EVIDENCE ON THE DEVELOPMENTAL AND REPRODUCTIVE TOXICITY OF

Methyl Isocyanate

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Reproductive and Cancer Hazard Assessment Branch
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PREFACE

Proposition 65[1] requires the publication of a list of chemicals "known to the state" to cause cancer or reproductive toxicity. It specifies that "a chemical is known to the state to cause reproductive toxicity … if in the opinion of the state's qualified experts the chemical has been clearly shown through scientifically valid testing according to generally accepted principles to cause reproductive toxicity ...." The "state's qualified experts" regarding findings of reproductive toxicity are the members of the Developmental and Reproductive Toxicant Identification Committee (DART IC) of the Office of Environmental Health Hazard Assessment (OEHHA) Science Advisory Board[2]. OEHHA, a department within the California Environmental Protection Agency, is the lead agency for implementing Proposition 65.

After consultation with the DART IC, OEHHA selected methyl isocyanate (MIC) as a chemical for consideration for listing by the DART IC. Upon selection, the public was given the opportunity to submit information relevant to the assessment of the evidence on the reproductive toxicity of MIC. No submissions were received.

OEHHA developed this document to provide the DART IC with comprehensive information on the reproductive toxicity of MIC for use in its deliberations on whether or not the chemical should be listed under Proposition 65.

[1] The Safe Drinking Water and Toxic Enforcement Act of 1986 (California Health and Safety Code section 25249.5 *et seq*.)
[2] Title 27 Cal. Code of Regs. Section 25302

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A. Executive Summary

Methyl isocyanate (MIC) is a highly reactive volatile and flammable chemical used to produce carbamate pesticides and other industrial chemicals. It is a severe pulmonary irritant and is extremely toxic to humans from acute (short-term) exposure. The available human data on the effects of MIC on reproduction and development are based on exposures that occurred in December 1984 in Bhopal, India from an accidental release of MIC that resulted in the deaths of over 2,500 people and debilitating health effects in more than 200,000 survivors. While the exact composition of the gas cloud created by the accident is not known, the presence of very high levels of MIC in the cloud is not disputed.

Many animal studies were conducted after the Bhopal disaster in an attempt to understand the effects of this chemical seen in the exposed population in Bhopal. In this report, the data from the animal studies are evaluated along with the findings of studies of the survivors of the Bhopal disaster.

Animal data suggest an effect on fetal loss subsequent to *in utero* exposure. This is consistent with the reports of pregnancy losses, particularly spontaneous abortions, seen in studies of women exposed during the gas leak in Bhopal. There are consistent findings across human studies of elevated rates of pregnancy loss in the first trimester (spontaneous abortion). Neonatal mortality was also elevated in multiple studies, which together with the increased rate of spontaneous abortion observed provides strong evidence of developmental effects of MIC. Additionally, a trend has been observed of persistent increased rates of fetal loss in the years that followed the Bhopal incident among women exposed to the gas cloud, suggesting an overall reproductive effect in females in the affected area.

Adverse skeletal effects in the animal studies, manifested as a shortening of bones, provides biologic plausibility that supports the findings of the only human study that evaluated postnatal growth of children exposed *in utero*. In that well-controlled study, boys born to mothers who were exposed during pregnancy were found to be significantly shorter in stature.

The two studies that continued to follow women in Bhopal in the years following the accident found that these women continued to experience higher rates of spontaneous abortion for years after the exposure. This was demonstrated most strongly in the one study that was able to differentiate groups of women based on the extent of exposure they experienced, and found higher rates of spontaneous abortion in women who resided in areas more severely affected by the gas cloud. The increased rates of spontaneous abortion seen in these women in the years after the exposure occurred would reflect female reproductive toxicity instead of direct effects on the fetus. These findings, along with the lower mean weight of placentae from full-term pregnancies in gas-exposed women, are indicative of effects on the female reproductive system. Decreases in placental weight in

animals exposed to MIC were also reported; an animal study did not find effects on reproduction after exposure of female rats to MIC 70 days prior to mating.

The data in animals suggest that exposure to MIC resulted in disappearance of spermatozoa in seminiferous tubules 3 days post exposure with recovery at 8 days and normalization at 15 days. Additionally, some researchers have concluded that MIC may alter the integrity of extra- and intratubular blood testis barriers by binding with specific membrane proteins, and cause reversible testicular damage with protein deficiency potentiating the effect of MIC. No dominant lethal effects were noted in the animal studies. The human data did not include any adequate study of male reproductive toxicity. Both studies that evaluated sperm parameters in men exposed to MIC were carried out too long after exposure (6 months and 100 –120 days, respectively) to have detected an effect on spermatogenesis and were essentially inconclusive for sperm counts or other parameters measured.

