OFFICE OF ENVIRONMENTAL HEALTH HAZARD ASSESSMENT

Proposition 65

Reconsideration of Methyl n-Butyl Ketone Listed under Proposition 65 as Known to Cause Reproductive Toxicity

(Chemical Listed via the Labor Code Mechanism)

2015 Update

and

Consideration of 2,5-Hexanedione for Listing under Proposition 65 as Known to Cause Reproductive Toxicity

August 2015

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

Contents

Introduction

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 the lead agency for implementation of Proposition 65[2].

Methyl n-butyl ketone (MnBK, CAS No: 591-78-6, also known as 2-hexanone) was added to the list as known to cause reproductive toxicity (male endpoint) on August 7, 2009 because it was identified by reference as such in California Labor Code section 6382(d)[3]. Specifically, this identification was the Threshold Limit Value (TLV) for MnBK, developed by the American Conference of Governmental Industrial Hygienists (ACGIH) based on male reproductive toxicity – "testicular damage". Until 2012, the federal Hazard Communication Standard (HCS) incorporated TLVs as a definitive source for establishing that a chemical is hazardous.

In March 2012, the federal HCS was amended to remove reference to ACGIH TLVs as a mandatory basis for establishing that chemicals are hazardous. Consequently, TLVs based on reproductive or developmental toxicity no longer provide the basis for listing a chemical as known to the state to cause reproductive toxicity under Proposition 65.

Because the basis for listing under the Labor Code provision was no longer applicable, MnBK was presented to the Developmental and Reproductive Toxicant Identification Committee (DARTIC), the state's qualified experts for reproductive toxicity under Proposition 65[4], on March 19, 2014 for a decision as to whether it has been clearly shown through scientifically valid testing according to generally accepted principles to cause reproductive toxicity.

At that meeting, the DARTIC deferred a decision on MnBK and requested that OEHHA attempt to procure additional information on studies of the reproductive toxicity of MnBK, in particular from the study conducted by the National Institute for Environmental Health Sciences (NIEHS) (Peters et al., 1981). The DARTIC also identified concerns

[1] The Safe Drinking Water and Toxic Enforcement Act of 1986, codified at Health and Safety Code section 25249.5 *et seq.,* commonly referred to as Proposition 65.
[2] Health and Safety Code section 25249.12, Title 27, Cal. Code of Regs., section 25102(o)
[3] Health and Safety Code section 25249.8(a) requires that substances identified in Labor Code section 6382(d) as causing reproductive toxicity be included on the Proposition 65 list. Labor Code section 6382(d) captures any chemicals within the scope of the federal Hazard Communication Standard that are identified as reproductive toxicants.
[4] Title 27, Cal Code of Regs, section 25302(a)

about 2,5-hexanedione (2,5-HD), a metabolite of MnBK, and requested that information on that metabolite be provided to them when they again considered whether MnBK should be retained on the Proposition 65 list.

Accordingly, OEHHA contacted NIEHS to ascertain whether additional data from the study in question were available. OEHHA also, through a contract with the Sheldon Margen Public Health Library at the University of California, Berkeley, conducted literature searches to identify studies that potentially provide information on the reproductive toxicity of MnBK and 2,5-HD. The searches covered the three major reproductive toxicity endpoints; namely, developmental toxicity, male reproductive toxicity and female reproductive toxicity. Additionally, a search was conducted to identify studies that describe the metabolism of MnBK to 2,5-HD.

The results of these searches were reviewed by OEHHA staff and all studies that provided data on reproductive toxicity were identified. The design parameters and results of these studies on male reproductive, female reproductive and developmental toxicity are summarized in this document in separate tables for each chemical and endpoint, and the complete study reports for these chemicals have been provided to the DARTIC and are available to the public upon request. Information on the metabolism of MnBK to 2,5-HD is also provided. Because MnBK is metabolized to 2,5-HD in the body, data on the reproductive and developmental toxicity of 2,5-HD is relevant to the potential identification of MnBK as causing reproductive toxicity under Proposition 65. The DARTIC will also consider whether 2,5-HD itself has been clearly shown through scientifically valid testing according to generally accepted principles to cause reproductive toxicity.

Methyl n-Butyl Ketone Metabolism to 2,5-Hexanedione

The pharmacokinetics and metabolism of the hexacarbon solvent MnBK have been reviewed by the US Environmental Protection Agency (US EPA, 2009). Reviews by Boekelheide and Schoenfeld (2001) and Boekelheide et al. (2003) also summarize the information which demonstrates that 2,5-HD is the predominant metabolite of MnBK.

Studies of ^{14}C-labeled MnBK in rats have helped characterize the metabolic fate and disposition of this water-soluble compound (Couri and Milks, 1982). Following oral doses in the rat, MnBK was almost completely absorbed, extensively metabolized and rapidly eliminated in expired air and urine. These authors reported that 48 hours after dosing approximately 83% of the radioactivity was recovered in the breath and urine with about 16% remaining in the carcass and 1% excreted in the feces. Bus et al. (1981) reported that in inhalation studies in F344 rats metabolism of MnBK to 2,5-HD proceeded rapidly, while further metabolism of 2,5-HD and its elimination proceed much more slowly. In rats dosed by intraperitoneal (i.p.) injection the peak blood level of MnBK was reached in 30 minutes and declined biphasically - the half-life of MnBK for the rapid elimination phase was about 10 minutes and about 7 hours in the following slow phase (Abdel-Rahman et al., 1976).

In the guinea pig, the amount of MnBK found in the blood compartment at 1 hour after i.p. injection was 1.4% of the original dose, suggesting rapid and extensive distribution (DiVincenzo et al., 1976). The half-life and clearance time of MnBK in serum was 78 minutes and 6 hours, respectively (DiVincenzo et al., 1976).

Several studies in rats and guinea pigs have demonstrated that MnBK undergoes metabolism by a variety of pathways, including reduction to 2-hexanol, or cytochrome P450-mediated ω-1 oxidation to 5-hydroxy-2-hexanone (5H2H) (DiVincenzo et al., 1976; Scala, 1976; Krasavage et al., 1980; Couri and Milks, 1982; Huang, 2008). Similar metabolism also is thought to occur in humans (DiVincenzo et al., 1978). The specific Phase I and Phase II enzymes involved in MnBK metabolism are not known. Metabolites of MnBK which have been identified in serum include 5H2H and 2,5-HD (Krasavage et al., 1980; Couri and Milks, 1982). The predominant metabolite identified in serum is 2,5-HD (DiVincenzo et al., 1976). Urinary metabolites of MnBK have been reported to include 2-hexanol, 2,5-hexanediol, 5H2H, and 2,5-HD (Couri et al., 1978, Abdel-Rahman et al., 1976; Eben et al., 1979).

As outlined in the metabolic scheme in Figure 1 below, MnBK can be oxidized or reduced, leading to the formation of 5H2H or 2-hexanol, respectively. 2,5-HD can be

formed from both of these initial metabolites as a result of further oxidation reactions. From the work of DiVincenzo et al. (1976), oxidation appears to proceed by hydroxylation of the ω-1 carbon, forming 5H2H, while reduction occurs at the carbonyl group to form the secondary alcohol, 2-hexanol. DiVincenzo et al. further concluded that 5H2H either undergoes oxidation to 2,5-HD or reduction to 2,5-hexanediol. The initial reductive metabolite of MnBK, 2-hexanol, can be converted back to MnBK (via oxidation), or metabolized to 2,5-hexanediol (via ω-1 oxidation). 2,5-hexanediol, in turn, can be oxidized to form 5H2H.

US EPA (2009) concluded that although the proportions of metabolites may differ across species, ω-1 oxidation and carbonyl reduction appear to be initial steps in the metabolism of MnBK in rats, cats, dogs, guinea pigs, and humans.

