).

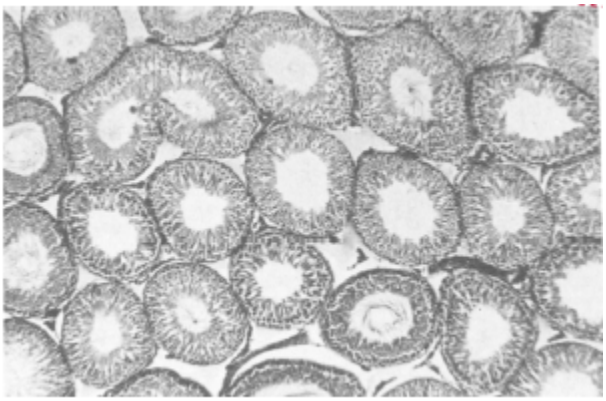

Figure D.1. Section of the rat testes after saline painting on skin for 30 days. Note normal histology, shape of the seminiferous tubules and interstitium (x 80)

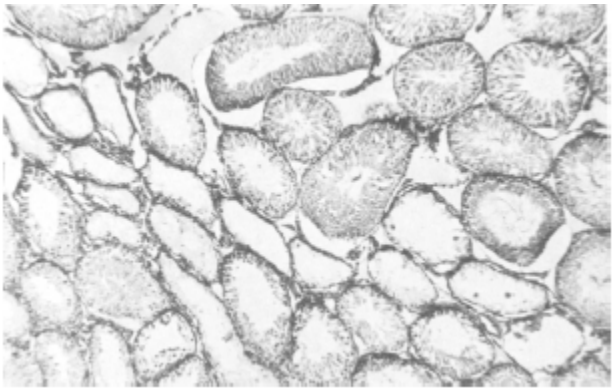

Figure D.2. Section of the rat testes after nickel (100 mg Ni/kg/day) painting on skin for 30 days. Note seminiferous tubules, shrunken, necrosed and tubules devoid of spermatogenic cells (x 80).

The testis showed degeneration and edema of seminiferous tubules. The dermal application of three different concentrations of nickel sulfate for 15 or 30 days induced varied tissue damage in rats which indicate absorption of the metal through intact skin. Furthermore, the extent of tissue damage appears to be directly proportional to the

concentration of $NiSO_4$ as well as to the duration of its application and the authors also note that the effect of temperature and moisture may be significant in contributing to the absorption of $NiSO_4$ through the skin. This study was conducted at room temperature and the authors suggest the possibility that workers at nickel refineries (who work at elevated temperatures) and others exposed to nickel during hot weather (or under conditions with increased sweating) may have a higher rate of dermal absorption, leading to more severe skin and internal organ damage.

Mathur et al. (1977b). Biochemical and morphological changes in some organs of rats in nickel intoxication.

The goal of this paper was to investigate the biochemical changes in vital organs in rats after exposure to $NiSO_4$ and to ascertain if these changes had a relationship to the duration of $NiSO_4$ exposure (Mathur et al. 1977b). A total of 40 male albino rats were used. Thirty animals were dosed with 3 mg Ni/kg as $NiSO_4$•6 H_2O dissolved in 1 ml of 0.9% NaCl by intraperitoneal (ip) injection daily for 90 days and the rest received an equal volume of normal saline. The animals were weighed every 6th day and the dose adjusted accordingly. Five rats from the experimental and two from the control groups were sacrificed by decapitation 48 h after the last injection at 7, 15, 30, 60 and 90 days. Liver, kidney, testis and myocardium were removed and processed for biochemical and histological examinations. The activity of succinic oxidoreductase, adenosine triphosphatase, and acid phosphatase were determined in liver, kidney and testis.

There were no biochemical or histological alterations on experimental days 7 and 15. Five animals from the experimental group died during the course of the experiment; however, the authors were unable to establish the cause of death. The activity of succinic dehydrogenase did not change by day 30 in the liver or kidney of $NiSO_4$ dosed rats but decreased thereafter. The activity of succinic dehydrogenase remained unaltered in the testis throughout the experiment. The activity of adenosine triphosphatase significantly increased in the testis at days 30, 60 and 90 of nickel administration. The activity of acid phosphatase remained unaffected up to 30 days in the testis. However, it was decreased in the testis at days 60 and 90 (see Table D.3 for summary of data).

Table D.3. Activity of enzymes in the testes of rats after daily ip injection of Ni (3 mg/kg)*

Enzyme	Control	Day 30	Day 60	Day 90
Succinic dehydrogenase	3.2 ± 0.16	3.2± 0.22	3.2 ±0.23	3.3± 0.38
Adenosine triphosphatase	71.2 ± 2.22	109.6 ± 3.46***	105.2 ± 2.40***	1116.4 ± 7.51***
Acid phosphatase	13.3 ± 0.57	12.9 ± 0.93	11.0 ± 0.97*	10.0 ± 0.32***

**Note*: Modified from Table 1 of Mathur et al 1997b to reflect activity of enzymes only in the testes Each value represents mean ± S.E. of 10 rats in control and 5 rats in experimental groups *p< 0.05, **p<0.01, ***p <0.001 when compared with control as evaluated by the Student *t* test.

In rats treated with $NiSO_4$ the histopathology of testicular tissue at days 30 and 60 was normal but at day 90, degenerative changes (not described) were seen in a few seminiferous tubules.

The testis appears to be less sensitive to nickel treatment than the other organs investigated since comparatively fewer histopathological changes were observed. conclude that repeated intraperitoneal administration of small quantities of nickel produce significant histological changes in the vital organs of experimental rats which appears to be directly related to the duration of treatment.

Xie et al. (1995). Effects of chelating agents on testicular toxicity in mice caused by acute exposure to nickel.

The effects of chelating agents on testicular toxicity in mice caused by acute nickel exposure were evaluated in this study (Xie et al. 1995). Male ICR mice were injected intraperitoneally (ip) with $NiCl_2•6H_2O$ at doses of 0, 0.5, 1.0, 3.0, or 5.0 mg Ni/kg bw and sacrificed 24 hr after injection. Nickel administration resulted in dose-dependent increases in testicular lipid peroxidation (LPO), and Ni, calcium (Ca) and iron (Fe) concentrations (all p< 0.05, N=5). Lesser increases in testicular copper (Cu) and zinc (Zn) were also seen. Treatment with 5.0 mg Ni/kg and observation for seven days showed increasing LPO with a peak at two days after Ni administration followed by a gradual decrease. Testicular weight decreased from about 0.65% of body weight to 0.4% over the same period (p< 0.05, N = 5).

N-Benzyl-D-glucaminedithiocarbamate (BGD), diethyldithiocarbamate (DDTC), dihydroxyethyldithiocarbamate (DHED), trans-1,2-cyclohexanediamine N,N,N',N'-tetraacetic acid (CDTA), and meso-2,3-dimercaptosuccinic acid (DMSA) were studied for their protective effects against the testicular toxicity in mice induced by acute exposure to nickel. Mice were injected ip with $NiCl_2$ (5 mgNi/kg) and 30 min or 24 h later, they were injected ip with chelating agents (400 µmol/kg). At 30 min after Ni treatment, the chelating agents other than CDTA effectively depressed Ni concentration

in the testes. At 24 h after Ni treatment, DMSA, BGD, and DDTC were effective in mobilizing Ni from the testes. (Table D.4).

Table D.4. Effects of chelating agents on the concentration of Ni in testes of mice pretreated with Ni*

Treatment	Nickel µg/g wet tissue	
	30 minutes	24 hours
Control (Saline)	ND	ND
Nickel	2.69 ± 0.40	2.23 ± 0.17a
Nickel + BGD	N.D.	0.72 ±0.06b
Nickel + DDTC	0.96 ± 0.07b	0.93 ± 0.08b
Nickel + DHED	1.06 ± 0.08b	1.56 ± 0.16
Nickel + CDTA	2.06 ± 0.19	1.40± 0.09
Nickel + DMSA	ND	0.73 ± 0.16b

*values represent the mean ±SD. for 6 animals. ND:Not detected.
[a]Significantly different from control ($p < 0.05$); [b]Significantly different from Ni ($p < 0.05$).

Male mice were injected i.p with Ni (5 mg Ni/kg) or saline (control). Thirty min or 24 h later, they were injected ip with saline or chelating agents (400 µmol/kg). The mice were mated with virgin female mice for 7 days, starting 7 days after Ni administration. The authors stated that the number of females tested versus the number of males was 2: 1 and described the fertility rate as the number of females impregnated versus the number of females tested. The number tested appears to mean the number of animals included in the study and for fertility rate this would mean the number of females impregnated versus the number bred (Table D.5).

Table D.5. Effect of chelating agents on fertility of mice after nickel administration

Treatment	Fertility	
	30 minutes	24 hours
Control (Saline)	10/10	10/10
Ni 0.5 mg/kg +Saline	10/10	10/10
Ni 1.0 mg/kg +Saline	8/10	8/10
Ni 5.0 mg/kg +Saline	5/10	5/10
Ni 5.0 mg/kg +BGD	10/10	10/10
Ni 5.0 mg/kg +DDTC	10/10	9/10
Ni 5.0 mg/kg +DMSA	10/10	10/10

In this study, injection with Ni increased lipid peroxidation, and decreased the testicular weight and the fertility rate, indicating the toxic effect of Ni on testes. Among five chelating agents tested, DMSA and BGD were the most effective in removing nickel from the testes and, protecting against LPO and Ni-induced sterility.

The findings from a series of studies published in 1997, 2000 and 2002 by Das and Dasgupta are summarized below.

Das and Dasgupta (1997). Alteration of testicular biochemistry during protein restriction in nickel treated rats.

This study investigated the effect of nickel sulfate ($NiSO_4$) • (H_2O) on total protein, glycogen, and cholesterol content of the testes, lactate dehydrogenase, and glutamate oxaloacetate transaminase activities, as well as organ weights of testes and accessory sex glands during a protein-restricted dietary regime (Das and Dasgupta 1997).

Twenty-four male albino Wistar rats were separated into four groups of six. Two groups were fed normal protein diet (18% casein) and the other two were fed protein-restricted diets (5% casein). One set from each group served as control and the other two sets were dosed with $NiSO_4$ at 2 mg/100 g of body weight intraperitoneal (ip) on every alternate day until the 10^{th} dosage. The body weights of all rats were recorded on day 1 of the dietary treatment, on day 1 of $NiSO_4$ injection, and just before sacrifice. Animals were sacrificed by decapitation after the last injection and overnight fasting. The testes, seminal vesicles, epididymis (cauda and caput), and prostate of each group were separately dissected out, trimmed of fat, wiped clean, and weighed immediately. Testes were frozen immediately for further analysis. Lactate dehydrogenase (LDH) and glutamate oxaloacetate transaminase activity was measured. Glycogen content and cholesterol was also measured.

In both $NiSO_4$ groups, there was a significant reduction in body weight, with a more significant reduction in body weights in those rats that had a restricted protein diet. There was a significant decrease in testes weight in both groups that were treated. There were nonsignificant decreases in the weights of seminal vesicles, epididymis (caput), and prostate in both groups. Significant reduction of LDH activity in the testes occurred after nickel sulfate administration in both experimental groups in comparison to their respective controls. Testicular glycogen and cholesterol content were elevated significantly in both experimental groups, whereas there was a significant reduction of testicular protein. All the biochemical parameters are summarized in Table D.6 below.

Table D.6. Biochemical Parameters in the Testes after Nickel Sulfate Treatment (2.0mg/100g ip10 days every alternate day)*

Biochemical Parameters	Normal Protein Diet	Normal Protein Diet with NiSO4	Protein Restricted Diet	Protein Restricted Diet with NiSO4
Glycogen (mg/g)	117.72 ±5.08[a]	129.39 ±4.06[b]	125.99± 3.01[b]	149.09 ±7.57[c]
Lactate dehydrogenase (LDH) activity (m.I.U. /mg of protein)	334.04 ±5.26[a]	210.89± 8.56[b]	320.71 ±2.11[a]	179.12±7.99[c]
Total Proteinmg/g)	21.42 ±1.70[a]	15.38 ±2.02[b]	17.08± 0.84[b]	11.29 ±0.68[c]
Glutamate oxaloacetate transaminase (mg/g/h)	26.23 ±0.68	29.57± 1.75[NS]	27.49 ±1.65	28.99 ±1.36[NS]
Total Cholesterol (mg/g)	84.05± 4.30[a]	141.19± 6.16[b]	110.3± 8.29[c]	171.62± 9.80[d]

*Each value is mean ± SEM of 6 observations in each test group. In each column, values with different superscripts were significantly different from each other ($p < 0.05$).
m-I.U. = mμmol/mL/min where mμ = millimicron (unit of measure for optical density)

The authors state that the reduction in weight of the testes and accessory glands is most likely due to lowered production of testicular androgen because of loss of mass of Leydig cells and/or interference with protein synthesis in those cells. $NiSO_4$ also alters testicular metabolism in both the regular and protein deficient treatment groups. The increase in glycogen content was observed in the testes of animals treated with nickel in both the normal and protein-restricted groups. Glucose is the major substrate for metabolism in spermatids and spermatocytes in rat testes; therefore, an efficient glycolytic pathway ensures sufficient energy yield. The authors state that $NiSO_4$ induced glycogen accumulation in the testes is probably the result of inhibition or lowering of the rate of glycolysis.

According to the authors the decreased LDH activity corroborates the above discussion of a lowered rate of glycolysis leading to the accumulation of glycogen and since anaerobic glycolysis plays a major role in the maintenance of energy yield in the testes, the nickel-induced reduction of LDH activity in both treated groups (normal protein and protein-restricted) is most likely the result of inhibition or reduction of glycolysis. Significant changes in total protein content in both nickel-treated experimental groups indicates that nickel reduces protein biosynthesis in the testes.

The authors suggest that the increase in cholesterol content in the testes of nickel-treated rats of both dietary groups may be due to the inhibition of testosterone synthesis. Since testicular cholesterol is the main source of androgen production, alteration of cholesterol levels in the testes affects spermatogenesis. Therefore, the

authors postulate that the increase of cholesterol content in the testes after nickel treatment may reflect a reduction of androgen production as well as spermatogenesis. Thus, the authors conclude nickel sulfate adversely affected the biochemical micro-environment of the testes of albino rats fed normal protein, which was further aggravated in protein-restricted diets.

Das and Dasgupta (2000). Effect of nickel on testicular nucleic acid concentrations of rats on protein restriction.

The effects of $NiSO_4 \cdot H_2O$ was evaluated on male reproductive health by measuring DNA and RNA concentrations in the testes of albino rats during protein restriction (Das and Dasgupta 2000). Forty adult male Wistar rats were divided into four groups of 10. Two groups were maintained on a normal diet (18% casein) and the other two were protein-restricted (5% casein). They were dosed with 2.0mg/100 g bw ip on alternate days until the tenth dosage. Animals were sacrificed by decapitation at the end of the last dose after overnight fasting. The number and motility of sperm from samples collected from cauda epididymis were examined and testicular DNA, RNA, and total protein content were measured. Sperm concentration and motility were significantly reduced by nickel in both protein-restricted and normal diet rats; however, the percentage decreases in sperm concentration and motility induced by nickel were greater under protein restricted diet conditions, compared to normal diet conditions. (see Figure D.3 below).

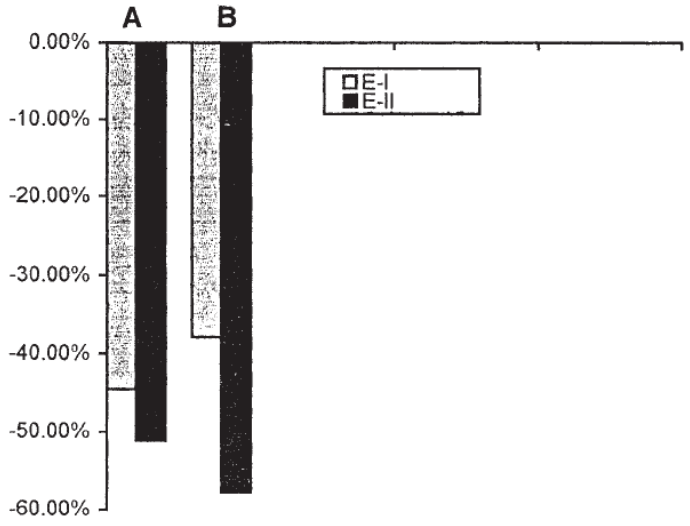

Figure D.3. Percentage change of sperm concentration and sperm motility after nickel sulfate treatment. E-I: normal dietary control; E-II: protein-restricted dietary control

A = SpermConcentration
B = Sperm Motility
Percentage change of sperm concentration and sperm motility with respect to normal dietary control (E-I) and protein-restricted dietary control (E-II) after nickel sulfate treatment.

$NiSO_4$ exposure was associated with a statistically significant decrease in testicular DNA, RNA, and protein content in both protein-restricted and normal diet rats (see Table D.7).

Table D.7. Effect of Ni Sulfate (2.0 mg/100g bwt ip 10 days every alternate day) on Testicular Nucleic Concentrations in Rats*

Groups of Animals	DNA (mg/gm)	RNA (mg/gm)	Total Protein (mg/gm)
Normal Protein Diet + Vehicle	0.66± 0.6[a]	17.99 ±1.48[a]	20.38± 1.24[a]
Normal Protein Diet + $NiSO_4$	0.50± 0.03[b]	12.48 ±1.49[b]	14.32 ±2.21[b]
Protein Restricted + Vehicle	0.54 ±0.02[b]	0.54± 0.02[b]	16.29± 1.21[b]
Protein Restricted + $NiSO_4$	0.33 ±0.01[c]	0.33 ±0.01[NS]	10.23± 0.82[c]
	F=5.86 p<0.05	F=8.25 p<0.05	F=5.34 p<0.05

* Each value is the mean ±SEM of 10 observations in each group. In each column, values with different superscripts were significantly different from each other. Significance of intergroup difference is presented by Fischer's Distribution (F); intragroup differences presented by Duncan's multiple range test fixed at $p < 0.05$

The authors conclude that $NiSO_4$ is a male gonadotoxin resulting in altered sperm count, motility, and reduced testicular DNA, RNA, and total protein concentrations ($p< 0.05$).

Das and Dasgupta (2002). Effect of nickel sulfate on testicular steroidogenesis in rats during protein restriction.

The effect of nickel sulfate on testicular steroidogenesis was assessed to ascertain whether such alterations are reversible with normal protein and protein-restricted dietary regimes (Das and Dasgupta 2002). In this report, adult male Wistar rats were divided into six equal groups of 10 each. Three groups were fed a normal protein diet of 18% casein and the other three were fed a protein restricted isocaloric diet of 5% casein. Groups 1 and 4 served as controls and groups 2 and 5 were dosed with nickel sulfate ($NiSO_4$) intraperitoneally (ip) at 2 mg/100 g body weight every other day until 10 doses Groups 3 and 6 received the same dose of $NiSO_4$ as those in groups 2 and 5; however, they were given a recovery period of 15 days after the 10^{th} dose. There were four components of male reproductive toxicity that were measured: testicular steroidogenic enzymes, cholesterol, ascorbic acid content, and plasma testosterone. Testes tissue

was analyzed to estimate the activity of the steroidogenic enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) and to estimate cholesterol and ascorbic acid content.

$NiSO_4$ treated rats showed a significant decrease in body weight. In addition, the relative weights of the testes significantly decreased in all the experimental groups (groups 2, 3, 5, 6) compared to their controls (groups 1 and 4) and recovery groups in both protein-restricted (group 3) and normal protein diets (group 6) showed significant improvement in testicular weight. There was a significant increase in both ascorbic acid and cholesterol levels and a significant decrease in the activities of 3β-HSD and 17β-HSD in all $NiSO_4$ treated groups in comparison to their controls. Plasma testosterone levels were also decreased in all experimental groups compared to their controls. However, after the 15 day recovery period, there was a significant increase in activity of the steroidogenic enzymes and in plasma testosterone levels in animals fed both protein restricted and normal protein diets (Figures D.4 and D.5).

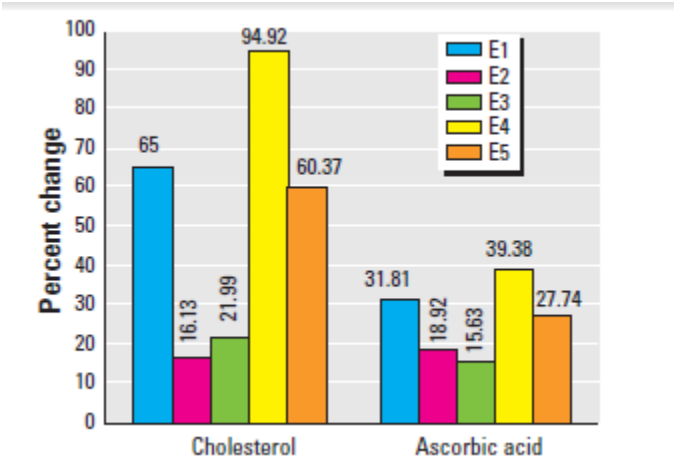

Figure D.4. Percent change in testicular cholesterol and ascorbic acid concentration after nickel treatment. E1, group 1 vs. group 2; E2, group 1 vs. group 3; E3, group 1 vs. group 4; E4, group 1 vs. group 5; E5, group 1 vs. group 6.

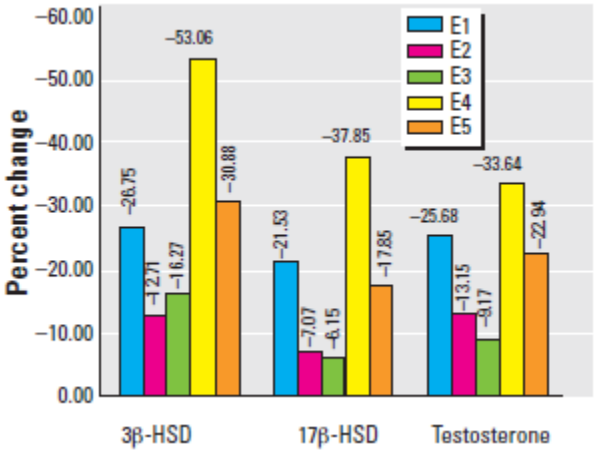

Figure D.5. Percent change in testicular 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase and plasma testosterone concentration after nickel treatment. E1, group 1 vs. group 2; E2, group 1 vs. group 3; E3, group 1 vs. group 4; E4, group 1 vs. group 5; E5, group 1 vs. group 6.

The authors state that reduction in weights of testes may be due to the lowered production of testicular androgen as a result of the loss of Leydig cell mass since $NiSO_4$ has been shown to cause germinal epithelium degeneration in testes. Ascorbic acid and cholesterol steroidogenesis in the testes and altered activity of either suggests altered steroidogenesis. In this study, there was an increase in cholesterol levels in the testes suggesting that cholesterol is being accumulated corroborating nickel sulfate-induced reduction in steroidogenesis. Ascorbic acid is also crucial for testicular hormonogenesis and its altered levels after $NiSO_4$ exposure in both diet groups suggests that it was not used for steroidogenesis like cholesterol leading to overall lowered levels of testosterone in the blood. Das and Dasgupta conclude that $NiSO_4$ adversely affects the biochemical microenvironment of the testes of rats fed normal protein diet and is further aggravated in protein-restricted diets. According to the authors, these effects of nickel may be reversible, since 15 days after cessation of nickel sulfate treatment, testicular steroidogenic enzyme activity and plasma testosterone levels, increased significantly in both normal protein-fed and protein-restricted dietary groups.

Käkelä et al. (1999). Effects of nickel chloride on reproduction of the rat and possible antagonistic role of selenium.

In this study as described in detail in previous sections, nine experimental groups, of Wistar rats (females only, males only or both males and females were exposed to nickel in drinking water at 10-100 ppm, added as $NiCl_2$•6 H_2O (Kakela et al. 1999). In one female group and one male group, the drinking water was also supplemented with 0.3 ppm selenium (added as Na_2SeO_3). The female exposures started 14, 28 or 100 days before copulation and continued during pregnancy and lactation. Breeding success and the growth and viability of pups were recorded and these details have been described

previously. When only the males were exposed (for 28 or 42 days before copulation), $NiCl_2$ reduced both the number of pregnancies and the number of pups born (Table D.8).

Table D.8. Breeding Success in Experimental Groups with Nickel-Exposed Males (*n*= 6 dams)

	Fertility index (%)	Born-dead pups (n)	Gestation index mean ± SE	Litter Size 21 days mean ± SE	Mean Pup weight, 21 days (g) mean ± SE	Sex Ratio 0--21days (M/M+F)	Viability Index (%)	Weaning Index (%)
Control	100	3	10.2±1.5	9.2±1.5	31.4±0.9	51-46	100	90
Male 30 ppm 28 days	50	0	2.7±1.4**	1.3±0.9**	40.5±0.5	38-63	56	89
Male 30 ppm+Se 28 days	67	1	7.0±5.7	7.0±5.7	34.5±0.5	62-62	100	100
Male 30 ppm 42 days	83	0	7.8±2.0	6.2±2.0	35.9±1.0	40-38	98	80
F+M 30 ppm 28 days	50	1	6.0±2.8	2.7±1.3**	39.6±1.3	33-13	100	44

** Differs from control at p<0.01, Mann Whitney's U-test.

In all the groups in which males were exposed, the fertility index decreased while in all the female only-exposure groups, and the control group, the fertility index was 100%. When both parents were exposed to $NiCl_2$, the fertility index was 50% and pup mortality during lactation was high with a weaning index of 44%, Effects in the groups where females were exposed to nickel are presented in other sections of this document. In the male-exposure groups, the 28-day exposure to 30 ppm nickel was associated with severe pup mortality during the first days after birth and by weaning, but milder mortality was found in the corresponding but longer exposure. Again, when the males received 30 ppm nickel plus selenium, no pups died. So in males, selenium supplementation of the drinking water protected those pups that were born; but fertility was lower than the controls demonstrating that addition of selenium to the drinking water of $NiCl_2$-exposed males did not significantly improve the fertility index. In summary, selenium seemed to counteract the deleterious effects of $NiCl_2$ on the reproduction of rats. In addition to effects of nickel on the gestation index, and litter size, the sex-ratio data suggest a decrease in the proportion of males in the litters.

Examining the histology of the testes, $NiCl_2$ induced shrinkage of the seminiferous tubules, as evidenced by a significant reduction in the mean diameter of seminiferous

tubules in the treated group, as compared with controls. The authors reported that nickel treatment 'seemed to close some of the tubules' and decreased the number of basal spermatogonia within the tubules. Figure. D.6 below shows the cross-section of a seminiferous tubule (40X magnification, allochrome staining): (A) from nickel-exposed testis, (B) from control testis. Examining the middle of the testes, the seminiferous tubules of the exposed males had shrunk compared with the control group, and there were empty spaces between the tubules. In addition, the connective tissue surrounding these shrunken tubules was in folds.

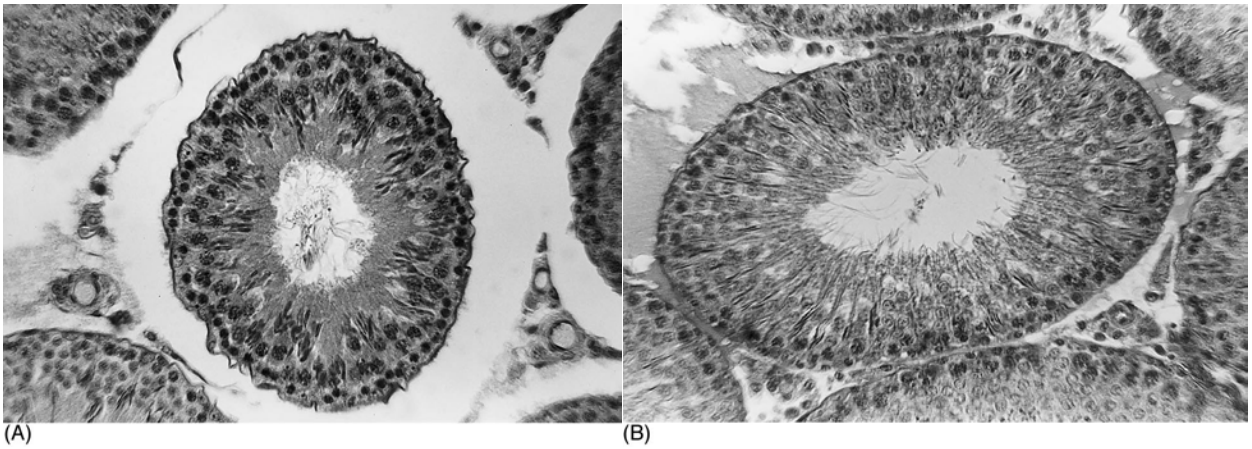

Figure D.6. Cross-section of a seminiferous tubule (40X magnification, allochrome staining): (A) from nickel-exposed testis, (B) from control testis.

Additionally, the authors reported that 42-day exposure of the males to 30 ppm nickel caused less severe changes in testis histology and defects in reproduction than 28-day exposure, suggesting that with continued exposure some recovery may occur. According to the authors, the clear shrinkage of the seminiferous tubules noted in the middle of the testes may have caused functional defects as seen in the Table D.9 below.

Table D.9. Diameters of seminiferous tubules, number of basal spermatogonia and ratio of open and closed tubules in the mid-region of the testes (mean ± SE)

	Tubule diameter (mm) (*n*=180)	**Basal spermatogonia per 250 mm (*n*= 60)**	**Closed:open tubules (*n* =6)**
Control	264.391.9	18.9 ±0.4 0	0.24±0.04
30 ppm, 28 days	240.3±2.0***	17.6±0.3*	1.32±0.34*
30 ppm, 42 days	251.7 ±1.9***	18.7±0.4	0.56±0.14**

* p ≤0.05, Student's *t*-test. *** p ≤0.001, Student's *t*-test. **p ≤0.10, Student's *t*-test.

In the exposure groups there were n=6 males; plus one control group (six unexposed males). In each rat, the means of the largest and smallest diameters of 30 seminiferous tubules (selected at random) were determined and expressed as the mean of those 30

mean diameters. In each group, 60 mature tubules (10 for each male) were selected at random; and the basal spermatogonia were counted along a segment 250 mm long.

The protective effect of selenium was hypothesized by the authors to result from increased synthesis of glutathione peroxidase, a selenium-containing enzyme that catalyzes the reaction of glutathione with hydrogen peroxide and organic peroxides.

Obone et al. (1999). Toxicity and bioaccumulation of nickel sulfate in Sprague-Dawley rats following 13 weeks of subchronic exposure.

In this study, adult male Sprague-Dawley rats were given 0, 0.02, 0.05, or 0.1% nickel sulfate ($NiSO_4 \bullet 6H_2O$) (corresponding to 0, 44.7, 111.75, and 223.5 mg Ni/L) in drinking water for 13 weeks (Obone et al. 1999). Twenty-four hours following the end of treatment, all animals survived with no apparent clinical signs of toxicity. The mean of body weight of nickel sulfate-treated rats was not significantly decreased compared to control animals, except for the 0.1% nickel sulfate treated group. Total plasma proteins, plasma albumin and globulins, and plasma glutamic pyruvic transaminase activity were all significantly decreased in 0.1% nickel sulfate-treated rats. The data in Table D.10 below show that exposure of rats to three different low-level exposures of nickel sulfate in drinking water for 13 weeks failed to produce any damage to the testes, as verified by measurements of the activities of some testicular enzymes, such as alkaline phosphatase, acid phosphatase, and lactate dehydrogenase, which showed no significant changes in their activities.

Table D.10. Biochemical Parameters of Testicular Toxicity in Rats Following Subchronic Exposure to Nickel Sulfate ($NiSO_4 \bullet 6H_2O$) in Drinking Water for 13 weeks*

Biochemical parameter	**Control**	**0.02% $NiSO_4$**	**0.05% $NiSO_4$**	**0.1% $NiSO_4$**
Alkaline phosphatase (U/g)	109.23 ± 10.26	105.33 ± 8.73	99.88 ± 4.79	92.71 ± 5.83
Acid phosphatase (U/g)	49.08 ± 5.54	45.62 ± 0.97	48.74 ± 0.59	40.28 ± 1.10
Lactate dehydrogenase (U/g)	474.61 ± 40.70	440.15 ± 30.88	418.31 ± 10.21	433.16 ± 12.85

*Values are averages ± SE of eight rats. Parameters were measured 24 h after termination of exposure. U in U/g refers to units of enzyme activity, where activity means the amount of enzyme that catalyzes 1 mole of its respective substrate.

According to the authors, the present data have shown that sub-chronic exposure of rats to low levels of nickel sulfate for 13 weeks does not have the potential to induce testicular toxic effects as verified by both biochemical and morphological studies, as well as by comparing relative testicular weights of treated and control rats. Similarly, the total testicular protein contents also were not modified. Hence they concluded that nickel sulfate under such sub-chronic exposure conditions cannot induce any destruction of epithelial cells of type I and type II of seminiferous tubules, nor could it

produce any perturbations of cellular permeability or rupture of cells of seminiferous tubules and this is in agreement with the studies of Mathur et al. (1977a, 1977b). No gross or microscopic changes were seen in any of the various tissues examined including the testis. The relative order of bioaccumulation of nickel in different organs of rats when treated at 0.1% nickel sulfate (223.5 mg Ni/L) was kidneys > testes > lung = brain > spleen > heart = liver. Further, the authors state that toxic effects due to nickel sulfate are related to exposure concentrations and not nickel tissue burden and the order of toxicity did not follow the order of bioaccumulation of nickel in different organs/tissues.

The findings from the work of Pandey and coworkers published in 1999, 2000 and 2001 are presented below.

Pandey et al. (1999). Male reproductive effect of nickel sulfate in mice.

The effects of orally administered nickel sulfate ($NiSO_4$) on the histoarchitecture of different compartments of the testes and accessory organs (epididymis and seminal vesicles), and on sperm count, sperm motility, and abnormalities of spermatozoa were investigated in this study (Pandey et al. 1999). The authors also described the bioaccumulation pattern of nickel in reproductive organs, effects of nickel on enzymes considered to be marker of testicular function, and male mediated developmental effects of nickel.

Sixty male adult Swiss albino mice were divided into 3 groups of 20 each. $NiSO_4$ was dissolved in distilled water and 0.2 ml of the solution was administered by gavage. The animals of groups II received 5 mg of $NiSO_4$ per kg body weight per day and III received 10 mg $NiSO_4$ per kg body weight per day by gavage, 5 days per week for 35 days. The mice in Group I received an equal volume of distilled water and served as control animals. The body weights of animals were recorded at the beginning and end of the experiment. Mice were fasted overnight, weighed and sacrificed by cervical dislocation on the 36th day of experimentation. The testes, epididymides, prostate glands and seminal vesicles were removed and weighed. The testes, epididymides and seminal vesicles of ten mice from each group were used for determination of nickel contents. The tissues from the remaining ten mice of each group were used for histopathological and biochemical studies. For male mediated developmental toxicity, 20 fertile male mice received daily gavage doses of $NiSO_4$ at 10mg/kg body weight, 5 days a week for 35 days; 20 male mice were given an equivalent amount of distilled water and served as control mice. After mating with fertile female mice, the number of pregnant females were recorded from both experimental and control groups for determination of fertility index. On the 18th day of gestation laparotomies were performed, number of corpora luteal were counted and fetuses were removed by uterine section. The number of live and resorbed fetuses (embryo) and total number of implantations were recorded. Fertility index, preimplantation loss and post implantation loss were calculated. Fetal weight and crown rump length were also recorded. The effects of treatment on organ weights are summarized below in Table D.11.

Table D.11. Effect of $NiSO_4$ Exposure on Organ Weights of Adult Male Mice

Group		Testis	Epididymis	Accessory Sex Organs	
				Seminal Vesicle	Prostate Gland
I Control (*n* = 10)	A	0.199 ± 0.077	0.056 ± 0.008	0.175 ± 0.009	0.035 ± 0.012
	B	0.787 ± 0.158	0.0266 ± 0.054	0.694 ± 0.165	0.135 ± .001
II 5mg/kg (*n* = 10)	A	0.165 ± 0.021*	0.045 ± 0.005	0.173 ± 0.041	.021 ± 0.025
	B	0.676 ± 0.236*	0.218 ± 0.075	0.485 ± 0.095*	0.101 ± 0.001*
III 10 mg/kg (*n* = 10)	A	0.153 ± 0.023*	0.048 ± 0.005	0.223 ± 0.012*	0.018 ± 0.006*
	B	0.625 ± 0.262*	0.191 ± 0.034*	0.328 ± 0.232*	0.072 ± 0.005*

*p< 0.05; A–Absolute organ weight (g); B–Relative organ-to-body weight (g); Mean ±S.E. of requisite number of mice in each group.

The above table indicates that there was a decrease in absolute and relative weight of testis, epididymis, seminal vesicle and prostate gland.

Nickel sulfate also was associated with changes in sperm motility and total sperm count in the treated group receiving 10 mg/kg of $NiSO_4$ (Table D.12).

Table D.12. Effect of $NiSO_4$ Exposure on Sperm Motility and Total Epididymal Sperm Count

Group	Sperm Motility (%)	Total Sperm Count (per epididymis) x 10^7
I Control (*n* = 10)	82.5 ± 2.88	9.30 ± 1.97
II 5 mg/kg (*n* = 10)	80.5 ± 3.90	8.11 ± 2.14
III 10 mg/kg (*n* = 10)	76.75 ± 3.94*	5.87 ± 1.18*

Mean ± S.E; *p < 0.05.

There was a significant increase in morphological abnormalities in different regions of the spermatozoa of mice exposed to 5 and 10mg/kg of $NiSO_4$ (data not shown) seen as abnormalities in the head, neck and tail of spermatozoa.

The effects of $NiSO_4$ on testicular enzymes are summarized below in Table D.13.

Table D.13. Effect of nickel sulfate exposure on activity of marker testicular enzymes in mice

Enzyme	Group I (Control)	Group II (5 mg/kg)	Group III (10 mg/kg)
ɣ-glutamyl transpeptidase	28.76 ± 2.16	35.23 ± 4.50	38.44 ± 2.24*
Sorbitol dehydrogenase	7.88 ± 0.78	6.00± 0.86	4.01 ± 0.86*
Lactate dehydrogenase	194.22 ± 3.15	236.7 ± 0.023	243.86 ± 0.017*

Enzyme activities are expressed as specific activities (n moles substrate oxidized or product formed/min/mg protein). *p< 0.05

There was a significant decrease in the activity of sorbitol dehydrogenase and an increase in the activities of ɣ -glutamyl transpeptidase and lactate dehydrogenase at 10 mg/kg of $NiSO_4$. Sorbitol dehydrogenase is associated with germ cell maturation. ɣ-Glutamyl transpeptidase and lactate dehydrogenase are associated with Sertoli cells and germinal epithelium respectively.