In summary, the evidence for the developmental toxicity of MIC comes from:
Animal studies
Finding fetal loss after *in utero* exposure
Finding shortening of bone length following in utero exposure
Studies of women exposed to the Bhopal disaster:
Pregnancy losses
Elevated neonatal mortality
Shorter stature in boys exposed *in utero*

Evidence for the female reproductive toxicity of MIC comes from
Animal studies finding decreased placental weight
studies of women exposed to the Bhopal disaster:
Lower mean weight of placenta
Increased spontaneous abortion years after the disaster

Evidence for the male reproductive toxicity of MIC comes from animal studies finding disappearance of spermatozoa in seminiferous tubules.

B. Introduction

Methyl isocyanate (MIC) is a highly reactive volatile and flammable chemical used to produce carbamate pesticides. It is a severe pulmonary irritant with LC50 levels in rodents in the 6-12 parts per million (ppm) range (California Department of Pesticide Regulation [CDPR], 2004) and is extremely toxic to humans from acute (short-term) exposure. The available human data on the effects of MIC on reproduction and development are based on exposures that occurred in December 1984 in Bhopal, India. Introduction of water into a storage tank at a pesticide manufacturing facility resulted in an uncontained release of an estimated 27 tons of MIC. Within one week more than 2,500 people died. Debilitating health effects have been related to the disaster in greater than 200,000 survivors (Mishra *et al*., 2009b; U.S. EPA, 1986).

For each of the major endpoints – developmental, male reproductive and female reproductive toxicity – the review begins with a discussion of effects seen in studies of humans who were exposed during the Bhopal incident. Then the data on effects seen in animal studies are described, followed by a discussion of other data relevant to these toxicities. The discussion of each endpoint concludes with an integrative evaluation of the human, animal and other relevant data.

B.1. Chemical Structure and Main Physical Characteristics

MIC is a colorless liquid at ambient temperatures and has a sharp odor. It has a chemical formula of C_2H_3NO, and the molecular weight is 57.05 g/mol (Amoore and Hautala, 1983). The Chemical Abstracts Service (CAS) Registry Number is 624-83-9.

The chemical structure of MIC is shown in Figure 1, and its physicochemical characteristics are summarized in Table B1.

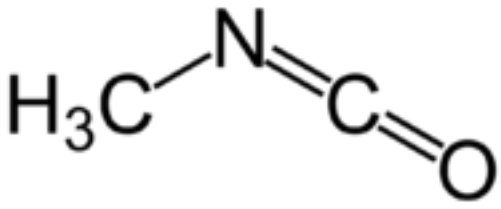


Figure 1. Chemical structure of MIC.

Table B1. Chemo-physical properties of MIC

Property	Value
Odor threshold	2.1 ppm
Boiling point	39.5ºC
Melting point	-45ºC
Specific gravity	0.9599 at 20ºC
Solubility in water	6.7% at 20ºC
Vapor pressure	348 mm Hg at 20 °C

Values from: http://www.atsdr.cdc.gov/mhmi/mmg182.pdf

When released to air, a vapor pressure of 348 mm Hg at 20 deg C indicates MIC exists solely as a vapor in the atmosphere. MIC will be degraded in the atmosphere by reaction with photochemically-produced hydroxyl radicals; the half-life for this reaction in air is estimated to be 118 days (HSDB, 1995).

B.2. Use and Exposure Information

MIC is a breakdown product of some pesticides and is used to a lesser extent in the production of polyurethane foams and plastics (U.S. EPA, 1986). It is also a carbamylating intermediate and this is the basis for its use in the manufacture of carbamate-based insecticides and herbicides. The chemistry of MIC suggests that hydrolysis to methylamine and dimethylurea is rapid, such hydrolysis in moist air is probably slow, and the reaction with photochemically produced hydroxyl radical is also slow (chemical $t_{1/2}$ about 3 months) (U.S. EPA, 1986). Brown *et al.* (1987) have shown that the alkylisocyanates (e.g., MIC) are relatively resistant to hydrolysis in water.