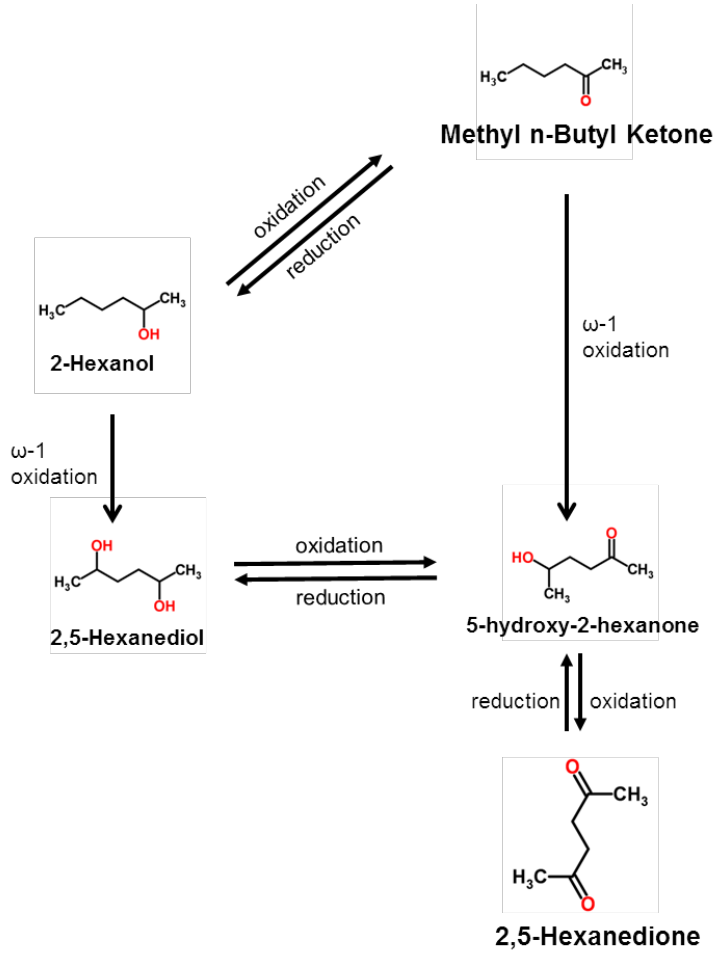
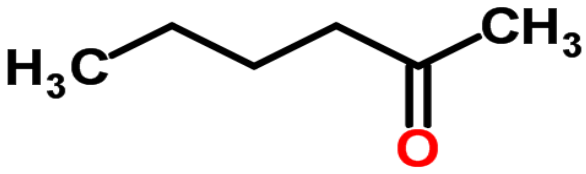


Figure 1. Schematic metabolic pathway for MnBK, modified from Krasavage et al., 1980



Methyl n-butyl ketone (MnBK)

Molecular Formula: $C_6H_{12}O$

MnBK is a solvent used in a wide variety of materials including paints, lacquers, ink thinners, nitrocellulose, glues, resins, oils, fats and waxes, and in printing of plasticized fabrics.

Relevant Studies

Katz GV, O'Donoghue JL, DiVincenzo GD and Terhaar CJ (1980). Comparative neurotoxicity and metabolism of ethyl n-butyl ketone and methyl n-butyl ketone in rats. *Toxicology and Applied Pharmacology* **52**(1): 153-158.

Krasavage WJ, O'Donoghue JL, DiVincenzo GD and Terhaar CJ (1980). The relative neurotoxicity of methyl-n-butyl ketone, n-hexane and their metabolites. *Toxicology and Applied Pharmacology* **52**(3): 433-441.

Peters MA, Hudson PM and Dixon RL (1981). The effect totigestational exposure to methyl normal-butyl ketone has on postnatal-development and behavior. *Ecotoxicology and Environmental Safety* **5**(3): 291-306.

Tables 1 and 2 below include the same studies presented at the March 19, 2014 DARTIC meeting with additional details from the published studies added. OEHHA attempted to retrieve additional information from NIEHS as requested by the committee. No additional information on the Peters et al. (1981) study was available from NIEHS.

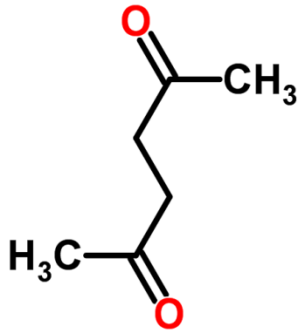
An additional literature search did not identify any other reproductive or developmental toxicity studies of MnBK.

Table 1. MnBK: Study Reporting on Developmental Effects

Reference	Experimental Parameters					Endpoints Assessed	Results (Effects/NOEL/LOEL)		Comments
	Chemical (Source/ Purity/ Preparation)	Animal Model (Species/ Strain/Sex/ Age) N	Study Design	Exposure (Route/Period/ Frequency/ Vehicle)	Doses/ Concen-trations		Parental	Developmental	
Peters et al., 1981	Methyl n-butyl ketone Source/purity not stated	Fischer 344 rats Female Weight 150g N = 25/group	Developmental neurotoxicity study; pups weaned at PND 28; One control group for each dose group, plus one pair-fed to the high dose group. Testing for several neurological endpoints. Also noted was the duration of time animals slept after a hypnotic dose of pentobarbital (45 mg/kg in 14-week old or pubertal animals and 25 mg/kg in 18-month old or geriatric animals).	Inhalation GD0-GD20 6 h/day	0, 500, 1000, 2000 ppm.	Daily maternal weights; pregnancy outcome at birth; PND2 behavior observation of pups; postnatal developmental indices; week 4, 8, 12 and month 18-20 gross and histopathology and behavioral test battery; (Not all tests at all ages). Righting reflex and inclined screen tests; open-field activity; food maze behavior; activity wheel, swimming stress test; shock avoidance learning. Indicators of normal development included time of eye opening, and weight gain.	↓ maternal weight gain 1000 ppm (10%), 2000 ppm (14%); clinical signs 2000 ppm hair loss, lack of muscular coordination and weakness by GD20; statistics not given; NOEL 500 ppm	↓litter size, birthweight (2000 ppm); ↓ postnatal and adult weights (males, 1000, 2000 ppm); dose-dependent ↓ in weight gain in male offspring persisting though out life. ↓ grip strength, maze latency, activity (1000, 2000 ppm male and/or female, at least one age). Pentobarbital ↑ sleeping time (2000 ppm, adult males). ↓ testes weights in weanlings (at 1000 ppm); ↑ ovarian cysts (at 1000 ppm.and 2000 ppm at 18 months; ↓neurological activity (running behavior, measured using the activity wheel) at 1000 ppm suggests possible premature aging of offspring	Methods well reported; data not all reported. According to the authors, maze behavior suggests an alteration in motivation, goal-oriented pursuit and/or ability to learn a simple task. No statistics.

Table 2. MnBK: Studies Reporting on Male Reproductive Effects

Reference	Experimental Parameters					Endpoints Assessed	Results (Effects/NOEL/LOEL)		Comments
	Chemical (Source/ Purity/ Preparation)	Animal Model (Species/ Strain/Sex/ Age) N	Study Design	Exposure (Route/Period/ Frequency/ Vehicle)	Doses/ Concen-trations		Systemic Toxicity	Reproductive Toxicity	
Katz et al., 1980	Methyl n-butyl ketone Source not stated 96.1% pure	CD rats Male N = 5/group	Adult neurotoxicity study with terminal necropsy	Inhalation 72 h of exposures per week for 81 days (2 20-hour and 2 16-hour exposure periods/week)	0, 700 ppm	Body weights, clinical chemistry, gross and histopathology, neurotoxicity	Markedly ↓ weight gain; ↓ WBCs	↓ testes weights; atrophy of the testicular germinal epithelium described, no other data presented	2,5-hexadione in serum; authors report the effects are significant but p-values not presented
Krasavage et al., 1980	Methyl n-butyl ketone Source not stated 96.1% pure	CD rats Male N = 5/group	Adult neurotoxicity study with terminal necropsy	Gavage 5 days/week for 90 days	0, 660 mg/kg	Body weights, gross and histopathology, neurotoxicity	↓ body weight gain	Atrophy of the testicular germinal epithelium described, no data presented	No statistics or data for testes



2,5-Hexanedione (2,5-HD)

Molecular Formula: $C_6H_{10}O_2$

2,5-Hexanedione (2,5-HD, CAS No. 110-13-4) is used as a starting reagent in the synthesis of trans-2,5-dimethylpyrrolidine and other pyrroles. It is also described as a "reagent used for the protection of amino groups in amino sugars and nucleosides; for the synthesis of five-membered heterocycles, in particular 2,5-dimethyl aminopyrroles, indane-type and benzannulated systems" [5].