There were also histopathological changes. At 10mg/kg of $NiSO_4$ there was congestion in the peripheral region of most of the seminiferous tubules. They also appeared to be atrophied with an increase in inter-tubular spaces. There was also evidence of disturbed spermatogenesis. The cauda epididymis showed degeneration of epithelial cells and sperm were either absent or few in number. Principal cells in the epithelium were vacuolated and lacked nuclei. The authors postulate that the alteration in the activities of marker testicular enzymes associated with the histopathological changes may be responsible for production of decreased number of spermatozoa. The observed histopathological changes in secretory folds of epithelium (see Figures D.7- D.12) and reduction in their size suggest the secretory surface area of seminal vesicles following exposure to nickel is reduced. The authors state that such an effect could adversely affect the secretion from the seminal vesicle which are essential for maintenance of the sperm. They conclude that the decrease in weight of testis may be due to degeneration of germinal epithelium and that the observed loss in weight of epididymis, and the seminal vesicle may be due to decrease in sperm count and morphological changes in these organs.

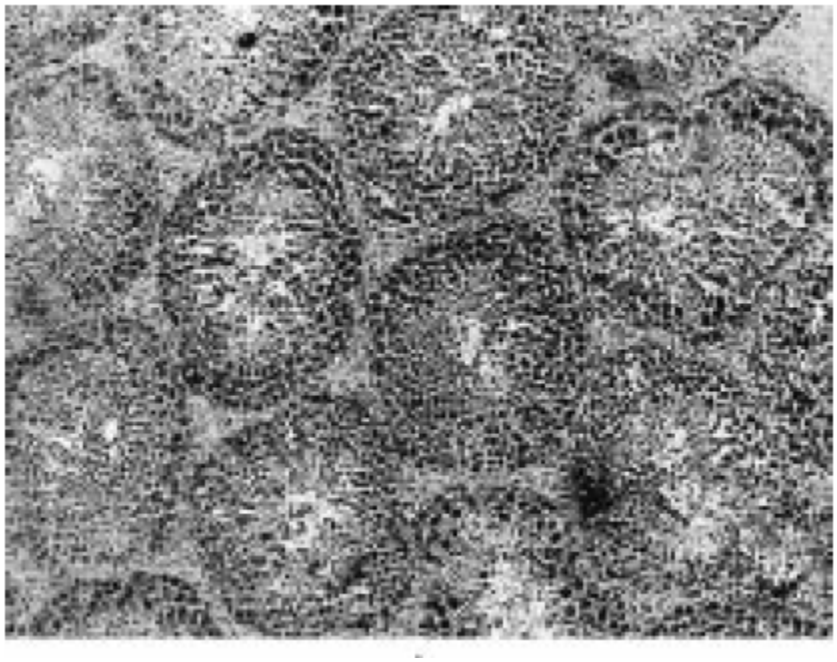

Figure D.7. Control testes show compact seminiferous tubules, and the tubules have well developed germinal epithelial cells.

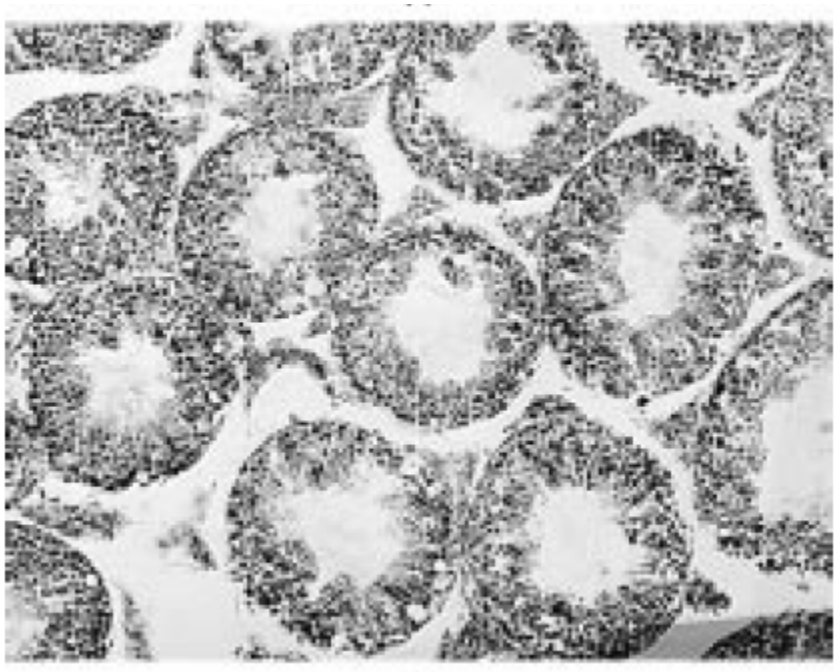

Figure D.8. At 10mg/kg $NiSO_4$ most of the seminiferous tubules located centrally appeared atrophied with increase in intertubular spaces, and there was evidence of disturbed spermatogenesis (loss of spermatids).

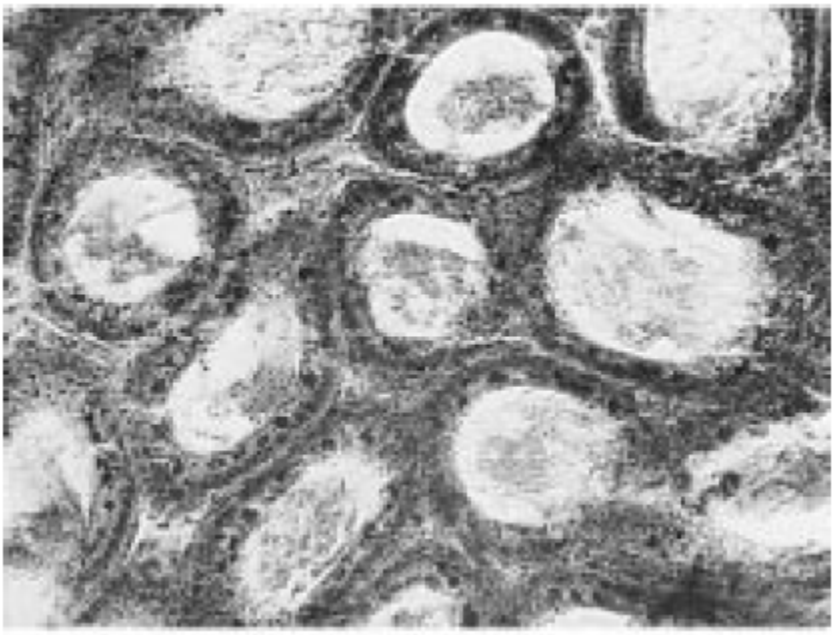

Figure D.9. Control cauda epididymis with well-developed columnar epithelium and few granules in cytoplasm of epithelial cells

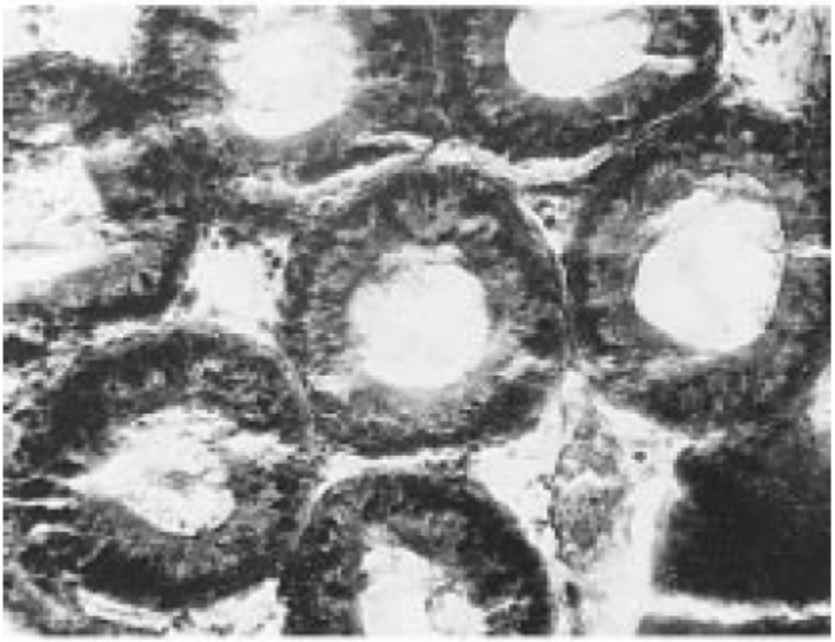

Figure D.10. 10 mg/kg $NiSO_4$ resulted in loss of columnar epithelium and granules in cytoplasm of epithelial cells.

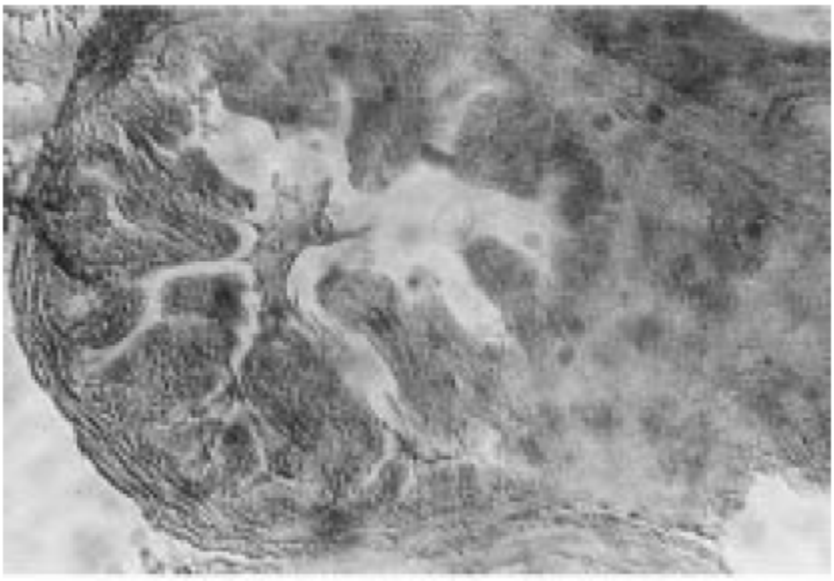

Figure D.11. Control seminal vesicle with well-developed vesicular epithelium

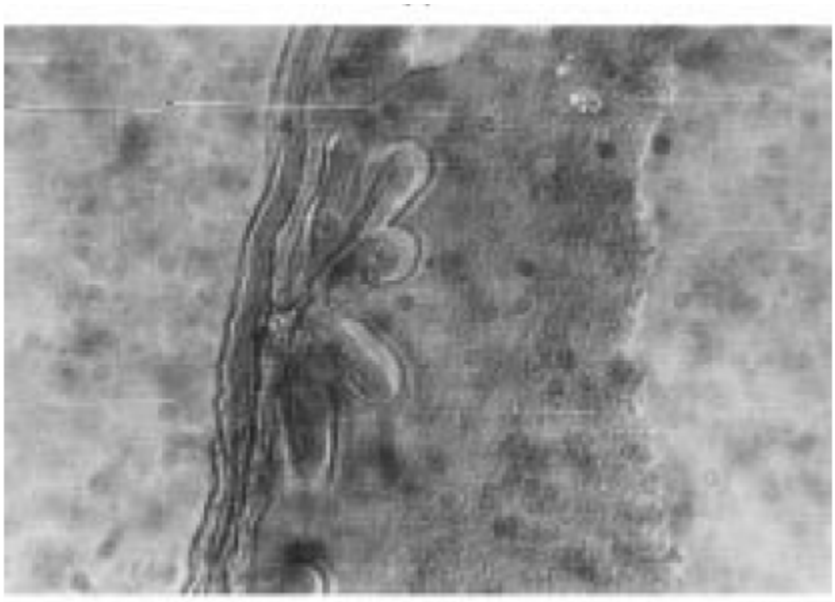

Figure D.12. 10mg/kg $NiSO_4$ associated with loss in size of vacuolar folds

The authors calculated the fertility index of exposed male mice based on their ability to impregnate non-exposed female mice and described this as male-mediated developmental toxicity. At 10 mg/kg $NiSO_4$ the fertility index was 46.6% in comparison to the control (66.6%). A significant increase in pre and post implantation loss was observed in untreated females bred to the treated males (Table D.14). The authors

stated abnormal sperm morphology has been associated with spontaneous abortion and abnormal sperm flagellar activity may be responsible for reduced fertilizing capacity. Male mice exposed to $NiSO_4$ were able to impregnate unexposed females, but the rate of pregnancy was lower than in unexposed females mated to unexposed male mice. An increase in abnormal sperm and a decrease in epididymal sperm count in the 10 mg/kg group were associated with a decrease in fertility.

Table D.14. Male mediated embryotoxicity studies with $NiSO_4$

	Control	**$NiSO_4$ 10mg/kg**
Number of dams	15.00	15.00
Corpora lutea	12.00 ± 1.64	13.53 ± 1.18
Implantation	12.40 ± 0.82	10.93 ± 1.16*
Pre Implantation Loss (%)	3.33 ± 0.82	19.20 ± 1.16*
Live Fetuses	11.83 ± 1.63	9.13 ± 1.16*
Number of Resorptions	0.57 ± 0.05	1.80 ± 0.77*
Post Implantation Loss	4.59 ± 1.63	16.46 ± 1.16*
Fetal Weight (g)	0.889 ± 0.16	0.417 ± 0.13*
Fetal Crown-rump Length (mm)	3.60 ± 0.16	3.41 ± 0.31

*p < 0.05 considered to be statistically significant.
Pre implantation loss (%) (Corpora lutea-implantation/Corpora lutea)x100.
Post implantation loss (%)(implantation – Live fetuses/implantation)x100.

Pandey et al 1999 conclude that the increases in pre and post implantation losses and resorbed fetuses, and the decreased fetal weight in the mice mated with the $NiSO_4$ exposed males may be due to developmental toxicity of this metal.

Pandey and Srivastava (2000). Spermatotoxic effects of nickel in mice.

The effect of nickel on sperm motility, morphology, and count in mice on male fertility was evaluated in this study (Pandey and Srivastava 2000). Forty-eight male mice were divided in two main groups (one treated with nickel sulfate ($NiSO_4$), the other treated with nickel chloride ($NiCl_2$)), consisting of 24 animals in each. Each main group was then divided into 4 sub-groups. Sub-groups II, III and IV received the nickel compound e ($NiSO_4$) or ($NiCl_2$) at 5, 10 or 20 mg/kg-d, orally 5 days a week for 35 days, in 0.2 mL distilled water. Group I in both the sub groups received 0.2 mL distilled water (vehicle controls). Body weight was recorded at initiation and completion of the experiment. The mice were fasted overnight and killed by cervical dislocation on the 36th day of the experiment.

No overt toxicity or mortality was observed. Dose-related effects on body weight gain were observed at 10 and 20 mg/kg for both $NiSO_4$ and $NiCl_2$ The absolute and relative weights of testes, epididymis, seminal vesicles, and prostate gland were significantly decreased at 20 mg/kg for both $NiSO_4$ and $NiCl_2$ (data not shown). For effects of $NiSO_4$ and $NiCl_2$ on sperm motility and count, see Table D.15.

Table D.15. Effect of nickel on motility and total epididymal sperm count in mice treated with nickel chloride and nickel sulfate for 35 days

	Motile Sperm (%)		Sperm Count Epididymis (10^7)	
Sub-group Number	**$NiSO_4$ Treated**	**$NiCl_2$ Treated**	**$NiSO_4$ Treated**	**$NiCl_2$ Treated**
I Control	88.3 ± 2.11	86.0 ±2.39	8.0 ± 0.32	8.0 0.17
II 5mg/kg	85.5 ± 2.3 (2.8%)	85.10 ±1.29 (1.2%)	8.5 ±0.31 (6.2%)	8.2± 0.08 (2.5%)
III 10mg/kg	75.0 ±4.91 (15.1%)*	65.0 ±1.29 (24.4%)*	7.0 ±0.24 (12.5%)	6.0 ±0.07 (25%)*
IV 20mg/kg	65.0 ±4.8 (26.4%)*	49.1 ±1.35 (42.9%)*	6.0±± 0.21 (25%)*	5.0 ±0.05 (37.5%)*

*p<0.05, Mean±S.E.M. of 6 mice per group.

There were significant dose-related increases in abnormal sperm (Figures D.13 and D.14). The abnormalities were in the head, neck and tail region of the sperm observed with both $NiSO_4$ and $NiCl_2$. Curved neck and curved, bent, round, loop and folded tails were seen at both the higher doses of each nickel compound.

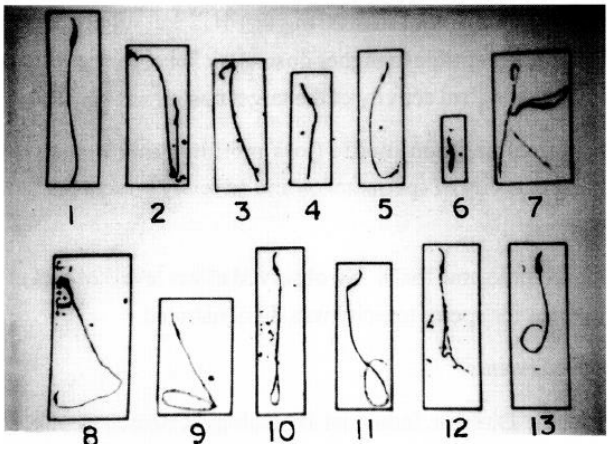

Figure. D.13. Sperm Shape Abnormalities
1. Normal sperm; 2. Acrosome up; 3. Acrosome down; 4. Acrosome absent; 5. Banana head; 6. Detached head; 7. Curved neck; 8. Bent neck; 9. Bent tail; 10. Round tail; 11. Loop tail; 12. Folded tail; 13. Signet tail.

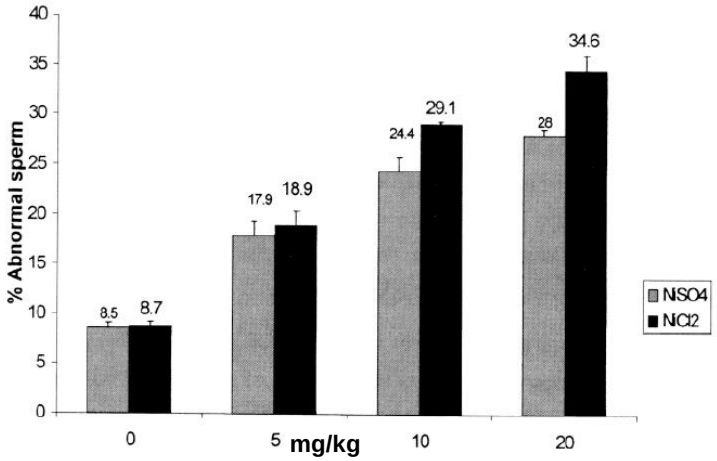

Figure D.14. Effect of $NiSO_4$ and $NiCl_2$ on sperm abnormalities in mice

The authors state that sperm morphology also has an important relationship to sperm motility and the reduced motility observed may have been due to the morphological aberrations. They postulate that the loss in number of motile sperm may be due to greater number of abnormal sperm. The authors stated that the abnormal less motile sperm may reduce fertilizing capacity and adversely affect the fertilization of the ovum.

Pandey and Singh (2001). Seminal toxicity of nickel sulfate in mice.

Pandey and Singh (2001) investigated the possibility that decreased production of testosterone may be an early effect of long-term nickel exposure, by studying the morphology of the seminal vesicles, which is influenced by testosterone (Pandey and Singh 2001).

Ten male Swiss albino mice were given a daily oral dose of 20 mg/kg of $NiSO_4$ five days/week for 6 months and 10 mice served as controls and were dosed with saline (0.9% NaCl). Urine was collected before and after the treatment at 6 weeks and 25 weeks post experimentation.

There were no outward signs of toxicity in any of the treated animals. However, there were significant changes in the weight and size of the seminal vesicles in mice exposed to $NiSO_4$, as shown in Table D.16.

Table D.16. Weight and Size of Seminal Vesicles in Mice Exposed to $NiSO_4$

	Control	**Ni Exposed (20mg/kg/d)**
Weight (mg)	14.58 ± 4.07	9.15 ± 2.84*
Largest diameter (mm)	3.80 ± 0.22	3.17 ± 0.48*
Smallest diameter (mm)	2.46 ± 0.20	2.25 ± 0.29*

As seen in Table D.16, there was a statistically lower mean weight and diameter of the cross-sectioned seminal vesicles in mice exposed to $NiSO_4$ in comparison to controls. However, testicular weight and morphology did not differ between the control and $NiSO_4$ treated groups. The urinary excretion of protein (mg protein/mg creatinine) was lower in the treated mice after 6 weeks, and more prominently after 25 weeks, but these changes were not statistically significant and the authors attribute the lower values in the control group to be due to seasonal variations in testosterone production. There was a difference in the secretory activity (presence of high columnar epithelium) of the cells of the vesicular epithelium (Figures D.15 and D.16); however, the authors could not establish a scoring system and statistical evaluation was not possible.

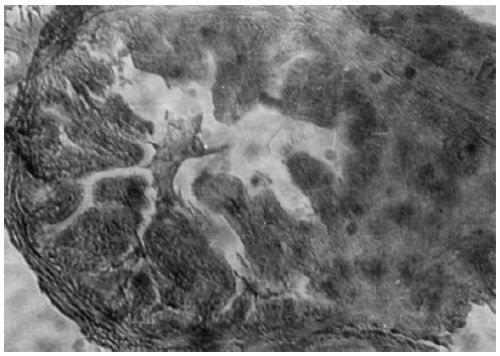

Figure D.15. A control animal, showing the high columnar epithelium indicative of secretory activity

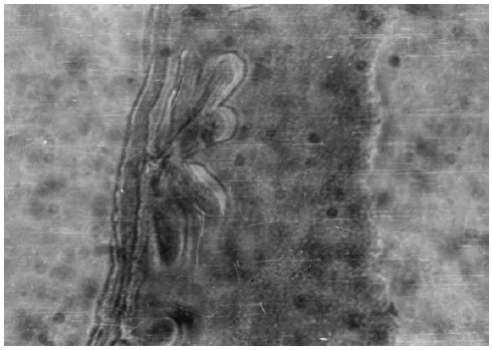

Figure D.16 An animal exposed to $NiSO_4$ for 6 months, showing a low cuboidal epithelium.

Nevertheless, Pandey and Singh conclude that the decrease in seminal vesicle weight, diameter and the change in vesicular epithelium indicative of lowered secretory activity of epithelium in $NiSO_4$ exposed animals is treatment-related, and consistent with effects associated with decreased testosterone levels.

Siglin (2000a) A one-generation reproduction range-finding study in rats with nickel sulfate hexahydrate. and Siglin (2000b) An oral (gavage) two-generation reproduction toxicity study in Sprague-Dawley rats with nickel sulfate hexahydrate.

Protocols for these studies have been described in detail in previous sections.

Springborn Laboratories, Inc. (SLI) conducted a range-finding 1-generation study (Siglin 2000a), followed by a two-generation reproductive toxicity study (Siglin 2000b) In both studies, groups of male and female Sprague-Dawley rats were gavaged daily with nickel sulfate hexahydrate. In the range-finding study, the rats received doses of 0, 2.2, 4.4, 6.6, 11, or 17 mg Ni/kg-day as nickel sulfate hexahydrate, beginning two weeks prior to mating, through the day of scheduled sacrifice (males on PND 0 and dams on PND 21). In the two-generation study, daily doses of 0, 0.2, 0.6, 1.1 or 2.2 mg Ni/kg-day were administered beginning 70 days prior to mating, and continuing through mating and parturition. Litters were culled to eight pups/litter on postnatal day (PND) 4, and dosing of the F1 rats started on PND 21, at the same dose of nickel sulfate as their parents. F1 rats were mated after a minimum of 70 days of treatment. Male rats were sacrificed after 16-18 weeks of treatment, and dams were sacrificed on PND 21. All the F1 pups not selected for breeding and all the F2 pups were sacrificed on PND 21. In addition to evaluation of pup viability and growth, reproductive measures included estrous cyclicity and sperm parameters, as well as histopathology focusing on the male and female reproductive tracts.

No toxicologically meaningful differences were noted among the groups for sperm parameters, copulation and fertility indices, precoital intervals, gestation lengths, gross necropsy findings or onset of sexual maturation in F1 rats and histopathological evaluations did not reveal any changes in reproductive organs examined in these studies.

Doreswamy et al. (2004). Nickel-induced oxidative stress in testis of mice: evidence of DNA damage and genotoxic effects.

In this study adult albino mice (CFT-Swiss) were given single doses of nickel chloride via the intraperitoneal route (individual doses not provided but highest dose was 5 µmol/100 g of body weight) and subsequent, multiple sub-lethal doses (1.25, 2.5 or 5.0 µmol/100 g of body weight per day) for 3 or 5 consecutive days. Mice were killed either 24 hours after the last dose (biochemical studies, DNA damage, and fragmentation experiments) ort 1, 2, 3, or 5 weeks after the first dose (sperm head abnormality study). Immediately after euthanasia, testes and epididymis were excised and weights were recorded. One testis and its correspondent epididymis were fixed in Bouin's fixative and processed for histopathological examination. Effects on testicular histoarchitecture, lipid peroxidation (LPO) in testis (homogenates, microsomal or mitochondrial fractions) and epididymal sperm, DNA damage, induction of apoptosis in testis, and the incidence of sperm head abnormalities were examined (Doreswamy et al. 2004). The authors investigated the propensity of nickel to induce oxidative stress in testis and epididymal

sperm (ES) of mice following administration of single or multiple sub-lethal doses. After single-exposure regimen, only induction of oxidative stress was ascertained (by measuring the extent of LPO). Using similar multiple doses, induction of DNA damage in testis or ES and apoptosis (in terms of DNA fragmentation) in testis was also investigated. Furthermore, nickel-induced genotoxic effects were ascertained by examining their effects on sperm in the cauda epididymis (counts and head abnormalities) and their ability to induce male-mediated dominant lethal (DL) mutations. For this,10 adult males were administered (intraperitoneally) nickel chloride (2.5 mmol/100 g of body weight) for 5 consecutive days and mated with untreated (1:1) virgin females each week sequentially for 5 weeks. Successful mating was ascertained by the presence of vaginal plugs, and all the pregnant females were humanely killed 16 to 17 days after detection of plugs and assessed for the degree of postimplantation embryolethality (a single dose of cyclophosphamide at 100 mg/100 g of body weight served as the positive control).

Short-term doses of nickel were reported to induce only a minimal LPO response (data not presented), but multiple doses elicited a moderate (15% to 30%) increase in LPO in whole homogenates and higher dose-related increases in both mitochondrial (20% to 50%) and microsomal (25% to 60%) fractions measured at 24 hours after the last dose. The degree of induction of LPO was relatively low in homogenates, but significant increases were evident in both mitochondrial and microsomal fractions, suggesting the higher susceptibility of these membranes to nickel. A significant increase in DNA damage in the testis (increased single-strand breaks) was observed in the fluorimetric analysis of the DNA unwinding assay and this led to a decreased percentage of double-stranded DNA evident only at higher doses (2.5 and 5 µmol/100 g of body weight). The effects of multiple doses of nickel chloride on DNA damage in testis and epididymal sperm of mice quantified at 24 hours after the last dose are presented in Table D.17 below. The percentage of double-stranded DNA was decreased in the mid- and high-dose groups, compared to controls.

Table D.17. Percentage of double-stranded DNA in testis and epididymal sperm of mice administered (intraperitoneally) multiple doses of nickel chloride

Group	Double-Stranded DNA %	
	Testis	**Epididymis**
Control	83 ± 5	90 ± 4
1.25 µmol/100 g of body weight	80 ± 6	85 ± 5
2.5 µmol/100 g of body weight	65 ± 8 *	82 ± 4 **
5.0 µmol/100 g of body weight	62 ± 6 *	80 ± 5 **

The values are mean ± SD of 4 animals each. Data analyzed by Student's t test.
* p ≤0.05; **p ≤0.02

At higher doses of 2.5, and 5.0 µmol of nickel chloride per 100 g of body weight per day, marked apoptosis (as ladder patterns of DNA fragmentation) was observed in the testis from mice treated with nickel chloride for 3 days. Sperm counts from the cauda epididymis at all sampling weeks (at 1, 2, 3, and 5 weeks) showed no alterations, but

there was a nearly 3- to 4-fold increase in the percentage of abnormal sperm (head abnormalities) among the nickel-treated males during the first 3 weeks. The major head abnormalities consisted of amorphous heads, balloon heads, and big heads and hammerheads. Table D.18 below provides information on the incidence of abnormal sperm in mice administered multiple doses of nickel chloride.

Table D.18. Incidence of abnormal sperm in mice administered (intraperitoneally) multiple doses of nickel chloride

Group	Week			
	1	2	3	5
Control	2.0 ± 0.12	2.1 ± 0.15	1.8 ± 0.22	2.3 ± 0.16
1.25 µmol/100 g of body weight	2.1 ± 0.18	2.2 ± 0.21	1.9 ± 0.18	2.2 ± 0.15
2.5 µmol/100 g of body weight	5.9 ± 0.24†	7.3 ± 0.25†	5.0 ± 0.28†	3.6 ± 0.19‡
5.0 µmol/100 g of body weight	5.9 ± 0.26†	5.7 ± 0.27†	6.9 ± 0.18†	4.2 ± 0.25‡

The values are mean ± SD of 4 animals each. Data analyzed by Mann-Whitney U test. † $p < 0.001$; ‡ $p < 0.002$.

Also, mating of nickel-treated (2.5 µmol/100 g of body weight per day for 5 days) males sequentially for a period of 5 weeks with untreated females resulted in a significant increase in male-mediated dominant lethal-type mutations (the frequency of dead implantations) during the first 3 weeks, suggesting a stage-specific effect on postmeiotic germ cells. In the present study, moderate elevations in the activities of the antioxidant enzymes glutathione peroxidase, glutathione S-transferase, and catalase in the testis was observed, suggesting the induction of oxidative stress. According to the authors, these findings suggest that testicular toxicity of nickel compounds may be related to enhanced production of reactive oxygen species, probably mediated through oxidative damage to macromolecules, including damage to DNA. They also speculated that oxidative stress mechanisms play a significant role in nickel-induced toxic effects and the associated genotoxic implications in vivo. Since nickel-induced accumulation of iron in hepatic tissue may be directly responsible for the oxidative damage to macromolecules, the authors speculate that similar mechanisms may also be operating in germ cells, since their previous work noted significant increases in iron in the rat testis following nickel intoxication. Overall, the authors concluded that multiple doses of nickel exposure produced moderate oxidative stress in testis of mice, apparently associated with apoptotic cell death and DNA damage in testis and epididymal sperm. The specific genotoxic effects (i.e., increased frequency of epididymal sperm with abnormal heads and higher percentage of DL-type mutations) can be interpreted as a specific effect on spermatozoa and spermatids (early or late), discernible only during specific post-treatment weeks, which can play a significant role in the development of male infertility. The authors suggest that nickel-induced testicular dysfunction at lower sublethal doses is wholly or partly mediated through oxidative damage to macromolecules, including damage to DNA.

Gupta et al. (2007). Effect of l-ascorbic acid on antioxidant defense system in testes of albino rats exposed to nickel sulfate.

In this study the effect of oral supplementation with L-ascorbic acid (50 mg/100 g body weight) on nickel sulfate (2.0 mg/kg body weight, ip)-induced lipid peroxidation (LPO)in the testes of Wistar strain male albino rats was examined (Gupta et al. 2007). The levels of testicular LPO and glutathione (GSH) and the activities of the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) were measured, and sperm count and motility of sperm samples collected from cauda epididymis was assessed one day after the last exposure. The authors reported that nickel sulfate treatment significantly increased the level of testicular LPO and decreased all antioxidant enzymes activities and GSH concentration along with decreases in sperm count and motility (Table D.19). Simultaneous treatment of L-ascorbic acid exhibited a slight protective role on the toxic effect of nickel sulfate on testicular LPO and GSH concentration, as well as the antioxidant enzymatic defense system (details in article, but not shown here).

Table D.19. Effect of nickel sulfate (2.0 mg/kg bw) on sperm count and sperm motility in rats

Treatment Group	Sperm count (million/mL)	Sperm motility (%)
Untreated control	51.12 + 2.45[a]	70.55+5.44[a]
Nickel sulfate	27.77 + 2.1 2[b]	47.34 + 4.34[b]
L-ascorbic acid	49.84 + 3.22[a]	67.98 +3.45[a]
Nickel sulfate + L-ascorbic acid	36.88 + 2.87[c]	50.66 + 3.65[b]

Values are Mean ± SEM of six observations in each group. Values with different superscripts (a, b, c) were significantly different from each other ($p < 0.05$).

Zemanova et al. (2007). Nickel seminal concentrations in various animals and correlation to spermatozoa quality

This study analyzed the concentration of nickel in the semen of stallion, bull, ram, boar and fox and its relation with morphological abnormalities of spermatozoa (Zemanova et al. 2007). The highest number of total abnormal spermatozoa was observed in stallions followed by rams, bulls, boars and foxes. In stallion semen, knob-twisted flagellum, separated flagellum and effects on the flagellum torso were found in increased number and the predominant effects in the semen of the bull, ram and boar were separated flagellum, flagellum torso and knob-twisted flagellum, while broken flagellum was noted in fox semen. The concentration of nickel in semen was 0.20 mg/kg in stallion, 0.12 mg/kg in bull, 0.31 mg/kg in ram, 0.06 mg/kg in boar and 0.36 mg/kg in fox. Seminal nickel concentration was significantly higher ($p < 0.05$) in foxes than that in bulls and significantly higher ($p < 0.01$) in rams and foxes in comparison with boars. Correlation analysis in bulls indicated a high positive correlation between seminal nickel and separated flagellum ($r = 0.76$) and medium positive correlation between nickel and flagellum torso ($r = 0.62$) and in rams a high positive correlation between nickel and

separated flagellum (r = 0.77). Medium positive correlation was found between nickel and separated flagellum (r = 0.43) and between nickel and other pathological spermatozoa (r = 0.45) in boars. According to the authors, this study revealed that there are different levels of nickel in the semen of various animal species (stallion, bull, ram, boar, fox) and even in normal concentrations, there are correlations between the concentration of nickel and the occurrence of pathological spermatozoa, most evidently in bulls and rams. The authors further suggest that alterations described in this study are associated with cellular changes mainly related to abnormalities of the membrane and nucleus.

Massanyi et al. (2007). Effect of nickel administration in vivo on the testicular structure in male mice.

Effects of nickel ($NiCl_2$) on murine testicular structure were examined in this study. Experimental animals (n = 5/group were injected intraperitoneally with a single dose of 20 mg $NiCl_2$ per kg of body mass (bm) or 40 mg $NiCl_2$ per kg bm. The control group received no injection(n = 5) (Massanyi et al. 2007). Animals were killed 48 hours after administration of nickel. The body mass of animals, the mass of testes and the testes: body mass ratio were not significantly affected by treatment. In both experimental groups a significant ($p< 0.001$) decrease of germinal epithelium in comparison with the control group was observed and the decrease of the relative volume of the germinal epithelium reached almost 10%. The relative volume of the interstitium was increased in both experimental groups, but not significantly. An increase in the relative volume of the lumen was noted in both experimental groups in comparison with the control group. The qualitative analysis detected a dilatation of blood vessels in the interstitium, undulation of the basal membrane and several empty spaces in the germinal epithelium. The diameter (n = 150) of the seminiferous tubule was markedly ($p< 0.05$) decreased in both experimental groups compared to the control group. The height of the germinal epithelium showed a significant decrease ($p< 0.05$-0.001) after nickel administration. TUNEL assay detected a higher frequency of localized apoptosis in the interstitium of nickel-administered animals compared to the control group. According to the authors, the results clearly document the negative effect of nickel on spermatogenesis and the decrease in the relative volume of germinal epithelium indicates that spermatozoa production is altered. Previous studies from this group (Massanyi et al. 2003) (Massanyi et al. 2004a) examined the levels of nickel in the semen of several species other that mice and reported that the level of seminal nickel is significantly higher in the fox and ram (0.31 ± 0.19 mg/kg) in comparison with the bull (0.12 ± 0.07 mg/kg) and boar (0.06 ± 0.08 mg/kg) and the concentration of nickel in the semen of stallion was 0.20 ± 0.24 mg/kg. Similar to the work of Zemanova et al., 2007 (also from this group), correlation analysis in bulls as well as rams showed a high positive relation between nickel and sperm abnormalities like separated tail (Massanyi et al. 2004b). Citing these findings, the authors state that all detected results suggest that nickel has a negative effect on the testicular structure, affecting mainly spermatozoa development as well as steroidogenesis.

Su et al. (2011a). Protective effects of grape seed procyanidin extract against nickel sulfate-induced apoptosis and oxidative stress in rat testes

The protective effects of grape seed proanthocyanidin extract (GSPE) against Ni toxicity in the testes were examined (Su et al. 2011). The authors speculated that GSPE could attenuate nickel-induced reproductive damage because of its anti-oxidative effects. Sperm motility, testicular apoptosis, the testicular cell cycle, oxidative stress, and the expression of related proteins were investigated to explore their possible involvement in reproductive damage induced by nickel in male rats. Additionally, this study examined whether Ni-induced reproductive damage occurs via apoptosis and oxidative stress and also examined the effect of nickel on expression of Bax and c-kit. The proapoptotic protein Bax is an apoptotic promoter, and the signal receptor c-*kit* is a transmembrane protein that can regulate germ cell proliferation, apoptosis, and adhesion. Male Wistar rats (six groups with eight rats each) were treated for 30 days with normal saline (control group), Ni alone (1.25, 2.5 or 5 mg/kg/day), or Ni (2.5 mg/kg/day) plus GSPE (50 or 100 mg/kg/day). Sperm motility parameters were assayed using computer-aided sperm analysis (CASA) systems, which generate an accurate profile of sperm motility. Parameters included curvilinear velocity (VCL), straight line velocity (VSL), and average path velocity (VAP), mean angular deviation (MAD), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), linearity (LIN), wobble (WOB), straightness (STR), and density of spermatozoa (ρ).

The authors report that after 30 days, nickel sulfate (Ni) significantly decreased sperm motility and the percentage of S-phase testicular cells and enhanced testicular apoptosis in the 2.5 and 5 mg/kg/day dose groups. Also levels of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and nitric oxide (NO) significantly increased. The decreased activity of glutathione peroxidase and catalase in the Ni groups showed that Ni could increase oxidative stress, especially at 2.5 and 5 mg. Western blot analysis showed that the expression of Bax protein and c-kit increased in 2.5 and 5 mg Ni groups compared to controls. The authors reported that these changes were partially attenuated in rats simultaneously administered GSPE, especially at the 100 mg/kg/day GSPE dose level. According to the authors, these results demonstrate that Ni can cause reproductive toxicity in rats at concentrations of 2.5 and 5 mg by decreasing sperm motility. Also intratesticular apoptosis, oxidative stress, and c-kit overexpression play pivotal roles in reproductive damage induced by Ni; and GSPE enhances sperm motility by down-regulating c-kit expression and offsetting the apoptosis and oxidative stress induced by Ni by directly decreasing MDA and NO, scavenging H_2O_2, and down-regulating Bax expression.

Jargar et al. (2012). α-Tocopherol ameliorates nickel induced testicular oxidative and nitrosative stress in albino rats.