Tobacco smoke from some brands of cigarettes also contains MIC (about 4 micrograms (µg) per cigarette) and the general population may be exposed to MIC through inhalation of cigarette smoke (HSDB, 1995). At the MIC 8-hour threshold limit value of 0.02 ppm (46 µg/m^3) exposure would be approximately 460 µg MIC in a workday. Occupational exposure may occur through inhalation and dermal contact with this compound at workplaces where MIC is produced or used. The concentration of MIC measured in a single air sample collected during a welding operation in a car repair shop was 290 µg/ m^3 (Karlsson, 2000).

Exposure to MIC may also occur following applications of the soil fumigant, metam sodium due to photolysis of the metam sodium breakdown product methyl isothiocyanate (MITC). The yield of MIC from MITC has been reported to be about 7 percent (%) in laboratory experiments (Geddes *et al*., 1995). Preliminary measurement of MIC after agricultural use of metam sodium in Kern County, CA revealed levels between 0.09 and 2.5 parts per billion (ppb) (CDPR, 2004) and the potential highest level of MIC to be found under the conditions of this study was estimated to be 5 ppb, exceeding by about 12-fold the OEHHA chronic

reference exposure level (REL) of 0.43 ppb (1 μg/m^3) for exposures to the general public and by 5-fold the 1-hour acute REL of 0.98 ppb calculated by CDPR (CDPR, 2004). It is below the OSHA and Cal OSHA Permissible Exposure Limit (PEL) of 20 ppb for judging exposures to workers. Factors influencing the microclimate during such an exposure to MIC include inversion and air temperature which could have an impact on the exposure to MIC (CDPR, 2004).

B.3. Pharmacokinetics

The uptake and distribution of MIC was studied in guinea pigs and mice. MIC-derived ^{14}C activity was detected in venous and arterial blood within minutes of exposure; however, blood MIC concentrations in animals exposed by way of tracheal cannula were much lower than in those which breathed normally. MIC was cleared slowly from the blood within 3 days. In female mice the highest concentrations at 2 hours after exposure were found in the lung, fetus, spleen, uterus, and kidney. After 24 hours, the highest concentrations were in the lung, spleen, and fetus (Ferguson *et al*., 1988). Since administration of ^{14}C-labeled MIC to mice by inhalation demonstrated that radioactivity rapidly distributed to all tissues including the uterus, placenta and fetus, it has been suggested that placentally-transferred MIC can directly affect the fetus (Varma *et al*., 1990).

A transport system for MIC via reduced glutathione (GSH) has been suggested by the discovery of the MIC-adduct, S-(N-methylcarbamoyl) glutathione (SMG), in the bile and the MIC-adduct of N-acetylcysteine (mercapturic acid, AMCC) in the urine of rats exposed to MIC by non-inhalation routes (Pearson *et al.*, 1990; Slatter *et al.*, 1991). Metabolites of MIC include monomethylamine (MMA), dimethylamine (DMA) and trimethylamine (TMA) (Varma et al., 1990).

B.4. Non-DART toxicities

B.4.1. Acute Toxicity

In the 1984 Bhopal incident, ground level concentrations were estimated to range between 0.12 and 86 ppm (Dhara and Dhara, 2002). MIC exposure resulted in the deaths of more than 2,500 people in the first few days and adverse health effects in greater than 170,000 survivors (U.S. EPA, 1986). Pulmonary edema was the cause of death in most cases, with many deaths resulting from secondary respiratory infections such as bronchitis and bronchial pneumonia (U.S. EPA, 1986; HSDB, 1995). Other effects noted from acute inhalation exposure to MIC in humans are respiratory tract irritation, difficulty breathing, blindness, nausea, gastritis, sweating, fever, chills, and liver and kidney damage. Survivors continue to exhibit damage to the lungs (e.g., bronchoalveolar lesions and decreased lung function) and the eyes (e.g., loss of vision, loss of visual

acuity, and cataracts). Similarly, animal studies have reported pulmonary edema, upper respiratory tract irritation, respiratory lesions, and weight loss from acute inhalation exposure to MIC. LC50 levels in rodents following a 6 hour exposure were in the 6-12 ppm range (CDPR, 2004)

B.4.2. Subchronic and Chronic Toxicity

Three years after the Bhopal disaster, loss of vision and loss of visual acuity were more prominent among exposed residents than among unexposed people, and the losses appeared to be dose-dependent (Andersson *et al.*, 1990). Similarly, cataracts were reported more often among the exposed than among the unexposed group.