Relevant Studies

Two publications on female reproductive toxicity, four publications on developmental toxicity, and 38 studies on the male reproductive toxicity of 2,5-HD were identified. The scientific literature on the male reproductive toxicity of 2,5-HD, the primary bioactive metabolite of MnBK, is extensive because the compound is a model chemical for testicular toxicity.

Studies have established that bioactivation of MnBK to 2,5-HD is required for its neural (Scala, 1976; Krasavage et al., 1980; Couri and Milks, 1982; Huang, 2008) and most likely testicular toxicity (Boekelheide et al., 2003). Consequently, 2,5-HD is a model chemical used to induce testicular toxicity in rodents and for comparative studies with other chemical agents.

Two reviews by the Boekelheide group (Boekelheide and Schoenfeld, 2001; Boekelheide et al., 2003) summarize the effects of 2,5-HD. The 2001 review refers to 2,5-HD as the toxic metabolite resulting from oxidation of the commonly-used solvent MnBK, and describes the experimental model (rats exposed to 1% 2,5-HD in the drinking water for a period of 3 - 5

[5] On-line encyclopedia of reagents for organic synthesis (http://onlinelibrary.wiley.com/doi/10.1002/047084289X.rn00460/abstract).

weeks) and its toxic effects, especially in the rat testes. The toxicity resulting from exposure to 2,5-HD or its precursor (MnBK) is a slowly progressive peripheral polyneuropathy as well as testicular injury that has the Sertoli cell as a target. Specifically, the 2,5-HD testicular effects are the loss of germ cells by apoptosis and testicular atrophy. In addition, the second review (Boekelheide et al., 2003) summarizes the direct toxicity of 2,5-HD in the rat testes, concentrating on discussion of the mechanism of action that explains the toxic effects of 2,5-HD in the testes.

Study design parameters and findings of each of these studies[6] on 2,5-HD are summarized in the following tables:

Table 3. 2,5-HD: Studies Reporting on Female Reproductive Effects
Table 4. 2,5-HD: Studies Reporting on Developmental Effects
Table 5. 2,5-HD: Studies Reporting on Male Reproductive Effects

Citations for the tabulated studies are provided in the References section following the tables.

[6] Reports published only as abstracts are not tabulated.

Table 3. 2,5-HD: Studies Reporting on Female Reproductive Effects

Reference	Experimental Parameters					Endpoints Assessed	Results (Effects/NOEL/LOEL)		Comments
	Chemical (Source/ Purity/ Preparation)	Animal Model (Species/ Strain/Sex/ Age) N	Study Design	Exposure (Route/Period/ Frequency/ Vehicle)	Doses/ Concen-trations		Systemic Toxicity	Reproductive Toxicity	
Siracusa et al., 1992	2,5-HD Fluka AG, Buchs, Switzerland Re-purified by reflux distillation Purity not stated	Swiss CD1 mice Virgin female 8 weeks old N = 15/group	Effects on female reproduction: Ovarian biochemistry and histology. Fertility: Effect on total reproductive capacity	Drinking water for 4 or 6 weeks	0, 1.5% (v/v) in water	BW; Morphology: Light microscopic observation and oocyte morphometry Fertility: Presence of newly born mice	BW 10% ↓ at 4 weeks ($p< 0.01$) and 14% ↓ at 6 weeks ($p< 0.0001$)	31% ↓ in protein content/ovary ($p<0.01$) and 21% ↓ in the DNA content/ovary ($p <0.05$). 25% fewer medium (growing) oocytes after 6 weeks ($p<0.01$). The 6-week treatment regression line differed significantly from the control group ($p<0.00001$), which indicates a faster ↓ in litter size with time in the treated animals. The 4-week regression line did not significantly differ from control	
Zhang et al., 2013	2,5-HD Sigma, St Louis, MO, USA > 98% pure	Wistar rats Female 21 days old N = 45	In vitro study: Granulosa cell viability	10^6 cells/ml plated in different wells and exposed to 2,5-HD in the culture medium, for 0, 12, 24 or 36 h exposure	0, 20, 40, 60 mmol/L	Cell viability; Apoptosis after a 12 h exposure by TUNEL assay	N/A	12h exposure: ↓ cell viability at 60mM ($p<0.05$). 24h exposure: ↓ cell viability in all treated groups ($p<0.05$). Dose and time had a negative correlation with cell viability ($p<0.01$). ↑ apoptotic index (assessed at 12h) in all treated groups ($p<0.05$)	Control had ↓ viability at 36h ($p<0.05$) compared to 0 h.

Table 4. 2,5-HD: Studies Reporting on Developmental Effects

Reference	Experimental Parameters					Endpoints Assessed	Results (Effects/NOEL/LOEL)		Comments
	Chemical (Source/ Purity/ Preparation)	Animal Model (Species/ Strain/Sex/ Age) N	Study Design	Exposure (Route/Period/ Frequency/ Vehicle)	Doses/ Concen-trations		Parental	Developmental	
Moretto et al., 1991	2,5-HD Source/purity not stated	Dorsal root ganglion (DRG) cells from aborted human fetuses (gestational age of 9-11 weeks) cultured in vitro	In vitro study to assess morphological and morphometric changes of developing ganglia	In vitro exposure of cultured dissociated DRG cells for 2 weeks	0, 2.8 mM	Daily examination of living cells for sprouting and elongation of dendritic processes Ultrastructural examination of cross-sections for axonal effects, number of neurofilaments (NF) per unit area and microtubule (MT) density	N/A	Diffuse modifications of cytoskeletal components occurred in treated human DRG cells in culture. Focal NF-containing enlargements in distal, preterminal regions of unmyelinated fibers, ↓ density of NF and ↓ axonal size in more proximal segments. 30% lower mean cross-sectional area of axons, corresponding to a 50% ↓ in NF density	Induced re-organization of the whole axonal cytoskeleton resulting in longitudinal re-distribution of NF polypeptides

Reference	Experimental Parameters					Endpoints Assessed	Results (Effects/NOEL/LOEL)		Comments
	Chemical (Source/ Purity/ Preparation)	Animal Model (Species/ Strain/Sex/ Age) N	Study Design	Exposure (Route/Period/ Frequency/ Vehicle)	Doses/ Concen-trations		Parental	Developmental	
Ogawa et al., 1991 and 1993	2,5-HD Source/purity not stated As reported in Ogawa et al. (1993): Tokyo-Kasei-Kogyo, Tokyo, Japan >97% pure	Sprague-Dawley rats Pregnant female 12 weeks old weighing 256-293 g N = 6 Control N = 6 (340 mg/kg) N = 5 (680 mg/kg)	Exposure of pregnant rats Dams sacrificed on GD20	Subcutaneous injection (s/c) (volume: 0.68 ml/kg body weight) once a day from GD12 - GD16, or GD12-GD19 (340 mg/kg dose) 0.9% saline vehicle	0, 340, 680 mg/kg	Maternal BW, Fetal BW, sex, viability, external abnormalities; sciatic nerves (fetal axons) examined.	Significant ↓BW in exposed pregnant rats. No dose-dependent differences in reproductive status such as the number of implants, the number of resorptions, and the number of live fetuses per pregnant female	Significant ($p < 0.01$) ↓ in the mean live fetal BW of treated group. No external abnormalities observed in any live fetuses. Degeneration in fetal sciatic nerves. Irregularly-shaped large axons. Vacuoles and irregularly-distributed NF in the large axons. Fusion of axons and axonal enlargement without aggregation of NF. The severity of morphological changes was dose-related.	Axonal fusion may be explained by the selective ↓ in the cholesterol content of the axolemma
Cheng et al., 2012	2,5-HD Sigma–Aldrich, MO, USA Purity not reported	Fertilized Leghorn chicken eggs from Avian Farm of the South China Agriculture University N =13 Control N =10 (10 and 1000 nM) N =14 (100 mM)	Chick embryo direct exposure study.	Direct injection (100 µl volume) into the air chamber of the egg. After treatment, embryos were incubated for a further 10 hrs or 4 days and then harvested for analysis on day 6 Phosphate-buffered saline (PBS) vehicle	0 (vehicle), 10, 100, 1000 mM	BW; malformation; cell viability NTD (neural tube defect); NF expression; Neurite growth assay		Significantly ↓ BW at 10, 100 and 1000 mM. Approximately 70% embryo lethality in 1000 mM group. Various types of CNS deformities seen at 10 mM. ↑ NTD at all doses weaker NF expression at 100mM. Abnormal forebrain ventricle following treatment "…vivid disorganized structure of the neural tubes..."	