This study presented the effect of supplementation of α-tocopherol (10 mg/100 g body weight, im) on nickel sulfate (2.0 mg/100 g body weight, ip) induced testicular oxidative and nitrosative stress in Wistar strain male albino rats (Jargar et al. 2012). Since free

radicals and intermediate products of lipid peroxidation contribute to oxidative and nitrostative stress, biochemical parameters such as serum and testicular nitric oxide, L-ascorbic acid and serum α-tocopherol concentrations were evaluated. Also sperm count, motility and histopathology of testes were examined. Nickel treated rats showed significantly decreased body weight, testicular somatic index, sperm count, sperm motility, serum and testicular L-ascorbic acid concentration and serum α-tocopherol levels as compared to their controls. Nickel also significantly increased serum and testicular nitric oxide concentrations compared to controls. When rats were given α-tocopherol along with nickel sulfate, a remarkable improvement of these parameters was noted along with significantly decreased nitric oxide concentrations in both serum and testes. Histopathology of the testes revealed tortuous seminiferous tubules, and as seen in Figures 1& 2 of the article (reproduced below as Figures D.17 and D.18), loss of spermatogenesis (> 75%), congestion and necrosis in nickel sulfate treated rats, whereas rats simultaneously treated with nickel sulfate and α-tocopherol had almost normal seminiferous tubules and near normal spermatogenesis as compared to rats treated with nickel alone.

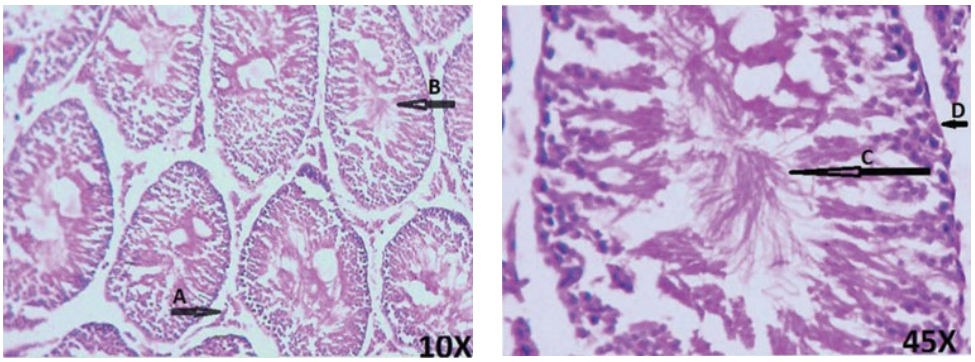

Figure D.17. Testis section of Group I (healthy control rats) showing normal architecture. The fibrovascular stroma (A) present between the seminiferous tubules contains the varying number of Leydig cells. The seminiferous tubules (B), spermatogenesis process (C) and basement membrane (D) appeared normal.

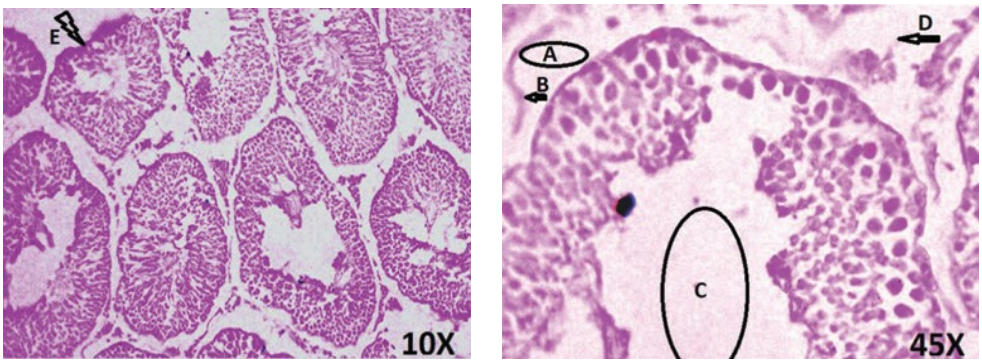

Figure D.18. Testis section of Group III (nickel sulfate treated rats) showing distorted normal architecture. Decreased Leydig cells (A), foci of fibrosis (B), loss of spermatogenesis process (C), foci of interstitial edema (D) and tortuous seminiferous tubules (E).

Based on these findings (decreased testicular somatic index, sperm count, sperm motility; increased damage to the seminiferous tubules; loss of spermatogenesis (> 75%), and increased congestion and necrosis in the testes) the authors concluded that nickel sulfate treatment causes testicular oxidative and nitrosative stress in albino rats.

Murawska-Cialowicz et al. (2012). Oxidative stress level in the testes of mice and rats during nickel intoxication.

Protamines are small, arginine-rich, nuclear proteins that replace histones late in the haploid phase of spermatogenesis and are believed essential for sperm head condensation and DNA stabilization. The authors state that researchers analyzing the ratio of protamines P1 and P2 in the semen of fertile male mice, hamsters and humans, concluded that the ratio of P1 : P2 is constant for a given mammalian species but differs between species and damage to protamine or a changed P1/P2 ratio results in fertility reduction. Also, P2 is indispensable for the production and maturation of sperm (since it allows binding with zinc), and nickel-protamine P2 interaction can prevent normal chromatin condensation thus modulating DNA damage. The lowered level of the protein can cause male infertility, and oxidative activity of Ni(II) increases its interaction with P2, leading to changes in the DNA structure and appearance of oxidation products. According to the authors, other researchers have stated that rats have hardly any protamine 2 in comparison with mice, with P2 in rats accounting for only 2–5% of the amount found in mice. The aim of the study was to find out if rats lacking spermatic protamine 2 are less susceptible to Ni(II) than mice (Murawska-Cialowicz et al. 2012). In this study, 25 male rats (*R*) of the Buffalo strain, and 25 male mice (*M*) of the Balb/c strain, were divided into four groups: 10 rats and 10 mice in the controls (*C*) and 15 rats and 15 mice in the exposed groups. Control groups received ip injections of 0.9% NaCl once and exposed animals received an ip injection of 5mg Ni(II)/kg b.w. in the form of $NiCl_2$. Forty-eight hours after exposure the animals were sacrificed and their testes were removed for analysis. The levels of malondialdehyde + 4 hydroxynonenal (MDA+4HDA) - markers of lipid peroxidation, as well as the level of reduced glutathione (GSH) were measured within the testes of both species (rat and mouse). The levels of lipid peroxidation markers were elevated in testicular homogenates of exposed mice but not in rats ($p < 0.001$). Similarly, GSH levels were significantly lower than controls in mice but not rats as noted in the Table D.20 below.

Table D.20. GSH concentration (*μ*mol/g of the tissue) in the groups of Nickel-exposed rats and mice and in the control groups

ANIMAL	Control	Nickel	Significance
Rat	56.92 ± 2.96	52.88 ± 3.25	*n.s*
Mice	64.56 ± 1.35	51.80 ± 1.85	p = *0.0029*
Significance R:M p = *0.05*			

These results suggest that Ni(II) can initiate oxidative stress in the testes of mice but not of rats and the antioxidative defense (as evidenced by the GSH level) of the testes is reduced in mice.

The findings present the differences in the level of stress in animals that either have or lack protamine 2 and the authors suggest that lowered level of human protamine 2 in sperm may be the cause of male infertility. According to the authors, Ni(II) causes oxidative stress in the testes and may also contribute to infertility.

Toman et al. (2012). Quantitative histological analysis of the mouse testis after the long-term administration of nickel in feed.

In this study, the effects of nickel chloride ($NiCl_2$) on testis histopathology and morphometry of mice were investigated (Toman et al. 2012). Effects on the male reproductive system after long-term administration of nickel to young mice at the beginning of their sexual maturity are presented in this study. Male mice (4 weeks old) were given 10 mg $NiCl_2$/kg bw in pellets. After 3, 6, 9 or 12 weeks of exposure, the relative volume of whole seminiferous tubule, germinal epithelium, tubule lumen, interstitium and blood vessels as well as the diameter of seminiferous tubules were assessed. Authors reported that microscopic examination of testis showed significant changes in all nickel-exposed groups with no significant alterations in the relative weights of testis (data not shown in this report). Degeneration of germinal epithelium, and the relative volume of empty spaces in the seminiferous epithelium significantly increased ($p < 0.001$) in all experimental groups when compared with the corresponding control. The relative volume of seminiferous epithelium was significantly decreased after 6 and 12 weeks of nickel exposure with increased luminization of the tubules ($p < 0.001$) after 6, 9 and 12 weeks and the seminiferous tubule diameter significantly ($p < 0.001$) decreased after 12 weeks. Thus the changes noted in the testes after nickel exposure were time-dependent and occurred mainly in the germinal epithelium. The interstitial tissue significantly decreased after 6 and 9 weeks of nickel exposure but increased after 12 weeks (Table D.21, Figure D.19). According to the authors, the most vulnerable site of the testis was the seminiferous epithelium and apoptosis of the germinal cells could be a possible mechanism for loss of germinal epithelium followed by increasing occurrence of empty spaces in the epithelium resulting in alterations in testis structure and damage of spermatogenesis.

Table D.21. Morphometry analysis of relative volume of the mouse testis structure and tubule diameter after 12 weeks of experiments

	Control (n = 5)			12 weeks of Ni exposure (n = 5)		
Testis structure	**x**	**SD**	**CV**	**x**	**SD**	**CV**
Seminiferous tubule (%)	90.07	3.30	3.66	88.22*	2.64	3.00
Seminiferous epithelium (%)	84.80	3.29	3.87	66.28***	4.23	6.38
Tubule lumen (%)	5.27	1.94	36.73	21.94***	3.20	14.57
Empty spaces in the epithelium (%)	0.70	0.66	94.82	6.25***	3.02	48.30
Interstitium (%)	9.93	3.30	33.24	11.78*	2.64	22.45
Blood vessels (%)	0.13	0.35	262.28	0.47*	0.66	139.82
Diameter of seminiferous tubule (μm)	216.17	21.79	10.08	125.21***	35.35	28.24

x = mean; SD = standard deviation; CV = coefficient of variation; *p < 0.05; ***p < 0.001.

The authors report that the longer the period of nickel intake, the more visible changes occur in the testis.

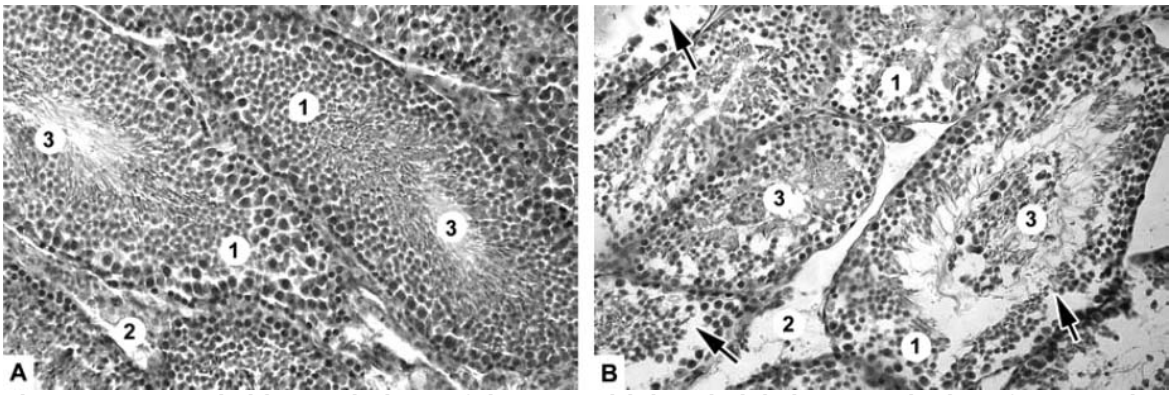

Figure D.19. Testis histopathology of the control (A) and nickel-exposed mice after 3 weeks of administration (B). Normal seminiferous epithelium (1) with spermatozoa in the lumen (3) and interstitial tissue (2) occurs in the control males. Degeneration of the epithelium (1), germ cells desquamation from the epithelium with empty spaces (→) and lumen containing the germ cells (3) are found in experimental mice [H-E, 200×].

Kong et al. (2014). Nickel Nanoparticles Exposure and Reproductive Toxicity in Healthy Adult Rats.

The authors reported that nickel micro-particles (Ni MPs) have been shown to have reproductive toxicity, and this study attempted to determine the association between exposure of nickel nanoparticles (Ni NPs) and reproductive toxicity (Kong et al. 2014). In characterizing the Ni NPs, the authors state that SEM and TEM images showed that the Ni NPs were spherical and had an average diameter of 90 nm with slight agglomeration and Ni MPs had an average diameter of 3μm, without agglomeration. In the one-generation reproduction toxicity study, 50 male F0 parental rats (10 per group)

and 100 female rats (20 per group) were administered different doses of Ni MPs and 0.9% sodium chloride solution (control group) by oral gavage for 10 weeks before the initiation of the mating period. The groups were control group, Ni NPs (high dose 45mg/kg, mid-dose 15 mg/kg, low dose 5 mg/kg/day), and Ni MPs (45 mg/kg). The male rats were killed at the end of the 14-day mating period and necropsied. Sex hormone levels, sperm motility, histopathology, and reproductive outcome were examined. Serum hormone concentrations were measured by enzyme-linked immunosorbent assay (ELISA) and reported in Figure D.20 below. Authors reported a gradual decrease in body weight and the ratio of epididymis weight over body weight increased after exposure to nickel with both Ni NPs (mid-dose and high-dose) and Ni MPs. The levels of FSH and testosterone (T), in serum were significantly lower while the level of LH was significantly higher in the high dose of Ni NPs compared with Ni MPs. The male rat serum FSH, LH and T content analysis showed that the levels of FSH and T were decreased significantly by Ni NPs treatment as seen in Figure D.20. According to the authors, the findings suggest that the decreased level of T, which resulted from testicular damage, affected testicular spermatogenesis and the testicular damage was exacerbated by reduced FSH. The authors state that the effect of Ni NPs on testicular function in male rats was severe with lowered levels of FSH and T, and the change of reproductive hormone levels indicates the abnormal reproductive axis function, which correlated with male infertility. The effects of the Ni MPs on serum sex hormone levels are similar to the effects of Ni NPs, but to a lower extent.

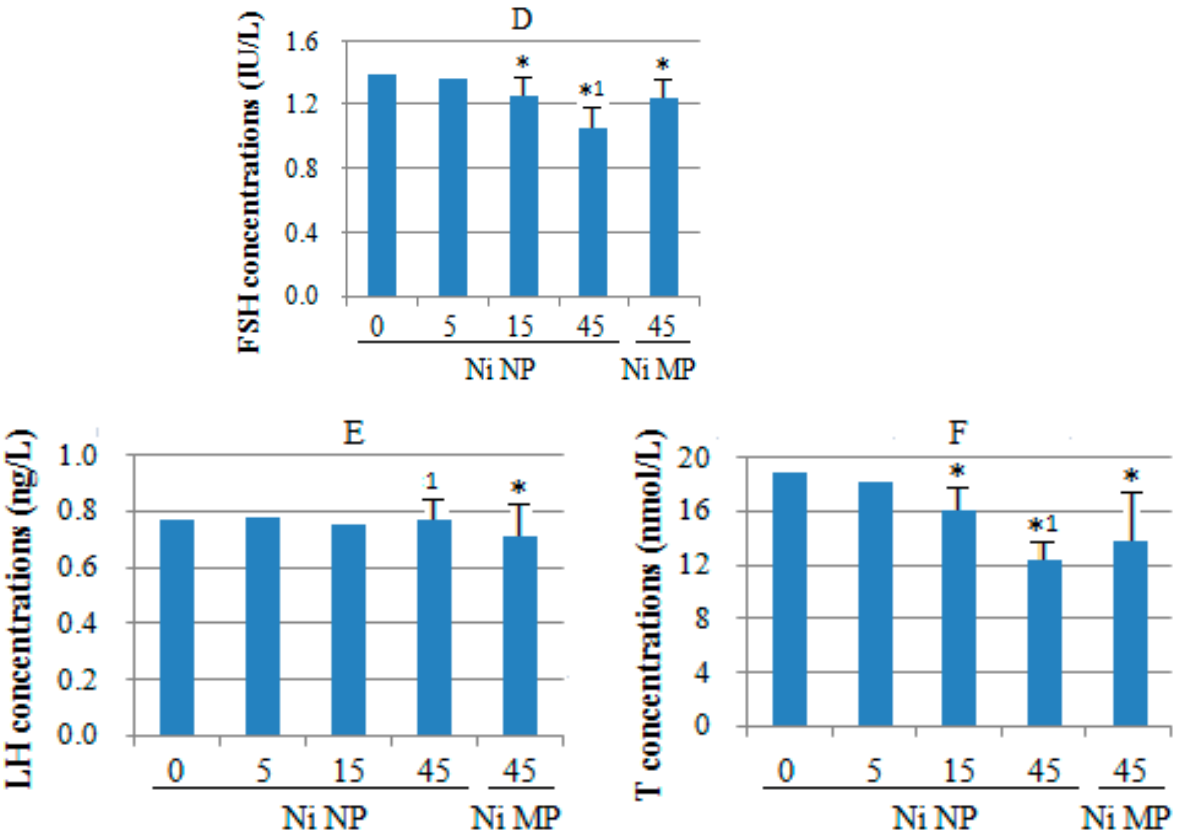

Figure D.20. Effects of Ni NPs and Ni MPs on serum hormone concentrations in male rats. Values represent the mean ± SD (*n* = 7). * p < 0.05, compared with control group (0 mg/kg BW); 1 p< 0.05, compared with Ni MPs (45 mg/kg BW).

The motility parameters of sperm were analyzed with a computer-assisted sperm analysis (CASA) system and authors reported that Ni NPs exerted adverse effects on the sperm motility in a dose-dependent manner (Table D.22). The values of curvilinear velocity (VCL) and linearity (LIN) were progressively reduced with increasing dose of Ni NPs and may contribute to diminished fertility associated with Ni NPs in males.

Table D.22. Effects of nickel nanoparticles (Ni NPs) on rat sperm motility

Group	Control	Low Dose	Mid Dose	High Dose	Ni MPs
Average path velocity (VAP) (μm/s)	210 ± 15	211 ± 14	209 ± 10	207 ± 8	204 ± 8
Curvilinear velocity (VCL) (μm/s)	410 ± 24	405 ± 25	398 ± 18	382 ± 21 *	384 ± 29 *
Straight line velocity (VSL) (μm/s)	145 ± 9	144 ± 9	144 ± 6	141 ± 6	140 ± 8
Beat cross frequency (BCF) (Hz)	19 ± 1	20 ± 1 *	20 ± 1 *	20 ± 1 *	20 ± 1 *
Straightness (STR) (%)	67 ± 1	68 ± 1	68 ± 1	67 ± 1	67 ± 1
Linearity (LIN) (%)	37 ± 1	37 ± 1	36 ± 1 *	36 ± 1 *	36 ± 1 *
Amplitude of lateral head displacement (ALH) (μm)	18 ± 1	18 ± 0	19 ± 1	19 ± 1	19 ± 1
elongation (ELON) (%)	68 ± 1	69 ± 1	68 ± 2	68 ± 1	68 ± 2

* $p < 0.05$, compared with control group.

The data on motility parameters of sperm were consistent with the testicular histopathological changes of the male rats displayed below in Figure D.21

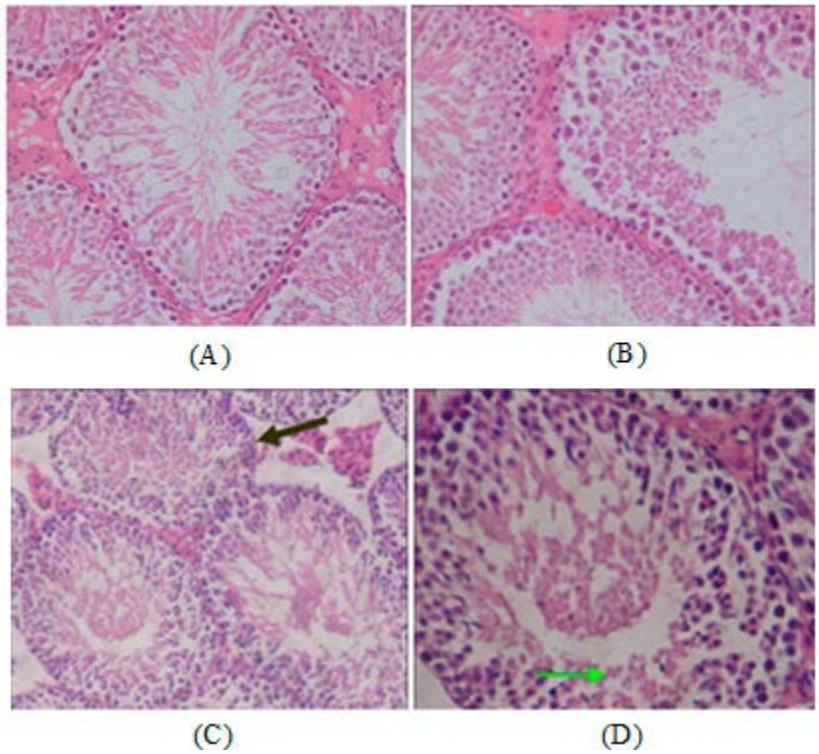

Figure D.21. Testicular histopathological changes.
Control group (**A**); 45 mg/kg BW (**B**–**D**). Original magnification 200× and 400× (200× refers to picture **A**, **B** and **C**; 400× refers to picture **D**). The arrow on (**C**) points to disordered arrangement of cells in the tube and on (**D**) it points to cell apoptosis

Examination of histopathology showed shedding of epithelial cells of seminiferous tubule, disordered arrangement of cells in the tube, and the appearance of cell

apoptosis and death in rats exposed to nickel nanoparticles. According to the authors, Ni NPs give a product many new characteristics (including a high level of surface energy, high magnetism, low melting point, high surface area, and low burning point), and compared with Ni MPs, the toxicity of Ni NPs was more severe in reproductive toxicity studies due to the change in particle size and surface area.

D.2.1. Related articles

Lindemann et al. (1980). A selective effect of Ni^{2+} on wave initiation in bull sperm flagella.

This study examined the effects of Ni^{2+} on motility in bull sperm (Lindemann et al. 1980). The authors tested the effects of Ni^{2+} on microtubule sliding, axonemal wave propagation, and sustained motile activity. Three main experiments were performed. Each experimental method and the corresponding results are summarized below:

Impalement studies were performed by rupturing the plasma membrane of the sperm with a drawn glass microprobe. $NiSO_4$ at a concentration of 0.01M was added to the samples to reach a concentration of 0.3mM and the cells were impaled in order to study the effects of Ni.

Demembranation studies were performed by adding 0.01M $NiSO_4$ to samples reaching a total concentration of 0.5mM in the sperm suspensions after motility of the sperm was verified.

Microtubular sliding studies were tested using the same extraction medium as given in the demembranation studies. The sperm were divided into two equal aliquots, $NiSO_4$ was added to one aliquot to a final concentration of 0.5 mM. After a 5-min incubation, ATP was added to both samples to a final concentration of l mM. The sperm in each aliquot were then observed.

Bull sperm that were demembraned, impaled, or dissected lost all spontaneous wave motility after exposure to Ni^{2+}. A concentration of 0.5 mM Ni^{2+} was enough to stop endogenous wave generation and even after a 30 fold dilution into new media, no motility was restored (Figure D.22). The authors repeated this experiment three times and there was no recovery of motility. Sperm that were added to the same suspension in the diluted sample were still motile while the nickel inhibited sperm did not regain motility. They found that the sperm are irreversibly inhibited by the original exposure to Ni^{2+}.

The beat frequencies of Ni^{2+} -treated sperm were recorded to measure the level of flagellar activity in comparison to untreated sperm.

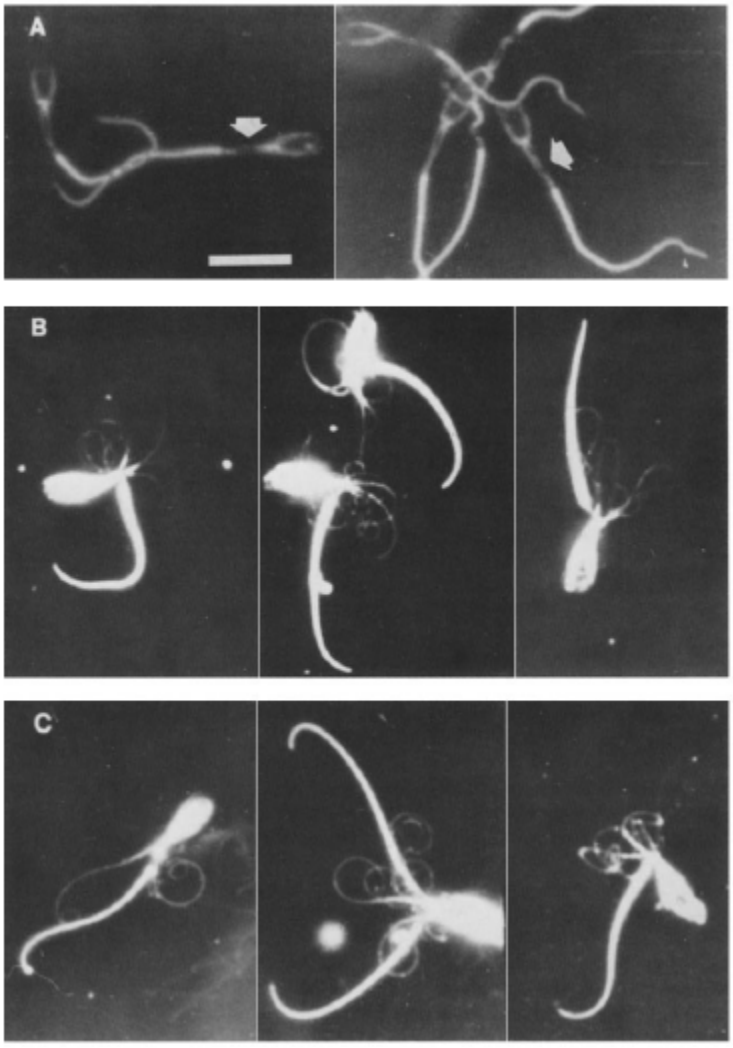

Figure D.22. Microtubular sliding in Ni^{2+}-inhibited bull sperm. **(A)** Triton X-100-treated bull sperm after 48 h of frozen storage at -20°C. **(B)** Sperm prepared as in (A) + 1mM ATP. **(C)** Sperm prepared as in (A) + 5 min incubation in 0.5mM Ni^{2+}.

The authors concluded that the balance of ATP and Mg^{2+} produced a pattern of behavior in sperm that is identical to that produced by Ni; however, unlike Ni, it is reversible. They state that the wave initiation in bull sperm is not a consequence of an intact microtubular sliding mechanism and that the process of wave propagation and initiation have a different basis in origin. The authors conclude that Ni treated sperm are incapable of initiating bending waves.

Jacquet and Mayence (1982). Application of the in vitro embryo culture to the study of the mutagenic effects of nickel in male germ cells.

In vitro embryo cultures were utilized to determine the mechanism of preimplantation loss of embryos (Jacquet and Mayence 1982). Two groups of 10 male mice were

injected i.p with a single dose of 40 or 56 mg/kg nickel nitrate (Ni $(NO_3)_2.6H_2O$). There was an untreated control group, and males that were treated with nickel nitrate and were negative in the dominant lethal test were also considered controls (40 mg/kg). Matings were allowed to occur 2 days after nickel treatment. Females with vaginal plug were isolated and killed 44 hours after HCG injection. Mating of the males with new superovulated females was repeated weekly for 5 consecutive weeks (for each of the 5 weeks tested, the experiment was repeated 2-3 times so that a total of 4154 embryos were obtained, of which 2260 were cultured). The embryos were flushed from the oviducts and classified into cleaved and noncleaved eggs and the number of cleaved eggs as well as development of embryos to blastocysts and implantation were determined.

Fertilizing capacity of the spermatozoa as well as development of the cultured embryos were not influenced by a dose of 40 mg/kg nickel nitrate. A dose of 56 mg/kg significantly reduced the fertilization rate 3 and 4 weeks after treatment but did not affect development of 2-cell embryos. The authors conclude that the preimplantation loss induced by nickel treatment of males is due to toxic effect on spermatids and spermatogonia and not due to a clastogenic action.

The authors conclude that nickel induces preimplantation loss due to a toxic effect on spermatids and spermatogonia.

Price et al. (1988). Fertility and reproductive performance of the F1 generation; Final study report (III of III) Two-generation reproduction and fertility study of nickel chloride administered to CD rats in the drinking water.

The protocol for this study has been described in detail in previous sections. In a two-generation study (Price et al. 1988), nickel chloride (as nickel chloride hexahydrate) was administered in drinking water to male and female CD rats (30/sex/dose) at dose levels of 0, 50, 250, or 500 ppm (2 matings per generation). Data from the F1 generation animals and their offspring (F2a pups and F2b fetuses) were presented in this Final Report, III of III. After delivery of an F2a litter, each dam was housed with her own litter throughout the 21-day lactational period. Dams were allowed to rest for 14-28 days prior to cohabitation of F1 breeding pairs to produce the F2b litters. At the end of the second cohabitation period, all F1 males were sacrificed and designated tissues saved for histopathology. Blood was collected from 2 F1 males each in the vehicle and 250 ppm dose groups for the animal health surveillance diagnostic panel.

Exposure of F1 breeders to nickel chloride was initiated indirectly via administration to their parents (P0 generation) and reported in a different document (part II of III) that was not available to OEHHA. Direct exposure from the drinking water began for F1b pups before weaning, and continued during the post weaning period until scheduled sacrifice (21 to 24 weeks of age for males and 27 to 30 weeks of age for females).

No adverse effects were noted during evaluation of the F1 generation at 50 ppm. Adverse effects during juvenile development and parturition were observed in the 250 and 500 ppm groups. During the non-reproductive portions of the study (weeks 32-38 and 40-49), F1 males showed a pattern of decreasing fluid intake (g/kg/day) with increasing age. Average Ni^{2+} intake for the low- through high-dose groups, was 8, 36 and 63 mg Ni^{2+}/kg/day during the first week, as compared to 4, 19 and 41 mg/kg/day just prior to the first cohabitation, and 3, 14 and 28 mg/kg/day just prior to the second cohabitation. Water intake at 50 ppm was significantly reduced only on weeks 36 (91% of control intake) and 38 (92% of control intake). At 250 ppm, water intake was significantly reduced on weeks 33-38 (81-87% of control}, and 41-44 (87-89% of control). The water intake for the 500 ppm group was significantly below controls on weeks 33-38 (77-95% of control), weeks 41- 44 (88-92% of control), and week 48 (86% of control). For F1 males, the predominant effects on water intake occurred at 250 and 500 ppm prior to study week 45 and food intake (g/kg/day) for nickel-exposed males did not differ significantly from controls for weeks 33-37. For the remaining weeks (38 and 41-49), transient increases occurred at 250 ppm (105% of control on week 45), and 500 ppm (108-121% for weeks 38 and 41-48): a significant trend on week 49 reflected a non-significant increase at 500 ppm (108% of control). The absolute amount of food consumed (g/day) was significantly decreased in the 500 ppm group throughout the study due to the persistence of decreased body weight for males in that group. The F1 male body weight for the 500 ppm group was significantly reduced (71-86% of control weights) at all times.

During PND 22 to 42, a total of 23 F1 males (2/60, 4/67, 8/60, and 9/31) died in the control through high-dose groups, respectively with a significant increase in the incidence of deaths at 250 and 500 ppm for males. When necropsied, F1 breeder males showed statistically significant changes for the absolute or relative weights of all weighed organs (including prostate, seminal vesicles, testicles, liver, kidneys. heart. lung and pituitary), except for the adrenals. A clear association with nickel exposure was noted only at 500 ppm for increased relative lung weight (114% of control) and relative pituitary weight (123% of control). The authors report that the P0 generation males showed increased pituitary weight (both absolute and relative) at 250 and 500 ppm, but other relative organ weights were not affected. For F1 males, treatment-related pathology was not noted upon gross examination at necropsy and treatment-related microscopic findings were limited to an increase in histiocytic cellular infiltration of the lungs at the high dose (0%, 3%, 3% and 18% of males examined from the control through high-dose groups, respectively). The authors report that the F1 generation histopathology did not differ remarkably from the P0 data. Thus, exposure levels of 50, 250 and 500 ppm Ni^{2+} in the drinking water were generally well-tolerated by F1 animals other than reduced water intake at 250 and 500 ppm, and an increase in deaths between weaning and PND 42 at 250 and 500 ppm for F1 males.

Ng and Liu (1990). Toxic effect of heavy metals on cells isolated from the rat adrenal and testis.

This study was conducted to determine if selected heavy metals have a direct toxic effect on steroidogenesis of cells isolated from testes and adrenal glands of the rat (Ng and Liu 1990). Rat Leydig cells were isolated and dosed with various concentrations of metals; however, here, we will only focus on nickel. Rat Leydig cells were exposed to nickel chloride ($NiCl_2.6H_20$), at concentrations of 1, 10, and 100 µM.

There were no effects seen on corticosterone and testosterone production nor on steroidogenesis by Ni at the concentrations of 1, 10 or 100 µM tested.

Kanous et al. (1993). Inhibition of microtubule sliding by Ni^{2+} and Cd^{2+}: evidence for a differential response of certain microtubule pairs within the bovine sperm axoneme.

This study examined the effects of Ni^{2+} and Cd^{2+} on microtubule sliding in bull sperm (Kanous et al. 1993). Only the effects of nickel are presented below. Extracted bovine bull sperm were used to determine if Ni^{2+} would inhibit sliding of particular microtubules. Bovine sperm ejaculate, collected in an artificial vagina, was diluted and the sperm de-membraned and prepared in multiple dishes. Cell preparations were used and tested for bilateral sliding. If bilateral sliding was apparent, that particular sample was used as the control and a parallel sample was also run in which Ni^{2+} was added to the cell preparation and then observed for microtubule sliding. Samples were incubated with 0.25mM Ni^{2+} for 1 minute. The results are summarized below (Figure D.23):

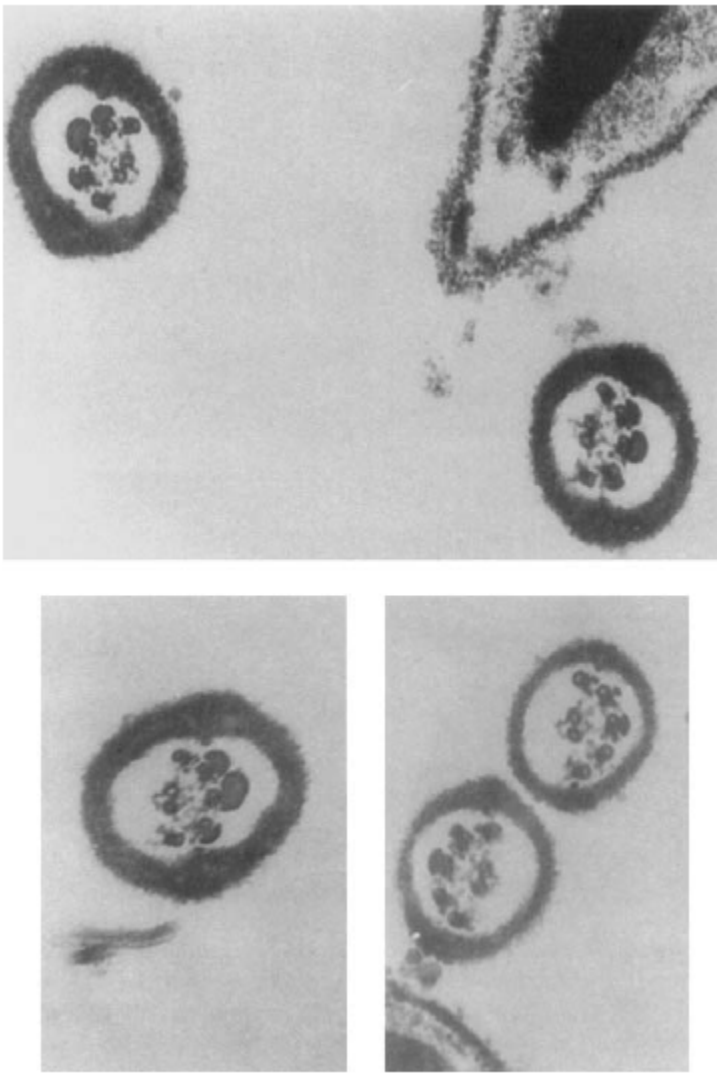

Figure D.23. Transmission Electron Microscopy (TEM) micrographs of the residual axonemal structures following microtubule sliding of Ni^{2+}-treated sperm (2 minutes with 0.25mM Ni^{2+}). Incubation with Ni^{2+} reduced the percentage of two-sided sliding, and increased the percentage of one-sided sliding.

The authors conclude that the dissociation of bull sperm flagellar axoneme followed a pattern. They state that Ni^{2+} is known to selectively block spontaneous flagellar oscillation in detergent-extracted bull sperm models. Their current findings indicate that Ni^{2+} significantly exerts a selective inhibition of sliding of the 9, 1, 2 bundle from the bull sperm axoneme. They conclude that Ni^{2+} has an inhibitory effect on both polarities.

Lindemann et al. (1995). Ni^{2+} inhibition induces asymmetry in axonemal functioning and bend initiation of bull sperm.

The hypothesis of this study was that the dynein arms driving the two phases of the flagellar beat cycle in sperm are unequally sensitive to Ni^{2+} (Lindemann et al. 1995). In

this study a decrease in beat amplitude of bovine sperm flagella was almost immediately observed, upon treatment with 0.66 mM $NiSO_4$. As shown in the Figure D.24 below, as the amplitude decreased, asymmetry in one bend direction increased, until motility finally ceased. At that point the flagella exhibited a sickle-shaped curvature (see Figure D.25 below).

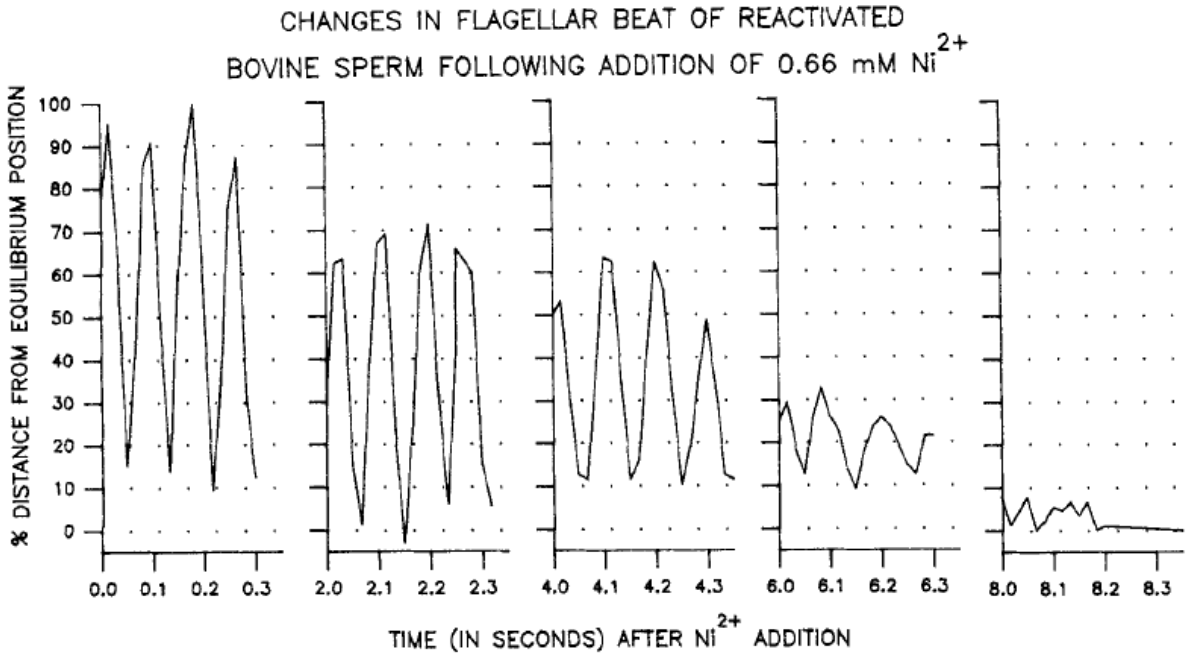

Figure D.24. Decay of spontaneous motility during Ni^{2+} inhibition (0.66mM $NiSO_4$). The cell continued to beat, but there was a decrease in beat amplitude. The graphed data show increasing asymmetry in one bend direction as the amplitude decreased, until motility completely stopped at one extreme of the beat cycle.