Delayed MIC inhalation toxicity has been observed in experimental animals studies (Dodd and Fowler, 1986, Mitsumori *et al.*, 1987). The chronic REL of 0.43 ppb (1 $\mu g/m^3$) derived by OEHHA was based on endpoints observed within 1 day of cessation of exposure. Post-exposure evaluation showed that at a higher exposure level (3.1 ppm), progressive changes, including death, occurred.

The long term pulmonary effects of a single exposure to MIC were examined in rodents (Bucher et al., 1989). Groups of 50 or 100, 6 to 8 week old F344 rats and B6C3F1 mice were exposed to MIC by inhalation at 0, 1, 3, or 10 ppm for 2 hours. After 2 years, animals were sacrificed and tissues and organs were examined microscopically. No differences in survival rates or body weight gains were found in the MIC-exposed animals versus controls. Male and female rats exposed to 10 ppm MIC had 42% and 36% incidence, respectively, of intraluminal fibrosis of secondary bronchi; no evidence of this lesion was seen in controls or animals exposed to lower concentrations.

B.4.3. Mutagenicity and Cancer

In the study described immediately above (Bucher et al., 1989), the long term carcinogenic effects of a single exposure to MIC were also examined. For male and female mice and female rats, no neoplastic lesions were significantly associated with this single acute MIC exposure. Male rats exposed to MIC had marginally increased rates of pheochromocytomas of the adrenal medulla and adenomas of pancreatic acinar cells (Bucher et al., 1989). It is unknown whether progression of these tumors to malignancies would be seen after chronic exposure.

In vitro evidence of genotoxicity has been reported by Shelby *et al.* (1987). In four experiments involving exposures on 4 consecutive days to 0, 1, 3, or 6 ppm MIC, delays in bone marrow cell cycle were observed. Increases in sister chromatid exchanges (SCE) and chromosomal aberrations were observed in

bone marrow cells, and a dose-related increase in SCE occurred in lung cells but not in peripheral blood lymphocytes. A significant increase in micronucleated polychromatic erythrocytes in the peripheral blood was observed in male mice in one experiment. From these results, it appears that MIC has the capacity to affect chromosome structure but not to induce gene mutation. Induction of these chromosomal effects by MIC was not dependent on an exogenous source of metabolism. As discussed below in section E.2.2., no evidence of a dominant lethal effect was observed in male mice exposed to MIC (Schwetz *et al*., 1987). Whether the lack of effect in this study is related to the failure of MIC to reach critical target cells is unknown.

There are only a few reports available based on conventional cytogenetic profiles of exposed humans. These reports document substantial linkage of MIC exposure to somatic mutagenesis, but one researcher noted several shortcomings in the methodological approach (Mishra, 2009b).

Several studies have been conducted to evaluate the genotoxic potential of MIC in cultured mammalian cells after *in vitro* exposure. These studies investigated cellular DNA damage response through qualitative phosphorylation states of ATM, γH2AX proteins and quantitative state of p53 phosphorylation; DNA cell cycle analysis and cellular apoptotic index. According to Mishra *et al*. (2009b), by negatively regulating the DNA damage response pathway, MIC might promote cell cycle arrest, and apoptosis in cultured mammalian cells. Additionally, induction of genomic instability in cultured human colonocytes following exposure to MIC demonstrated that many treated cells were arrested at the G2/M phase of the cell cycle and had an increased apoptotic index and elevated inflammatory cytokine levels. Cytogenetic analyses revealed varied chromosomal anomalies, with abnormal expression of pericentrin protein. Also, increased microsatellite instability due to variable amplification of simple inter-sequence repeats has been demonstrated (Mishra *et al*., 2009b). Morphological transformation and stress-induced senescence was noted in cultured human kidney epithelial cells after treatment with 0.005 µM concentration of MIC and along the time course, an increase in DCF fluorescence indicative of oxidative stress, depletion of superoxide dismutase (SOD), glutathione reductase (GR) and consistent accumulation of 8-oxo-dG were noticed. According to the authors, MIC exposure *in vitro* appears to result in aberrant expression of p53, p21, cyclin E and CDK2 proteins, suggestive of deregulated cell cycle, chromosomal aberrations, centromeric amplification, aneuploidy and genomic instability (Mishra *et al*., 2009b).