Table 5. 2,5-HD: Studies Reporting on Male Reproductive Effects

Reference	Experimental Parameters					Endpoints Assessed	Results (Effects/NOEL/LOEL)		Comments
	Chemical (Source/ Purity/ Preparation)	Animal Model (Species/ Strain/Sex/ Age) N	Study Design	Exposure (Route/Period/ Frequency/ Vehicle)	Doses/ Concen-trations		Systemic Toxicity	Reproductive Toxicity	
Gillies et al., 1981	2,5-HD Eastman Kodak, Rochester, NY Redistilled before use Purity not stated	Fischer-344 rats Male Weight 187±5g N = 8/group	Subchronic reproductive study on testicular effects: Relation between lipid metabolism and 2,5-HD induced testicular atrophy	Oral in the drinking water for 6 weeks. The control group was pair fed to the 2,5-HD-treated group	0, 1% in water	Lipid metabolism in the testis, sciatic nerve, and liver	Peripheral neuropathy including everted and flat foot placement and hindlimb weakness. Lipid metabolism was significantly altered in sciatic nerve, but not liver	Testicular atrophy: testes from treated rats were 30-60% smaller and weighed threefold less ($p<0.05$) Lipid metabolism was significantly altered in testis ($p<0.05$)	

Reference	Experimental Parameters					Endpoints Assessed	Results (Effects/NOEL/LOEL)		Comments
	Chemical (Source/ Purity/ Preparation)	Animal Model (Species/ Strain/Sex/ Age) N	Study Design	Exposure (Route/Period/ Frequency/ Vehicle)	Doses/ Concen-trations		Systemic Toxicity	Reproductive Toxicity	
Chapin et al., 1982	2,5-HD Eastman Kodak, Rochester, NY Redistilled before use Purity not stated	Fischer 344 rats Male Weight 160-180g N = 3-6/ group/time	Subchronic male reproductive toxicity studies. Exp1: Effect on testicular enzyme markers, luteinizing hormone (LH) and testosterone.	Oral in the drinking water for 6 weeks	0, 1% in water	After 1, 3, and 6 weeks blood, testis and liver samples were collected. Cellular marker enzymes in the testis and liver, Spermatocyte: SDH and Sertoli gamma-glutamyl transpeptidase (GGT) Hormone levels; Morphologic changes in the testes.	Both liver lysosomal enzymes: beta-glucuronidase and acid phosphatase were ↓ at all times	64% ↓ in testes wt. At 3 weeks Sertoli cells' enzymes activity, beta-glucuronidase and GGT were ↓ and ↑ at 6 weeks. The spermatocyte marker, SDH was ↑at 1 week and ↓ at 6 weeks. No changes in testosterone levels at any time. No changes in LH levels at 1 and 3 weeks, but ↑at 6 weeks.	
			Exp2: LH release in response to gonadotropin releasing hormone (GnRH) after 6 weeks exposure	Oral in the drinking water for 6 weeks	0, 1% in water	Hormone levels		No effect on the response of pituitary (LH) to GnRH.	
			Exp3: effects on beta-glucuronidase and sorbitol dehydrogenase (SDH) activities in vitro	Direct treatment of testicular homogenates from untreated males	0, 0.5-20 nM	Cellular marker enzymes in the testis,		No effect on the activities of beta-glucuronidase or SDH at anyconcentration	

Reference	Experimental Parameters					Endpoints Assessed	Results (Effects/NOEL/LOEL)		Comments
	Chemical (Source/ Purity/ Preparation)	Animal Model (Species/ Strain/Sex/ Age) N	Study Design	Exposure (Route/Period/ Frequency/ Vehicle)	Doses/ Concen-trations		Systemic Toxicity	Reproductive Toxicity	
Chapin et al., 1983	2,5-HD Eastman Kodak Redistilled before use Purity not reported.	Fischer 344 rats Male Weight 160-180g N = 30 total (n per group not provided)	Sub chronic male reproductive toxicity study	Oral in the drinking water Control= tap water Treated for 3, 4, 5 and 6 weeks	0, 1% in tap water	At the end of treatment, testes were prepared for histologic analysis: Light and electron microscopy (EM)	N/A	Testes lesions: Vacuolation at 4 weeks Chromatin margination, epithelial disruption and multinucleated giant cells at 5 weeks. Intratubular cellular debris at 6 weeks. EM: Enlarged SER; Some degenerating giant cells consisted mainly of electron-dense cellular debris	
Boekelheide, 1987 *	2,5-HD Eastman Kodak Co., Rochester NY >98% pure	CD rats Male Weight 200g N = 8 Control N = 17 Treated	Subchronic male reproductive toxicity study: Effects on microtubules in Sertoli cells resulting in testicular atrophy	Oral in the drinking water for 4 weeks	0, 1% in water	Clinical neurotoxicity; brain and testes weight. Crude supernatants to determine tubulin Light and electron microscopy	↓ BW ($p<0.001$). Nervous system impairments: moderate and severe degrees of hindlimb weakness. Brain tubulin assembled earlier and faster in treated rats	↓ testicular wt. ($p< 0.001$) Larger and numerous vacuoles; occasional giant cells, and chromatin margination in spermatids. No alteration in tubulin content of crude supernatants. Alterations in the kinetics of tubulin assembly	

Reference	Experimental Parameters					Endpoints Assessed	Results (Effects/NOEL/LOEL)		Comments
	Chemical (Source/ Purity/ Preparation)	Animal Model (Species/ Strain/Sex/ Age) N	Study Design	Exposure (Route/Period/ Frequency/ Vehicle)	Doses/ Concen-trations		Systemic Toxicity	Reproductive Toxicity	
Boekelheide, 1988a	2,5-HD Eastman Kodak Co., Rochester, NY >98% pure	CD rats Male Weight 200g N = 5-6/ group N1(Low) = 4 or 5/ time N2 (moderate) = 5 or 6/ time N3 (high)= 60 total 5/group (10 groups) and 10 animals reserved	Subchronic male reproductive toxicity study: Dose and time course effects at three exposure levels	Low: intra peritoneal (ip) injection, 5 days/week for 2 weeks. Moderate: Oral exposure. Rats were exposed ad libitum for 2 or 3 weeks in drinking water High: ad libitum for 5 weeks in drinking water	0, Low: 4 mmol/kg/day for a total dose of 40 mmol/kg. Moderate: 1% (v/v) in drinking yielding average total doses of 90 or 138 mmol/kg, High: 1% (v/v) in drinking yielding average total dose of 211 mmol/kg.	Weekly assessment: BW, Clinical neurotoxicity. Average testis wt. /animal at sacrifice. Testes histopathology. Controls: rats were killed at 0,4,5,7, or 22 weeks for testis weight and histology 1. Low: Groups of rats were killed at 2, 4 or 7 weeks of first injection 2. Moderate: oral exposure, Groups of rats were killed at 7 or 22 weeks 3. High: oral exposure. Groups of rats were killed at 2, 4, 5, 6, 7, 8, 10, 12, 16, or 22 weeks	↓ BW gain ($p<0.05$) Low: no signs of neurotoxicity at 2 weeks Moderate: No clinical signs of neurotoxicity in the 2- week exposure group. The 3-week group had unsteady gait but no deficit in hindlimb retraction High: 8 animals lost >40% of initial BW and were replaced. Moderate to severe neurotoxicity. BW rose during the recovery period	↓ testicular wt. at 7 weeks after 2-week exposure ($p<0.001$) for all treated animals (n=17) Low: normal testes wt. at 4 weeks. Large Sertoli cell vacuoles At 7 weeks: normal testes wt., tubule histology, and Sertoli cell. Moderate: at 7 weeks, the 2- and 3-week exposure groups had severe testicular atrophy: half of the testes were depleted of germ cells. The less severely damaged testes had similar injuries to the ones in the low dose group. At 22 weeks ↓ in testes wt. of the 2 groups (ranging from 40% of control to control values). Variable germ cell repopulation in the two exposed groups. High: ↓ testes wt. by 40% at 7 weeks and did not recover. Germ cell loss by necrosis and sloughing, disruption of the seminiferous epithelium. Vacuoles in Sertoli cells first at 4 weeks. At 22 weeks, germ cell repopulation, but testes wt. <50% of controls	