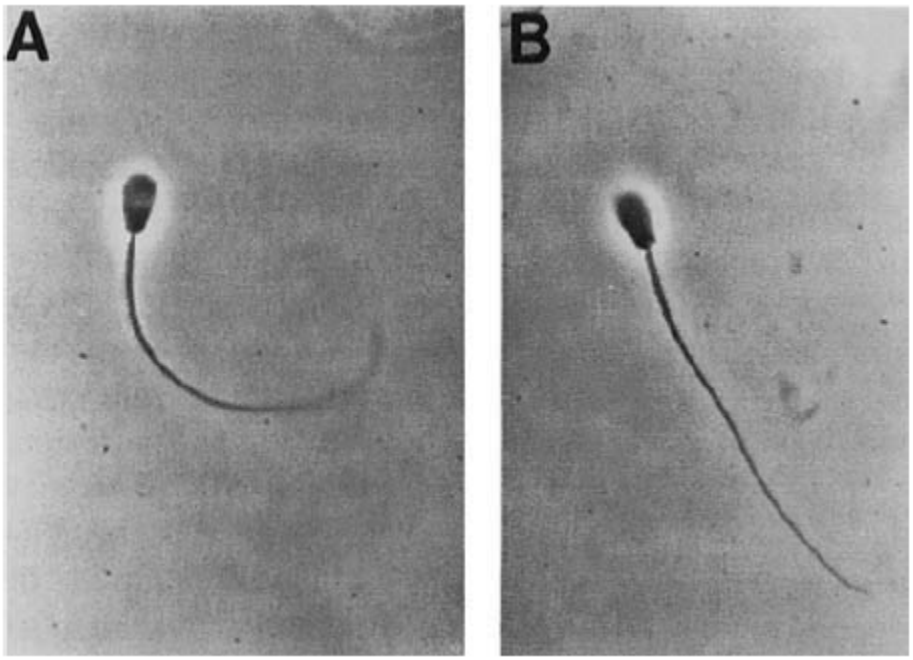

Figure D.25. Equilibrium positions of Ni^{2+}-treated (0.66mM) and vanadate-treated (2 µM) bull sperm models. Those cells which had been Ni^{2+} -inhibited were observed to arrest in a sickle-shaped curvature **(A),** while vanadate inhibited flagella exhibited a straight, resting configuration **(B).**

The authors conclude that Ni^{2+} selectively impairs the function of one set of dynein bridges of the flagella.

Forgács et al. (1998). Effect of Ni^{2+} on the testosterone production of mouse primary Leydig cell culture.

This study evaluated the effects of Ni^{2+} on testosterone (T) production of mouse Leydig cells *in vitro* following an *in vivo* or *in vitro* exposure to nickel (Forgacs et al. 1998). Five CFLP mice per dose were subjected to repeated exposure to $NiSO_4 \cdot 7H_2O$ (4 treatments, subcutaneously, every 3 days) at 10, 20 or 40 mg/kg body weight of $NiSO_4$ or 1.0 mL of 0.9% NaCl solution. One day after the last dosing mice were anesthetized with 60 mg/kg ip phenobarbital and testes, epididymides, adrenals, and kidneys were removed, cleaned, and weighed. Interstitial (Leydig) cells obtained from animals treated with equivalent dose were isolated in one block and this mixture of cells was cultured at each dose. After a 48-hr incubation in the presence or absence of 1 IU/ml human chorionic gonadotropin (hCG) in the culture medium, the basal and maximal hCG-stimulated testosterone production of Leydig cells after 48-h incubation was measured.

In order to evaluate the *in vitro* concentration-response action of Ni^{2+} on 48-h Leydig cell culture, $NiSO_4$ was added to the cells to achieve a Ni^{2+} concentration of 62.5, 125, 250, 500, or 1000 µM. This study was performed 7 times with 4–15 replicate samples at each concentration. In each experiment, the mixture of testicular interstitial cells obtained

from 15–20 mice was cultured. The results are summarized below in Figures D.26 - D.29:

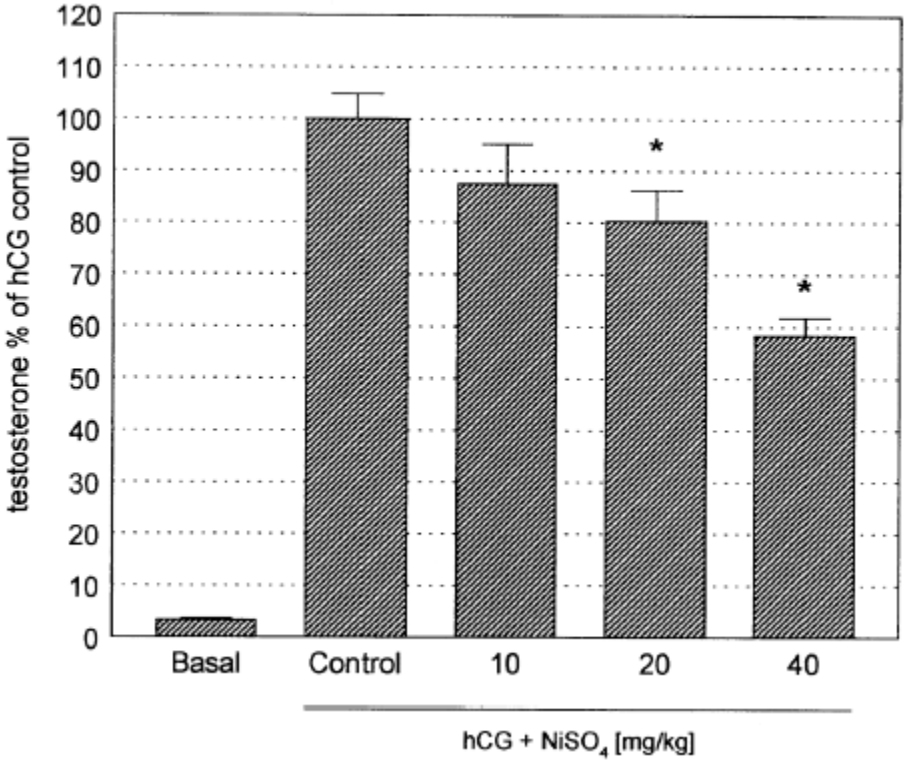

Figure D.26. Effects of in in vivo $NiSO_4$ treatment on hCG-stimulated T production. (±SEM), *p<0.05

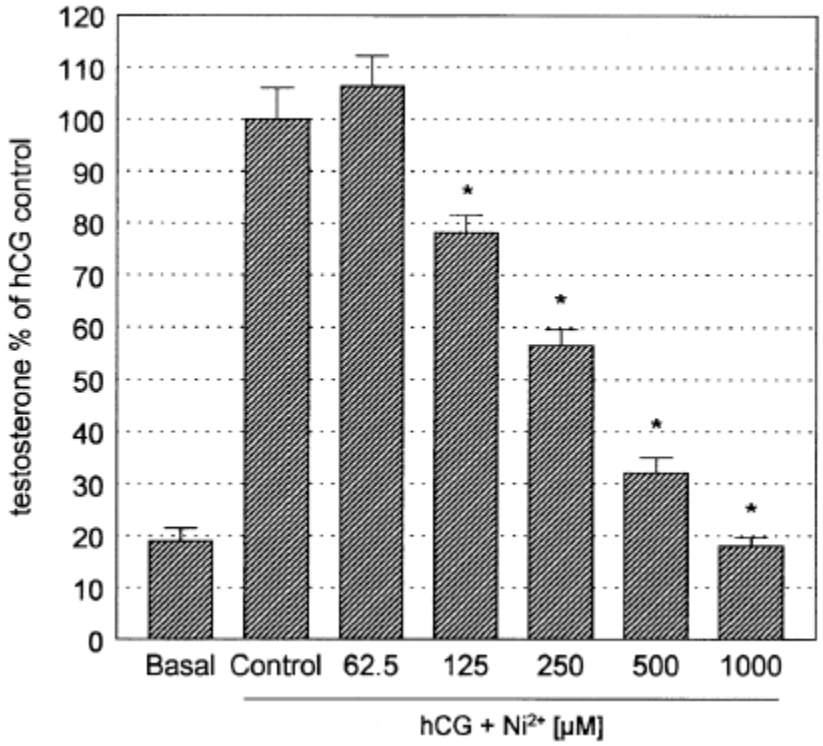

Figure D.27. Effect of increasing concentrations of Ni^{2+} on hCG-stimulated T production. *p<0.05

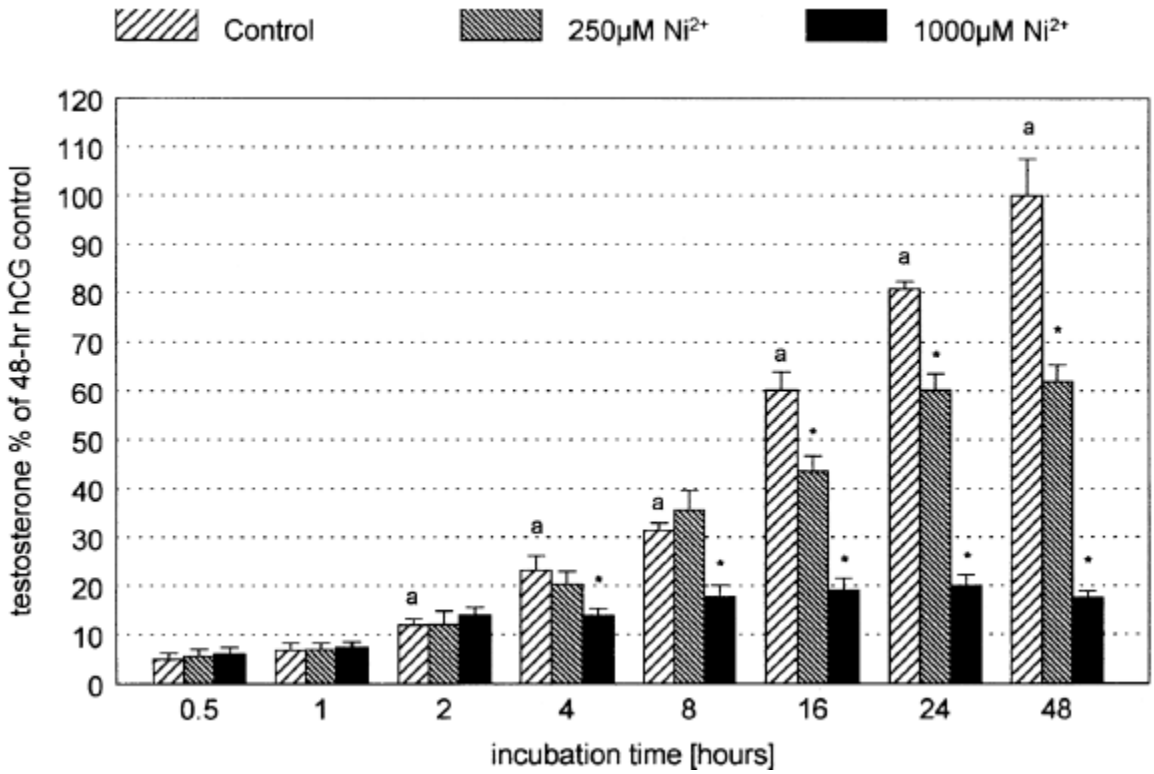

Figure D.28. Effects of 250 or 1000 µM Ni^{2+} on hCG-stimulated T production at different incubation times on mouse Leydig cells in culture.
The bar values represent the mean (±SEM) testosterone as a percentage of 48-h hCG control of four repeated experiments (*n* = 4), number of replicate wells was three to five at each point per experiment.
* = comparison of each dose to its respective control for the same incubation time point
a = comparison of control group for each incubation time point to control group at 0.5 h.
$p < 0.05$.

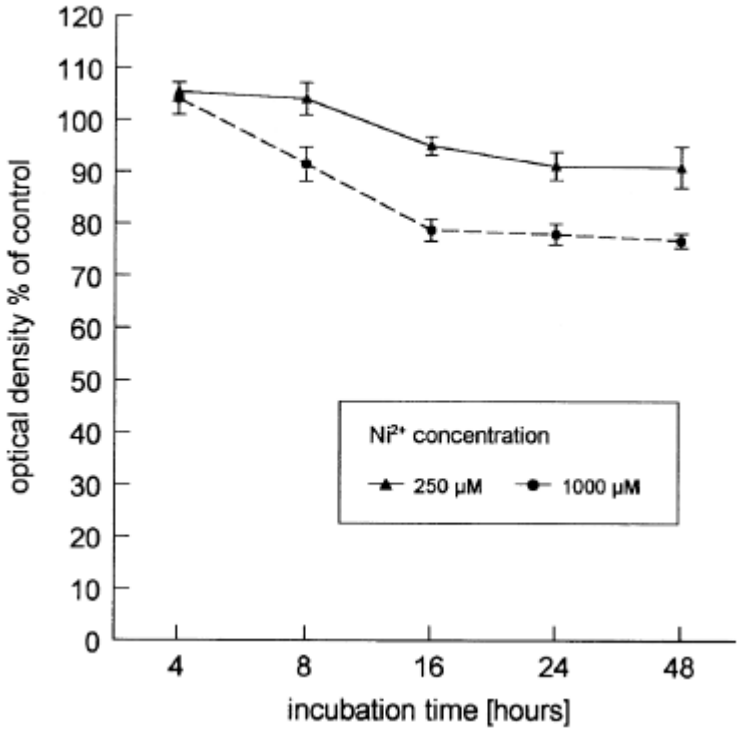

Figure D.29. Effects of 250 or 1000 µM Ni^{2+} on the viability of mouse testicular interstitial cell culture assessed by MTT assay. Cell viability was unaffected.

The authors state that the results of this study indicates a dose-dependent depression in stimulated testosterone production of mouse Leydig cells in culture following either *in vivo* or *in vitro* Ni^{2+} treatment at a dose that does not cause general toxicity or cytotoxicity. The findings also indicate that the effect of Ni on hCG-stimulated testosterone production is not the result of impairment of steroidogenesis, since basal (i.e., non-stimulated) testosterone production was unaltered following either *in vivo* or *in vitro* Ni^{2+} treatment (data not shown). The study findings indicate that the Ni-induced Leydig cell T production is time- and dose-dependent.

Although this study shows effects on altered testosterone production, the authors fail to provide the dosing time-frame for the *in vivo* study. They state that mice were dosed in 4 treatments every 3 days, but do not state for how long.

Forgács et al. (2001). Specific amino acids moderate the effects on Ni^{2+} on the testosterone production of mouse Leydig cells in vitro.

The purpose of this investigation was to study the effectiveness of histidine (His) and cysteine (Cys), to prevent the inhibitory action of Ni^{2+} on testosterone (T) production in mouse primary Leydig cell culture (Forgacs et al. 2001).

Interstitial cells were isolated from 15-20 male CFLP mice and the Leydig cell cultures prepared were incubated with 62.5, 125, 250, 500, or 1000 µM of $NiSO_4$ and co-incubated with equimolar (62.5, 125, 250, 500, or 1000 µM) or twice the equimolar (125, 250, 500, 1000, or 2000 µM) concentrations of His or Cys. The results indicate that His and Cys protect against Ni induced inhibition of T production, in a dose-dependent manner. The protective effect of His and Cys can be overcome by increasing the dose of Ni. Protection is greatest when His or Cys are added simultaneously with Ni (Figures D.30 - D.32).

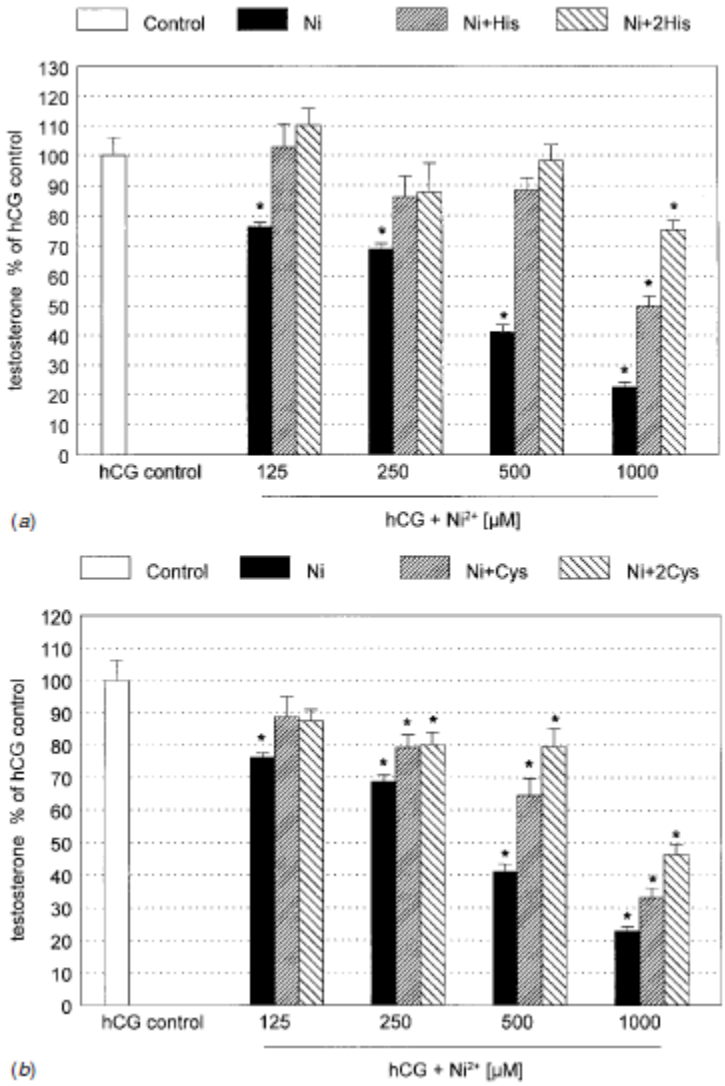

Figure D.30. Effect of increasing concentrations of Ni^{2+} on hCG-stimulated testosterone (T). in the presence or absence of equimolar or twice the equimolar concentration of (*a*) His or (*b*) Cys. *p< 0.05.

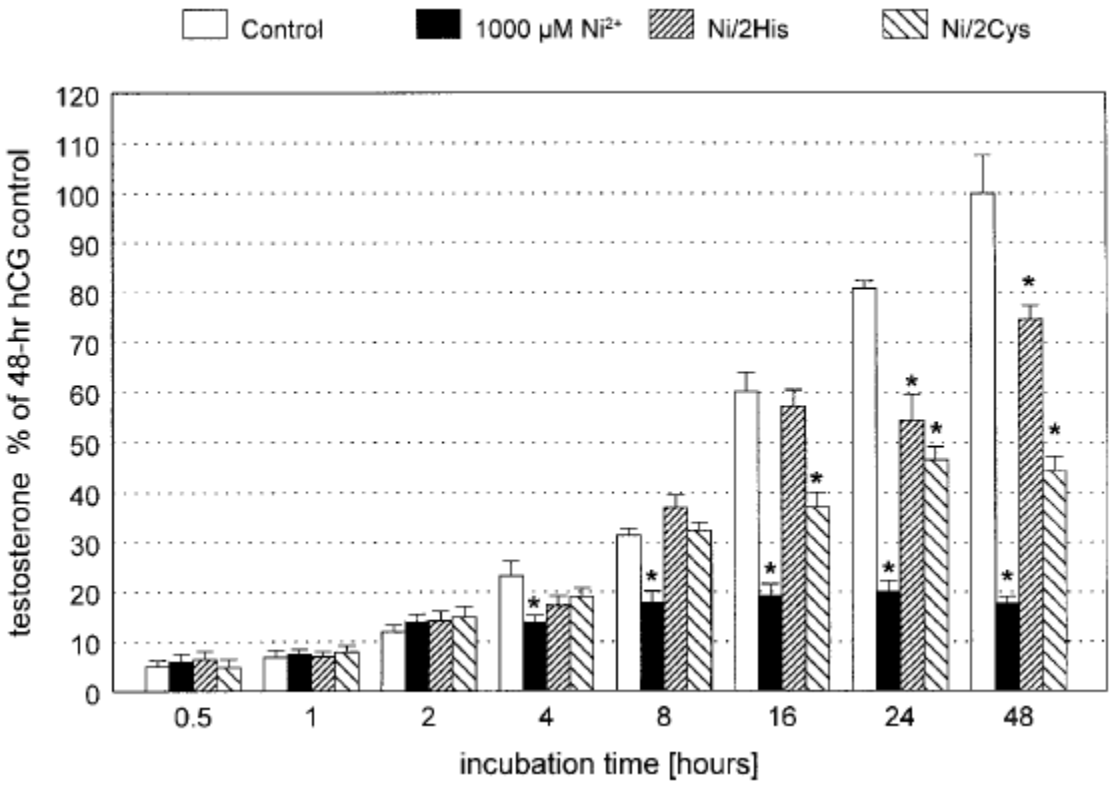

Figure D.31. Protective effect of twice the equimolar concentration (2000 μM) of His or Cys against 1000 μM Ni^{2+} action on hCG-stimulated T production. (±SEM) *p < 0.05.

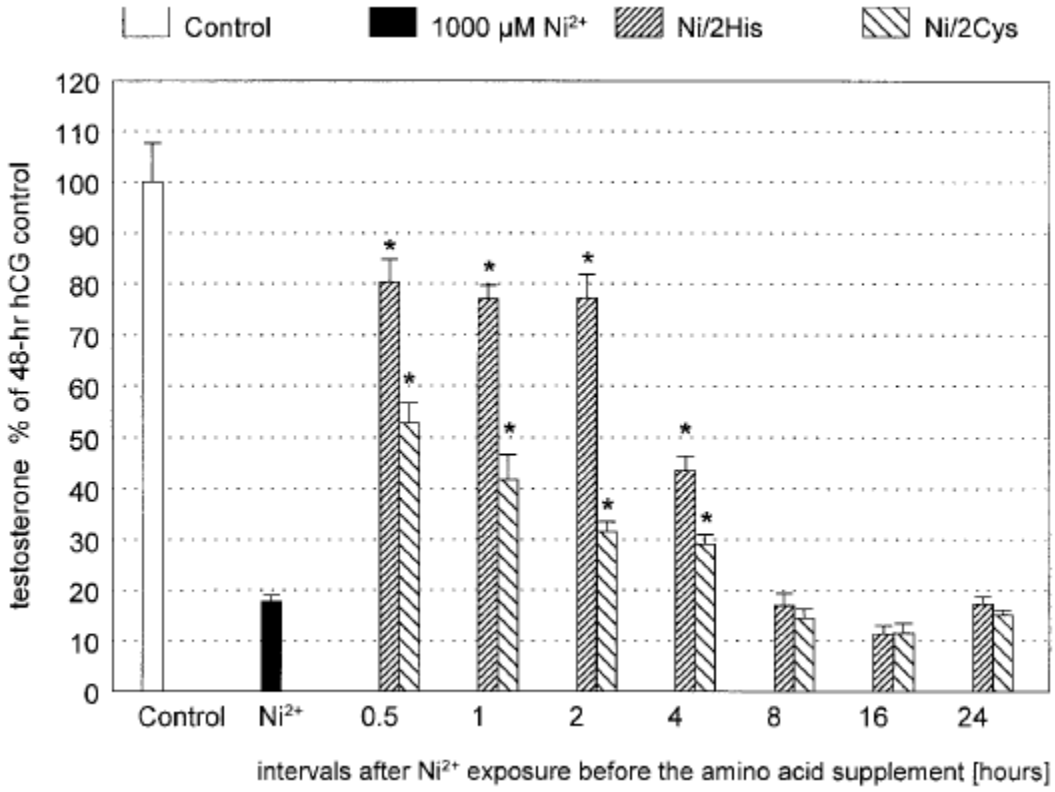

Figure D.32. Effect of increasing the time interval between administration of 1000 µM Ni^{2+} and 2000 µM His or Cys supplement on the restoration of hCG-stimulated T production. (±SEM) *p < 0.05.

The findings in this study are consistent with the previous work of the authors (Forgacs et al. 1998) that demonstrated the effects of Ni^{2+} on the testosterone production of primary mouse Leydig cell cultures. They postulate that the reduced effectiveness at longer incubations may possibly be due to these amino acids only slowing the speed of transport, but not completely preventing the uptake of Ni^{2+} into the cells.

Lukac et al. (2011). In vitro effect of nickel on bovine spermatozoa motility and annexin V-labeled membrane changes.

This study examined the toxic effect of Ni on spermatozoa motility parameters and spermatozoa membrane integrity *in vitro* (Lukac et al. 2011).

Bovine semen was collected from Holstein-Freisian breed bulls and in an *in vitro* culture, were dosed with $NiCl_2$ at 7.8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 µM for four time periods (30, 60, 120 and 240 min) at 37 °C. The results of the *in vitro* culture of bovine spermatozoa with $NiCl_2$ on motility and progressive motility (sperm that swim in a straight line or in very large circles) are presented in Table D.23 below:

Table D.23. Average parameters of bovine spermatozoa motility and progressive motility at different nickel concentrations and time periods

Parameter	Time (min)	Control	7.8	125	250	500	1000
Motility (%)	30	95.81	95.47	96.60	93.95	91.64	95.61
	120	95.88	95.47	94.09	92.91	94.34	90.05*
	240	84.15	87.02	85.61	82.70	76.45**	73.26**
Progressive Motility (%)	30	89.30	85.46	90.44	86.85	88.28	82.67**
	120	90.31	91.40	87.05	84.33	89.59	80.12**
	240	65.00	67.44	60.54	49.93**	45.89**	45.09**

Concentrations presented as µM Ni ml^{-1}
*p <0.05; **p <0.001

During the following time periods: 30, 120, and 240 min of culture, there was a concentration-dependent inhibitory effect of high $NiCl_2$ concentration on motility as well as progressive motility. After culture of spermatozoa with addition of 125 µM Ni ml^{-1} for 240 minutes, fluorescence in the mitochondrial segment of bovine spermatozoa was detected; at higher concentrations, the Annexin V-positive reaction was also seen on the spermatozoa head membrane. At a concentration of 500 µM Ni ml^{-1} necrotic processes were detected. In the group with the highest concentration and the longest exposure time (1000 µM Ni ml^{-1}; 240 min), apoptotic Annexin-positive regions were detected not only in the mitochondrial region, but also in the spermatozoa heads), showing significant alteration of spermatozoa membrane integrity (please see original paper for florescent images).

The authors conclude that nickel causes significant alteration of spermatozoa membrane integrity and that their study confirms previous findings describing the toxic effect of nickel on male reproductive abilities.

Sun et al. (2011). Binding of nickel to testicular glutamate-ammonia ligase inhibits its enzymatic activity.

This study aimed to investigate the ability of nickel to cause testicular damage by binding to testicular proteins, specifically glutamate-ammonia ligase (GLUL), which Sun et al. identified as a prominent nickel-binding protein via mass spectrometry (Sun et al. 2011). Protein quantification was determined by the Bradford assay (Bradford 1976).

For histology studies, forty 8-week old male Wistar rats were randomly divided into four groups. One group was treated as control and treated with saline, and the other three were dosed with $NiSO_4$ intraperitoneally at 1.25, 2.5 or 5.0 mg/kg for 30 days. For the nickel concentration and GLUL enzyme activity assay, 36 rats were divided into two groups: a mock treatment control group of 9 animals (3 animals per time point of 10, 20, and 30 days of treatment) and an experimental group of 27 animals (9 animals treated for each time point of 10, 20, and 30 days with 5.0 mg/kg nickel). After treatment, the animals were euthanized, and the testes of the animals were removed for histological and GLUL activity examinations as well as nickel concentration measurement. The

authors conducted several different experiments which included: screening of Ni binding proteins in the testes, protein quantification, cloning of glutamate-ammonia ligase cDNA, expression of recombinant protein using E. coli cells, Ni-binding assay, assay for glutamate-ammonia ligase enzymatic activity, western blot analysis for GLUL in rat testes, rat testis histology, immunohistochemistry, cryo-sectioning and immunofluorescence, and determination of Ni concentration in rat testes. Selected results are summarized below in Figure D.33:

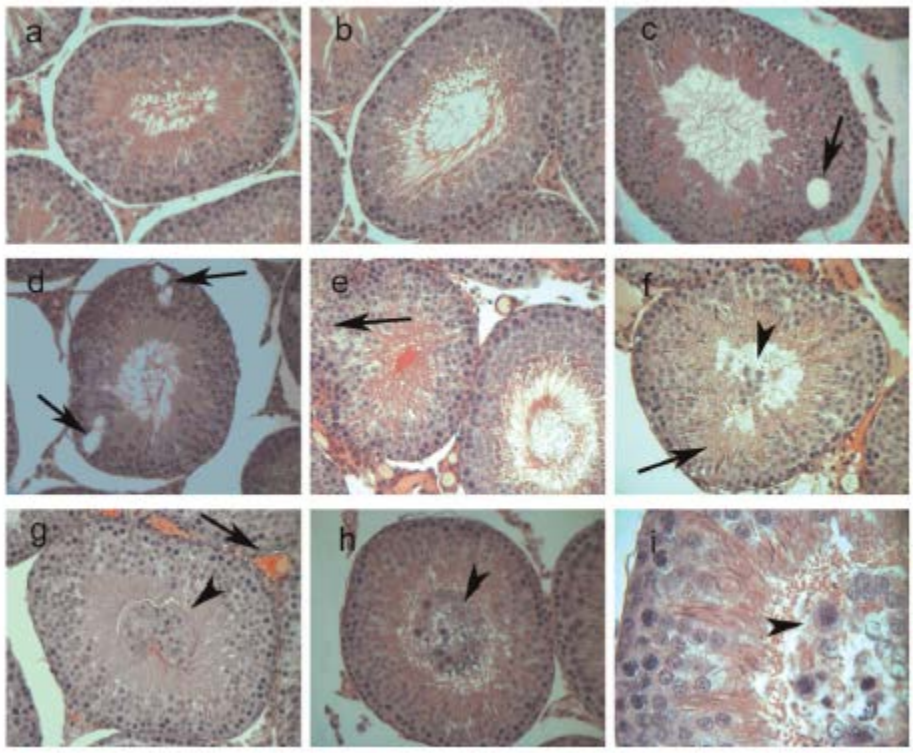

Figure D.33. Pathology of rat testis after 30 days of ip treatment. A,**B**: Control C–I: 5 mg/kg nickel sulfate. **C**,**D**: Arrows indicate vacuoles. **E**: Arrow indicates occasionally observed interruption in the germ cell layer. **F**: Arrow denotes disarrangement of spermatocytes. **G**: the arrow denotes blood vessel expansion in the interstitium. **F**–**H**: Arrowheads indicate detached germ cells into the lumen of the seminiferous tubules. **I**: The arrowhead points to detached germ cells present in the lumen of seminiferous tubules displaying abnormal staining of chromatin. A–H: 40× magnification. I: 100× magnification.

The authors reached several conclusions based on the results of their experiments. They found that nickel inhibits testicular glutamate-ammonia ligase activity. They also found that the testis accumulates nickel and GLUL activity is significantly reduced after 30 days of treatment. They observed pathological changes in the rat testis at a high dosage which included disturbance of the germ cell layer close to the basement membrane of the seminiferous tubules and the presence of detached germ cells inside the lumen of seminiferous tubules. They found that in the rat testis, GLUL is a protein that binds Ni and catalyzes many different reactions; it plays an important role in the

removal of glutamate and/or ammonia, which contributes to detoxification in the testis, and is important in cell proliferation (inhibition can trigger cell apoptosis) and when bound to Ni, its activity is greatly reduced.

The authors state that they do not know if or how the inhibition of GLUL may be correlated to the observed Ni-induced testicular abnormalities, but they show that in the rat testis, GLUL is present at high levels in interstitial cells and in elongating spermatids.

Kročková et al. (2011). Nickel induced structural and functional alterations in mouse Leydig cells in vitro.

The present study was aimed at investigating the effects of nickel ($NiCl_2$) on secretion of testosterone (T), cell viability, ultrastructure and apoptosis in mouse Leydig cells (Krockova et al. 2011).

Thirty male NMRI mice were used to prepare the Leydig cell culture. Interstitial cells were isolated and treated with 15.67, 31.25, 62.5, 125, 250, 500 or 1000 µmol/L of $NiCl_2$ in a 48-hour Leydig cell culture. The following parameters were evaluated: cytotoxicity, testosterone, and apoptosis. Selective results are summarized below in Figures D.34 - D.36.

Following 48 h of culture of Leydig cells in presence of nickel a concentration-dependent depression of the testosterone release was observed. The lowest testosterone production was detected after administration of 1000µmol/L $NiCl_2$ (50.74±11.77%, $p \leq 0.01$).

The percentage of apoptotic cells was increased in each of the experimental groups except at 62µmol/L $NiCl_2$ (40.71±17.56%). The highest percentage of apoptotic cells was found after administration of 125µmol/L $NiCl_2$ (80.29±13.90%, $p \leq 0.05$)

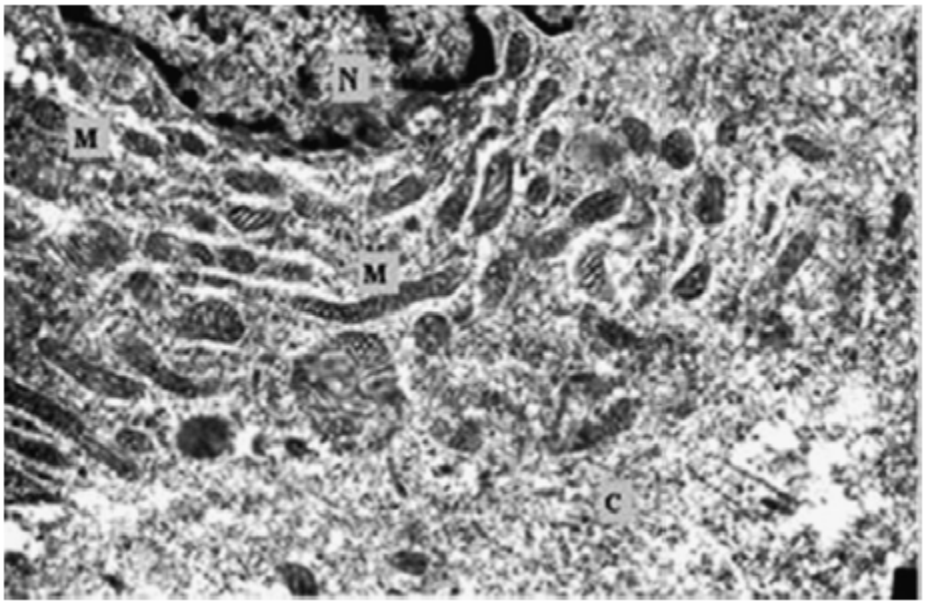

Figure D.34. Ultrastructure of Leydig cell in control group. The visible nucleus (N) and tubular mitochondria (M) in cytoplasm (C) (TEM; 14,000×).

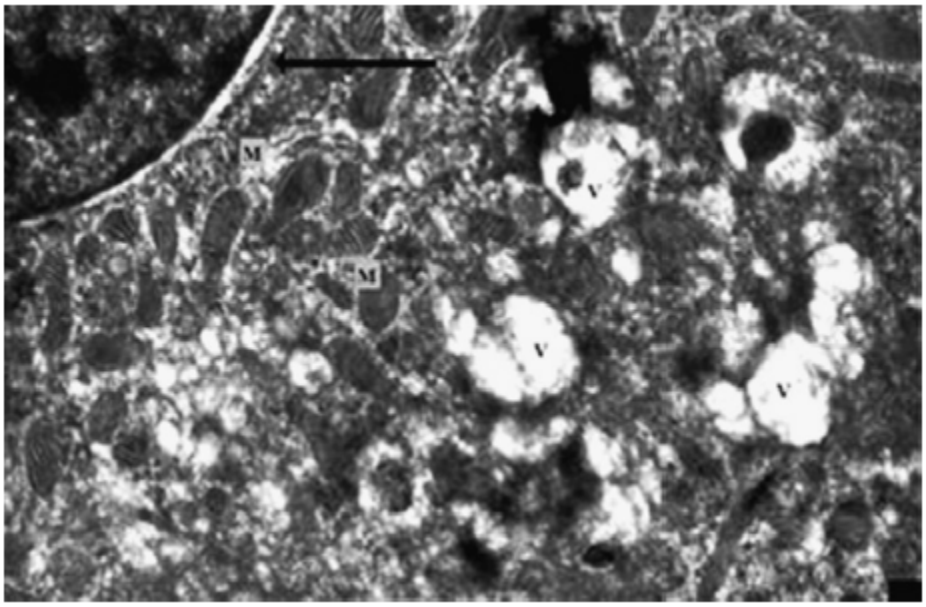

Figure D.35. **Ultrastructure of Leydig cell after a lower dose of nickel administration**. Dilated space between (arrow) outer and inner nuclear membrane was detected. In some of the mitochondria (M) the tubular structure was altered. Also, increased occurrence of vacuoles (V) in cytoplasm was observed (TEM; 14,000×).

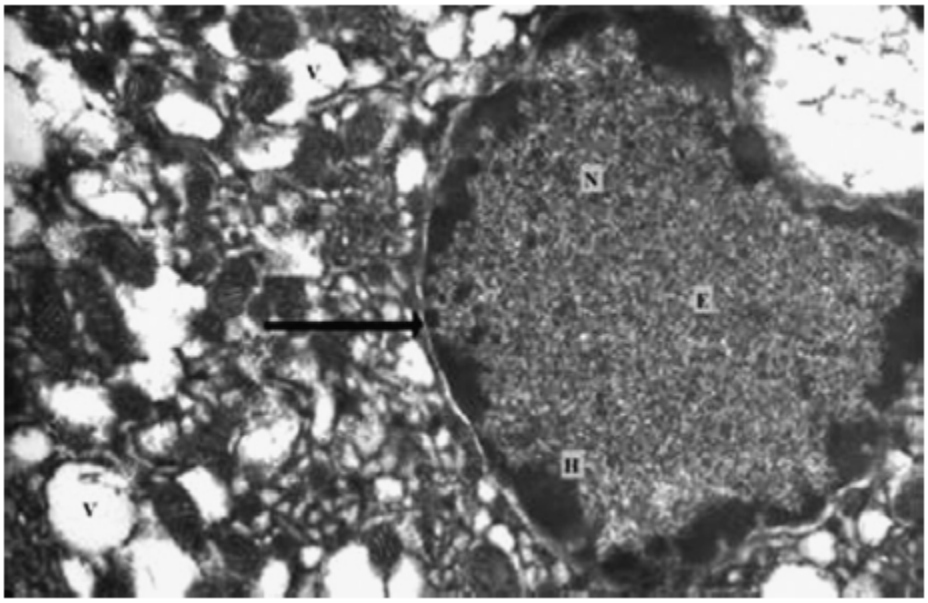

Figure D.36. The ultrastructure of Leydig cell in the group with nickel administration. The marginal localization of heterochromatin (H) and central localization of euchromatin (E) in nucleus were observed. Undulation of nuclear membrane (arrow) and many vacuoles (V) in cytoplasm was detected (TEM; 14,000×).