No information is available on the carcinogenic effects of MIC in humans. Increases in relatively rare gallbladder adenocarcinomas in Bhopal survivors are being examined (Mishra *et al*., 2009c). In a study in which animals were exposed once by inhalation, no tumors were significantly associated with MIC exposure in mice and female rats. Over 20 years ago U.S. EPA classified MIC as a Group D, not classifiable as to human carcinogenicity because of insufficient data on which

to make the evaluation (U.S. EPA, 1986). The U.S. EPA has not updated this evaluation, but as noted above there are few data with which to judge the carcinogenic potential of the compound.

C. Developmental Toxicity

C.1. Human Studies

C.1.1. Introduction to Human Studies of MIC

The available human studies of the effects of MIC on reproduction and development are based on exposures that occurred in December 1984 in Bhopal, India. These exposures occurred due to an accidental release from a pesticide manufacturing plant operated by Union Carbide. "MIC is an intermediate product in the manufacture of carbaryl… a carbamate pesticide" (Dhara and Dhara, 2002). Nearly 30 of the 42 metric tons of MIC stored in a tank at the plant escaped within a matter of 45-60 minutes (Varma and Varma, 2005). This accidental release occurred as a result of the entry of water into the tank, which resulted in an uncontrollable reaction (Dhara and Dhara, 2002). The contents of the tank spread "as a cloud over a large, densely populated area of approximately 40 km^2" (Mehta *et al*., 1990). Atmospheric inversion and a low wind speed prevented dispersion of the gas (Dhara and Dhara, 2002). Because of the wind direction, the area with the largest number of dead and severely injured was approximately 7 km^2 south of the plant (Mehta *et al*., 1990).

Bhopal was a city of more than 800,000 people at the time of the accident, according to the report of the Indian Council of Medical Research (ICMR, 2001). Estimates of the number of people exposed to the gas vary. Varma and Varma (2005) note that approximately 200,000 were exposed; a recent review article (Mishra *et al*., 2009b) notes that more than 500,000 people have registered as survivors with the authorities, a value consistent with the ICMR's estimate of the number of residents affected by the gas. The ICMR estimated that more than 100,000 people were living in the areas they define as "severely affected" or "moderately affected" by the gas, and more than 400,000 were living in the areas they define as "mildly affected".

The release killed several thousand people in the first three days. Estimates of the number killed in the 48-72 hours following the release vary from a low of 1800 (Mehta *et al*, 1990) to a high of 10,000 people (Sharma, 2005), with most accounts citing a number between 2500 and 5000 (e.g., ICMR, 2001; Dhara and Dhara, 2002; Varma and Varma, 2005; Mishra *et al*., 2009b). According to the ICMR (2001), part of the reason for the uncertainty was due to the large number of people who might have died but had no residential address, what they call "the floating population of city like visitors, daily wage labour and passengers at the Railway Station." Another 50,000 to 250,000 people were injured. In the first 24-

hour period, 90,000 patients were seen at nearby health facilities. The ICMR developed a tracking system for a long-term cohort study, in which they classified the exposure of 80,021 persons as severe, moderate or mild and assigned each household a tracking number. This assignment of numbers helped individuals to be tracked in later studies conducted by the ICMR and other investigators who made use of these already-assigned numbers.

The only report published on the extensive studies conducted from 1985 through 1994 by the ICMR (2001) provided limited information on the extent of health impacts found in the survivors of the gas leak. That report, although apparently released publicly, is not available in the open literature. OEHHA was able to review a copy obtained via interlibrary loan by the University of California library from the Library of Congress; the entire volume was examined but only a limited number of pages were permitted to be copied. The ICMR conducted symptom prevalence surveys but did not publish the results of these studies (Dhara and Dhara, 2002). Follow-up studies conducted by the ICMR of the prevalence of symptoms associated with the accidental release continued to find elevated rates of respiratory, ocular and gastrointestinal symptoms several years afterwards (1988-1990), with respiratory, ocular, and neurological symptoms reported in later surveys (1994), showing "a strong gradient by exposure category", as reported by Dhara and Dhara (2002). According to Mishra *et al*. (2009b), researchers working in Bhopal Memorial Hospital and elsewhere in India, "[s]urvivors continue to experience higher incidence" of a range of health problems, including respiratory, neurologic and ophthalmic symptoms.