Reference	Experimental Parameters					Endpoints Assessed	Results (Effects/NOEL/LOEL)		Comments
	Chemical (Source/ Purity/ Preparation)	Animal Model (Species/ Strain/Sex/ Age) N	Study Design	Exposure (Route/Period/ Frequency/ Vehicle)	Doses/ Concen-trations		Systemic Toxicity	Reproductive Toxicity	
Boekelheide, 1988b*	2,5-HD As in Boekelheide, 1988a: Eastman Kodak Co., Rochester, NY >98% pure	CD rats Male Weight 200g N for Controls not reported N = 15 Treated	Biochemical studies complement the light microscopic observations reported earlier (Boekelheide, 1988a)	Oral in the drinking water for 5 weeks, followed by 17 weeks recovery	0, 1% (v/v) in water	Determinations of tubulin content and pyrrole reactivity. Purified testis tubulin from treated rats at weeks 0, 2, 5,8, or 12 and from controls at week 12	N/A	Severe testicular atrophy: loss > 50% of control testis wt., in all rats from weeks 7 through 22. ↑ pyrrole formation during treatment and then ↓ in the recovery phase. ↑ tubulin content after testicular atrophy	Companion paper to (Boekelheide, 1988a)*

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Boekelheide and Eveleth, 1988 *	2,5-HD Eastman Kodak Co., Rochester, NY >98% pure	CD rats Male Weight 220g N = 6/group Companion control for each treatment group N = 5-6/group treated	Subchronic male reproductive toxicity study: Mechanism of action in testis injury, effects on microtubule formation	Oral in the drinking water for 21, 35, or 69 days	0, 131± 2 mmol/kg [administered as either 1.0, 0.5, or 0.25% (v/v) for 21, 35, or 69 days, respectively, producing dose rates of 6.1, 3.8, and 1.9 mmol/kg/day]	Weekly BW Euthanasia at 4 weeks after ending exposure. Pyrrole formation in association with microtubule nucleation in testes. Testes histopathology	No clinical signs of nervous system injury. ↓ BW at 6.1mmol/kg/day that did not recover. ↓ BW at 3.8mmol/kg/day only during exposure. Dose response in pyrroles formation and microtubule formation	↓ testis wt. (p=0.001) in the 6.1 mmol/kg/day group. No differences in the other treatment groups. Germ cell depletion at 6.1 mmol/kg/day, similar to previous references (Chapin et al., 1983) and (Boekelheide, 1988a). In the 3.8mmol/kg/day group only 2/6 of the animals had germ cell depletion	
Boekelheide et al., 1990*	2,5-HD As in Boekelheide, 1988a: Eastman Kodak Co., Rochester, NY >98% pure	Charles River CD rats Male Weight 200g N = 6-7/group for protocol (P)1 N = 5-8 for P2	Subchronic reproductive toxicity study: Comparison between experimental cryptorchidism and 2,5-HD exposure. Control groups: cryptorchidism control and sham operated.	Oral in drinking water for 3 or 5 wk, as previously described (Boekelheide, 1988a). Two protocols (P): P1: cryptorchid surgery first then 2,5- HD for 5 weeks P2: 2,5-HD for 3 weeks then cryptorchid surgery	0, 1% (v/v) in water P1: Dose rate of 0, 6.7 mmol/kg/day P2: Dose rate of 0, 6.5 mmol/kg/day	Weekly BW Average seminiferous tubule diameter Pyrrole content The effect of 2,5-HD alone was evaluated in sham operated controls Testes histopathology	P1: ↓ BW in 2,5-HD-treated groups P2: BW initially ↓ with a later recovery in all 2,5-HD-treated groups	2,5-HD-treated sham-operated rats in both protocols had ↓ testicular, epididymal wt., and tubule diameter ($p<0.05$). No recovery. Recovery of germ cells by cryptorchidism in P1 only. 2,5-HD-treated cryptorchid rat testes had less pyrrole formation ($p<0.05$)	

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Boekelheide and Hall, 1991*	2,5-HD Source/purity not stated	Charles River CD rats Male Weight 208g N = 39, (On day 10 of exposure, 13 of the 39 treated rats were euthanized because of excessive weight loss] N = 18 control (5/time) N = 26 treated (7-9/time)	Subchronic male reproductive toxicity study: Examined the possibility of germ cell recovery over a long period after acute 2,5-HD exposure.	Oral in drinking water for 5 weeks	Average dose rate of 3.1 mmol/kg/day	BW (weekly until week 20, then every 3 weeks). 5 weeks oral exposure. Killed at 27, 60 or 75 weeks. Testes histopathology LH, FSH and Testosterone in blood at week 27	↓ BW until week 60, then no differences until the end of experiment (week 75)	Testicular atrophy at 27, and 60 weeks, and in all nine treated rats at 75 weeks. ↑ LH and FSH but normal Testosterone. Week 27: seminiferous tubule epithelium recovered: 2 partial; 1 complete 3 complete atrophy Week 60: one full restoration the rest had atrophic tubules Week 75: no tubules recovered	

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Hall et al., 1991*	2,5-HD Aldrich, Milwaukee, WI 97% pure	Charles River CD rats Male Weight 200-250g N = 5 control N = 7 treated	Subchronic male reproductive toxicity study: Distribution of filaments during testicular injury	Oral in drinking water for up to 5 weeks followed by untreated water	Average consumption: 5.4 mmol/kg/day	BW. Testes wt. and Histopathology Testes filaments Immunohisto-chemistry Assess the re-expression of keratins, crosslinking of intermediate filaments in testis	↓ BW at week 3 and remain low at week 8 ($p<0.05$)	↓ testes wt. from week 3 to 8 ($p<0.05$) Vacuolation of Sertoli cells and elongated spermatids. Nuclear margination of round spermatids and atrophic tubules with no germ cells. Altered distribution of filaments in Sertoli cells	

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Johnson et al., 1991*	2,5-HD Eastman-Kodak, Rochester, NY 98% pure	Charles River CD rats Male Weight 200g N = 3-6 Control N = 6-9 Treated	Subchronic male reproductive toxicity study: Effects on seminiferous tube fluid (STF) secretion and testicular tubulin distribution	Oral in drinking water for up to 5 weeks, followed by water only	0, 1% in water. The average rate was 4.9 mmol/kg/day	BW. Testis weight and histopathology. Staging of seminiferous tubules Sertoli cell morphology STF secretion at several times up to 8 weeks	BW changes as in (Boekelheide, 1988a) Tubulin immuno-histochemistr y as in (Hall et al., 1991)	No changes in fluid secretion up to 25 days. ↓ STF at 28 days ($p<0.05$) and recover by week 8 Testes wt. as described in (Boekelheide, 1988a) and correlated to STF secretion. ↑ histological abnormalities around 3.5 weeks. About 90% of the testes manifested Sertoli-cell-plus elongate spermatid changes. Testes with round spermatid changes by week 4 and inhibited STF secretion. ↓ tubulin immunodetection	