The results show that there was a decrease in the volume of smooth endoplasmic reticulum after incubation with the highest nickel concentrations (≥500µmol/L). The granular endoplasmic reticulum was present in the control group only. There was a low occurrence of the tubular type of mitochondria after administration of the highest nickel concentrations (≥500µmol/L). There was also a low occurrence of nuclear heterochromatin and subsequent intense presence of euchromatin in the nickel-exposed cultures. There were also a great number of lipid droplets and vacuoles at the highest nickel concentration in comparison with the control group.

The authors conclude that the results of this study indicate a significant depression in testosterone production and decrease in smooth endoplasmic reticulum of mouse Leydig cells following *in vitro* treatment with ≥31µmol/L of $NiCl_2$, in comparison with the control group. They state there was an increase in the percentage of apoptotic cells after $NiCl_2$ administration and electron microscopy detected degenerative changes of nuclei, mitochondria, and higher vacuolization in the cytoplasm.

Zou et al. (2017) Nickel sulfate induced apoptosis via activating ROS-dependent mitochondria and endoplasmic reticulum stress pathways in rat Leydig cells.

In a recent study by Zou et al., nickel-induced reactive oxygen species (ROS) generation in mitochondria and endoplasmic reticulum stress (ERS) mediated apoptosis pathways in rat Leydig cells were investigated (Zou et al. 2017). Leydig cells from adult

Wistar rats were isolated and cultured. Cells were treated with 0, 250, 500, or 1000 µmol/L $NiSO_4$ for 12 hours for concentration response studies. For time course experiments, the cells were treated with 500 µmol/L of $NiSO_4$ for 0, 6, 12, or 24 hours. The following assays were utilized to measure various parameters including cytotoxicity, apoptosis, ROS generation and ERS indicators:

1. Cytotoxicity studies were conducted via an MTT assay (viability) and LDH assays (cytotoxicity)
2. Determination of ROS generation by fluorescent dichlorofluorescein (DCF) assay where intracellular ROS was indicated by the fluorescence intensity.
3. $NiSO_4$-induced apoptosis from morphological nucleolus deformation by DAPI assay.
4. Cell apoptosis assay via Annexin-V FITC/PI assay
5. RNA isolation and RT-qPCR
6. Protein expression levels of mitochondria and ERS key indicators via Western blot assay

The results showed that nickel sulfate induced ROS generation leading to nucleolus deformation and apoptosis in testicular Leydig cells. This was attenuated by ROS inhibitors of N-acetylcysteine (NAC) and 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) (selected data shown in Figure D.37). Nickel sulfate-triggered Leydig cells apoptosis via mitochondria and ERS pathways was characterized by the upregulated mRNA and proteins expression of Bak, cytochrome c, caspase 9, caspase 3, GRP78, GADD153, and caspase 12, which were inhibited by NAC and TEMPO respectively (see Figure D.38).

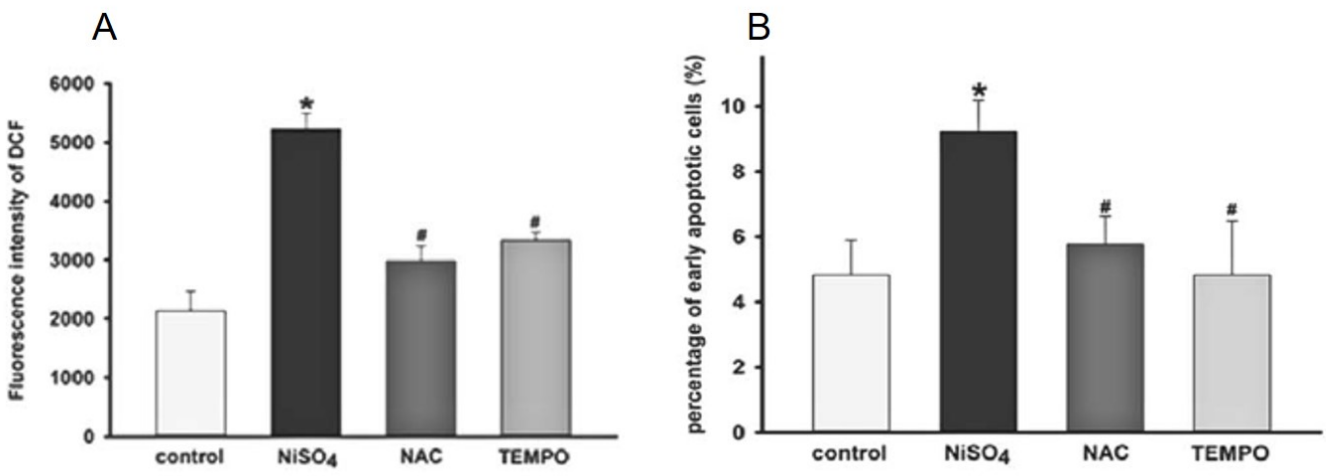

Figure D.37. Effect of NAC and TEMPO on $NiSO_4$-induced ROS generation and apoptosis in rat Leydig cells. (A) Semiquantitative analysis of intracellular ROS activity in Leydig cells from DCF fluorescence intensity (n=5). (B) Percentage of early apoptotic cells (n=3).
*p< 0.05 from the control group, #p <0.05 from the 500 µmol/L $NiSO_4$ group.
Modified from Zou et al. (2017)

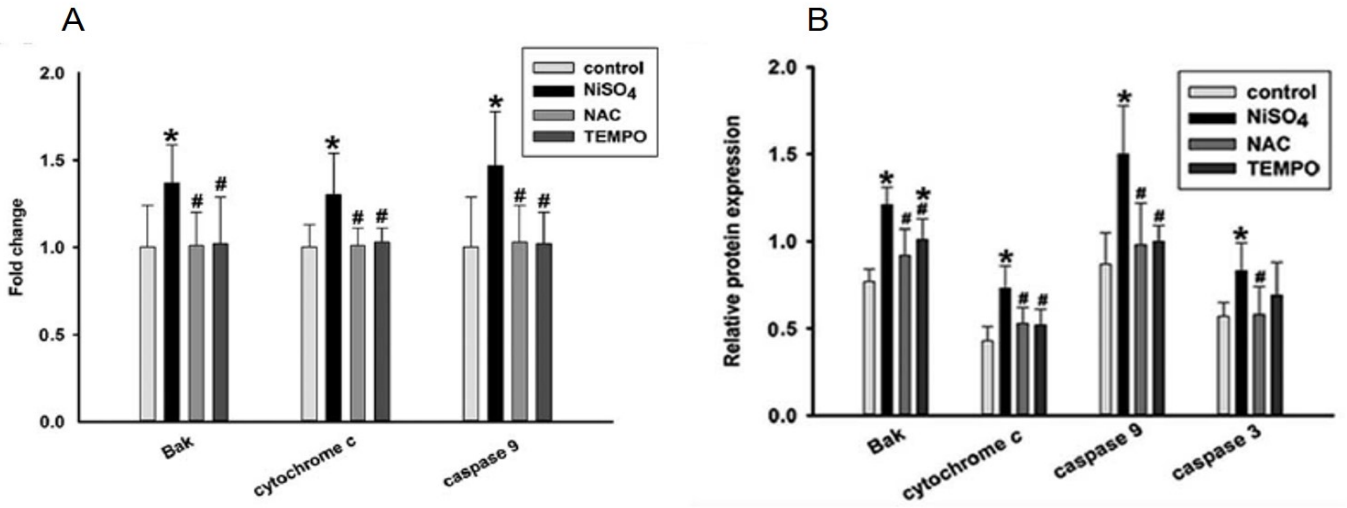

Figure D.38. Effects of NAC and TEMPO on $NiSO_4$-induced upregulation of mitochondria related genes and proteins in rat Leydig cells. (A) Relative mRNA expression levels in rat Leydig cells by RT-qPCR, n=5
(B) Relative protein expression levels by Western blot (B) and semiquantitative analysis n= 5. Data are expressed as mean± standard deviation.
* p<0.05 from control group. #p < 0.05 from the 500 µmol/L $NiSO_4$ group.
Modified from Zou et al. (2017)

The authors state that the results of their study suggest several points. The first is that ROS plays a critical role in an early event of apoptotic cascades induced by $NiSO_4$. Second, $NiSO_4$ could induce ROS generation and consequently lead to apoptosis mediated by mitochondria pathways in rat Leydig cells. Third, $NiSO_4$-induced ROS generation could result in apoptosis elicited by ERS in rat Leydig cells.

Based on these results, the authors concluded that in rat Leydig cells, ROS-dependent mitochondria and ERS mediated apoptotic signal pathways are involved in $NiSO_4$-induced apoptosis.

Han et al. (2018). ROS generation and MAPKs activation contribute to the Ni-induced testosterone synthesis disturbance in rat Leydig cells.

This recent study investigated the role of reactive oxygen species (ROS) and mitogen-activated protein kinases (MAPKs) in Ni-induced disturbance of testosterone synthesis in rat Leydig cells (Han et al. 2018) . To better understand the roles of MAPK signal pathways in Ni-induced Leydig cell dysfunction the authors examined the phosphorylation of members of the MAPK super family such as the extracellular signal-regulated kinases (ERK1/2), p38 and the c-JUN NH2-terminal protein kinase (JNK) MAPKs.

Rat Leydig cells in culture were incubated with $NiSO_4$ (250, 500, 1000 μM) for 0, 6, 12 or 24 h in the presence of hCG (a hormone that activates the LH receptor and is frequently used instead of LH). After incubation, the culture medium from each group was collected, and the levels of testosterone were measured using ELISA. Data are presented below in Figure D.39. Dose-dependent decreases of testosterone levels in culture media and especially significant decreased testosterone contents in the groups treated with 500 μM $NiSO_4$ for 24 h and 1000 μM $NiSO_4$ for 12 or 24 h ($p < 0.05$) were observed.

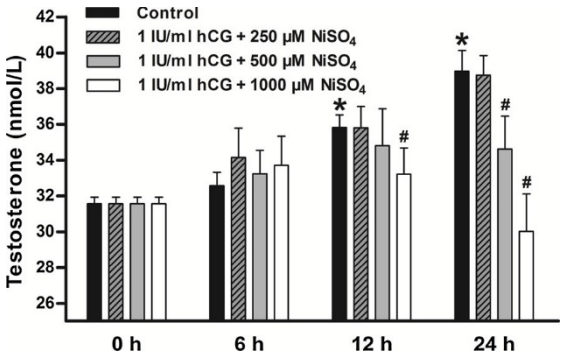

Figure D.39. Effect of $NiSO_4$ on testosterone production in Leydig cells. Cells were treated with different concentrations of $NiSO_4$ (0, 250, 500 or 1000 μM).
Data are expressed as means ± SD of five separated experiments.
*p < 0.05 compared with the 0 h group at the same NiSO4 concentration;
#p < 0.05 compared with the respective control group at the same time.

The intracellular ROS levels, represented by dichlorofluorescein (DCF) fluorescence intensity, are summarized in Figure D.40.

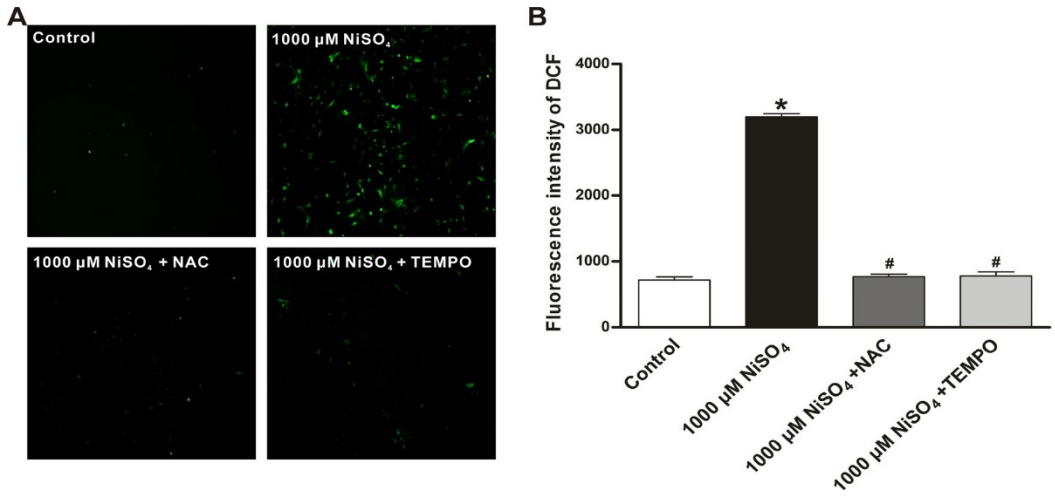

Figure D.40. Effect of ROS scavengers, NAC and TEMPO on $NiSO_4$-induced ROS generation in rat Leydig cells. Intracellular ROS represented by DCF fluorescence intensity (A) and semiquantitative analysis (B).
Data are expressed as mean ± SD of five separated experiments.
*p < 0.05 compared with the control group; #p < 0.05 compared with 1000 μM $NiSO_4$ group.

Additionally, the effects of $NiSO_4$-induced ROS generation on the mRNA and protein expressions of testosterone synthetase were evaluated. Also mRNA levels for target genes, StAR, CYP11A1, CYP17A1, 3β-HSD and 17β- HSD were analyzed by RT-qPCR and protein expression levels were analyzed by Western blot. The authors reported that in Leydig cells, Ni triggered ROS generation, decrease of testosterone synthetase

expression and testosterone production. Also Ni inhibited testosterone synthesis through activating ERK1/2 and p38 MAPK signal pathways in Leydig cells, suggesting that “Ni causes testosterone synthesis disorder, partly, via ROS and MAPK signal pathways”.

The authors concluded that Ni-induced ROS generation and the activation of ERK1/2 and p38 MAPK pathways contributed to the downregulation of mRNA and protein expression levels of targeted genes, which ultimately reduced testosterone production in rat Leydig cells in culture.

D.3. Integrative Evaluation of Male Reproductive Toxicity

D.3.1. Male Reproductive Toxicity in Humans

Table D.24 shows a very broad overview of the human male reproductive studies of Ni. Danadevi et al. (2003) found that blood Ni was associated with poorer linear progressive motility, nonlinear progressive motility, tail defects, and vitality in sperm in a small sample of welders in south India. However, analyses included Ni and Cr separately, and blood Cr was more strongly associated with three of those four outcomes. In the studies by Slivkova et al. (2009) and Skalnaya et al. (2015), Ni in semen was not reported to be associated with effects on sperm or semen parameters. However, Zafar et al. (2015) examined Ni in seminal plasma and found that Ni was negatively correlated with sperm concentration, volume, and motility. Sancini et al. (2014) found that among police working outdoors, personal air exposures affected urinary Ni, and urinary Ni was associated with decreased plasma testosterone. Zeng et al (2013) also found some evidence of an association between urinary Ni and lower testosterone, though the association was not robust in models with other metals. The other studies from the fertility clinic in Tongjing also observed associations between urinary Ni and reproductive outcomes; namely, sperm morphology (abnormal head; (Zeng et al. (2015)), lower T/LH ratio (Wang et al. (2016)), and increased comet assay tail length, indicating DNA damage (Zhou et al. (2016)).

Table D.24. Overview of studies of Nickel and male reproductive effects in humans

Study	Exposure assessment	Outcome measured	Result
Danadevi et al. (2003)	Blood	Sperm parameters	Poorer sperm quality
Slivkova et al. (2009)	Semen	Sperm parameters	No reported findings
Zeng et al. (2013)	Urine	Plasma testosterone	Lower testosterone associated with 3rd quartile exposure but Ni was not retained in final model with other metals.
Sancini et al. (2014)	Urine, air	Plasma testosterone	Lower testosterone
Skalnaya et al. (2015)	Semen	Semen parameters	No associations
Zafar et al. (2015)	Seminal plasma	Semen parameters	Negative associations with sperm concentration, volume, and motility
Zeng et al. (2015)	Urine	Semen parameters	Trend for sperm abnormal head
Wang et al. (2016)	Urine	Reproductive hormones, sperm DNA integrity, sperm apoptosis	Lower testosterone/ luteinizing hormone ratio
Zhou et al. (2016)	Urine	Sperm DNA damage	Increased comet assay tail length (indicating DNA damage)

D.3.2. Male Reproductive Toxicity in Animals

In several animal studies, after nickel exposure, a number of effects on the male reproductive system have been noted. These include effects on the testis and epididymis (histopathological effects and biochemical effects such as lipid peroxidation and oxidative stress), serum hormone changes and effects on sperm motility and mortality. The studies reporting these effects are briefly summarized in Table D.25.

Reported histopathological effects include changes in the seminiferous epithelium, Leydig cells and interstitium, and apoptosis and cell death (Kong et al., 2014; Doreswamy et al., 2004). Degeneration of germinal epithelium along with a reduction in interstitial tissue (Toman et al. 2012) and lesions such as congestion in seminiferous tubules and disrupted spermatogenesis (Pandey et al 1999) were also observed. Additionally, epithelial changes in the cauda epididymis (Pandey et al., 1999 and Hoey, 1966) were reported. The marked testicular damage noted in rats may be related to the dose and route of exposure to nickel. The action of the metals on the epididymis varies from that on the testis, and the damage produced in the epididymis and ductuli efferentes shows less tendency to recover.

A significant increase in testicular lipid peroxidation in rodents was observed after exposure to nickel (Murawska-Cialowicz et al., 2012, and Gupta et al., 2007). According to these researchers, oxidative stress in the testes may contribute to infertility and animals that have protamine 2 such as mice and humans are more sensitive than species that do not. Decreases in sperm count and motility and effects on the structure

and function of the seminiferous epithelium, the site of spermatozoa production, have been reported after nickel exposure in many species (Massanyi et al., 2007 and Zemanova et al., 2007).

A decrease in testosterone level, resulting from testicular damage, appears to have affected testicular spermatogenesis and was exacerbated by reduced FSH (Kong et al., 2014). Simultaneous supplementation of α-tocopherol has been shown to ameliorate the effects of nickel on testicular histopathology (Jargar et al., 2012) and adverse effects on the biochemical microenvironment of the testes of rats is further aggravated in protein-restricted diets. Abnormal sperm and lowered motility of spermatozoa after nickel exposure have been reported. While some multigeneration studies have demonstrated male-mediated effects on development (reductions in the number of pregnancies and pups born), others have not; however, histopathologial changes such as shrinkage of the seminiferous tubules and a decrease in the number of basal spermatogonia have been reported in studies with reduced fertility index after male-only exposure.

These studies are further supported by the findings in related articles (*in vitro* and *in vivo*) pertaining to histopathology, sperm motility, and hormonal changes. Selected studies reporting these effects are also summarized in Table D.25. Overall, the *in vitro* studies report that nickel has two major male reproductive toxicological effects. The first is that nickel inhibits sperm motility (Kanous et al. 1993; Lindemann et al. 1980; Lindemann et al. 1995; Lukac et al. 2011). The second is that nickel causes a concentration dependent depression in testosterone production (Forgacs et al. 1998; Sun et al. 2011) (Krockova et al. 2011). Two recent *in vitro* studies by Zou et al. 2017 and Han et al. 2018 focused on the mechanisms of toxicity involved in nickel-induced effects. Zou et al. 2017 concluded that reactive oxygen species (ROS) generation caused apoptosis in cultured rat Leydig cells and Han et al. 2018 concluded that nickel ultimately reduced testosterone production in cultured rat Leydig cells via ROS generation.

Further, the *in vivo* studies report dose-related decreases in testosterone production in the mouse (Forgacs et al. 1998) and testicular damage by affecting the histopathology of the germ cell layer of the rat testes (Sun et al. 2011).

Table D.25. Studies Reporting Male Reproductive Toxicity

Reference	Compound	Animal Model (Species/ Strain/Sex/Age N	Study Design Exposure (Route/Period/ Frequency/ Vehicle	Doses/ Concentrations	Results	Comments
Hoey et al., 1966	Nickel Sulfate (Other metals - Silver, copper, tin, nickel and cobalt as aqueous salts)	Rats n= 3-5 per group	5 different experimental designs Subcutaneous injection Single dose or as daily injections from 1 to 30 days	0.04 mmol/kg.	Histological changes in testis, altered spermatogenesis. Changes in epididymis.	All tissues showed recovery, less so in epididymis and ductuli efferentes.
Schroeder and Michener 1971	Nickel (soluble salt)	Mice and Rats (10-11 litters/group)	Multi-generational study Oral via drinking water	5 ppm Nickel	↓ size of the litters with each generation, with two failures to breed, ↓number of rats and few males were born in the third generation.	
Mathur et al. 1977a	$NiSO_4$	Male albino rats n=8/group	Dermal, daily for 30 days. Vehicle: saline	Groups painted with0.25 mL containing 0, 40, 60, or 100 mg Ni/kg	Tubular degeneration and edema of seminiferous tubules. NOEL = 40 mg/kg	
Mathur et al. 1977b	$NiSO_4 \cdot 6H_2O$	Male albino rats Treated: n=30 Control: n=10	ip injection for 90 days	3 mg/kg	At day 90 there were degenerative changes in the seminiferous tubules; Significant increase in adenosine triphosphatase at 30, 60, and 90 days; significant decrease in acid phosphatase after day 30.	5 rats from the treated group died during the course of the experiment, but authors weren't able to establish cause of death.
Xie et al, 1995	$NiCl_2$	ICR Mice n=5-6/group	ip injection (one time and killed after 24 h)	0, 0.5, 1.0, 3.0, or 5.0 mg Ni/kg bw	↑ lipid peroxidation, and ↓ testicular weight and fertility rate.	

Das and Dasgupta 1997	$NiSO_4$	Male Wistar rats n=6/group 2 Control groups Ni + normal protein Ni + protein-restricted	ip injection on every alternate day until the 10^{th} dosage	2.0 mg/100 g body weight	Significant decrease in the relative weights of the testes in $NiSO_4$ treated rats; significant decrease in LDH activity in the testes and in testicular protein; significant increase in glycogen and cholesterol content in both normal protein and protein restricted groups.	
Das and Dasgupta 2000	$NiSO_4$	Adult male Wistar rat n=10/group	ip injection on alternate days for 10 days	2.0 mg/100g body weight	Significant decrease in nucleic acids and total protein concentration in experimental animals. Also decrease in sperm concentration and motility.	
Das and Dasgupta 2002	$NiSO_4$	Male Wistar Rats n=10/ group	ip injection	2 control groups; 2 groups given 2 mg/100 g bw every other day for 10 doses; 2 groups were also administered 2 mg/100g bw but were given an additional recovery period of 15 days after the 10^{th} dose	Significant reduction in activities of steroidegenic enzymes and plasma testosterone levels. Significant decrease in the relative weights of the testes in all experimental groups compared to control; significant increase in ascorbic acid and cholesterol levels.	
Obone et al., 1999	$NiSO_4$	Male Sprague-Dawley rats n=8/group	Oral via drinking water for 13 weeks	0, 44.7, 111.75, and 223.5 mg Ni/L	No biochemical or morphological damage to testes. No effect on relative testicular weights	
Pandey et al. 1999	$NiSO_4$.	Adult male albino Swiss mice n= 20/group	Oral (gavage) 5 days per week, for 35 days; also tested for male mediated dev	0, 5 and 10 mg/kg/day	↓ absolute and relative weight of testis, epididymis, seminal vesicle and prostate gland. ↓ sperm count and motility at 10 mg/kg/day; also a significant ↑ morphological abnormalities in different regions of the	

			toxicity effects: 10 mg/kg/d 5 days/wk for 35 days		spermatozoa in 5 and 10 mg/kg/day groups. Significant increase in ɣ-glutamyl transpeptidase and lactate dehydrogenase at 10 mg/kg. Significant decrease in sorbitol dehydrogenase at 10 mg/kg. Congestion in peripheral region of seminiferous tubules at 10 mg/kg and disrupted spermatogenesis; regressed epithelium of cauda epididymides at 10 mg/kg; decrease in fertility index and number of corpora lutea and significant increase in percent pre and post implantation loss at 10 mg/kg	
Pandey and Srivastava, 2000	$NiSO_4$ or $NiCl_2$	Young male mice n=24/group	Oral (gavage) 5 days per week for 35 days, in 0.2 ml distilled water	0, 5, 10, or 20 mg/kg	Significant decrease in absolute and relative weights of testes, epididymides, seminal vesicles, and prostate gland at 20 mg/kg; dose dependent decrease in sperm motility and sperm count at 10 and 20 mg/kg. Dose related ↑ abnormal sperm. Abnormalities were in the head, neck and tail region of the sperm. Curved neck and curved, bent, round, loop and folded tails seen at both the higher doses of each nickel species. NOEL = 5 mg/kg	
Pandey and Singh 2001	$NiSO_4$	Young male Swiss albino mice n=10/group	Oral 5 days/week for 6 months	20 mg/kg/day	Statistically significant lower mean weight and diameter of vesicles; difference in the secretory activity of the cells of the vesicular epithelium in the two groups (scoring system and stats were not reported)	Testicular weight and histology did not differ in the two groups; Authors suggest ↓ in seminal vesicle weight, diameter, and activity of epithelium in Ni exposed animals is similar to that expected when organ is subjected to decreased testosterone levels

Kakela et al., 1999	$NiCl_2$	Wistar Rats n=6/group	Oral via drinking water Male only exposure: for 28 or 42 days before copulation	30 ppm in males	Fertility index ↓ when males were exposed. Shrinkage of the seminiferous tubules, and ↓ number of basal spermatogonia	
Doreswamy et al., 2004	$NiCl_2$	CFT Swiss Mice n= 4/group	ip injection for 3 or 5 consecutive days	Single and multiple sub-lethal doses (1.25, 2.5 or 5.0 µmol/100 g bw per day)	Significant ↑ in LPO in both mitochondrial and microsomal fractions, significant ↑ in DNA damage, single-strand breaks and apoptosis in the testis. ↑ in abnormal sperm and male-mediated dominant lethal-type mutations.	Suggestive of a stage-specific effect on post-meiotic germ cells.
Gupta et al., 2007	Nickel sulfate	Wistar strain male albino rats n=6/group	ip injection on alternate days until the tenth dose.	20 mg/kg body weight,	↑level of testicular lipid peroxide and ↓ all antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase) activities and GSH concentration along with changes (↓) in sperm count and motility	
Zemanova et al., 2007	Nickel	stallion, bull, ram, boar and fox	Nickel concentration in the semen determined and the occurrence of pathological spermatozoa analyzed to find associations with spermatozoa quality		Positive correlation between seminal nickel and sperm abnormalities in bull, ram and boar.	Correlation analysis
Massanyi et al., 2007	$NiCl_2$	Mice n=5/group	Single ip injection	20 mg/kg or 40 mg $NiCl_2$/kg; Control group not injected	Negative effect of nickel on the structure and function of the seminiferous epithelium. ↑ frequency of	

					localized apoptosis in the interstitium compared to control group.	
Su et al., 2011	Nickel sulfate	Wistar rats n=8/group	Oral gavage once per day for 30 days	Ni alone (1.25, 2.5, or 5 mg/kg/day), or Ni (2.5 mg/kg/day) plus GSPE (50 or 100 mg/ kg/day).	↓ sperm motility at 2.5 and 5 mg.	GSPE protects against Ni-induced decrease in sperm motility.
Murawska-Cialowicz et al 2012	Ni (II)	Rats (Buffalo strain) and Mice (Balb C) n=10 (Control) n=15 (Nickel)	ip injection (one time)	5 mg Ni(II)/kg b.w	↑levels of lipid peroxidation markers in testicular homogenates of exposed mice but not in rats. ↓GSH level in mice but not rats.	Authors conclude that Ni(II) can initiate oxidative stress in the testes of mice but not of rats and the anti-oxidative defense of the testes is reduced in mice.
Jargar et al., 2012	nickel sulfate	Wistar strain male albino rats n=6/group	ip injection on alternate days for 10 doses	Nickel: 2.0 mg/100 g body weight α-tocopherol:10 mg/100 g body weight, im	Nickel alone: ↓ body weight, testicular somatic index, sperm count, sperm motility, serum and testicular L-ascorbic acid concentration and serum α-tocopherol levels. ↑ serum and testicular nitric oxide concentrations. Histopathology of the testes -tortuous seminiferous tubules, loss of spermatogenesis, congestion and necrosis in testes.	Nickel sulfate treatment causes testicular oxidative and nitrosative stress and simultaneous supplementation with α-tocopherol is protective.
Toman et al., 2012	$NiCl_2$	Male mice n=5/group; 4 control groups	In pellets. 3, 6, 9 or 12 weeks of exposure	10 mg/kg bw	Degeneration of germinal epithelium, with germ cells released into tubule lumen, empty spaces in seminiferous epithelium. Changes in the testes were time-dependent. ↑ Relative volume of empty spaces in the seminiferous. ↓ in the relative volume of seminiferous epithelium observed after 6 and 12 weeks. ↑ luminization of the tubules after 6, 9 and 12 weeks. Interstitial tissue significantly ↓ after 6 and 9 weeks exposure and ↑ after 12 weeks.	

					Seminiferous tubule diameter significantly ↓ after 12 weeks	
Kong et al., 2014	Ni NPs: average size 90 nm, Surface area ≥ 8 m^2/g Ni MPs: average size 3μm, Surface area ≥ 3 m^2/g	Rat n=10/group	Gavage Administered for 10 weeks before the initiation of the mating period	Control group, Ni NPs (90 nm), high dose 45 mg/kg, mid-dose 15 mg/kg, low dose 5 mg/kg/day, and Ni MPs (3μm)	Ratio of epididymis weight over body weight increased. Changes in sperm motility and diminished levels of FSH and testosterone. Histopathology: shedding of epithelial cells of seminiferous tubule, disordered arrangement of cells in the tube, cell apoptosis and death in rats exposed to nickel nanoparticles.	According to the authors, compared with Ni MPs, the toxicity of Ni NPs was more severe in reproductive toxicity studies due to the reduction of particle size and greater surface area.
RELATED ARTICLES						
Lindemann et al. 1980	$NiSO_4$	Bull sperm	*In vitro*	0, 0.3, 0.5 mM	Bull sperm lost all spontaneous wave motility in the presence of Ni^{2+}. 0.5 mM Ni^{2+} stopped endogenous wave generation	
Lindemann et al. 1995	$NiSO_4$	Bovine sperm	*In vitro* bovine sperm resuspended and incubated with $NiSO_4$	0.66 mM	Decrease in beat amplitude and loss of spontaneous motility of sperm cells	
Forgacs et al. 1998	$NiSO_4$	Mouse n= 5/group	*In vivo* and *in vitro* experiments	*In vivo* experiment: sc injection, 0, 10, 20, or 40 mg/kg bw every three days and then mouse interstitial cells were cultured; *in vitro* experiments: $NiSO_4$ was added to cultured interstitial cells: final conc: 62.5, 125, 250, 500, or 1000 μM.	Dose-dependent depression in hCG-stimulated T production in both *in vivo* and *in vitro* studies. Authors state that there was no change in basal T production; however, we cannot confirm this since there was no data provided.	

Kročková et al. 2011	$NiCl_2$	Mouse n=30	*In vitro* Leydig cell culture	15.67, 31.25, 62.5, 125, 250, 500 and 1000 µmol/L of $NiCl_2$ in a 48-hour culture	Concentration-dependent depression of the testosterone release; lowest T production at 1000µmol/L $NiCl_2$; percentage of apoptotic cells increased at each concentration except at 62.5 µmol/L (highest concentration at 125µmol/L); altered mitochondrial structure; decrease in the volume of SER (≥500µmol/L)	
Lukac et al. 2011	$NiCl_2$	Cultured bovine semen	*In vitro*	7.8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 µM for (30, 60, 120 and 240 min) at 37 °C.	Concentration-dependent inhibitory effect of high $NiCl_2$ concentration on sperm motility as well as progressive motility; alteration of spermatozoa membrane integrity	
Sun et al. 2011	$NiSO_4$	Male Wistar rats Control group: (3 animals per time point of 10, 20, and 30 days of treatment) experimental groups: (9 animals treated for each time point of 10, 20, and 30 days with 5.0 mg/kg nickel).	ip injection for 30 days	Histology: 1.25, 2.5, or 5.0 mg/kg	Testis accumulates nickel; pathological changes in the rat testis at a high dosage; nickel inhibits testicular glutamate-ammonia ligase activity. Also affects the germ cell layer of the rat testes resulting in detached germ cells with abnormal staining of chromatin into the lumen of the seminiferous tubules.	

D.3.3. Integrative Evaluation of Human and Animal Male Reproductive Toxicity

Human studies of Ni on male reproductive endpoints found that inhaled Ni was associated with Ni in urine and urinary Ni was in turn associated with lower plasma testosterone, T/LH ratio, sperm morphology, and DNA damage. Blood Ni was associated with the following effects on sperm: reduced vitality, slow/nonlinear progressive motility, and tail defects (although other exposures, such as Cr, may at least partly account for this finding). Observations from animal studies include effects of nickel on sperm motility and mortality as well as histopathological effects and biochemical effects on the testis and epididymis. These effects may contribute to serum hormone decreases that are observed in animal studies and are consistent with the findings noted in some studies in humans.

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Appendix 1: Pharmacokinetic and Maternal-Fetal Distribution of Ni Studies

Studies discussed in detail in this appendix are relevant to the pharmacokinetics of Ni and maternal-fetal transfer of Ni.

Lu et al. (1981). Placental transfer and body distribution of nickel chloride in pregnant mice.

A study by Lu et al. examined the maternal body distribution of Ni and its transfer to fetal mice along a time course after an injection of $NiCl_2$ was made to pregnant mice (Lu et al. 1981). Pregnant ICR mice were used for this study (n=120 treatedl). The day when a copulation plug was observed was defined as GD 0. The experimental groups had n=15/group, while the control group had n=18. A single ip injection of $NiCl_2$, (4.6 mg/kg) was administered on GD 16. Animals were killed at intervals of 2, 4, 6, 8, 10, 24, 30, and 48 hr after the injection and tissues were obtained for measurement of Ni concentration. Concentration of Ni in blood and placentas were found to be at maximum level (19.8 and 3.9 µg/g) 2 hr after injection, and those in liver, spleens, and kidneys reached maximum levels of 4.9, 1.3 and 56.2 µg/g, respectively, 4 hr after injection. The maximum concentration in fetal tissues (1.1 µg/g) occurred 8 hr after injection. The relative concentrations of Ni in blood,organs, and tissues of pregnant mice at 24 hr after injection were found in order from highest to lowest concentration: kidneys > blood > placentas > fetuses $\underset{\sim}{}$ liver > spleen. It was estimated that the Ni would be excreted in about 42 to 84 hr after injection from the calculated biological half-times (Lu et al. 1981). Notably, the kinetics of $NiCl_2$ in fetal tissues showed a different pattern from that in maternal tissues.

Mas et al. (1985). The acute toxicity and teratogenicity of nickel in pregnant rats.

Mas et al. conducted a study of Ni to investigate: (a) Ni distribution during pregnancy, (b) the influence of changes in body weight during pregnancy on the toxicity of Ni, and (c) the effect of a teratogenic dose of Ni on the uptake, incorporation and/or transport of certain essential metabolites in the placenta and fetuses of the pregnant rat (Mas et al. 1985). Pregnant Wistar Porton rats were from an inbred laboratory colony.

For studies on Ni distribution, groups of 12- and 19-day pregnant rats were injected ip with ^{63}Ni (5 µCi/mg Ni; 4 mg Ni/kg BW). The animals were killed by decapitation at 0.25, 1, 4 or 24 h after dosing. After ip administration, Ni was cleared rapidly from the blood of 12- and 19-day pregnant rats. The data for 15 min, 1 hr, 4 hr, and 24 hr suggest that the concentrations of Ni in the maternal tissues of both groups were maximal at about 1 h after dosing and then decreased progressively. Concentrations of Ni in the placenta and fetuses were maximal at 1 h and 4 h in the 12-day and 19-day pregnant rats.

LD_{50} determinations were made on virgin, 12-day, and 19-day pregnant rats. Four animals per group were given $NiCl_2$ ip in a constant volume of solution (1.0 ml/kg BW) as described in Table 1.1.

Table 1.1. LD_{50} values for Ni administered ip to non-pregnant and pregnant rats (Adapted from Mas et al. 1985)

Animal group (n=4)	LD_{50} (mg Ni/kg BW)	95% Confidence limits (mg Ni/kg BW)
Virgin	9.33	8.51 – 10.23
12-day pregnant	6.30	5.62 – 7.08
19-day pregnant	5.96	5.45 – 6.50

Teratogenic effects were determined on the survivors from these experiments and also with other groups of animals that were injected on day 8, day 12 or day 16 of pregnancy with 1, 2 or 4 mg Ni/kg BW. Many of the fetuses of dams that survived the administration of Ni at or near the LD_{50} on day 12 of pregnancy, when removed on day 20, were malformed. The survivors of the group treated on day 19 of pregnancy, however, delivered normal young and at 5 days postpartum, the litter sizes and weights were similar to those of control, untreated females. Although treatment of the dam with Ni during organogenesis decreased the incidences of fetal reabsorption and skeletal retardation (which are high in this inbred colony), it induced specific malformations; e.g. hydrocephalus, hydronephrosis and heart defects, and other effects; e.g. bleeding into the subachranoid spaces and pin-point hemorrhages in various organs, particularly in the lungs (Mas et al. 1985). These abnormalities were common to fetuses of dams that were dosed with Ni on either day 8 or day 12 of pregnancy, but fetal weight (at day 20) was reduced significantly only in the latter group. When injected on day 16 of pregnancy, Ni (1–4 mg/kg BW) was neither teratogenic, nor highly fetotoxic. Mas et al. noted there may be species differences in Ni toxicity, as some of their findings in their inbred rats were different from results in mice and golden hamsters (Ferm 1972; Lu et al. 1979).

Mas et al. (1986a). Distribution and kinetics of injected nickel in the pregnant rat.

In a subsequent study by Mas et al., the distribution and kinetics of injected Ni was examined in Wistar female virgin rats initially weighing 180 to 200 g (Mas et al. 1986a). Females were mated with adult males until impregnation. Three groups of animals were selected as follows: virgin controls, day 12 and day 19 pregnant rats. To determine the mobility of Ni and its distribution, the rats were injected ip with $^{63}NiCl_2$ (370 kBq/kg BW) carrier free (dose 0), or in the presence of 4 mg/kg BW of Ni in the form of $NiCl_2$ (dose 4) in a volume of 1 ml/kg BW of sodium acetate buffer (0.2 mol/l, pH = 7.0). At exactly 15 min, 1, 2, 4 or 24 h after injection, groups of 5-7 rats from each of the control, day 12, and day 19 pregnant rats were selected for assessment. In all groups, tissues with the maximal Ni uptake or fixation corresponded to kidney, pancreas and blood plasma, irrespective of the dose, with most other tissues following at a lower level of fixation.