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Larsen et al., 1991	2,5-HD Source/purity not stated	Mol/Wist SPF rats Male Weight 150g Female Weight 250g divided into 16 groups N = 10 rats/group	Subchronic male reproductive toxicity study: Morphological testicular changes and fertility. Interaction with acetone effects and reversibility within a period of 10 weeks	Oral in drinking water for 6 weeks	Male rats were exposed to 0%, 0.13%, 0.25% or 0.50% 2,5-HD alone or in combination with acetone 0.50%. Based on water intake the 2,5-HD in the three dose groups was about 170, 270 and 440 mg/kg/day	Weekly BW, food and water consumption for males. Fertility: half of males at the end of exposure. The other half 10 weeks after. Females sacrificed on GD20: number of pregnant dams and number of fetuses were recorded. Testicular histopathology: number of vacuoles and tubule diameter	↓Food and water consumption with treatment in a dose dependent manner. ↓BW gain in the same way ($p<0.005$) from the 3rd week.	↓ in pregnancies, number of fetuses and testis wt. with 0.50% 2,5-HD ($p<0.05$) (all the effects were potentiated with acetone). Small recovery after 10 weeks but still effects ($p<0.05$) The tubule diameter was the most sensitive parameter. There was a dose dependently ↓ diameter ($p< 0.01$) at all three 2,5-HD doses	

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Hall et al., 1992*	2,5-HD Sigma Chemical Co. St. Louis, MO Purity not stated	Charles River CD rats Young adult male Weight 150-175g N = 2 with 2 replicates	In vitro study: from 21 day-old untreated, adult, cryptorchid and 2,5-HD-exposed rat testes.	Oral in drinking water for 5 weeks followed by a 3-5 week recovery period	0, 1% in water	morphological observation of Sertoli cell-cultures: Cytoskeleton proteins	N/A	Sertoli cells exhibited a spindle-like shape with long bipolar processes, appeared as disorganized masses of cells and never reach confluence. Complex microtubule and vimentin filament networks versus a well-defined pattern of filament networks in control cells	
Linder et al., 1992	2,5-HD Eastman Kodak Co., Rochester, NY Purity not stated	Sprague-Dawley (SD) rats Male 90 days old N = 6/group	Subchronic male reproductive toxicity study: Sensitivity and utility of multiple endpoints Sacrificed on day 2 or 14 after beginning of treatment.	Oral by gavage in water. 1 to 5 days exposure:	0, 2000 mg/kg	Weight of testis, epididymis, seminal vesicles, and prostate. Testicular histopathology Sperm characteristics on the contralateral testis	N/A	No effects on organ weight. Affect spermiation: Digested spermatids in the basal Sertoli cell and retained at the lumen in Stage IX to XIII tubules at 2 weeks post treatment	

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	Chemical (Source/ Purity/ Preparation)	Animal Model (Species/ Strain/Sex/ Age) N	Study Design	Exposure (Route/Period/ Frequency/ Vehicle)	Doses/ Concen-trations		Systemic Toxicity	Reproductive Toxicity	
Richburg et al., 1994*	2,5-HD Aldrich Chemical Co., Milwaukee, WI >98% pure	SD, CD rats Adult male Weight 200-400g N = not stated	Subchronic male reproductive toxicity study	Oral in drinking water for 3 and 4 weeks.	0, 1% in water	Seminiferous tube fluid (STF) secretion, seminiferous tubule histology and testicular histopathology	N/A	↓ in STF secretion after 3 weeks ($p<0.05$) with nearly normal testicular histopathology At 4 weeks of exposure: greater decline in STF secretion, testes showed marked alterations in the seminiferous epithelium including abnormally elongate spermatid heads and the presence of germ cell nuclear debris	
Allard et al., 1995*	2,5-HD Aldrich Chemical Co., Milwaukee, WI Purity not stated	Charles River CD rats Male Young adult Weight 150-175g 42-46 days old N = not stated	Subchronic male reproductive toxicity study	Oral in drinking water for 5 weeks followed by a recovery period	0, 1% in water	Assessment of stem cell kinetics in treated rats 7 and 35 weeks following exposure. Labeling index of stem spermatogonia detected by immunohistochemistry.	N/A	Testes from treated rats showed variable atrophy, as previously described (Boekelheide and Hall, 1991) Stem cells of exposed rats had a longer cell cycle time of 8 through 14 days (normal cell cycle time is 42h) at both 7 and 35 weeks after exposure	

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Hall et al., 1995*	2,5-HD Aldrich, Milwaukee, WI 97% pure	Crl:CD(SD)BR rats Male Young adult Weight 175-200g N = 4-6	Subchronic male reproductive toxicity study: Immuno-distribution of dynein and kinesin during and after exposure, correlation to seminiferous tube fluid (STF) secretion	Oral in drinking water for 5 weeks followed by a recovery period. Autopsy at 3, 4, or 5 weeks after the beginning of exposure or 3 weeks after the rats were returned to normal water	0, 1% in water	Testes histopathology; immunohistochemistry for dynein and kinesin	N/A	↓ in apical Sertoli cell dynein ($p < 0.05$) At 5 weeks, the seminiferous epithelium became disorganized with a diffuse dynein staining pattern. ↓ Kinesin but recovered at 3 and 4 weeks after exposure. Golgi complex became vesiculated and dispersed in the Sertoli cell	

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Rosiepen et al., 1995	2,5-HD As in Gillies et al., 1981): Eastman Kodak, Rochester, NY Redistilled before use Purity not stated	SD rats Adult male Weight 250-300g Start: N = 20 control N = 40 (2,5-HD) End: N = 13 control N = 22 (2,5-HD)	Subchronic male reproductive toxicity study: Relation among alterations in stage frequencies and the duration of the seminiferous epithelial cycle	Oral, in drinking water for 29 days	0, 1% in water	BW, testis and epididymis weights were recorded.at 17 (left testis) and 29 days (right testis).FSH and testosterone levels Testes histology and cell cycle stage	↓ BW at 17 and 29 days ($p<0.01$)	↓ testicular wt. at 29 days ($p<0.05$), ↓ epididymis wt. at 17 and 29 days ($p<0.01$) Testicular effects at 29 days: presence of vacuoles in the seminiferous epithelium. ↓ frequency of stage VII on day 17 and 29; stage VIII frequency was reduced on day 29. Stage IX frequency had ↑ on days 17 and 29. ↑ frequency of stage X on day 17 No changes in serum T or FSH concentration	

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Allard et al., 1996*	2,5-HD Aldrich Chemical Co., Milwaukee, WI Purity not stated	SD CD rats Male 21 days old to isolate Sertoli and germ cells SD CD or Fischer rats Adult male Weight 150 and 175g for 2,5-HD exposure Northern blot analysis N = 3 SD rats/group RTPCR N = 2 Fischer Rats/group	Subchronic male reproductive toxicity study: Stem cell factor (SCF) function in 2,5-HD testes effects. In vitro coculture to find effective dose of SCF, and an in vivo intratesticular delivery of SCF to rats with 2,5-HD-induced atrophy	Oral in drinking water for 5 weeks	0, 1% in water	Northern blot analysis (SCF expression), from rats killed at 22 weeks after the start of exposure. RTPCR analysis (from animals killed at 13 weeks.)	N/A	↑ SCF mRNA expression 13 wk. after the start of exposure. SCF mRNA was preferentially expressed in the soluble form (the control testes expressed primarily the transmembrane form) Exogenous SCF ↑ germ cell survival and/or proliferation in 2,5-HD treated ($p<0.05$)	

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Blanchard et al., 1996*	2,5-HD Aldrich Chemical Co., Milwaukee, WI Purity: "highest quality"	Fischer F344 rats Male Weight 150–175g upon arrival N = 9 Controls (3 each at 0, 4 and 8 weeks) Treated: N = 24 total (Weight 200g 60 days old). 3 rats/group for each timepoint for sacrifice	Subchronic male reproductive toxicity study: Test the hypothesis that apoptosis plays a major role in the initiation of 2,5-HD dependent testicular atrophy	Oral in drinking water up to for 5 weeks	0, 1% (v/v) in water	Killed at 2, 3, 3.5, 4, 5, 6, 8 or 12 weeks from beginning exposure. Testes wt. DNA isolation on left testis. Right testes for histopathology and apoptosis determination (TUNEL)	N/A	↓ testis wet wt. starting at 4 weeks ($p<0.01$). DNA fragmentation at 5 and 6 weeks. Basal vacuoles at 4 weeks. Alteration in germ cell population: multinucleated giant cells and sloughing of germ cells. Cells with chromatin condensation and fragmentation. At 6 weeks atrophic tubules with only Sertoli cells, spermatogonia, and spermatocytes with few spermatids. At week 8 and 12 Sertoli cells and few spermatogonia. ↑ germ cell apoptosis starting at week 4 ($p<0.05$), returned to low levels at 8 -12 weeks	

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Allard and Boekelheide, 1996*	2,5-HD Aldrich Chemical Co., Milwaukee, WI Purity not stated	SD rats Male Weight 150 - 175g N = 5/group Apoptosis Germ cell counting: N = 4 (12 weeks) N = 2 (40 weeks)	Subchronic male reproductive toxicity study: Testicular atrophy characterization	Oral in drinking water for 5 weeks	0, 1% in water	Testes wt. Apoptosis: animals were killed at 12 weeks. One testis for TUNEL the other for histopathology. Germ cell counting: spermatogonia types on both testes	N/A	↓ testis wt. by 42% at both 12 and 40 weeks. ↓ spermatogonia population, the majority of spermatogonia were of type A	
Lee et al., 1999*	2,5-HD Aldrich Chemical Co., Milwaukee, WI As reported in Blanchard et al., 1996: Purity: "highest quality"	Fischer rats Male Weight 150g and 175g N not stated	Subchronic male reproductive toxicity study: Interaction with the Fas system (apoptosis signal transduction pathway)	As previously described (Blanchard et al., 1996): Oral, in the drinking water for 5 weeks	0, 1% (v/v) in water	Testes for frozen sections and isolation of RNA. Were collected at various times after initiating exposure (0, 2, 4, 5, 7, or 13 weeks). Apoptosis (Fas marker) by TUNEL and RT-PCR	N/A	↑ germ cell apoptosis by week 5. Fas and Fas ligand mRNA also increased at the same time	

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Horimoto et al., 2000	2,5-HD Wako Chemical Co., Osaka, Japan, lot No. LEL4798 Purity not stated	SD rats Mature male 10–15 weeks of age N = 4 control N = 5/group, 2,5-HD treated	Subchronic male reproductive toxicity study: Use of CASA system to determine, epididymal sperm motility	Oral by gavage	0, 100, 250 mg/kg/day for 28 days	BW. On day 29, testes and epididymides wt. Sperm motility and count Motion parameters: percentage of motile sperm, and progressively motile sperm, Path (VAP), Straight line (VSL), and curvilinear (VCL), velocity Beat cross frequency (BCF), Amplitude of lateral head displacement (ALH), Straightness (STR), Linearity (LIN)	↑ BW gain in the 250 mg/kg/day group ($p<0.01$) (from Table 1, page 58)	↑ testes relative wt. in the 250 mg/kg/day group ($p<0.05$). No differences in sperm count. ↓ motile sperm. ↓ progressive motility values, and velocity parameters (VAP, VSL and VCL). ↑ BCF values. No differences on ALH values. ↓ STR values only at the 250 mg/kg/day dose. ↓ LIN values (most at $p<0.01$)	The text described a "decreased BW gain" but OEHHA noted that in Table 1 the numbers are 34.4 for control and 35.4 for 2,5-HD treated in the 250 mg/kg/day group

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Aoki et al., 2004	2,5-HD Source/purity not stated	Wistar rats Male 10 weeks old N = 10 control N = 6/group	Subchronic male reproductive toxicity study: Effect of 2,5-HD on principal sperm parameters	Subcutaneously for 5 days per week for 12 weeks	0, 100, 200, 400 mg/kg/day	Evaluation of sperm motility and morphology on treatment day 90	N/A	↓ sperm motility at 100 mg/kg. No motility at higher doses. ↓ normal sperm morphology and spermatid count at the two higher doses ($p<0.05$). No germ cells at 400 mg/kg/day in 2/3 of the rats	

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Markelewicz et al., 2004*	2,5-HD Sigma-Aldrich Chemical Company, St. Louis, MO Highest purity available	Fischer rats Adult male Weight 150–175g N = 15/group	Subchronic male reproductive toxicity study: Interaction between 2,5-HD and carbendazim (CBZ) on testicular damage	Oral in the drinking water for 2.5 weeks (18 days) (CBZ by gavage, at a dose of 200 mg/kg BW in corn oil on day 17)	0, 1% in water	BW. On day 18: testes (including ligated ducts) were, weighed, and immersion fixed in 10% neutral-buffered formalin for histopathology and seminiferous tubule diameters	↓ BW ($p<0.05$)	↓ testes wt. 2,5-HD-exposed testis revealed qualitatively normal spermatogenesis. ↓ tubule diameter and ↑ vacuolization. 2,5-HD/CBZ exposure: ↑ in seminiferous tubule diameter and disruption of the epithelium with vacuolization and sloughing	
Fukushima et al., 2005	2,5-HD Sigma, St. Louis, MO Purity not stated	CD rats Male 12 weeks old N = 5/group	Acute male reproductive toxicity study: Testicular gene expression 6h after the single exposure	Oral (once by gavage)	0, 60, 1000 mg/kg	Testes and epididymides wt. Histopathology on the left testes. Frozen right testes for gene expression		No changes on testis or epididymis wt. Several genes (involved in testicular physiology) were up and others down regulated after exposure	

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Kihaile et al., 2005	2,5-HD Source/purity not stated	Wistar rats Male 3 weeks of age N = 10/group	Subchronic male reproductive toxicity study: Use of sperm parameters as indicator of reproductive toxicity in male rats	Subcutaneously for 5 days/week over 12 weeks	0, 100, 200, 400 mg/kg-day.	BW and limb paralysis were monitored weekly. On day 90, testes and epididymides were weighed. Evaluation of sperm count, motility and morphology	↓ BW at the two higher doses ($p<0.05$ and $p<0.01$ respectively) All 10 rats in the 400 mg/kg group displayed quadrapelegia and could not move. 5 rats in the 200 mg/kg day group showed mild paralysis	Two higher doses: ↓ testes ($p<0.05$ and $p<0.01$), and epididymides wt.; ↓ spermatid and sperm count ($p<0.05$). ↓ motility at all doses ($p<0.05$). 70% of rats with Sertoli cell-only syndrome at 400 mg/ (kg day)	
Yamamoto et al., 2005	2,5-HD Sigma, St. Louis, MO, USA Purity not stated	SD rats Mature male 12 weeks old N = 5/group	Acute male reproductive toxicity study: Protein expression related to 2,5-HD exposure (and other chemicals).	Oral by gavage. Single dose Controls received distilled water	0, 250, 1000 mg/kg	Sacrifice at 6h and 24h after exposure. BW and testes wt. Blood sample for 2,5-HD concentration. Histopathology on left testis. Gene and protein expression on the right testes	No changes in BW	No changes in testis or epididymis wt. No remarkable histopathological findings after 2,5-HD treatment. Specific protein expression found after 2,5-HD exposure	Identification of biomarkers (specific proteins) but no histologic or anatomic effect of 2,5-HD

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Moffit et al., 2007*	2,5-HD Sigma-Aldrich Corporation, St. Louis, MO Purity: "reagent grade or better"	Fischer 344 rats Adult male Weight 250–275g N = not stated	Subchronic male reproductive toxicity study: Dose-response for Sertoli cell toxicants (2,5-HD and others) and histopathology endpoints	Oral in drinking water for 18 days	0, 0.125, 0.21, 0.3125, 0.625% in water	BW, testis wt. Histopathology, Apoptosis by TUNEL. Real-time PCR to measure α-tubulin mRNA levels	No changes in BW	No changes in testis wt. ↑ % of tubules with >3 spermatid retained heads ($p<0.05$). ↑ α- tubulin mRNA levels for the 0.125% and 0.21% 2,5-HD doses. No apoptosis and tubule diameter changes	
Bryant et al., 2008*	2,5-HD Source not stated ≥98% pure	Fischer 344 rats Adult male Weight 200 –225g N = 9 control N = 6 for 0.33% and 1.0% 2,5-HD N = 3 for dose response, time course and recovery experiments	Subchronic male reproductive toxicity study: Marker for testicular toxicity:	Oral in drinking water for 18 days Time course:11, 13, 15, or 18 days (0.3%2,5-HD) Recovery: 1, 2, or 4 weeks (water) after the 18 weeks of 2,5-HD exposure	0, 0.33%, 1% in water Dose response: 0.08%, 0.14%, 0.21%, 0.33%, 0.625%, or 1% in water	BW and testis wt. at time of necropsy Determination of retained spermatid heads (RSH).	↓ BW at 1% exposure	0.33% : ↑ RSH (>3) at Stages IX–XII; and 1% does it at Stages IX–II/III Dose-dependent ↑ in RSH at Stages IX–XII. Exposure to 0.33% for 11 to 18 days showed a steady ↑ in RSH. Recovery occurred after 4 weeks	