The effects of a load of unlabeled Ni on radioactive Ni uptake by the tissues were grossly different in the three groups studied. Values were higher for the higher dose

than in controls except for blood and pituitary (a single pooled value), whereas on day 12 of pregnancy, rats showed practically no differences in radioactive Ni incorporation between tissues, except for higher values for kidney and lower for ovary. On day 19, this uniformity was lost, and a significant number of tissues showed increases in Ni uptake with higher doses of unlabeled Ni (liver, lung, kidney and pituitary), and there were significantly lower values for whole fetus and lower specific radioactivity in the fetal blood. The values found for $t_{1/2}$, were remarkably similar for all tissues tested, with values between 2.9 and 6.6 h, and most tissues between 3 and 5 h. The effects of a higher dose of Ni are reflected in equal or even lower $t_{1/2}$ values except for the day 19 pregnant rats when values in the ovary and whole fetus were significantly higher. Pregnancy affected the $t_{1/2}$ values for Ni differently, dependent on the dose of unlabeled Ni injected. No changes were found for blood and liver at dose 0, but the speed of elimination of liver Ni was increased in 12-day pregnant rats. The age of fetuses resulted in higher $t_{1/2}$ values, which were grossly increased in 19-day-old fetuses by increased amounts of unlabeled Ni. The half-life for nickel in fetal blood was 6.3 hours following treatment on gestation day 19, and the half-life of Ni in fetal plasma was actually the highest among the organs studied.

Mas and Sarkar. (1988). The metabolism of metals in rat placenta.

Mas and Sarkar examined the status and transfer of metals across the rat placenta by subcellular and molecular fractionations of this organ at 2 and 24 h after iv injection of radiolabeled metals (Mas and Sarkar 1988). Several metals evaluated by Mas et al., will not be discussed here, include copper, zinc, and cadmium. Pregnant Sprague-Dawley rats were injected iv on PD 19 with 200 microcurie (µCi) of radioactive solution in an effort to evaluate materno-fetal transfer of metals through the rat placenta to understand the mechanism involved in placental transfer and retention. The 0.2 M sodium acetate solution used as treatment contained tracer amounts of radioactive $^{63}NiCl_2$ (specific activity 11.7 mCi/mg). Animals were sacrificed by decapitation 2 and 24 h after the injection. The placenta is the organ that shows higher accumulations (after the kidney) among all the organs tested for Ni at both times of study (Table 1.2) (Mas and Sarkar 1988).

Table 1.2. Relative activity (specific activity in organ or tissue/specific activity in kidney) in different organs of pregnant rats at 2 and 24 hr after administration of $^{63}NiCl_2$ (Adapted from Mas and Sarkar 1988)

	$^{63}NiCl_2$	
Organ	**2 hr**	**24 hr**
Kidney	1	1
Liver	0.09	0.3
Plasma	0.02	0.2
Placenta	0.22	1
Fetal liver	0.03	0.02

The results of the subcellular fractionation experiment showed the soluble fraction retains the highest content of nickel (77.4% of total radioactivity in placenta at 2 hr, 81.2% of total radioactivity in placenta at 24 hr) (Mas and Sarkar 1988). The distribution of Ni among the components of the soluble fraction showed poor binding to proteins and most of the Ni was found associated with low molecular weight components . Mas et al. stated this does not reflect the distribution in plasma where it is known to be associated with low molecular weight components and albumin. The distribution was akin to that of kidney cytosol, where most of the Ni was associated with low molecular weight components.

Mizejewski et al. (1990). Effects of heavy metals on alpha-fetoprotein in maternal sera and amniotic fluid of pregnant mice.

Mizejewski et al. also studied the distribution of Ni, in the form of $NiCl_2$, in pregnant mice (Mizejewski et al. 1990). Specifically, the effect of low dose administration of Ni on levels of alpha-fetoprotein (AFP) in the sera and amniotic fluid (AF) of pregnant mice during mid-gestation period prior to the rise of fetal metallothioneins were investigated. Heavy-metal-binding substances, such as metallothionein, are useful in the study of trace-metal metabolism, interaction and distribution in fetal and neonatal tissues. However, the low molecular-weight fetal metallothionein is difficult to detect during mid-gestation since it rises sharply only at late pregnancy (18–21 days) in rodents and is most prominent during postnatal development. Adult Nya:Nylar outbred pregnant female mice were used in this study. A total of 173 pregnant mice were used; and pregnancy was untimed. Fetal crown-rump length was used to estimate gestational age. The dams were injected with $NiCl_2$ solution (1 mg/kg) on Monday and Wednesday and autopsied on Friday. The determination of metal concentrations in biological specimens by X-ray fluorescence spectroscopy were measured and reported in units of keV. The authors stated nickel-treated mice displayed increasing serum AFP levels as gestation progressed, while AF-AFP remained somewhat constant (Table 1.3).

Table 1.3. Nickel and AFP concentrations in individual sera and amniotic fluids as determined by x-ray fluorescence spectroscopy (Adapted from Mizejewski et al. 1990)

Dose (mg/kg)	**Serum ions (µg/ml)**	**Serum (AFP µg/ml)**	**Amniotic fluid (AFP mg/ml)**	**% Resorb./litter**	**Gest. age ****
Maternal Sera: *Ni: 7.22 – 7.25 keV					
1.0	8.8	19	0.84	0	14.5
1.0	17.0	25	0.56	0	15.0
1.0	11.0	57	0.44	0	16.0
Dose (mg/kg)	**AF-metal (µg/ml)**	**Serum (AFP µg/ml)**	**Amniotic fluid (AFP mg/ml)**	**% Resorb.**	**Gest. Age**
Amniotic Fluid:					
1.00	Ni – 2.9 *	36	0.44	0	14.0

*Corrected for spectral bandwidth overlap and background
**Determined by fetal crown-rump measurement

Serafini and Romeu. (1991). Nickel interactions with glutathione and related enzyme in 11-day embryo and yolk sac in rat.

Nickel interactions with glutathione (GSH) and related enzymes have been investigated in pregnant Wistar albino rats (Serafini and Romeu 1991). Groups of 8-10 pregnant rats were given 4 mg $NiCl_2$/kg by ip injection. Control rats received vehicle (sterile NaCl solution). Both treated and control rats were killed on GD 11 at 1 or 24 hours after treatment. This study showed a significant effect on GSH metabolism in rat embryo and visceral yolk sac of dams that were injected with Ni^{2+} (4 mg/kg BW). This dose was about 60-65% of the LD_{50} of Ni^{2+}, administered ip as $NiCl_2$, in the 11-day pregnant rat LD_{50}: 6.30 mg Ni/kg BW (Mas et al. 1986b). The effects of Ni^{2+} on GSH concentrations in the embryo and visceral yolk sac differed over time. At 1 hour after Ni-administration embryo GSH levels were 128% that of control, however, at 24 hour post-treatment embryo GSH levels were 77% that of control. In visceral yolk sac at 1 hour post-treatment, GSH levels were about 146% that of control while at 24 hours post-treatment GSH levels were comparable to control levels.

Szakmary et al. (1995). Haemodynamic effect of nickel chloride in pregnant rats.

Szakmary et al. investigated the hemodynamic effect of $NiCl_2$ in pregnant rats (Szakmary et al. 1995). Groups of non-pregnant and pregnant CFY rats were given either 8 ml/kg physiological saline or 3 mg $NiCl_2$/kg dissolved in 10 ml/kg physiological saline daily by gavage during organogenesis on GD 7-14. The blood of non-pregnant animals, and maternal and fetal blood of pregnant animals and amniotic fluid were examined in the 24th hour following the last treatment.

Treatment groups: non-pregnant female controls, n=14
non-pregnant females + Ni, n = 11
pregnant female controls, n = 11
pregnant females + Ni, n = 13

Nickel chloride did not influence the values of the systemic (blood pressure, total peripheral resistance (TPR), cardiac index), or the organs' (including the placenta) circulation parameters, in either non-pregnant or pregnant animals (Szakmary et al. 1995). Pregnancy increased the cardiac index, decreased the arterial blood pressure and the TPR; increased the blood flow and decreased the vascular resistance of the heart, lungs, and the ovaries.

Further two non-pregnant and two pregnant animals received 0, 12, 25 or 50 mg $NiCl_2$/kg on GD 19, and 24 hours later the Ni concentrations in the maternal and fetal blood, and amniotic fluid were determined by atomic absorption spectrophotometry. Concentrations of Ni measured in maternal and fetal blood, and amniotic fluid 24 hours after treatment on GD 19 are presented in Table 1.4.

Table 1.4. Concentration of Ni in the maternal and fetal blood and in amniotic fluid. (Adapted from Szakmary et al. 1995)*

Exposure (mg $NiCl_2$/kg BW)		**Fetal**	
	Maternal blood (µg/L)	**Blood (µg/L)**	**Amniotic fluid (µg/L)**
0	3.8	10.6	2.5
12.0	18.5	14.5	16.5
25.0	90.0	65.5	20.0
50.0	91.5	70.5	17.0

*Values are means (2 mothers, and 3 members of 3 litters each, respectively)

Wang et al. (2010a). Gestational age and dose influence on placental transfer of ^{63}Ni in rats.

In a study by Wang et al. placental uptake, retention and transfer of ^{63}Ni following administration of various doses at different gestational ages were investigated (Wang et al. 2010). Pregnant Wistar rats on GD 12, 15 or 20 were injected ip with saline, 64, 320 or 640 kBq/kg body weight of ^{63}Ni. The radioactive $^{63}NiCl_2$ solution with a specific activity of 8.5 GBq/L ^{63}Ni (Radiopurity > 99%) was diluted with physiological saline solution to obtain the concentrations of 6.4, 32 and 64 kBq/mL for various doses in same volume. The control group had 3 rats, and each treatment group had 5 rats, for a total of 13 rats. Twenty-four hours after administration, samples were harvested. The average weight of placentas on GD 12, 15 and 20 in rats injected with various doses of ^{63}Ni for 24 h were 0.132, 0.344 (2.6-fold increase over GD 12, $p < 0.001$) and 0.664 g (1.9-fold increase over GD 15, $p < 0.001$) respectively and did not differ significantly from their respective controls. In all three dose groups, the ^{63}Ni concentrations in

fetuses increased markedly from GD 12 to 15, but decreased moderately on GD 20; in other words, the highest ^{63}Ni concentrations were detected in fetuses on GD 15. The radioactivity of ^{63}Ni was only concentrated within basal lamina of the placenta 24 hr after three doses of ^{63}Ni injection. The ^{63}Ni concentration in amniotic fluid was also found to increase dose- and gestational age-dependently, and reached a maximum on GD 20. The fetal membrane had the highest ^{63}Ni concentrations, which increased significantly in response to doses and gestational age. The ^{63}Ni concentrations detected in maternal blood on GD 12, 15 and 20 in rats injected respectively with the doses of 64, 320 and 640 kBq/kg BW of ^{63}Ni for 24 hr were not significantly different. However, the ^{63}Ni concentrations detected in maternal kidneys on GD 12, 15 and 20 in rats injected respectively with the dose of 64, 320 and 640 kBq/kg BW of ^{63}Ni for 24 hr exhibited a significant increase in relation to dose, but no significant difference in relation to gestational days. Overall, in placenta, amniotic fluid and fetal membrane, ^{63}Ni concentrations increased with increasing doses and gestational age. These findings suggest that the Ni uptake, retention and transport in placenta increase dose and gestation age-dependently, and Ni transfer through placental barrier is primarily from mother into the fetus, but hardly from fetus to mother.

Hou et al. (2011). The characteristics of placental transfer and tissue concentrations of nickel in late gestational rats and fetuses.

In a study examining the characteristics of placental transfer and tissue concentrations of Ni in late gestational rats and fetuses, a single dose of radioactive Ni was administered. Pregnant Wistar rats were injected ip with 640 kBq/kg of ^{63}Ni or saline as control (Hou et al. 2011). The radioactive $^{63}NiCl_2$ solution with a specific activity of 8.5 GBq/L ^{63}Ni (radiopurity > 99%) was diluted with physiological saline solution to obtain the radioactivity of 64 kBq/mL. The dose of ^{63}Ni was expressed as radioactivity in 10 mL per kilogram body weight. The dams were euthanized respectively at 0.5, 1, 3, 6, 9, 12 or 24 hr after each injection. The quickest uptake of Ni after administration was found in maternal blood, in which the ^{63}Ni radioactivity reached a maximum at 0.5 hr and then decreased gradually. Among all fetal organs and tissues, the ^{63}Ni radioactivity detected in fetal blood reached the maximum first at 3 hr, and then decreased slowly and was persistently higher than in maternal blood at corresponding time points. The highest ^{63}Ni radioactivity among all samples was consistently detected in fetal membranes from 0.5 to 24 hr, and the second highest value was detected in the placenta. The only maternal organ where ^{63}Ni was consistently higher from 0.5 to 24 hr than the corresponding fetal organ was the kidney. Maternal kidneys consistently exhibited the highest Ni concentrations, beginning at 1 hr after injection. The authors concluded that the placenta has a high affinity for Ni, and its barrier does not protect the fetus from Ni exposure.

Related Studies

Sunderman et al. (1978). Embryotoxicity and fetal toxicity of nickel in rats.

In an investigation by Sunderman et al., the embryotoxicity and fetal toxicity of nickel chloride and nickel subsulfide was studied in rats, and the distribution of ^{63}Ni in organs and tissues of pregnant rats was determined following im injection of $^{63}NiCl_2$ (Sunderman et al. 1978). Albino rats of the Fischer 244 strain were used in this study. Injection solutions of nickel chloride were prepared by dissolving the desired amount of reagent grade $NiCl_2$ in sterile NaCl solution (145 mmol/liter). Injection solutions of $^{63}NiCl_2$ were prepared by diluting a sterile solution of $^{63}NiCl_2$ (specific activity, 5.8 mCi/mg of Ni) to the desired concentration by addition of sterile NaCl solution. Nickel subsulfide dust (Ni_3S_2) with median particle diameter < 2 µm was suspended in penicillin G procaine (3×10^7 units/ml) immediately prior to injection. The nickel compounds were administered by deep injection into the thigh muscles of pregnant rats on specified days of gestation. Unless otherwise stated, the im injections were performed at 9:00 AM. In each experiment, control rats were treated identically, except that they received only the injection vehicle. In one experiment, pregnant rats were allowed to deliver and rear their pups until 8 weeks of age. Records were kept of (a) the date of delivery, (b) the numbers of live pups and (c) the weights of the pups at 4, 6, and 8 weeks. In other experiments, pregnant rats were anesthetized with ethyl ether on GD 20, and the fetuses were delivered by Caesarean section. The numbers of live and stillborn fetuses, early and late resorptions, and CL were recorded. Each fetus was weighed.

$NiCl_2$ was administered intramuscularly (im) to seven pregnant rats on GD 8 at a dosage of 16 mg of Ni/kg, which did not cause any maternal mortality. Control rats received an im injection of the vehicle. All of the rats were allowed to deliver and rear their pups. The length of gestation was not significantly affected by the administration of $NiCl_2$. The BW of pups in litters of $NiCl_2$-treated dams were significantly less than the BW of pups in control litters, based upon measurements at 4, 6, and 8 weeks after delivery. No specific behavioral or developmental abnormalities were detected in the pups of $NiCl_2$-treated dams based on weekly observations from birth until 8 weeks of age. Administration of im $NiCl_2$ at dosages of 12 and 16 mg Ni/kg significantly increased intrauterine mortality. The mean BW of live fetuses whose dams had received 16 mg Ni/kg was significantly lower than the mean BW of live fetuses in the control group. Administration of 16 mg $NiCl_2$/kg on GD 18 was associated with a significant increase in fetal mortality. However, this fetal mortality was anticipated since approximately one-half of the dams died within 24 hr after im injection of $NiCl_2$ at a dosage of 16 mg of Ni/kg.

In another experiment, $NiCl_2$ was administered im to pregnant dams at 9:00 am and 4:00 pm on GD 6, 7, 8, 9 and 10. Two dosage levels were tested: 1.5 mg Ni/kg (total dosage of Ni = 15 mg/kg) and 2.0mg Ni/kg (total dosage of Ni = 20 mg/kg). No maternal mortality occurred at either dose. The higher dose was associated with a

significant increase in intrauterine mortality, but not any reduction in mean BW of live pups.

Nickel subsulfide was administered to pregnant rats as a single im injection in penicillin on GD 6 at a dosage of 80 mg of Ni/kg. Intermuscular injection of Ni_3S_2 on GD 6 was associated with a significant increase in intrauterine mortality

Twelve mg Ni/kg radioactive $^{63}NiCl_2$ was administered im to (a) nonpregnant female rats, (b) pregnant rats on GD 8 and (c) pregnant rats on GD 18. At 24 hr after injection of $^{63}NiCl_2$, the rats were killed, and organs, tissues, and body fluids were obtained for measurements of ^{63}Ni. In order of decreasing concentrations of ^{63}Ni, the relative localization of ^{63}Ni in organs and tissues of nonpregnant and pregnant rats was kidneys > serum > adrenal $\underline{\sim}$ lungs $\underline{\sim}$ ovary > spleen $\underline{\sim}$ heart $\underline{\sim}$ liver > skeletal muscle. There was a greater accumulation of nickel in the pituitary of pregnant rats than in nonpregnant rats. The mean concentration of ^{63}Ni in placentas from rats on GD 19 was less than the mean concentrations of ^{63}Ni in the maternal kidneys, but was higher than the mean concentrations of ^{63}Ni in all of the other maternal organs and tissues that were studied. Appreciable concentrations of ^{63}Ni were also found in the fetuses and amniotic fluid from rats on GD 19. On GD 19, the fetal organ that contained the highest concentration of ^{63}Ni was the urinary bladder, which suggested that the ^{63}Ni which entered the fetuses on GD 18 was rapidly excreted by the fetal kidneys.

Jasim and Tjalve. (1984). Effect of thiuram sulfides on the uptake and distribution of nickel in pregnant and non-pregnant mice.

Another study examined Ni distribution with thiuram sulphides or sodium diethyldithiocarbamate in pregnant and non-pregnant mice. Thiuram sulphides are widely used in industry, agriculture and medicine. In the rubber industry, these substances are utilized as accelerators in the vulcanization process. In agriculture, they are used as fungicides and insecticides. Tetraethylthiuram disulphide (TETD) is used in aversion therapy for chronic alcoholism. Diethyldithiocarbamate, as well as other dithiocarbamates, are chelating agents. Based on this property sodium diethyldithiocarbamate (SDC) is used as a therapeutic agent in the treatment of nickel carbonyl poisoning in man. Jasim et al. examined the effects of tetraethylthiuram disulphide (TETD) and some other thiuram sulphides on the uptake and distribution of Ni, given orally by gastric intubation together with these compounds (1984). Female C57BL mice were mated overnight to obtain pregnant animals; vaginal plugs found the morning after mating was considered day 0 of pregnancy. Non-pregnant mice were given $^{63}Ni^{+2}$ (2 µCi/animal together with non-labelled Ni^{+2}) and one of the thiuram sulphides, sodium diethyldithiocarbamate (SDC), or corn oil only (controls) and were then killed by CO_2-asphyxiation, in groups of 4 animals, after 5 hr, 24 hr and 72 hr. Pregnant mice, on PD 18, were given $^{63}Ni^{+2}$ (10 µmol [0.58mg]/kg BW; the amount was 3 µCi/animal together with non-labelled Ni^{+2}) and one of the thiuram sulphides, SDC (1 mmol/kg BW), or corn oil only (controls) and were then killed, in groups of 4 animals, after 24 hr. The administration of $^{63}Ni^{+2}$ together with SDC or either of the thiuram

sulphides to the non-pregnant mice resulted in very markedly increased levels of radioactivity in most tissues at all survival intervals in comparison with the animals given $^{63}Ni^{+2}$ only. In the pregnant mice, which were killed 24 h after the administrations, increased levels of $^{63}Ni^{+2}$ in the fetuses were induced by all compounds (Table 1.5).

Table 1.5. Effects of thiuram sulphides and SDC on tissue-concentrations of $^{63}Ni^{+2}$ in fetuses and placentae of pregnant mice (Adapted from Jasim and Tjalve 1984)

Fetal Tissues	Tissue-concentration of $^{63}Ni^{+2}$ (pmol/100 mg wet tissue)[a]						
	$^{63}Ni^{+2}$	$^{63}Ni^{+2}$ + SDC	$^{63}Ni^{+2}$ + TMTD	$^{63}Ni^{+2}$ + TETD	$^{63}Ni^{+2}$ + TBTD	$^{63}Ni^{+2}$ + DPTM	$^{63}Ni^{+2}$ + DPTT
Whole fetus	15 ± 1	165 ± 8 [b]	115 ± 19 [b]	346 ± 61 [b]	125 ± 12 [b]	513 ± 45 [b]	98 ± 8 [b]
Placenta	76 ± 13	392 ± 40 [b]	231 ± 22 [b]	444 ± 47 [b]	908 ± 104 [b]	820 ± 71 [b]	240 ± 33 [b]

[a] Mean ± S.E. of 4 determinations of material obtained from 4 pregnant mice
[b] Significantly different from controls ($P < 0.05$)
Tetramethylthiuram disulphide (TMTD), Tetraethylthiuram disulphide (TETD), Tetrabutylthiuram disulphide (TBTD), Dipentamethylenethiuram monosulphide (DPTM), and Dipentamethylenethiuram tetrasulphide (DPTT)

The results showed that some of the thiuram sulphides in the pregnant animals induced a very high uptake of nickel in the fetuses. Markedly increased tissue-levels of $^{63}Ni^{+2}$ were observed both in the pregnant and the non-pregnant animals after the treatments with these compounds, in comparison with animals given $^{63}Ni^{+2}$ only. Treatments with the thiuram sulphides and SDC resulted in very markedly increased amounts of urinary $^{63}Ni^{+2}$. Thiuram sulphides or sodium diethyldithiocarbamate are able to form lipophilic complexes with Ni metal. A facilitated penetration through the cellular membranes of the lipophilic complexes between Ni and these substances can explain the effects on the fate of the Ni. Dithiocarbamates and thiurarn sulphides may give rise to teratogenic effects and embryotoxicity; it is possible that combined exposure to thiuram sulphides or dithiocarbamates and nickel may constitute a hazard for the fetus.

Jasim and Tjaelve (1986a). Effect of sodium pyridinethione on the uptake and distribution of nickel, cadmium and zinc in pregnant and non-pregnant mice.

Jasim et al. also examined the effect of sodium pyridinethione on the uptake and distribution of Ni in pregnant and non-pregnant mice (Jasim and Tjaelve 1986a). The pyridinethiones are biologically active as antibacterial and antifungal substances. Sodium pyridinethione and zinc pyridinethione are used as antiseborrheic agents in shampoos and as cosmetic and industrial preservatives. Female C57CL mice were mated overnight. The presence of a vaginal plug the next morning was taken as an indication of pregnancy (PD 1). In this study, the mice were given Ni in 0.1 ml physiological saline . The isotopes were added to solutions in which non-labelled metal chloride salts had been dissolved so that each mouse received 10 µmol of Ni/kg body weight. This corresponded to 0.58 mg/kg body weight for Ni. The sodium pyridinethione was given dissolved in 0.2 ml physiological saline and each mouse received 1 mmol/kg body weight (149 mg/kg body wt). The mice were given Ni orally by gastric intubation

and were then immediately given the sodium pyridinethione also by gastric intubation. Control mice were given the metal followed by 0.2 ml physiological saline. Non-pregnant mice were given $^{63}Ni^{+2}$ and sodium pyridinethione and were then killed by CO_2-asphyxiation, in groups of 4 animals, after 5 hr, 24 hr and 72 hr. Pregnant mice, on day 18 of pregnancy, were given metal and sodium pyridinethione as above and were killed, in groups of 4 animals, after 24 hr. Various fetal tissues and the placentae were taken out and the radioactivity determined by liquid scintillation counting or gamma spectrometry. Non-pregnant mice were given Ni and sodium pyridinethione or Ni only (controls). They were then placed in groups of 4 (in cages), which permit collection of urine. The administration of sodium pyridinethione together with $^{63}Ni^{+2}$ to the non-pregnant mice resulted in very markedly increased levels of the metals in most tissues at all survival intervals (Jasim and Tjaelve 1986a). The effects of sodium pyridinethione on the tissue-concentrations of $^{63}Ni^{+2}$ in fetuses and placentae of pregnant mice are summarized in Table 1.6. In the pregnant animals, sodium pyridinethione induced markedly increased levels of $^{63}Ni^{+2}$ in the fetuses. Generally, the disposition of the $^{63}Ni^{+2}$ was most profoundly affected, with completely altered localization of the metal in several tissues of the animals given sodium pyridinethione, compared with those given the $^{63}Ni^{+2}$ only.

Table 1.6. Effects of sodium pyridinethione on the tissue-concentrations of $^{63}Ni^{+2}$ in fetuses and placentae of pregnant mice (Adapted from Jasim and Tjaelve 1986a)

Fetal Tissues	Tissue-concentration of $^{63}Ni^{+2}$ (pmol/100 mg wet tissue)[a]	
	$^{63}Ni^{+2}$	$^{63}Ni^{+2}$ + sodium pyridinethione
Whole fetus	16 ± 3	539 ± 18 [b]
Placenta	91 ± 19	674 ± 40 [b]
Kidney	53 ± 4	974 ± 89 [b]

[a] Mean ± S.E. of 4 determinations of materials obtained from 4 pregnant mice
[b] Significantly different from controls ($P < 0.05$)

The administration of sodium pyridinethione greatly increased the urinary excretion of $^{63}Ni^{+2}$ (Jasim and Tjaelve 1986a). Nickel is excreted to a considerable extent via the urine. The $^{63}Ni^{+2}$-distribution-pattern in the kidney is characteristic with a strong labelling of small areas in the cortex, probably corresponding to the distal-convoluted tubuli.

Appendix 2: Summaries of Epidemiologic Studies of Ni Exposure and Reproductive and Developmental Outcomes

Table 2.1. Summaries of Epidemiologic Studies of Ni Exposure and Developmental Outcomes

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
Basu et al., 2014 California (Los Angeles, Riverside, El Cajon, San Jose, Simi Valley, Bakersfield, Sacramento, and Fresno)	Retrospective cohort N = 646,296 singleton infants of 37-44 weeks gestation, born in 2000-2006 Birth records data were used to examine associations between LBW and exposures to $PM_{2.5}$ and $PM_{2.5}$ constituents, with stratification by season and exploration of possible effect modification by maternal characteristics. Included subjects had full gestational exposure information available for total $PM_{2.5}$ mass and 23 $PM_{2.5}$ constituents,	Birth weight Term LBW	Authors used data on ambient $PM_{2.5}$ mass and $PM_{2.5}$ constituents from US EPA monitors. Monitoring frequency was every 3 or 6 days. Constituents were included if monitored continuously and detection levels > LOD at least 30% of sampling days. Exposure to $PM_{2.5}$ constituents was calculated based on the average weekly mean for all weeks in the trimester. Full pregnancy exposure was calculated as the mean of all trimesters; births missing exposure estimates for one or	For the whole cohort, mean (SD) gestational exposure to $PM_{2.5}$ Ni was 0.0033 (0.0040) µg/m^3 IQR of $PM_{2.5}$ Ni exposure was 0.001 µg/m^3. The lowest mean gestational exposure to $PM_{2.5}$ Ni was in Bakersfield (0.0010 µg/m^3) and the highest was in San Jose (0.0076 µg/m^3).	Changes in birth weight and risk of LBW associated with an IQR increase in $PM_{2.5}$ Ni, adjusted for individual demographic and birth characteristics: Birth weight (adj.) −1 (−2, −1) g Birth weight (adj. additionally for ZIP code tabulation area (ZCTA) community-level characteristics) −1 (−2, −1) g Risk of LBW (adj) 1 (0, 1)% Risk of LBW (adj. for ZCTA) 1 (0, 1)% Many of the $PM_{2.5}$ constituents had stronger effects on birth weight than $PM_{2.5}$ total mass, indicating that the observed	Individual and birth characteristics included in models: - maternal race/ethnicity - education - age - infant month of birth - infant year of birth - gestational age - sex - region (north, south) - apparent temperature. ZCTA community-level characteristics included in models: - unemployment - home ownership - employment - non-White race Effect modification assessment variables: - average apparent temperature, - season, - maternal age,	The authors note that previous research has not found smoking to confound the relationships between $PM_{2.5}$ and LBW in California. Maternal zip code was for the time of delivery, not the pregnancy, which may have resulted in some exposure misclassification. 20 km radius might be too large for $PM_{2.5}$ constituents that have more spatial heterogeneity than total $PM_{2.5}$; any misclassification might result in underestimates of associations. Alcohol consumption was not included in

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
	and had birth residence zip codes with population-weighted centroid within 20 km of a monitor.		more trimesters were excluded. Multi-pollutant models were not examined.		associations were not merely a result of correlations with $PM_{2.5.}$ $PM_{2.5}$ Ni was not among those most strongly associated with reductions in birth weight.	- education, - race/ethnicity. Maternal alcohol consumption, parity, and delivery method were also not available, but are not suspected to be associated with $PM_{2.5}$.	analyses due to reluctance of many respondents to report.
Bell et al., 2010. Hartford, New Haven, Bridgeport, and Danbury, Connecticut Springfield, Massachusetts	Retrospective cohort Birth weight, demographic, and other data were obtained from birth certificate data. Of 232,347 births in 1999-2000, the final dataset included 76,788 singleton births after exclusions. .	Birth weight Term low birth weight (term birth < 2500 g)	Air data came from $PM_{2.5}$ regulatory monitors from CT and MA Depts. of Environmental Protection for August 2000-February 2004. Authors used positive matrix factorization for each air monitor to estimate the contribution of $PM_{2.5}$ sources to constituent levels and produce estimates for each source for each day and location. Estimates were combined to estimate exposure over each trimester and entire pregnancy.	Mean (SD) gestational exposure to Ni was 0.0031 (0.0015) $\mu g/m^3$. Ni constituted 0.02% of $PM_{2.5}$ mass	An IQR increase in exposure to Ni $PM_{2.5}$ over the pregnancy was associated with a change (CI) in birth weight of −7 (−12, −3) g and change in risk of low birth weight of 11% (3, 19). In analyses of trimester exposures, an IQR change in 3rd trimester $PM_{2.5}$ Ni exposure was associated with a change in birth weight of −9 (−15, −2) g. 1st and 2nd trimester $PM_{2.5}$ Ni exposures were not associated with	Models adjusted for: - apparent temperature by trimester - infant's sex - parity - type of delivery (vaginal, primary cesarean, repeat cesarean) - trimester of 1st prenatal care - gestation length - year of birth - mother's age - marital status - education - tobacco use in pregnancy - alcohol use in pregnancy - race (white, African-American, other)	The authors note: - Due to spatial heterogeneity of PM2.5, use of county-wide exposure, estimates could have introduced exposure misclassification, and this could vary by constituent and source. - Monitors do not capture differences in exposures due to individual activity patterns, such as time spent outdoors or occupational exposure.

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
					changes in birth weight. $PM_{2.5}$ Ni exposure was associated with a change in birth weight of −6 (−12, −1) g in infants of white mothers, and −12 (−24, 0) g in infants of African-American mothers.		
Chashschin et al., 1994 Russia	Cross-sectional 356 mothers who worked in Ni hydrometallurgy (232 in Ni hydrolysis, 124 in purification). Referents were 324 unexposed female construction workers. Birth and pregnancy data were obtained from the municipal health care registration board.	Adverse pregnancy outcomes, including spontaneous and threatened abortion, gestational toxicosis, pregnancy-induced hypertension, anemia, prematurity, hypoxia, hypertrophy, still birth, malformations	Subjects received a thorough general clinical health examination, including pulmonary X-ray, ECG, lung function, routine blood count, and determination of Ni concentrations in 24-h urine samples. Exposure assessment methods are not described.	Ni sulfate aerosol levels mean, range, mg/m^3 Electrolysis operators: 0.201, 0.107 – 0.308 Purification operators: 0.136, 0.077 – 0.196 Ni in urine mean, range µg/l: Electrolysis operators: 15.6, 5.2 – 22.6 Purification operators: 10.4, 3.2 – 18.0.	Pregnancy complications among Ni workers vs. referents (% of pregnancies): Threatened abortion 17.2 vs. 7.6; Spontaneous abortion 15.9 vs. 8.5; Gestational toxicosis (early) 12.9 vs. 13.5; Gestational toxicosis (late) 32.8 vs. 27.2; Pregnancy-induced hypertension 6.0 vs. 8.2; Anemia 11.2 vs. 12.9; Other complications 29.7 vs. 20.6 Adverse birth outcomes among	The authors obtained information on potential confounders, such as smoking, alcohol use, and other diseases from a random sample of 60 Ni-exposed mothers of children with malformations and an unstated number of time- and place-matched controls, and state the examined factors, age, and parity do not confound the findings.	The authors acknowledge the possibility of recall bias and expectation bias. Very little detail regarding study design, data collection, and analytic methods is reported.

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
					Ni electrolysis, Ni purification, and both Ni occupations combined, vs. referents, respectively (% of live births): Prematurity 8.6, 4.8, 7.3, 4.1 Hypoxia 7.3, 7.3, 7.3, 9.4 Hypertrophy 15.5, 29.0, 20.2, 15.8 Still birth 0.9, 0.8, 0.8, 1.2 Structural malformations 16.4, 17.8, 16.9, 5.8 RR of exposure to Ni work compared with non-Ni work: Spontaneous abortion 1.8. All defects* 2.9 - Cardiovascular defects 6.1 - Musculoskeletal defects 1.9 *Reportedly significant, along with prematurity, but p-values or CIs were not reported.		
Ebisu and Bell, 2012	Retrospective cohort	Birth weight,	Data on $PM_{2.5}$ chemical components from	Mean (SD) gestational level of	Odds (CI) of LBW was 5.7% (2.7, 8.8) higher per IQR	Models adjusted for: - maternal race	The smaller associations with LBW among

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
Northeastern and Mid-Atlantic Regions, USA	Birth certificate data for CT, DE, MD, MA, NH, NJ, RI, VT, VA, Washington, DC, and WV from January 2000 – December 2007. Exclusions: unspecified or different county of residence and birth, plural deliveries, gestation >44 weeks or <37 weeks, birth weight <1,000 g or >5,500 g, impossible gestational age and weight combinations, missing or problematic LMP. The final dataset included 1,207,800 singleton births from 49 counties.	term low birth weight (term birth < 2500 g)	the US EPA Air Explorer. PM_{10}, $PM_{2.5}$ total mass, CO, NO_2, O_3, and SO_2 data from the US EPA Air Quality System for 1999-2007. PM was measured every 3-6 days. Gaseous pollutants were measured daily (O_3 was measured mainly during the warm season). Exposures were estimated based on county of residence at delivery. Pollutant measurements were averaged to estimate daily pollutant levels and combined with apparent temperature to estimate weekly exposures, which were averaged to estimate gestational and trimester exposures.	$PM_{2.5}$ Ni, $\mu g/m^3$: 0.006 (0.006), IQR: 0.0071. Mean (SD) gestational level of $PM_{2.5}$ total mass, $\mu g/m^3$: 13.41 (2.05), IQR: 2.71 Mean (SD) gestational level of PM_{10} total mass, $\mu g/m^3$: 22.34 (4.31), IQR: 4.93.	increase in $PM_{2.5}$ Ni, adjusted for confounders (not including co-pollutants). OR for LBW associated with an IQR increase in $PM_{2.5}$ Ni was 10.2% (7.9, 12.4) lower among African American than white mothers, and 4.6% (2.2, 7.1) lower for females than for males. Nickel was highly correlated (r > 0.5) with vanadium (r=0.63), Zn (r=0.64), NO_2 (r=0.72), O_3 (r=-0.68), SO_2 (r=0.61). Only associations between LBW and Ni, elemental carbon, Al, and Ti were robust to adjustment for all copollutants with correlation <0.5.	- marital status - tobacco and alcohol use during pregnancy - education level - age - trimester of first prenatal care - gestational age - infant's sex - first birth - delivery method (vaginal, cesarean section, unknown) - average apparent temp. for each trimester - season of birth - year of birth - regional indicators. Pollutants that showed associations with LBW in single pollutant models were tested in two-pollutant models with pairs of pollutants whose correlation was < 0.5.	African American mothers and female infants observed in this study differ from previous findings by the same authors (Bell et al., 2010). Among limitations of the study, the authors mention use of birth certificate data, especially for alcohol and tobacco use, prenatal care, pregnancy complications, and labor. Unknown smoking status was associated with increased odds of LBW. Authors cited other possible sources of misclassification, including: - Missing chemical exposures below detection limits - Use of residence at time of delivery, instead of during the pregnancy - Spatial heterogeneity of pollutants within a

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
							county, especially for large counties and those living far from monitors (the maximum distance to a monitor from the border of a county was 75.6 km). Alcohol consumption was not included in analyses due to many respondents' reluctance to report.
Friel et al., 2005 Newfoundland, Nova Scotia, and Ontario, Canada	Cross-sectional 33 anencephalic and 22 control fetuses ("term and preterm infants whose tissues had undergone minimal autolytic changes") from 6 hospitals in 3 regions were included. Sample selection criteria were not reported.	Anencephaly	Samples of liver, kidney, diaphragmatic muscle, sciatic nerve, and pancreas were collected at autopsy from the right side of the fetus. Tissue samples were analyzed by inductively coupled plasma mass spectrometry for Zn, Cu, Mn, Co, Ni, Mo, and Cd.	Mean (SEM) Ni conc. in anencephalic and control fetuses combined, (ppm): Liver 1.6 (0.5) Kidney 2 (0.6) Sciatic nerve 36 (26) Pancreas 7.5 (3.7) Muscle 10 (7)	No differences in Ni conc. between the two groups were observed. Zn and Cd conc. were different for the two groups.		This study is not reported in detail.
Fuertes et al., 2014 Italy, Spain, Sweden,	Meta-analysis of data from 7 cohorts	Pneumonia in early childhood	Between October 2008 and February 2010, particulate matter	Range of cohort-specific mean (SD)	AOR (CI) for pneumonia and 2 ng/m^3 increase in Ni in the fully	The authors decided *a priori* that:	A related study reported that the LUR models did not perform well for

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
Netherlands, Germany, United Kingdom	with births between 1994-2008 15,962 children were included in analyses for Ni PM_{10}. For Ni $PM_{2.5}$, land use regression (LUR) model data from one cohort (n=3,971) were unavailable. Questionnaire-based information was collected between 6 and 36 months of age. Random-effects meta-analysis models to account for potential within- and between-cohort variability in combined estimates of associations between elements and pneumonia.	(generally from birth to 2 years) The cumulative incidence of pneumonia was 5.6% in the total population	measurements were taken at 20 or 40 sites in each of 7 study areas for 14 consecutive days in cold, warm, and intermediate seasons as part of the ESCAPE project. Site-specific annual averages for the 3 measurement periods were adjusted for temporal variation. Data on traffic intensity, population /household density, and land use were used to develop LUR models for each area, which were used to estimate average annual exposures to each element for each participant's home address at birth.	concentrations, ng/m^3: Ni PM_{10} 0.8 (0.2) to 3.7 (0.6) Ni $PM_{2.5}$ 0.4 (0.1) to 2.6 (0.4)	adjusted "main models": Ni PM_{10} 1.09 (0.83, 1.43) Ni $PM_{2.5}$ 0.84 (0.67, 1.05). Models with fewer covariates estimated slightly higher AORs: Adjusted only for sex and municipality: Ni PM_{10} 1.22 (0.99, 1.49) Ni $PM_{2.5}$ 1.11 (0.93, 1.32). Adjusted for all covariates except PM_{10} /$PM_{2.5}$ mass: Ni PM_{10} 1.31 (1.06, 1.63) Ni $PM_{2.5}$ 1.15 (0.96, 1.39).	1) "crude models" would adjust for sex and municipality (a 4-level categorical variable used in one cohort); 2) "minimally adjusted models" would add adjustment for older siblings, breastfeeding, season of birth, atopy of either parent, daycare attendance, maternal smoking during pregnancy, secondhand smoke, parental SES, use of natural gas for cooking, mold/dampness in the home, intervention (vs. observational study); 3) "main models" would additionally adjust for and PM_{10} or $PM_{2.5}$ mass.	predicting Ni particulate concentrations (de Hoogh et al. 2013). The authors do not provide reasons for including all of the covariates in the models; the result may be overadjustment.

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
Heck et al., 2013 California	Case-control Neuroblastoma case and control ascertainment was similar to that for retinoblastoma reported in Heck et al., 2015. 52 cases and 9,730 controls lived within 5 km of an air monitor and had sufficient exposure data for Ni analyses. ORs were estimated using logistic regression for each pollutant separately. Factor analysis with principal components extraction was used to address correlated pollutants; the two factors consisted mainly of traffic-related pollutants and polycyclic aromatic hydrocarbons; Ni was not included in a factor, although Ni was correlated	Neuroblastoma	Air toxics data were provided by the California Air Resources Board, which collects 24-h samples every 12 days from each air monitor. For 1998 and later births, exposure was assessed using the home address on the birth certificate. For births before 1998, the population-weighted centroid of residence zip code was used. The nearest monitor to each subject's home was used to assign pollutant values. Time-specific exposure averages were calculated based on the gestational age and date of birth for each trimester and the entire pregnancy, and 1st year of life. Trimester-specific values did not differ substantially, so	<u>Air Ni concentration</u> Mean (SD) 4.851 (2.187) ng/m^3 ng/m^3 (among controls only) IQR 3.193 ng/m^3	AOR (CI) associated with neuroblastoma and an IQR increase in average Ni exposure over the pregnancy for births within 5 km was 1.08 (0.71, 1.66), and for births within 2.5 km was 0.67 (0.29, 1.56).	Covariates included in final model: - maternal age - maternal race/ethnicity - birth year - method of payment for prenatal care Covariates evaluated but not included in models: - maternal education - neighborhood socioeconomic index The authors also examined stratification by child's age.	Maternal smoking status was not available until 2007 and was not examined. The study was limited by multiple comparisons and collinearity among pollutants.

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
	with pollutants in the first factor.		results are reported for the entire pregnancy.				
Heck et al., 2015 California	Case-control Cases were ascertained from the California Cancer Registry (CCR) with diagnoses 1990 – 2007 and were <6 yrs. Controls were selected from birth records for the same period and frequency matched to all childhood cancer cases by birth year, had no cancer diagnosis in the CCR and no California death record by age 6. 62 cases and 18,351 controls lived within 5 mi of an air monitor and had sufficient exposure data for Ni analyses. ORs were estimated using logistic regression for each pollutant separately.	Retinoblastoma	For 1998 and later births, exposure was assessed using the home address on the birth certificate. For births before 1998, the population-weighted centroid of residence zip code was used. Air toxics data were provided by the California Air Resources Board , which collects 24-h samples every 12 days from each air monitor. The nearest monitor to each subject's home was used to assign pollutant values. The mean (SD) distance between participants' homes and the nearest monitor was 4.9 (2.0) km. Time-specific exposure averages were calculated	<u>Air Ni concentration</u> Mean (SD) 5.08 (2.27) ng/m^3 (among controls only) IQR 3.18 ng/m^3	Although trimester-specific estimates were examined, the authors observed little-to-no differences in point estimates across trimesters and report results for the entire pregnancy only. Adjusted OR associated with an IQR increase in average Ni exposure over the pregnancy = 1.48 (1.08, 2.01). In the factor analysis, only 2 factors had eigen values >1; neither factor included Ni. The adjusted OR for hexavalent Cr was 1.22 (0.97, 1.54). Ni was also associated with ortho-dichlorobenzene (OR 1.46 (0.90, 2.37)), chloroform	Covariates included in final model: - paternal age, - maternal race and birthplace, - birth year, - method of payment for prenatal care. Covariates evaluated as potential confounders: - maternal - race/ethnicity and nativity, - paternal age, - year of birth, - method of payment for prenatal care - (private health insurance vs. government-insurance/self-pay), - region, - time periods (1990–97 vs. 1998 –2007)	The authors note that risk estimates based on residence within 5 miles of a monitor might be more accurate for Ni than for less stable agents. Maternal smoking status was not available until 2007 and was not examined. The authors did not have information on whether RB1 mutations were inherited or de novo, and were not able to stratify by germline or somatic mutations. The study was limited by multiple comparisons and collinearity among pollutants.

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
	Factor analysis with principal components extraction was used to address correlated pollutants. .		based on the gestational age and date of birth for 3 months preconception, each trimester, entire pregnancy, and 1st year of life.		(OR 1.35 (1.07, 1.70)), para-dichlorobenzene (OR 1.24 (1.04, 1.60)), and total Cr (OR 1.29 (1.04, 1.60)). Point estimates were similar for most pollutants when 3 km and 5 km radii were evaluated.		
Hu et al., 2015 Beijing, Xiamen, Lanzhou, and Taiyuan, China	Pilot study; design not described 81 mother-newborn pairs were enrolled from 4 hospitals in Beijing, Xiamen, Lanzhou, and Taiyuan in 2011. Mothers were interviewed at delivery for lifestyle and demographic information and medical history. Infant birth date, gestation week, gender, placental weight, and birth weight and mother's previous pregnancy outcomes and	Birth weight Pilot study of maternal and cord blood conc. of heavy metals and infant birth weight.	Inductively coupled plasma mass spectrometry to measure heavy metals, including Pb, Tl, Cd, Se, As, Ni, V, Co, and Hg, in maternal and cord blood.	Ni was detected in 65.4% of maternal blood samples and 51.9% of cord blood samples. (LOD=0.5 ng/g) Blood conc. (ng/g) Maternal 25th %ile <LOD Median 1.4 75th %ile 2.1 Cord blood 25th %ile <LOD Median 0.9 75th %ile 2.4 The difference between maternal and newborn Ni conc. was not statistically significant.	Ni in maternal blood was positively but not significantly associated with birth weight: adjusted beta = 45.6 (-17.2 – 108.4), presumably representing grams birth weight associated with 1 ng/g change in maternal blood Ni concentration. Adjusted beta for cord blood = 32.2 (-19.8 – 84.1)	Infant gender, gestation week, maternal age, maternal BMI	The authors did not describe the sampling design and subject selection criteria or methods. Correlation among the metals was not addressed, and the regression analyses did not include more than one metal at a time. The authors did not state how they treated concentrations below the LOD in statistical analyses.

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	weight at delivery were obtained from medical records.						
Huang et al., 2011 Lvliang Region, Shanxi Province, China	Ecologic Study to model the association between 12 trace elements in cultivated soil and risk of neural tube defects (NTDs). All live and still births in 2002-2004, and therapeutic abortions with estimated delivery date in the same period, in selected villages in Lvliang Region were included. Data were collected from hospital records, clinics in villages, and homes to generate a registry. Each woman on the registry was interviewed about pregnancy history and the infant, and infants were examined for NTDs and other diseases.	NTDs	Soil samples were collected from a depth between 2 cm and 20 cm, of at least two patches of cultivated land in each village, in December 2004. All soil samples from a village were mixed thoroughly to represent the village and analyzed using inductively coupled plasma mass spectroscopy to measure levels of the trace elements of interest. All NTD cases were matched by geo-code of the villages' names and locations.	Soil Ni concentrations, µg/g : Mean (SD) 41.38 (6.39), variance 40.77.	Ni levels and corresponding prevalence of NTDs: 30-34 µg/g - low prevalence >34 µg/g - medium prevalence <30 µg/g - high prevalence. Definitions of low, medium, and high prevalence were not reported. The authors state that their results show that "both deficiencies in and excessive amounts of nickel cause an increased risk of neural tube defects" and that Ni (and Sn, Pb, Fe, Cu, and Al) had "layered level effects" on the occurrence of NTDs, while no effects were observed for As, Se, Zn, Sr, and V, and a "threshold value" was observed for the effect of Mo.	Village Other trace elements examined: Sn, Pb, Ni, Fe, Cu, Al, As, Se, Zn, Sr, V, Mo	The authors did not report important information, such as sample size and results of statistical tests aside from fitness of the model, nor did they address the relevance of a village's average element content in soil to each individual's exposure, other potential risk factors for NTDs, possible interactions among elements and other exposures, and confounding.

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Kalkbrenner et al., 2010 North Carolina (NC) and West Virginia (WV)	Case-control 383 Cases (206 NC, 177 WV) 2829 Controls (1,096 WV, 1,733 NC) Cases and controls were ascertained through the Autism and Developmental Disabilities Monitoring Network (ADDM) in WV (2000 & 2002) and selected counties in NC (2002 & 2004). Cases: - met American Psychiatric Association definition of autism spectrum disorders (ASD) Controls were all children in the ADDM in NC and WV with speech and language impairment without other serious developmental problems. In WV a 1/3 random sample was used. All participants were born in the study area, had a matching	ASD at age 8	Individual exposures were assigned using modeled concentrations based on 1996 National Air Toxics Assessment (NATA) program HAP assessments for the residence census tract at birth.	Ni concentration geometric mean (geometric SD), ng/m^3: <u>NC</u> Urban 1.9 (1.6) Not urban 0.8 (1.9) All NC 1.1 (2.0) <u>WV</u> Urban 1.5 (7.4) Not urban 0.1 (4.9) All WV 0.2 (6.3)	AORs (CI) for ASD and Ni compounds at the 80th vs. 20th percentile (1.7 vs. 0.1 ng/m^3) for the whole cohort: All 1.1 (0.6, 1.9) 100% urban 1.8 (0.6, 4.9) 100% rural 1.2 (0.4, 3.4) Mixed urban/rural 0.9 (0.4, 2.0) NC 0.8 (0.2, 3.3) WV 0.9 (0.4, 1.8)	Adjusted models included maternal age, smoking in pregnancy, marital status, maternal education, race, census tract median household income, urbanicity, HAPs (except coke oven emissions), and exchangeability variables. The authors also evaluated effect modification by state, urbanicity, sex, comorbid intellectual disabilities.	Pb was negatively associated with ASD. The authors note that other known developmental neurotoxicants had near-null associations with ASD, and that low exposure levels and choice of control group could have contributed to these findings. The authors suggest that other studies' findings for Ni and other metals in relation ASD may be due to confounding by urbanicity, other pollutants, or related factors. ORs in this study suggest some uncontrolled confounding associated with state. Reported numbers of subjects are inconsistent.

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
	birth certificate and idenitifiable census tract. All pollutants were included in a semi-Bayes hierarchical model.						
Laurent et al., 2014 Los Angeles County, California	Retrospective cohort Birth data came from birth certificate records for all births to women residing in LA County from 1/1/01 to 12/31/08. Exclusions: multiple births, known birth defects or defect status unknown, missing gestational age, implausible gestation/ weight combinations, <260 days or > 308 days gestation. 960,945 births were geocoded to tax parcel, street, zip code, or city. 704,148 births were available for analyses of $PM_{2.5}$ and $PM_{0.1}$ species.	Term low birth weight	The University of California Davis/ CIT_Primary (UCD_P) chemical transport model was used to estimate concentrations of primary ground-level $PM_{2.5}$ and $PM_{0.1}$ elements for 4 km x 4 km grid cells. Daily concentrations were based on the grid cell where the mother resided at delivery, averaged over each trimester and the pregnancy. Exposures to total $PM_{2.5}$, NO_2, and O_3 for 2000-08 were modeled using empirical Bayesian kriging, and the effects of exposure to traffic were also estimated.	Primary $PM_{2.5}$ Ni, $\mu g/m^3$: Mean (SD)= 0.0030 (0.0026) 1st trimester IQR= 0.0022 2nd trimester IQR= 0.0022 3rd trimester IQR= 0.0023 Pregnancy IQR= 0.0021 Primary $PM_{0.1}$ Ni, $\mu g/m^3$: Mean (SD) = 0.0004 (0.0005) 1st trimester IQR= 0.0003 2nd trimester IQR= 0.0003 3rd trimester IQR= 0.0003	ORs (95% CI) for LBW per IQR Ni 1st trimester $PM_{2.5}$ 1.010 (1.003, 1.016) $PM_{0.1}$ 1.009 (1.003, 1.014) 2nd trimester $PM_{2.5}$ 1.008 (1.002, 1.014) $PM_{0.1}$ 1.008 (1.003, 1.014) 3rd trimester $PM_{2.5}$ 1.010 (1.004, 1.016) $PM_{0.1}$ 1.010 (1.005, 1.015) Pregnancy-long exposure $PM_{2.5}$ 1.009 (1.003, 1.015) $PM_{0.1}$ 1.009 (1.004, 1.014) Effect modification Race/ethnicity: ORs for LBW and $PM_{2.5}$ and $PM_{0.1}$ Ni were	Covariates in the main models were: - maternal - race/ethnicity, - education level, - parity, - trimester prenatal care began, - infant's gender. The authors adjusted for maternal age, gestation length, and median household income by Census block group using smoothing splines due to their J-shape relationship with LBW. Effect modification was evaluated for: - maternal race/ethnicity, - education level, - median block group income,	Air pollution metrics were based on maternal address at delivery; the actual exposure of mothers was not measured. The resulting error might have generated bias. Data on maternal smoking during pregnancy were available for 2007-08 but were not used due to potentially serious underreporting; the authors state this is "probably the major limitation of this study" The completeness of maternal disease reporting is uncertain. Potential under-reporting of maternal diseases on birth certificates

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				Pregnancy IQR= 0.0003	somewhat higher for women of Asian and "other and multiple" race/ethnicity, and slightly lower for White non-Hispanic women. ORs for African-American and Hispanic women were intermediate. ORs for LBW and $PM_{2.5 \text{ and } 0.1}$ Ni were somewhat higher among women with chronic hypertension, though the numbers for women with hypertension were small and the ORs for these women were NS. ORs for LBW and $PM_{2.5 \text{ and } 0.1}$ Ni were somewhat higher, though NS, among women with pre-eclampsia.	- hypertension, - diabetes, - pre-eclampsia.	makes the extent to which these data represent women with chronic diseases questionable.
Manduca et al., 2014 Gaza, Palestine	Case-control 48 cases with birth defects born in succession May – October 2011 with sufficient hair for	Congenital defects Preterm birth	The main source of the metal exposure of concern was a 2009 military attack on Gaza known as Operation Cast Lead, which used	Not reported	No differences in Ni concentrations between the phenotype groups.	Elements analyzed: ^{7}Li, ^{9}Be, ^{48}Ti, ^{51}V, ^{52}Cr, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{64}Zn, ^{75}As, ^{78}Se, ^{85}Rb, ^{114}Cd, ^{120}Sn, ^{121}Sb, ^{138}Ba, ^{140}Ce, ^{184}W,	Authors state that newborn hair reflects total accumulation of metals in the period of in utero hair growth, from

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	testing (87.3%) at a hospital in Gaza. 77 cases of premature infants (<2.0 kg, <37 weeks) without birth defects and with enough hair; of these, 9 infants' hair was analyzed. Controls: 12 full term healthy infants, with no birth defects among siblings and parents' collaterals, were randomly chosen from 3,892 "normal children" (term & ≥ 2.5 kg at birth) for hair analysis.		phosphorus ammunitions. Hair was collected from the nape of the neck, when possible, or from the occipital area within minutes of birth. Blinded analyses of hair samples were conducted using dynamic reaction cell inductively coupled mass spectrometry for 25 metals, including Ni. Parents were interviewed in the delivery room about residence, occupation, gestation length, other children's health status, neighborhood birth defects, reproductive history, exposure to white phosphorus ammunition attacks, etc.			^{202}Hg, ^{205}Tl, ^{208}Pb, ^{238}U	approximately 22 weeks of gestation, excluding direct contamination from the external environment. The study is not clearly described.
McDermott et al., 2014	Retrospective cohort	LBW	Addresses for the 6th month of pregnancy (when	Kriged estimates for mean Ni conc.	Unadjusted OR for LBW and an IQR	Covariates: - maternal age - race	Alcohol consumption was not included in

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South Carolina	Subjects were 9,920 singleton live births in 10 residential study areas 1996 – 2002, insured by South Carolina Medicaid (<185% federal poverty level). Mothers were identified through Medicaid and followed using reimbursement files and birth certificates. Exclusions: Multiple births, birth defects, and known causes of disability. Authors examined associations between LBW and conc. of 8 metals (As, Ba, Cr, Cu, Pb, Mn, Ni, and Hg) in soil near the subjects' homes.		enrollment was highest) were obtained from a Medicaid eligibility file and geocoded. A grid was laid over the study area and the intersections of grid lines were soil sampling locations. Samples were taken after the pregnancy at 5 cm depth and 1.0 – 3.0 km apart. To estimate soil metal concentrations at the known residential addresses, the authors used data from approximately 100 nodes per area to conduct Bayesian Kriging and statistical methods including variable transformation.	in soil near subjects' homes: 4.58 mg/kg for LBW infants, 4.57 mg/kg for normal weight births; IQR 43.21 mg/kg (whether this IQR represents cases and control combined is not stated)	increase in Ni was 1.00 (0.98, 1.02).	- parity - tobacco use - alcohol use- infant sex - gestational age	analyses due to reluctance to report it. The authors note that people track soil and dust from outside their homes, the soil and dust accumulate on the floors in the homes, and metals in soils are bioavailable to humans. The authors did not directly measure soil around the mothers' homes. Soil sampling occurred after the pregnancies. However, due to long soil half-lives for inorganic chemicals, these estimates should be good estimates for the concentration during pregnancy. The study was conducted among low-income families and may not be generalizable.

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Ni et al., 2014 Guiyu (a major electronic waste [e-waste] recycling destination) and Jinping District in Shantou City (no e-waste recycling), China	Cross-sectional N=201 mother-infant pairs (126 from Guiyu and 75 from Jinping), 2012 – 2013 Eligibility criteria for the mothers were age ≥18 years, no diseases that could affect oxidative stress markers (e.g., systemic lupus, diabetes), and singleton pregnancy. Criteria for neonates: 37-42 weeks gestation, birth weight >2,500 g, no severe neonatal illnesses. Women were interviewed in the hospital after deliveries for socio-demographic information, possible exposure to metals, and medication use during gestation.	8-hydroxydeoxyguanosine (8-OHdG) as a marker of oxidative damage to DNA	Immediately after delivery, two UCB samples were collected from the placenta of every subject: one sample was used to measure metals, and the other was used to measure 8-OHdG concentration. LOD for Ni was 0.92 µg/L. The authors asked mothers about possible sources of exposure to the metals, including distance between home and street, environmental contamination sources around the home, and occupational exposures (e.g., residence was an e-waste recycling workshop, maternal and paternal occupation).	UCB Ni concentration median (range), ng/mL: Guiyu 8.63 (3.68 – 544.20) Jinping 9.09 (4.67 – 152.40)	UCB 8-OHdG median concentration, ng/mL: Overall 162.09 Guiyu 162.09 Jinping 153.69 Pearson correlation coefficient was 0.314 ($p<0.001$) for log-transformed Ni and 8-OHdG concentrations. Association between 8-OHdG and Ni conc. (ng/mL): $\beta = 0.215$ (95% CI 0.113, 0.317), adjusted for Pb, Cd, Cr and other listed adjustment variables. In adjusted analyses, 8-OHdG was more strongly associated with Ni than other metals. 8-OHdG concentrations were higher if a parent was occupied in e-waste recycling: - mother works in e-waste recycling: 179.77 ng/mL,	Variables included in multivariate analyses: - sampling area, - maternal age, - education level, - maternal smoking. - alcohol use, - tea drinking, - maternal occupation - paternal smoking, - paternal occupation. Other metals of interest: Pb, Cr, Cd	The authors do not mention correlations or collinearity among the metals.

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					- mother does not work in e-waste recycling: 159.00 ng/mL (p=0.03), - father works in e-waste recycling: 168.99, - father does not work in e-waste recycling: 158.63, (p=0.22).		
Odland et al., 1999 Arkhangelskk, Nikel, and Monchegorsk, Russia Kirkenes, Hammerfest and Bergen, Norway	Cross-sectional In each location, ≥50 consecutive women presenting to the hospital delivery department in 1993 and 1994 provided blood and urine samples. Birth outcome and other data were obtained from delivery department records. A midwife or gynecologist collected demographic, behavioral, and health data.	Birth weight Body mass index of the newborn child (BMIC)	Women provided urine and blood samples at 1-2 days post-partum to assess and compare urinary nickel excretion and other variables and their influence on pregnancy outcomes in 3 communities in Russia and 3 cities in Norway. In Nikel and Kirkenes, women also provided urine samples at week 20 of pregnancy. First-voided urine was obtained from 265 infants (137 in Russia, 128 in Norway). Concentrations <10 nmol/L (LOD) were	Median (range) post-partum urine Ni conc., (nmol/L): Russia = 85 (5-2108) Norway = 5 (5-85) Monchegorsk Ni refinery workers (subset of Russian group): = 66 (range not reported)nmol/L Median (range) infants' first-voided urine Ni conc., (nmol/L): Russia = 34 (5-561) Norway = 5 (5-48) Median (range) creatinine-adjusted urine Ni conc., (nmol Ni/mmol creatL): <u>Russian group</u> mothers = 9 (1-285) Infants = 11 (1-510) <u>Arkhangelsk</u>	In multivariate analyses of birth weight, maternal urinary Ni was not a significant predictor of birth weight or BMIC. Birth weight change (CI) per nmol/L maternal urinary Ni: -0.1 (-0.5, 0.3) g BMIC change (CI) associated with maternal urinary Ni: 0 (-0.03, 0.004) Categorizing urine Ni as below/above 34 nmol/L did not result in association with birth weight. Maternal BMI and country were associated with infant's BMI.	Analyses of maternal urine Ni conc. and birth weight adjusted for: - BMI - maternal height - maternal urine creatinine - smoking (0, 1-10, >10 cigarettes per day) - country Other variables considered in birth weight or infant BMI analyses: - maternal age - parity - maternal weight - BMI - pre-eclamptic conditions - local food intake - infant's urine Ni conc. - infant's urine creatinine	During the study, maternity leave in Russia began 56 days before the anticipated delivery date; thus, the postpartum urine samples did not represent recent occupational exposures. Russian responses lacked information on education, and in many cases, alcohol use, so analyses excluded these data. Local food consumption was also not collected in Monchegorsk. The authors mention inconsistency between

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			assigned the value of (1/2)(LOD) = 5 nmol/L. Tap water from randomly selected homes in Nikel, Monchegorsk, Arkhangelsk, Zapolyarniy, and Umba was sampled and tested for Ni.	(subset of Russian group w/o Ni refinery) mothers = 6 (1-59) infants = 5 (1-325) <u>Norwegian group</u> mothers 1 (0.2-41) infants 2 (0.4-187) Post-partum and 20th pregnancy week urine Ni conc. did not differ. Water in communities with Ni refineries had higher levels of Ni in drinking water compared to than those w/o Ni point sources.		- urine Ni adjusted for creatinine - gender	interviewers and the possibility that translation of the questionnaire from Norwegian to Russian may have had a "steering effect" on some questions.
Odland et al., 2004 Arkhangelsk, Nikel, and Monchegork, Russia Kirkenes, Hammerfest, and Bergen, Norway	Cross-sectional Lifestyle information and specimens were collected from 50 consecutive mother-infant pairs from hospital delivery departments in each community between May 1993 and June 1994. Eligibility was based on	Birth weight Body Mass Index of Child (BMIC)	Women provided urine and blood samples at 1-2 days post-partum. First-voided urine was obtained from 265 infants (137 in Russia, 128 in Norway). Ni concentration from placenta samples was available for 220 births.	Median (range) Ni: Maternal urine, nmol/L Total 47.0 (4.3-2108) Russia 84.2 (4.3-2108) Norway 13.6 (4.3-96.9) Neonatal urine, nmol/L Total 18.7 (4.3-561) Russia 34.0 (4.3-561)	In univariate analyses, placenta Ni was the only element with an apparent significant negative effect on birth weight, but the association was not statistically significant after adjustment for country and gestational age: –1510 (–3191, 170).	Considered in multivariate models: - maternal age - smoking frequency - BMI - height - number of deliveries - urinary creatinine - gestational age - country - placental Pb, Ni, and Cu - maternal blood - cord blood Pb	The authors did not discuss why they adjusted for country. If country is a proxy for some mechanism in the causal pathway between exposure and fetal growth, adjustment for country may not be appropriate.

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	availability of maternal and cord blood Pb data. Women were interviewed for personal and morphometric information. Data on the birth and infant were taken from delivery records.		The authors tested for Ni, Cd, Pb, Se, Cu, Zn, Fe, and in a limited sample, Hg. Ni concentrations <10 nmol/L (LOD) were assigned the value of (1/2)(LOD)=5 nmol/L.	Norway 4.3 (4.3-37.0) Placenta, µg/g Total 0.017 (0.005-0.377) Russia 0.023 (0.005-0.119) Norway 0.012 (0.005-0.377)	The association between placental Ni and BMIC was –2.73 (–7.49, 2.02) kg/m^2 per µg/g increase in Ni concentration, adjusted for country and gestational age. Placental Ni was not significantly associated with birth weight or BMIC in final multivariate analyses.		
Pedersen et al., 2016 Europe (Sweden, Denmark, Lithuania, Netherlands, Germany, Italy, Spain),	Cohort (prospective personal and birth data collection with retrospective exposure assessment) 8 cohorts (2 from the Netherlands) from previous air pollution studies of mother-child pairs with singleton deliveries from 1994 – 2008. Inclusion criteria varied across cohorts.	Term LBW Term birth weight Head circumference at birth	Outdoor $PM_{2.5}$ and PM_{10} concentrations were measured during a 2-week period in summer, winter, and another season 1 year between 2008 – 2011 at 20-40 sites in each study area. $PM_{2.5}$ and PM_{10} filters were analyzed using X-ray fluorescence. Measurements were averaged and adjusted for temporal trends.	Mean (SD) Ni concentrations, ng/m^3: $PM_{2.5}$ 1.6 (0.8) PM_{10} 1.8 (1.2) Average air pollution exposure levels, ng/m^3: $PM_{2.5}$ 17.0 PM_{10} 26.9 The selected elements represented 6% of total mass of $PM_{2.5}$ and 7% of total mass of PM_{10}.	Results are reported for Ni $PM_{2.5}$ in 1 ng/m^3 increments and Ni PM_{10} in 2 ng/m^3 increments. AOR for term LBW (single pollutant models): Ni $PM_{2.5}$: 1.14 (1.00, 1.29) Ni PM_{10}: 1.29 (0.96, 1.75) OR for term LBW, 1 ng/m^3 increments (models with particle mass): Ni $PM_{2.5}$: 1.11 (0.94, 1.31)	Adjustment variables (selected *a priori)*: - gestational age, - sex, - parity, - season of - conception, - maternal age, - height, - prepregnancy weight, - education level (cohort-specific low, medium, high); - number of cigarettes/day during 2nd trimester.	Exposure assessments were limited to home addresses, and amount of time mothers spent at home was not known. Ni $PM_{2.5}$ exposures were not estimated for the Lithuanian and Danish cohorts due to a lack of predictors in the LUR model.

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	N=34,923 mother-child pairs Data on individual characteristics came from questionnaires and/or interviews during pregnancy. Gestational age, birth weight, head circumference at birth, and sex were obtained from birth records.		Land use regression (LUR) models were used to estimate annual ambient $PM_{2.5}$ and PM_{10} elemental concentrations at the mothers' addresses during pregnancy. Because exposure assessments did not precede pregnancies, data from routine monitoring stations were used to adjust the annual $PM_{2.5}$ and PM_{10} LUR estimates to the periods corresponding to each individual pregnancy.		Ni PM_{10}: 1.14 (0.90, 1.43). β (CI) for term birth weight Ni $PM_{2.5}$: 4 (-15, 22) Ni PM_{10}: 1 (-22, 24); head circumference at birth: Ni $PM_{2.5}$: -0.60 (-0.71, - 0.49) Ni PM_{10}: -0.46 (-0.57, -0.36); -head circumference, adjusted for particle mass: Ni $PM_{2.5}$: -0.49 (-0.61, - 0.36); Ni PM_{10}: -0.34 (-0.45, -0.22); -head circumference, adjusted for S: Ni $PM_{2.5}$: -0.31 (-0.44, -0.19); Ni PM_{10}: -0.05 (-0.20, 0.09)	The authors also performed analyses of two-pollutant models for each element adjusted for particle mass, other elements, or traffic density on the nearest street. Temperature, humidity, and atmospheric pressure were also evaluated as covariates.	
Roberts et al., 2013 USA	Case-control 325 Cases 22,098 Controls	ASD	Births were generally assigned to the mother's mailing address the year of or before the birth for	Median Ni conc., $\mu g/m^3$, for each quintile from the entire population: Quintile 1 0.0004	AORs (CI) (adjusted for sex and covariates indicated), by exposure quintile: 1 (Ref)	Other HAPs (not included in Ni analyses): Sb, As, Cd, Cr, Pb, Mn, Hg, diesel particulate matter, methylene	Although the authors refer to the exposures in this study as "perinatal", some of the exposure data

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	Subjects were drawn from the Nurses' Health Study II cohort. Cases were children of 756 women who had responded about having a child with ASD in the 2005 questionnaire and a follow-up questionnaire in 2007-08. Controls: -adequate address information -born 1987-2002 -mothers had provided information about live births and smoking during pregnancy -1 child per mother was randomly selected.		geocoding to a census tract identifier. HAP concentrations were obtained from the US EPA NATA program, which estimates average ambient air concentrations of pollutants for census tracts. HAP assessments were used to assign exposures to children as follows: HAP yr Birth yrs 1990 1987-93 1996 1994-97 1999 1998-00 2002 2001-02	Quintile 2 0.0012 Quintile 3 0.0024 Quintile 4 0.0045 Quintile 5 0.0159	2 1.3 (0.9,1.9) 3 1.6 (1.1, 1.2) 4 1.5 (1.0, 2.2) 5 1.7 (1.1, 2.5) AORs (CI) by sex Boys 1 (Ref) 2 1.4 (0.9, 2.0) 3 1.6 (1.1, 2.4) 4 1.7 (1.1, 2.6) 5 1.9 (1.2, 2.9) Girls 1 (Ref) 2 1.1 (0.5, 2.5) 3 1.2 (0.5, 3.0) 4 0.7 (0.3, 2.1) 5 0.7 (0.2, 2.2) HAPS most strongly associated with ASD were Pb, Mn, Cd, Ni, & methylene chloride; Pearson correlation coefficients for these HAPs with Ni: Cd 0.24, Pb 0.17, Mn 0.38, methylene chloride 0.12. In a model including all pollutants, ORs were attenuated compared with single pollutant models. Boys-only AORs (CI) for Pb	chloride, quinoline, styrene, trichloroethylene, vinyl chloride. Family & community SES indicators possibly associated with ASD ascertainment and included in models: census tract median income, census tract % college educated 6 yrs after birth; mother's parents' education during her infancy. Factors possibly associated with ASD or air pollutant exposure and included in models: year of birth, maternal age at birth, HAP year. Not included in models: family income in 2001, education level of mother's partner, smoking.	represent years after some of the births. The authors did not address the stability of the HAP concentrations. Roberts et al. note other possible sources of exposure misclassification, including the possibility that the mother relocated around the time of pregnancy, and the available address was not the residential address during and immediately following the pregnancy. Individuals' actual exposures, including indoor exposures and exposures when moving between areas, were not measured.

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					and Ni were the largest: Pb 1.6 (0.9, 2.9) and Ni 1.5 (0.8, 2.7). After false discovery rate adjustments, linear trend tests were not significant for both sexes, but remained significant for boys only.		
Togawa et al., 2016 Norway, Finland, and Sweden	Case-control Diagnoses 1978-2012, depending on country Data from the registry-based NORD-TEST Study to examine parental exposures to heavy metals and welding fumes and risk of testicular germ cell tumors (TGCT) in offspring. Cases (n=8,112): all males, 14 to 49	Testicular germ cell tumors (TGCT)	Authors obtained parental occupation codes from censuses and applied job exposure matrices (JEMs) to estimate exposures to Cr, Fe, Ni, Pb, Cd, and welding fumes for each parent. JEMs provide data on the proportion of workers exposed (P) and mean level of exposure (L). 2 methods of exposure classification:	Not available	ORs (CI) for TGCT and parental exposure to Ni with or without other metals/ welding fumes (referent is no exposure to Ni or welding): Father 1.07 (1.00, 1.16) Mother 1.07 (0.74, 1.51) ORs (CI) for TGCT and parental exposure to Ni based on exposure indices (P×L)	The following information from the various registries was available: maternal and paternal ages at birth, age at TGCT diagnosis, family history of testicular cancer, personal history of inguinal hernia, hypospadias, and cryptorchidism. Addition of covariates to models did not substantially alter the OR estimates, so the authors	Authors note that exposure misclassification is likely when applying JEMs, especially for jobs with low prevalence of exposure. Nearly 68% of parental occupational information was based on censuses administered two to nine years before the birth. Sensitivity analyses including occupational data for the year of or before the birth yielded similar or

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
	yrs, newly diagnosed with TGCT w/o a prior primary neoplasm (except non-melanoma skin cancer). Controls (n=26,264): randomly selected from population registry, and matched individually to each case by country and year of birth, at a ratio of 4:1.		1) Exposure groupings based on presence/absence of specific exposure (i. no exposure to Ni or welding fumes; ii. exposure to Ni, with or without other metals/welding fumes; iii. exposure to heavy metal(s)/welding fumes without Ni) 2) Exposure indices calculated as (P×L)		(referent is non-exposed): <u>Father</u> Low 1.08 (1.00, 1.18) High 1.03 (0.85, 1.24) <u>Mother</u> Low 1.00 (0.66, 1.51) High 1.27 (0.66, 2.44)	report unadjusted ORs.	somewhat greater ORs for Ni and other metals.
Vaktskjold et al., 2006 Monˇegorsk, Russia	Conditional logistic regression Cohort Infants were identified through the Kola Birth Registry for 1973-2001 23,141 live or still-births to 17,301 women; gestational age ≥28wks or weight ≥1kg, and mother resided in Monˇegorsk when	Genital malformations	Close to 500 workers participated in surveys of air and urine measurements of Ni in 1996-2001. Each occupation in the KBR was assigned a categorical exposure rating of background, low, or high Ni exposure.	Urinary Ni exposure categories (µg/L): background <10 low 10 - <70 high ≥70 See Vaktskjold et al. 2008a for mean levels.	AOR (CI) for pooled genital malformations and maternal Ni exposure (reference = background exposure): Ni exposure 0.81 (0.52, 1.26) (test for trend across 3 exposure categories)	Analyses adjusted for the following factors, selected *a priori* based on relevant associations in the literature: - parity, - maternal malformation, - exposure to solvents at work, - infectious diseases in the period from 2-3 months before	Malformations were identified in the 1st week after delivery; milder hypospadias might not have been detected in that time. Many female genital malformations would not be detected. The authors also acknowledge that

Study/ Location	Study Design/ Sample sizes	Outcomes of Interest	Exposure Assessment	Ni concentrations	Results	Covariates/ Confounders	Comments
	she became pregnant. 103 (44.5/10,000) infants with one or more malformations of the genital organs: - 8 girls (7.9/10,000), - 94 boys (82.0/10,000), - 1 sex undetermined		Background exposure included refinery occupations with exposure levels estimated to be comparable to that of the general population. Authors believed that the exposure assessments reflected past exposures. 87.3% of the births in the cohort were classified as having *background*, 7.4% *low*, and 5.3% *high* exposure. Individual subjects' actual exposure was not measured.		low exposure 0.71 (0.31, 1.64) high exposure 0.72 (0.26, 1.95). AORs (CI) for undescended testes: Ni exposure 0.76 (0.40, 1.47) (test for trend across 3 exposure categories) low exposure = 0.58 (0.16, 2.02) high exposure = 0.72 (0.17, 2.96) The authors were unable to fit a model for hypospadias.	pregnancy to 12 weeks of gestation, - influenza in the first 12 weeks of pregnancy, - gestational age (undescended testes sub-analysis only).	an assessment of incidence of malformations would require inclusion of all conceptuses and thus consideration of fetal loss, and that exposure may appear to be protective due to an increased risk of fetal loss. Inappropriate cut-off levels for exposure could bias the results toward the null. Any effect of paternal exposures on genital malformations would blur the findings of this study. The authors did not consider exposure to As, Cd, and Pb to be potential confounders.
Vaktskjold et al., 2007 Monçegorsk Russia	Cohort N = 25,245 singleton births with specified sex and	SGA SGA - birth weight <10th percentile for each week of	Air and urine measurements of Ni taken in 1995-2001 for the Monçegorsk secondary refining	Background exposure was < 10 µg/L, and high exposure was defined as ≥70 µg/L urinary Ni.	Mothers of 10.6% of SGA infants and 13.0% of the reference infants were employed at jobs with Ni	Analyses adjusted for: - first delivery, - regular maternal exposure to solvents at work,	The authors had limited insight into occupational exposures of women outside the Ni refinery; these

Study/ Location	Study Design/ Sample sizes	Outcomes of Interest	Exposure Assessment	Ni concentrations	Results	Covariates/ Confounders	Comments
	weight in the Kola Birth Registry. Inclusion criteria: 1) birth in 1973 - 2001 2) mother was a resident of Monchegorsk at start of pregnancy 3) gestational age 28-42 weeks 4) mother's workplace and job were registered 5) no diagnosis of trisomies 13,18, and 21, or Turner's syndrome. Newborns n = 22,836 SGA n = 2,096 (9.2%)	gestation in Monchegorsk. The 10th percentile was sex-specific only for infants ≥37 weeks.	processes were used to characterize subjects' exposure into background, low, and high levels of Ni, based on the water-soluble sub-fraction of the inhalable Ni-aerosol fraction and urinary Ni concentrations, and knowledge of refining processes and occupations. Background exposure includes Ni refinery work in areas with exposures estimated to be comparable to that experienced by the general population not employed at the refinery. Individual subjects' actual exposure was not measured.	See Vaktskjold et al. 2008a for mean levels.	exposure above the background level. Adjusted OR for SGA per unit increase in exposure category = 0.84 (0.75, 0.93). Excluding maternal height and previous induced abortions from the model resulted in a similar OR = 0.81 (0.73, 0.90). Among preterm births, the AOR for SGA = 0.61 (0.31, 1.20).	- maternal age >34 years, - maternal height, - smoking (yes/no), - pervious induced abortion (yes/no), - obvious signs of alcohol abuse in pregnancy (yes/no).	non-Ni workplace exposures could influence the ability to find an effect of Ni exposure. Russian women had the right to be transferred from potentially hazardous jobs when pregnant. However, the authors state that such voluntary job changes were unlikely for Ni production workers, as they would decrease salaries. The highest proportion of SGA births was observed among unemployed women and homemakers (11.8%), suggesting the possible role of the "healthy worker effect". Exposures at the refinery also included As, Cd, Pb. (Thomassen, 1999)