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Jiang et al., 2008	2,5-HD Source/purity not stated	SD rats Male 70–80 days of age N = not stated	Subchronic male reproductive toxicity study: Evaluation of the protective effect of Resveratrol (3,5,4'-trihydroxystilbene, RES) on germ cells after exposure to 2,5-HD	Oral in drinking water for 5 weeks RES was suspended in 5 g/L carboxy-methylcellulose	Average dose/rat of 3.1 mmol/kg/day	BW, testes wt. Right testes for histopathology, and the left testes were frozen at –80°C until protein analysis Immunodetection of c-kit protein, correlated with proliferation and differentiation of spermatogenic cells	2,5-HD effects: Abnormal changes of physical signs, rats were physically weak, loss of BW	2,5-HD: testis atrophy ($p<0.01$). Seminiferous tubules of 2,5-HD group appeared deformed, physiological progression of spermatogenesis was stagnant in undifferentiated type A spermatogonia. RES restored these effects of 2,5-HD. C-kit not detected in 2,5-HD treated (protein and mRNA)	
Qian and Zeng, 2008	2,5-HD Emuck, Germany Purity not stated	SD rats Male 2 months of age 175–200g N = 5/group	Subchronic male reproductive toxicity study: Relation with the effects of diethylstilbestrol (DES) in the re-establishment of spermatogenesis	Oral exposure in drinking water for 5 weeks	0, 1% in water	8th week plasma luteinizing hormone (LH) and testosterone (T) by RIA At 18 weeks the left testis was prepared for histopathology	No differences in BW	Testicular atrophy was observed following exposure (↓ testes wt. $p<0.05$) Testicular recovery with 3 and 30 ug/kg DES. 2,5-HD= ↓ T and ↑ LH ($p<0.05$)	

Reference	Experimental Parameters					Endpoints Assessed	Results (Effects/NOEL/LOEL)		Comments
	Chemical (Source/ Purity/ Preparation)	Animal Model (Species/ Strain/Sex/ Age) N	Study Design	Exposure (Route/Period/ Frequency/ Vehicle)	Doses/ Concen-trations		Systemic Toxicity	Reproductive Toxicity	
Campion et al., 2010a*	2,5-HD As in Markelewicz et al., 2004: Sigma-Aldrich Chemical Company, St. Louis, MO Highest purity available	Fischer 344 rats Adult male Weight 200–250g N = 4/group	Subchronic male reproductive toxicity study: Interaction of x-ray–induced gene expression alterations and 2,5-HD exposure	Oral in drinking water for 18 days	0, 0.33, 1% in water		N/A	Testes toxicological/morphologic effects were not described but referred to (Yamasaki et al., 2010)*	Companion paper to (Yamasaki et al., 2010)*
Yamasaki et al., 2010*	2,5-HD Purity not stated	Fischer 344 rats Adult male Weight 200–250g N = 10 for control N= 8 (2,5-HD)	Subchronic male reproductive toxicity study: Coexposure paradigm consisting of a 18-day exposure to 2,5-HD followed by an acute x-ray exposure	Oral in drinking water for 18 days	0, 1% in water	Necropsied on day 18, body and testis weights were recorded. Left testes fixed in 10% formalin for histology.	↓ BW	↓ testes Wt. ↑ Incidence of retained spermatid heads (RSH) in the basal compartment of seminiferous tubules. ↑ percentage of seminiferous tubules with greater than 3 RSH	

Reference	Experimental Parameters					Endpoints Assessed	Results (Effects/NOEL/LOEL)		Comments
	Chemical (Source/ Purity/ Preparation)	Animal Model (Species/ Strain/Sex/ Age) N	Study Design	Exposure (Route/Period/ Frequency/ Vehicle)	Doses/ Concen-trations		Systemic Toxicity	Reproductive Toxicity	
Campion et al., 2010b*	2,5-HD As in Markelewicz et al., 2004: Sigma-Aldrich Chemical Company, St. Louis, MO Highest purity available	Fischer 344 rats Adult male Weight 200–250g N = 4	Subchronic male reproductive toxicity study: Interaction of x-ray–induced gene alterations and exposure to 2,5-HD	Oral in drinking water for 18 days	0, 0.33%, 1% in water	Gene expression	N/A	Six genes were upregulated from 1 to 6 times while several were downregulated. IER3 (immediate early response gene): anti-apoptotic function	
Campion et al., 2012*	2,5-HD As in Markelewicz et al., 2004: Sigma-Aldrich Chemical Company, St. Louis, MO Highest purity available	Fischer 344 rats Adult male Weight 200–250g N = 3-4	Subchronic male reproductive toxicity study: 2,5-HD was used as a positive control on a carbendazim study	Oral in drinking water for 18 days	0, 0.33%, 1% in water	Testes histology. Gene expression underlying testicular pathology	N/A	"… co-exposure to the two Sertoli cell toxicants HD and CBZ revealed that they interact to produce synergistic effects on testicular toxicity, at the phenotypic level" decrease in gene expression for 5 genes at both doses	It does not show the toxic effect of 2,5-HD but makes reference to previous findings such as Campion et al. (2010a)

Reference	Experimental Parameters					Endpoints Assessed	Results (Effects/NOEL/LOEL)		Comments
	Chemical (Source/ Purity/ Preparation)	Animal Model (Species/ Strain/Sex/ Age) N	Study Design	Exposure (Route/Period/ Frequency/ Vehicle)	Doses/ Concen-trations		Systemic Toxicity	Reproductive Toxicity	
Pacheco et al., 2012*	2,5-HD Sigma Aldrich, St. Louis, MO 99% pure	Fischer 344 rats Male Weight 175-225g N = 20 control N = 19 (2,5-HD) N = 4-6 for time course	Subchronic male reproductive toxicity study: 2,5-HD is used as a positive control for testicular toxicity	3 months; oral by gavage in a corn oil vehicle	0, 0.33% in water (aproximately 234 mg/kg/d). Estimated average daily intake 0.973 g/mL	Body weights (BW) and reproductive organ weights were recorded at necropsy. Left testes were fixed in 10% neutral-buffered formalin for histological examination. Microarray on sperm RNA/ month. Inhibin B in serum	Decreased BW	↓ in testis, and epididymis weights after 3 months of exposure; a 14.5-fold ↑ in stage-specific spermatid head retention. All effects resolved after 3 months of post-exposure recovery No changes on inhibin B	
Erdos et al., 2013	2,5-HD Sigma-Aldrich, Saint Louis, MO ≥99% pure	Wistar-Han rats Male 10 weeks of age N = 8	Subchronic male reproductive toxicity study: Response of Inhibin B	Oral in drinking water (7 days) followed by gavage from day 8 to 28 Necropsy at: Days 21, 28 (300 mg/kg), Day 12–17 (600 mg/kg/day)	0, 300, 600 mg/kg/day	Plasma Inhibin B analysis at necropsy on study day 12 to 17. Terminal brain and testes weights. Testes histology	Significant adverse physical signs and/or body weight loss, which could have an unknown effect on Inhibin B levels	Seminiferous tubule degeneration, degeneration/necrosis of spermatids, Leydig cell degeneration, and vacuolation. Correlation ($p < 0.05$) between decreases in Inhibin B and seminiferous tubule toxicity (600 mg/kg/day). Testes wt not reported.	

* Boekelheide group (24 of the 38 references)

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Appendix A: Parameters for Literature Searches on the Reproductive Toxicity of Chemicals

Searches of the literature on the reproductive and developmental toxicity of the chemicals were 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 toxicity of the chemicals. The search sought to specifically 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 each substance 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.

Additional databases listed above were then searched for each chemical. 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.