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
Vaktskjold et al., 2008a Monˇegorsk, Russia	Case-control 1996-2002 Cases were women with a first self-recognized spontaneous abortion (SA), <28 wks gestation. Questionnaire study: cases = 184 (13.3%) recognized SA cases among 1,380 women in 14 workplaces in the Ni, Co, and Cu refinery complex and five outside the complex. Controls were women with a term delivery (≥37 wks) with no prior SA. Birth registry study: cases = 474 controls = 4571 from the Kola Birth Registry	Spontaneous abortion	Air and urine Ni measurements 1995-2001 with ~500 individual workers were used to characterize subjects' exposure based on the water-soluble sub-fraction of the inhalable Ni-aerosol fraction and urinary Ni concentrations (conc.), and knowledge of refining processes and occupations. Individual subjects' actual exposure was not measured.	Urinary Ni exposure categories (µg/L) based on previous measurements: background <10 low 10 -<70 high ≥70 Mean (SD), geometric mean urinary Ni conc. (µg/L) for male and female workers at Monˇegorsk, by department, 1995-2001 ranged from: rinsing (new) 77 (88), 53 to electrorefining (old) 293 (380), 179; new: 191(188), 127 (Thomassen et al., 1999) No other reporting of specific Ni exposure levels.	Questionnaire study: adjusted OR = 1.14 (0.95 – 1.37) per unit increase in exposure category Birth registry study: 11.4% of cases and 14.2% of controls had been exposed. Adjusted OR for SA across the 3 exposure levels = 0.87 (0.72, 1.06). When maternal smoking but not maternal age (which did not appear to change the OR) were included in the model, the adjusted OR = 1.10 (0.82, 1.47).	<u>Questionnaire study</u>: model adjusted for - previous delivery, - solvent or paint exposure, - heavy lifting, - age >34 years, - smoking. <u>Birth registry study:</u> model adjusted for: - previous induced abortion, - solvent or paint exposure, - maternal age >34 years.	The authors acknowledge that: - as induced abortions were common, the estimate of association should be skewed downwards compared to the true risk; - because the study design missed early SAs; "it might have been more appropriate to study the specific risks related to the different stages of pregnancy and limit the inclusion of cases accordingly". The inclusion criteria favor the most fertile women. Risks associated with Ni exposure may appear protective due to increased risk of fetal loss before a pregnancy is verified. If pregnancy outcomes influenced women's choice of work

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
							within the refinery, to leave the refinery, or participate in the study, results could be biased.
Vaktskjold et al., 2008b Monchegorsk Russia	Case control Kola Birth Register included data for all births ≥28 weeks' gestation in 1973-2001. 17,139 women delivered 22,965 newborns Cases = 341 (1.48%) Controls = 22,624 (98.5%). Data were registered retrospectively beginning in 1997. Logistic regression analysis to estimate the association between musculoskeletal malformations and exposure to water-soluble Ni, adjusted for variables selected *a priori* based on relevant	Musculoskeletal malformations	Air and urine Ni measurements 1995-2001 with ~500 individual workers were used to characterize subjects' exposure based on the water-soluble sub-fraction of the inhalable Ni-aerosol fraction and urinary Ni conc., and knowledge of refining processes and occupations. Individual subjects' actual exposure was not measured.	Urinary Ni exposure categories (µg/L): background <10 low 10 -<70 high ≥70 See Vaktskjold et al. 2008a for mean levels.	The observed incidence of musculoskeletal defects among newborns with no other malformation was 13.3/1,000 births, which is more than double the EUROCAT 2007 figure for all member registries. Adjusted OR for musculoskeletal defect = 0.96 (0.76, 1.21) per unit increase in exposure category over the three categories. The most frequent defect was deformities of the feet (48% of those with only musculoskeletal defects). Adjusted OR for feet deformity = 1.08 (0.78, 1.48)	Models adjusted for: - first delivery, - smoking during pregnancy, - exposure to paints or solvents at work, - alcohol abuse, - maternal age >34 years or <18 years.	The authors acknowledge that fetal malformations would increase the risk of fetal loss and stillbirth, though previous investigations did not reveal an increased risk of fetal loss. Most of the studied defects can be diagnosed prenatally, which could lead to interruption of pregnancy. However, the authors state that ultrasound was not available in the study area before the early 1990s and was likely non-differential in terms of Ni exposure. Many fathers of Monchegorsk neonates worked at the Ni refinery; any

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
	associations, and data availability.				per unit increase in exposure category. The incidence of polydactyly (1.6/1,000) was also relatively high.		potential effect of paternal exposure on risk of a musculoskeletal defect could blur the findings in the present study. Exposures at the refinery also included As, Cd, Pb (Thomassen, 1999).
Windham et al., 2006 San Francisco Bay Area, California	Case-control Cases = 284 Controls = 657 Cases were born in 6 San Francisco Bay Area counties in 1994, diagnosed with ASD by the 9th birthday and identified from records of the California Department of Developmental Services and the Kaiser Permanente Medical Care Program. Controls were born in the study area and randomly selected from the 1994 linked birth	Autism spectrum disorders (ASD)	US EPA estimated conc. of hazardous air pollutants (HAPs), including Ni, using a dispersion model. The 1996 HAPs conc estimates for Census tracts were used. Ni was grouped with other metals (other structural groups were aromatic solvents and chlorinated solvents; there were also groups of developmental toxicants and endocrine disruptors).	Mean ± SD estimated conc. of ambient Ni Cases: 0.0043 ± 0.0059 µg/m^3. Controls: 0.0037 ± 0.0038 µg/m^3	Referent groups for ORs are the lower quartiles of conc. AOR for autism and Ni exposure in single chemical models: 3rd quartile 1.11 (0.77, 1.59) 4th quartile 1.46 (1.04, 2.06). Ni was correlated with: As (r = 0.86) Cd (r = 0.77) trichloroethylene ($r \geq 0.77$) AORs for metals group: 3rd quartile 1.68 (1.17, 2.41) 4th quartile 1.50 (1.05, 2.12)	Final logistic regression models for Ni included child race, maternal age, and maternal education. Other covariates evaluated in statistical models: - maternal race, - parity; - paternal race and age; - LBW; - preterm delivery.	Due to correlations among chemicals, untangling the effects of specific chemicals was difficult. Actual personal exposures were not estimated. The residence-based estimates do not take into account mothers' specific activities, or mobility, although the authors state that US EPA found that modeled HAPs conc. were reasonable surrogates for personal exposure. HAP estimates were for 1996,

Study/ Location	Study Design/ Sample sizes	Outcomes of Interest	Exposure Assessment	Ni concentrations	Results	Covariates/ Confounders	Comments
	certificate file and matched 2:1 to original cases by sex and month of birth. Address, demographic data, and infant characteristics were obtained from birth certificates.		The authors calculated an index score for each chemical group by assigning a level of 1 to 4 for each quartile of conc. for each compound across the controls' census tracts, then summed across the compounds in each chemical group to obtain an overall score for that chemical group, in each census tract. The group score was assigned to all cases and controls born in that tract. Scores were then categorized into quartiles.		AORs for metals group, in models with all three structural groups: 3rd quartile 1.95 (1.23, 3.09) 4th quartile 1.74 (1.01, 3.01) (the authors noted that AORs estimated from models with all three structural groups may have been overadjusted).		whereas the subjects were born in 1994. The authors state that available air monitoring data suggest that it is unlikely that the relative conc. varied greatly between the estimates and the actual exposures. The authors note that the 1st trimester of pregnancy might be of the most concern etiologically. Other sources of chemical exposures, such as occupation, diet, and smoking, were not estimated. Aforementioned sources of exposure misclassification likely shifted effect estimates toward the null.
Zheng et al., 2011 Shanxi Province, China	Ecologic	Birth defects	The researchers collected soil samples from soil used for food	The mean (SD) concentration of Ni was 41.73 (6.67)	Adjusted RR and p-values for birth defects and quartiles of soil Ni,	Authors evaluated As, Cu, Pb, Sn, Sr, V, Zn, and Mo, and Ni.	The study was not described in detail.

Study/ Location	Study Design/ Sample sizes	Outcomes of Interest	Exposure Assessment	Ni concentrations	Results	Covariates/ Confounders	Comments
	379 cases of birth defects among 4,736 births A birth registry for the study area was created using hospital records of all live and still births in 2002-2004, and terminated pregnancies whose estimated delivery would have been in the same period. Cases were verified by doctors and all children (with or without birth defects) were examined and mothers interviewed and in a local clinic or in homes. Data were complete for 97 of 144 villages. Birth data were geocoded.		cultivation in ≥4 different locations in each village, and analyzed the samples by inductively coupled mass spectrometry. Measurements were averaged to determine each village's soil content. Soil data were geocoded. The authors focused primarily on heavy metals As, Cu, Pb, Sn, Sr, V, Zn, Mo, and Ni.	mg/kg in the study area. For analyses, soil Ni levels were grouped by quartile: 1st (referent) <37.54 2nd: 37.54 – 41.04 3rd: 41.04 – 44.86 4th: >44.86	compared to the 1st quartile: 2nd: 0.67; p = 0.0265 3rd: 0.54; p = 0.0034 4th: 0.44; p = 0.0003	Covariates in final model: As, Pb. No other covariates were specified, although the inclusion of other covariates is mentioned.	The relationship between human exposure and soil Ni content was not addressed. The authors did not report correlations among metals; if correlations among metals were high, then including them together in models may be inappropriate. Combining all birth defects together also may contribute to mixing of effects of different exposures. The authors comment that negative correlations of Ni with birth defects may be attributable to the antagonistic effect of alkaline soil on other poisonous heavy metals such as Hg, which they did not measure, and the form of Ni in the soil was not identified.

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
Zheng et al., 2014 Xiamen City, Xiamen, China	Cross-sectional 2010 1,106 pregnant women were recruited for UCB samples at the Maternal and Child Health hospital in Xiamen City, China. Cases = 73 women with adverse pregnancy outcomes. Controls = 106 women randomly selected from the remaining 1,033 women. Subjects with chronic hypertension, diabetes, or chronic renal or cardiac disease during pregnancy were excluded	Adverse pregnancy outcomes, including fetal distress, premature birth [<37 wks], macrosomia [≥4,000 g], and other unspecified outcomes. .	UCB was collected at delivery. Heavy metal and trace element concentrations were determined by inductively coupled plasma-mass spectrometry.	UCB levels, µg/L: Adverse pregnancy outcome n=58 Mean (SD) 38.82 (92.36) Median 11.63 IQR 5.07-41.28 p-value 0.732 Reference group n=68 Mean (SD) 46.32 (69.75) Median 12.65 IQR 4.53-47.62	There were no statistically significant differences in median Ni, As, Cd, Co, Cr, Cu, Mn, Pb, Sr, Ti, V, and Zn concentrations. Ti and Sb levels were higher among cases.	Maternal education and income, and infant sex were included in logistic regression models. Maternal age, height, prepregnancy weight and BMI were not associated with pregnancy outcome.	Up to 15 cases (20.5%) and 38 (35.8%) controls whose Ni levels were below the detection limit, which was not reported, appear to have been excluded. Alcohol consumption was not included in analyses due to reluctance of many respondents to report it. Many study details, e.g., how the sample was selected, are not described. Combining all adverse outcomes with various etiologies could make finding relationships between exposure and adverse effects difficult.

Table 2.2. Summary of Study Related to Ni Exposure and Developmental Outcomes

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
Yan et al., 2016 Shanxi and Hebei Provinces, China	Case-control Study area: 4 counties and city of Taiyuan in Shanxi, and 6 counties in Hebei. Women with NTD-affected pregnancies, including live and still births and terminated pregnancies, were recruited in 2003–2007. For each case, 1–2 women who delivered a healthy infant at the same hospital were matched by residence city or county and last menstrual period, as controls. 191 cases (and 261 controls were selected.	NTDs (anencephaly,spina bifida, and encephalocele)	Hair samples were cut near the scalp. ~3 cm samples of hair, to represent growth from a month before conception through 2 months after conception, were analyzed for concentrations of the 9 essential trace elements (ETMs; Fe, Zn, Cu, Co, Mn, Cr, Ni, Mo, and Sn) using inductively coupled plasma-mass spectrometry. Ni was detected in every sample.	Median (range) hair Ni conc., ng/mg: Anencephaly 0.138 (0.081 – 0.344) Spina bifida 0.139 (0.089 – 0.228) Encephalocele 0.152 (0.084 – 0.275) All NTDs 0.145 (0.089 – 0.306) Controls 0.189 (0.120 – 0.375)	AORs (CI) for Ni conc. (dichotomized with a cutoff at the control group median) and NTDs: All NTDs 0.53 (0.34–0.81) Anencephaly 0.50 (0.27–0.91) Spina bifida 0.42 (0.23–0.76) Encephalocele 0.82 (0.32–2.11) Ni concentrations above the 1st quartile were associated with decreased risk of NTDs. AORs for all NTDs and Ni by exposure quartile	Models adjusted for maternal age, education, history of previous defects, folate supplementation, fever or flu in early pregnancy, periconceptional active or passive smoking. Considered but not included in models: maternal occupation, gravidity, alcohol consumption	The authors report that Ni levels in this study were lower than levels in populations that are not near major sources of pollution, and interpret the results as indicating "deficiencies of …Ni were associated with an increased NTD risk." Ni and Zn were modestly correlated with consumption of fresh fruits and fresh green vegetables.

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
	Sample selection and case ascertainment methods were not reported. Within a week of delivery or pregnancy termination, health workers interviewed subjects about potential risk factors, including diet.				(compared to 1st quartile): 2nd 0.57 (0.32-1.03) 3rd 0.38 (0.21-0.69) 4th 0.45 (0.25-0.81) (supplemental information)		

Table 2.3. Summaries of Epidemiologic Studies of Ni Exposure and Female Reproductive Outcomes

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
Bloom et al., 2011 New York State, 1996-1997	Prospective cohort 80 nulliparous, 18-34-yr old women who were ceasing contraception to become pregnant were recruited from a cohort of anglers. Nurse-administered interview and daily diaries to record menstruation, sexual intercourse, behavioral and other risk factors. An 'at-risk' cycle was a cycle with sexual intercourse in the 'fertile window'. Effects on TTP were estimated using Cox proportional hazards models.	Time to Pregnancy (TTP)	A non-fasting blood sample was collected after interview. Additional samples were taken at other specified times. Whole blood specimens were pooled by subject and analyzed for metals	Whole blood Ni concentrations, µg/L: Non-pregnant women Mean (SD) 6.81 (1.44); Min-max 4.09-14.00 Pregnant women Mean (SD) 6.94 (1.47); Min-max 4.00-16.00 LOD = 0.003 µg/L	Ni levels were similar among women who became pregnant and those who did not. In the multivariable model, β for Ni = -0.176, p=0.79 (β < 0 suggests longer TTP). An IQR increase in blood Ni was associated with a NS 8.6% decrease in the conditional probability of pregnancy in an 'at-risk' cycle.	Models adjusted for: • Age • Parity (0, 1+) • Cigarette smoking • Alcohol use • Caffeinated beverage use • Groupings of PCB congeners (estrogenic, anti-estrogenic, other) • Serum lipids (mg/dL) • Frequency of intercourse during fertile window Metals: ^{75}As, ^{111}Cd, ^{114}Cd, ^{206}Pb, ^{207}Pb, ^{208}Pb, ^{25}Mg, ^{60}Ni, ^{62}Ni, ^{77}Se, ^{82}Se, ^{63}Zn, and ^{65}Zn	Ni (and other metal) levels were within population reference levels, with relatively low variability. The authors note that a single preconception measure of blood metals may have introduced exposure misclassification of nonpersistent metals. The sample may not be representative of the general population.

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
Maduray et al., 2017 South Africa	Case-control? (study design and subject identification and recruitment, and inclusion criteria were not reported) The authors compared hair and serum samples from pre-eclamptic and normotensive women in a large urban regional hospital in South Africa. The sampling design and subject identification and recruitment methods were not reported. Study population consisted of 43 pre-eclamptic women and 23 normotensive women. A research nurse collected clinical and demographic data.	Pre-eclampsia	Pubic hair and venous blood samples were collected from the same women for each study group. The authors analyzed the digested samples using inductively couple plasma-optical emission spectrometry.	Median ± SEM, µg/g <u>Hair</u> pre-eclamptic 6.86 ± 0.81 normotensive 8.40 ± 1.31 p=0.85 <u>Serum</u> pre-eclamptic 0.02 ± 0.0 normotensive 0.14 ± 0.0 p=0.16	Pearson correlations between hair Ni and maternal and fetal parameters were low in both groups of women. Serum Ni was also not correlated with maternal parameters; however, in the pre-eclamptic group, serum Ni was correlated with maternal age (r = 0.45), gestational age (r = –0.5), diastolic blood pressure (r=0.3), and infant weight (r= –0.35).	Other elements analyzed: As, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Pb, Se, and Zn No multivariate analyses Maternal and fetal parameters examined included maternal age, weight, BMI, diastolic blood pressure, and systolic blood pressure; and gestational age and baby weight.	

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
	Statistical analyses were T-tests, Mann-Whitney tests, and Pearson correlations.						
Zheng et al., 2015 Xiamen, China,	Case-control 96 non-smoking Polycystic ovary syndrome (PCOS) cases and 105 controls attending an army hospital's reproductive medicine center. PCOS definition: presence of ≥2 of: oligo-/amenorrhea, hyperandrogenism (&/or hirsutism), and polycystic ovaries. Exclusions: hypertension, diabetes, cardiovascular events; ≥6 months on oral contraceptives or drugs that might interfere with	PCOS and clinical characteristics: serum follicle stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E_2), prolactin, total testosterone (TT), progesterone, serum thyroid-stimulating hormone (TSH), sex hormone binding globulin (SHBG), dehydroepiandrosterone-sulfate (DHEAS), fasting glucose, fasting insulin, cholesterol, low and high density lipoprotein-C	Blood samples were collected on 2nd or 3rd day of the menstrual cycle if possible, Ni and other concentrations were determined by inductively coupled plasma-mass spectrometry.	Serum Ni concentrations, µg/L: Cases median 1.52; mean 1.96 Controls median 1.11; mean 1.23 µg/L; (P=0.000)	1-µg/L increase in Ni was associated with the following % changes: DHEAS 2.88 (unadjusted, p=0.109); 3.23 (adjusted, p=0.085) SHBG 13.19 (unadjusted, p=0.019); -12.60 (adjusted, p=0.032) fasting insulin 8.2 (unadjusted, p=0.009); 2.7 (adjusted, p=0.344) fasting glucose 1.35 (unadjusted, p=0.056); 0.98 (adjusted, p=0.175)	Analyses adjusted for: Age BMI Waist-hip ratio Other metals/elements examined: Ba, Cd, Pb, As, Cr, GA, Sr, V, Cu, Zn	Adjustment for age, BMI, and/or WHR may be inappropriate if they are causally associated with Ni, DHEAS, SHBG, insulin, or glucose concentrations. The authors state that previous research has shown elevated serum Ni concentrations in subjects with diabetes, and that the pathogenesis of PCOS has been linked to development of insulin resistance and hyperinsulinemia, which can progress to type 2 diabetes mellitus. The authors state that hyperinsulinism may cause

Study/ Location	Study Design/ Sample sizes	Outcomes of Interest	Exposure Assessment	Ni concentrations	Results	Covariates/ Confounders	Comments
	clinical and/or biochemical variables. Linear regression with natural log-transformed hormone levels.						increased androgen production and greater serum levels of free androgens. Increases in free androgens may also arise due to reduced hepatic synthesis of SHBG.

Table 2.4. Summaries of Epidemiologic Studies of Ni Exposure and Male Reproductive Outcomes

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
Danadevi et al., 2003 South India	Cross-sectional 57 welders and 57 unexposed workers who were matched to the welders on age, lifestyle, economic status, and lack of exposure to known harmful chemicals. Self-administered questionnaire for age, smoking, medication, and duration of exposure. Semen samples were collected weekly for 2 weeks after 3 days of sexual abstinence.	Sperm count, rapid linear progressive motility, slow/nonlinear progressive motility, vitality nonprogressive motility, immotility, normal morphology, head defects, tail defects, and mid-piece defects	Duration of exposure to welding fumes at the welding plant was self-reported (range 2-21 years). 28 welders and 27 unexposed workers were randomly selected from the sample for assays of Ni and Cr in blood. Ni and Cr in whole blood were quantified using inductively coupled mass spectrometry.	<u>Mean ±SD blood Ni, µg/l</u> Welders (n=28): 123.3 ± 35.2 Unexposed (n=27): 16.7 ± 5.8	Welders had poorer results for many sperm parameters. Among welders, blood Ni was associated with: • rapid linear progressive motility (linear coeffcient (r) =-0.381, p=0.045), • slow/nonlinear progressive motility (r=0.386, p=0.042), • tail defects (r=0.485, p=0.036), and • vitality (r=-0.420, p=0.026) Blood Cr was more strongly associated with the above parameters except slow/nonlinear progressive motility.	Statistical analyses did not appear to include covariates. <u>Mean ±SD</u> blood Cr, µg/l Welders: 131.0 ± 52.6 Unexposed: 17.4 ± 8.9 Smoking was not significantly associated with semen parameters.	No reported efforts to separate the effects of Ni and Cr. Other possible causes of adverse effects, including other occupational exposures, on semen parameters were not discussed.

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
Slivkova et al., 2009 Location not stated; possibly Slovak Republic	Cross-sectional 47 men who were 22-48 yrs and undergoing semen analysis at a state infertility center provided semen samples on-site. Specimens were analyzed for sperm morphology using a modified Papanicolau staining, with ≥500 sperm evaluated for each slide.	Pathological forms of spermatozoa: knob-twisted flagellum, separated flagellum, flagellum torso, broken flagellum, retention of cytoplasmic drop, acrosomal changes, large heads, small heads, flagellum ball, and other pathological forms.	Semen samples were assessed for concentrations of Ni and other metals of interest.	Semen Ni concentration, mg/kg: mean (SD) 0.40 (0.07) median 0.39 minimum 0.23 maximum 0.55	The authors do not report findings for Ni, although they do report correlations between pathological sperm and Pb, Fe, and Cd.	Pb, Cd, Fe, Cu, Zn	The study is not described in detail
Zeng et al., 2013 China	Cross-sectional The sample was selected from sub-fertile couples presenting at a hospital reproductive center in 2012 as part of another ongoing study. Of 149 men who agreed to participate, 118 men were selected after exclusions due to endocrine disease or other medical	Serum testosterone (T) LOD for T was 10 ng/dL Mean (range) T concentration 450.53 (114.95 – 941.65) ng/dL	Authors collected a single-spot urine sample and analyzed by inductively coupled plasma mass spectrometry. Urinary creatinine was determined using the picric assay.	The creatinine-adjusted urine Ni concentration (µg/g) was as follows: mean 3.55, range 0.36 – 91.16, 25th percentile 1.11, 75th percentile 2.98	Adjusted associations (β) between serum T and creatinine-adjusted urinary Ni exposure, relative to 1st quartile Ni exposure: 2nd quartile: –0.86 (–81.25, 79.53); 3rd quartile –83.79 (–163.85, –3.74); 4th quartile –36.35 (–116.31, 43.61)	Age, BMI, alcohol use, smoking status, and income were included in the models. Other potential confounders that were considered but not included: alcohol use, and education level	

Study/ Location	Study Design/ Sample sizes	Outcomes of Interest	Exposure Assessment	Ni concentrations	Results	Covariates/ Confounders	Comments
	conditions associated with infertility. Subjects completed a questionnaire and provided a 5-ml morning peripheral blood sample.				When simultaneously including multiple metals and potential confounders, Ni was not retained in the final model.	The authors included potential confounders in the multivariate model if they changed effect estimates for at least one metal exposure by 10% or more.	
Sancini et al., 2014 Italy	Cross-sectional 274 men were selected from 359 municipal police who worked in outdoor tasks in 8 areas of a large city. A subsample of 12 men (8 traffic police in different areas, and 4 police drivers) were selected for air monitoring. Exclusions: age >50 yrs; exposure to solvents, paints, or pesticides; other outdoor work; illicit drug use; heavy	Plasma testosterone	Subjects provided blood and urine samples at the end of a work week. Urinary Ni was determined by complexation with ammonium pyrrolidinedithiocarbamate and atomic absorption analysis in graphite furnace, with LOD 1.0 mg/g of urinary creatinine Air samples were collected for a 7-hour shift the same day as blood and urine sampling using cyclones with a cut-point for 5 micron diameter particles, attached at the workers' breathing area.	Urine Ni mean (SD), mg/100 mL Smokers: 4.78 (3.68) Non-smokers 4.62 (4.22) Air Ni conc. mean (SD), ng/m^3: Traffic police 178.4 (142.3) Police drivers 113.2 (123.4)	Log Ni in air significantly predicted urine Ni (β=0.924, p=0.000) in multiple regression. Log urinary Ni was a strong predictor of log plasma testosterone in multiple regression analyses; adjusted β= -0.466 (p=0.000) in the total sample.	Job position (traffic police, drivers, motorcyclists, and outdoor workers with other tasks), age, seniority, smoking habit were included in regression analyses (though the latter 3 covariates were not associated with urine Ni). Correlation analyses were stratified by age (20-35, 36-45, >45 yrs) and	Exclusion of workers with urinary Ni <LOD may not be appropriate. No other PM species, other air pollutants, or other potential exposures were explored.

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
	drinking; shift or night work; competitive sports; urinary Ni <LOD. Self-administered questionnaires for clinical case history and lifestyle data.					seniority (<10, 10-20, 21-40 yrs).	
Skalnaya et al., 2015 Russia	Cross-sectional 148 adult males (not otherwise described) provided semen samples.	Ejaculate volume, absolute and relative sperm count, motility, and vitality	ICP-MS was used to analyze ejaculate for metals.	<u>Ni in ejaculate, µg/mL</u>: Mean (SD) 0.026 (0.022) Median 0.021 Min 0.005 Max 0.197	Ejaculate volume <1.5 mL was associated with Ni (Mann-Whitney U-test p=0.015), Cu, Mn, and Mo. Dichotomized absolute sperm count, relative sperm count, progressive sperm motility, and sperm vitality were not associated with semen Ni.	20 metals: Al, As, Be, Bi, Cd, Co, Cr, CU, Fe, Hg, I, Li, Mn, Mo, Ni, Pb, Se, Sn, V, Zn	The study is not described in detail. Analyses are for single metals.
Zeng et al., 2015 China	Cross-sectional The sample was selected from sub-fertile couples presenting at a hospital reproductive center in 2011-2012 as part of	Semen quality (sperm count, concentration motility, morphology)	Urinary concentrations of 13 metals (As, Ca, Co, Cr, Cu, Fe, Pb, Mn, Mo, Hg, Ni, Se, Zn) were determined from a single-spot urine sample. Metal concentrations were adjusted for urine creatinine	Urine Ni conc., µg/g creatinine Geometric mean 1.46 Min 0.01 25th %ile 0.87 Median 1.53 75th %ile 2.47 Max 91.16	Urine Ni conc. was not associated with sperm count or conc. < reference level, or % normal morphology.	Age, abstinence time (3−5 and >5 vs <3 days), and smoking status (current and former vs never smoker)	Use of a single urine sample may result in misclassification of Ni exposure, given the short half-life of Ni in urine.

Study/ Location	Study Design/ Sample sizes	Outcomes of Interest	Exposure Assessment	Ni concentrations	Results	Covariates/ Confounders	Comments
	another ongoing study. 2,540 men were invited to participate regardless of knowledge of fertility status. After excluding men with azoospermia, medical conditions that might alter semen quality, or endocrine disease, 394 men were randomly selected. Sperm conc., motility, and count were dichotomized as < or ≥ World Health Organization (WHO) reference values. Men whose measurements ≥ WHO reference values for all of these parameters defined the comparison group. Multivariable linear and logistic regression				AORs for % motile sperm < reference level and quartiles of urine Ni conc: 1st quartile 1.00 2nd 0.80 (0.45, 1.44) 3rd 0.77 (0.43, 1.39) 4th 0.67 (0.37, 1.02) "Ni was significantly associated with an increasing trend for sperm abnormal head": regression coefficients (β) for % abnormal head and quartiles of urine Ni conc: 1st 1.00 2nd -1.65 (-3.9, 0.60) 3rd 0.92 (-1.32, 3.16) 4th 1.67 (-0.57, 3.92) p trend=0.03 When multiple metals were included to predict abnormal head, Ni and Cr remained in the	were included in the models. Potential confounders considered but not included were: BMI, alcohol use, education level, income, and tap water consumption.	Correlations between Ni and other metals ranged between 0.24 and 0.59.

Study/ Location	*Study Design/ Sample sizes*	*Outcomes of Interest*	*Exposure Assessment*	*Ni concentrations*	*Results*	*Covariates/ Confounders*	*Comments*
					model, and the trend test for the association between % abnormal head and Ni conc. was significant, p=0.01		
Wang et al., 2016 China	Cross-sectional The sample was selected from sub-fertile couples presenting at a hospital reproductive center in 2013 as part of another ongoing study. 1,247 men were invited to participate regardless of knowledge of fertility status. After excluding men with azoospermia, medical conditions that might alter semen quality, or endocrine disease, 1,052 participants were selected. Subjects provided a blood sample,	Spermatozoa apoptosis (n=460 samples) Sperm DNA integrity (n=516 samples) Reproductive hormones (n=511 samples): estradiol, follicle stimulating hormone (FSH), luteinizing hormone (LH), sex-hormone binding globulin (SHBG), total testosterone (T) Samples from 171 men were measured for all 3 sets of outcomes.	The two spot urine samples were separated by approximately four hours. Urine samples were analyzed for 18 metals: Al, As, Cd, Co, Cr, Cu, Fe, Pb, Mn, Mo, Ni, Sb, Se, Sn, Tl, U, W, and Zn Limit of quantification [LOQ] was not reported	Urine Ni conc., µg/L 1st sample (6.8% < LOQ) Geometric mean 2.0 Median 2.4 IQR 1.3–4.1 2nd sample (7.1% < LOQ) Geometric mean 1.8 Median 2.3 IQR 1.2–4.0 Values < LOQ were assigned LOQ/√2	The highest quartile of Ni exposure was associated with an adjusted 20% (CI −38, −4.2%) lower total T/LH ratio when compared with the lowest quartile. Further adjusted for other metals (Cr, Zn, Mo, Sn, Tl), the highest quartile of Ni was associated with 14% (CI 32, 2.0%) lower T/LH ratio. Cubic spline analysis of metals and T/LH indicated a non-linear relationship. Similar findings were reported for Zn and Mo.	Included in models: age, BMI, smoking status, daily cigarette consumption, and urinary creatinine. Some models also included other metals. Potential confounders that were considered but did not change effect estimates ≥10%, and were not included in multivariate models: alcohol use, education level, abstinence time (3−5 and	The authors state that lower total T/LH ratio indicates impaired Leydig cell function. Correlations among metals are not reported and could be an important consideration in analyses that include multiple metals.

Study/ Location	Study Design/ Sample sizes	Outcomes of Interest	Exposure Assessment	Ni concentrations	Results	Covariates/ Confounders	Comments
	semen sample and 2 spot urine samples, and answered a questionnaire during the clinic visit. Blood samples were analyzed for hormone levels. Multivariable linear models; variables with significant or suggestive associations after false discovery rate (FDR) correction were retained.				After FDR adjustment, Ni was not associated with sperm apoptosis parameters or sperm DNA integrity parameters.	>5 vs <3 days), income	
Zhou et al., 2016 China	Cross-sectional The sample was drawn from couples presenting at a hospital fertility clinic in 2012 as part of another ongoing study. 252 infertile men agreed to participate. The authors did not report criteria for determining	Sperm DNA damage Parameters evaluated: tail length, percent DNA tail, and tail distributed moment	A single urine sample was used to determine concentrations of 13 metals, including As, Cd, Cr, Co, Cu, Pb, Fe, Mn, Hg, Mo, Ni, Se, and Zn, using inductively coupled plasma mass spectrometry. LOD for Ni was 0.03 µg/L Urinary creatinine was analyzed by the picric acid assay. Urine Ni concentrations were adjusted for creatinine.	Urine Ni conc., µg/g creatinine: mean 2.29, geometric mean 1.03, 25th percentile 0.60, 50th percentile 1.05, 75th percentile 1.98, range 0.01–91.16	Urinary Ni and Hg concentrations were associated with significant increasing trends for tail length (both p for trend < 0.05). Men with Ni values in the 4th quartile of exposure had a significant increase in tail length of 2.74	Age, BMI, smoking status, and abstinence time were included in the multivariate models. Potential confounders that were considered but not included in multivariate models	Correlations among metals are not reported and could be an important consideration in analyses that include multiple metals.

Study/ Location	Study Design/ Sample sizes	Outcomes of Interest	Exposure Assessment	Ni concentrations	Results	Covariates/ Confounders	Comments
	infertility. After excluding men with azoospermia, medical conditions that might alter semen quality, or endocrine disease, 207 men were selected. Subjects provided a semen sample and a spot urine sample, and answered a questionnaire. Sperm DNA damage was measured using the neutral comet assay test.				µm (CI: 0.36, 5.12) compared to the 1st quartile. After inclusion of multiple metals, the effect was 2.95 µm (CI: 0.34, 5.56) The authors conclude that environmental exposure to Ni may result in increased sperm DNA damage.	because they did not change effect estimates ≥10%: race, alcohol use, education level, income	
Zafar et al., 2015 Pakistan	Study design, including population sampling and selection criteria were not stated. 75 male partners of infertile couples were selected. Time frame was not stated. Each patient completed a	Semen quality Parameters such as liquefaction time, volume, pH, viscosity, presence of pus or epithelial cells, semen quantity, sperm motility, sperm count and sperm	Concentrations of 17 different metals were measured in seminal plasma using inductively coupled plasma mass spectrometry.	Mean ±SD semen Ni conc., ppb, in the 3 groups were: normozoospermic (3.07 ±1.63), oligozoospermic (1.92 ±0.77), azoospermic (10.49 ±10.94).	Ni conc. was negatively correlated with sperm concentration (r = -0.26), sperm volume (r = -0.44) and motility (r = -0.33), $p < 0.05$ for each correlation. The authors reported that Ni	Although the authors collected questionnaire data on potential confounders, the covariates were not included in statistical analyses or further discussed.	Limitations include the generalizability of the results due to the nature of the sample population.

Study/ Location	Study Design/ Sample sizes	Outcomes of Interest	Exposure Assessment	Ni concentrations	Results	Covariates/ Confounders	Comments
	questionnaire concerning occupation, residence, diet, alcohol use, water source, smoking, height, weight, and medical history. Semen samples were obtained after 3-5 days of sexual abstinence and assessed for semen quality parameters. Seminal plasma samples from the participants were classified into 3 groups of 25 men: normozoospermia; oligozoospermia; and azoospermia. Normozoospermic men were designated as the control group.	morphology were measured.			and Cd concentrations in seminal plasma showed significant differences in all three groups (p = 0.01). Ni was highly correlated (>0.70) with Cd, Cu, Sn, and V. Concentrations of most metals were higher in azoospermic participants compared with the other two groups. Semen volume, sperm concentration, and sperm motility were statistically significantly different ($p<0.05$) among the three groups.		

Appendix 3: Parameters for Literature Searches on the Reproductive Toxicity of Nickel and Nickel Compounds

A search of the literature on the reproductive and developmental toxicity of nickel and nickel compounds was conducted under contract by the University of California, Berkeley (Charleen Kubota, M.L.I.S.). The goal was to identify peer-reviewed open source and proprietary journal articles, print and digital books, reports and gray literature that potentially reported relevant toxicological and epidemiological information on the reproductive and developmental toxicity of nickel and nickel compounds. The search sought to identify all literature relevant to the assessment of evidence on male reproductive, female reproductive and developmental toxicity.

Databases

The literature search utilized the following search platforms/database vendors:
ChemSpider (Royal Society of Chemistry)
MeSH (Medical Subject Headings) (National Library of Medicine)
Developmental and Reproductive Toxicology Database (DART/ETIC) (National Library of Medicine)
EMBASE® (Elsevier)
Environmental Sciences and Pollution Management (Proquest)
PubMed (National Library of Medicine)
National Technical Research Library (NTRL v3.0) (National Technical Information Service)
ReproRisk® System: **REPROTEXT®** Reproductive Hazard Reference**, REPROTOX®** Reproductive Hazard Information, **Shepard's Catalog of Teratogenic Agents**, **TERIS Teratogen Information System (RightAnswer® Knowledge Solutions OnSite™ Applications)**
Scifinder®: CAS (Chemical Abstracts Service)
TOXLINE (National Library of Medicine)
Web of Knowledge: BIOSIS Previews®, Web of Science® (Thomson-Reuters, Inc.)

Search Process

ChemSpider was searched first to gather chemical names, synonyms, CAS registry numbers, MeSH and Chemical Abstracts Service headings for nickel and nickel compounds before searching bibliographic databases. The MeSH database was used to identify relevant subject headings for reproductive and developmental toxicology endpoints. Relevant subject terms were entered into the PubMed Search Builder to execute a PubMed search.

The following is a typical DART chemical search strategy used to search PubMed:

("*chemical name*" [MeSh] OR CAS *registry number*[RN]) AND ("Congenital Abnormalities"[MeSh] OR "Pregnancy Complications"[MeSh] OR "Reproductive Physiological Phenomena"[MeSh] OR "Embryonic and Fetal Development"[MeSH])

In PubMed, MeSH (Medical Subject Headings) terms at the top of hierarchical lists of subject headings are automatically "exploded" in a search to retrieve citations with more specific MeSH terms. For example, the heading "Congenital Abnormalities" includes numerous specific conditions such as spina bifida and congenital heart defects. The broad subject heading "Pregnancy Complications" encompasses multiple conditions or pathological processes associated with pregnancy. Spontaneous abortion and many fetal diseases are listed under this term.

Staff checked citations from retrieved articles and relevant government documents to capture any additional references not identified in the primary search. Some of these studies are not available in the general literature.

Additional databases listed above were then searched. The search strategies were tailored according to the search features unique to each database. Web of Science, for example, was searched by entering chemical terms and refining the search by applying Web of Science categories Developmental Biology, Toxicology and/or Public, Environmental and Occupational Health. Sometimes other databases not listed here were searched as needed. For example, if there is a known behavioral endpoint linked to chemical exposure, a social science database such as PsycINFO® would be searched.