C.1.2. Exposure Information Relevant to Human Studies of MIC

Panic and disorientation caused by the irritant effects of MIC led people in Bhopal to run out of their homes into the gas cloud, increasing their exposure (Dhara and Dhara, 2002). These authors note that "[v]ariability in human exposure …resulted from distance of the residence to the plant, duration of exposure, and activity during exposure." Major organs affected by exposure include the eyes, the respiratory tract and the skin, with some oral exposure due to MIC dissolved in saliva, also likely (Dhara and Dhara, 2002).

Several investigators have suggested that there also may have been a variety of chemicals to which people were exposed. A news report (Crabb, 2004) published as part of the 20 year anniversary of the accident notes, "Early autopsy studies as well as analyses of the gooey residue left in the Bhopal storage tank found about two dozen chemical constituents." The ICMR (2001) report provides a list of MIC-related and other compounds identified in residues taken from sections of "the buried tank" some time after the disaster, including: methylisocyanate trimer; dimethyl urea; dimethyl isocyanate; trimethyl urea; dione; trimethyl biuret; tetramethyl biuret; mono methyl amine; dimethyl amine; chloride; metallic ions (Fe, Cr, Ni, Mo, Na, Ca & Mg). Relevance of these tank

residues to the composition of the gas cloud is unclear.

Because MIC pyrolyses to hydrogen cyanide (HCN) at high temperatures (427^0 C - 548^0 C) (Dhara and Dhara, 2002), the potential for co-exposure to this toxin has gained the attention of several investigators. In an early report, Lepkowski (1985) noted that scientists of India's Air Pollution Control Board detected cyanide at the MIC storage tank and 50 meters downwind three days after the accident. However, Varma (1989) reported that no HCN residue was detected in any part of the Bhopal plant, and most deaths occurred 12-72 hours after the accidental release. Varma also notes that this pattern was seen in studies of animals exposed to MIC and contrasts this with what would be expected from a lethal dose of HCN: death within three to four hours. Varma (1989) also notes the long-term effects seen in Bhopal of pulmonary lesions are not characteristic of HCN toxicity. According to Sharma (2005), VR Dhara, a member of the International Medical Commission on Bhopal, believes that a simulation of the accident in controlled conditions is needed to determine if other chemicals were generated.

The ICMR (2001) report provided findings from an investigation of the potential chemicals of interest by the "ICMR Task Force on Toxicological Study":

> "They demonstrated the presence of high cyanide levels in blood and preserved post-mortem tissues from persons who died between 3-6 December, 1984 [immediately following the accident]. This provided convincing evidence that hydrogen cyanide…was also one of the constituents of the Gases generated as a result of the pyrolation effects of MIC…."
>
> "This Group…confirmed that on entering the blood stream MIC causes irreversible N-Carbamoylation of end-terminal valine residues of haemoglobin… . This partly accounts for the reddish discoloration of blood. More importantly, the resultant diminished unloading of oxygen in tissues could be responsible for anoxic tissue damage of all organs but most seriously of the brain… . Instantaneous interaction of inhaled MIC seem [sic] to have resulted in destruction of alveolar membrane proteins; this seems to have caused massive exudation of fluid into the alveoli and compensatory emphysema. Passage of MIC down the respiratory tract seems to have caused destruction of the highly specialized alveolar membrane leading to wide spread systemic effects…"

While the exact composition of the cloud is not known, the presence of very high levels of MIC in the gas cloud is not disputed. The ICMR report (excerpted above) described the major role MIC played in causing the symptoms seen in those who were exposed to the gas, while at the same time acknowledging the uncertain identity of all constituents of the gas cloud. Mean MIC concentration in the cloud released from the Union Carbide plant that night in December 1984

was estimated as 27 ppm, with a range of concentrations estimated as 0.12 – 85.6 ppm (Dhara and Dhara, 2002). These authors cite the work of others for these estimates: the mean estimate was included in a report by the Central Water and Air Pollution Control Board published in 1985; the range was developed from a model simulating the accidental dispersion, published in 1987 by Singh and Ghosh.

As a comparison, the recommended threshold limit value (TLV) for exposure to MIC in an occupational setting is 0.02 ppm (0.047 mg/m^3) (ACGIH, 2001), a thousand times lower than the mean estimated concentration to MIC experienced by individuals exposed to the accidental release in Bhopal. According to the American Conference of Governmental Industrial Hygienists (ACGIH), "[t]his value is intended to minimize the potential for acute, severe mucous membrane irritation and possible pulmonary sensitization."

No measurements were made on the night of the accident, to verify the composition of the gas cloud or the level of MIC present. However, given the extremely large volume of MIC (~30 metric tons) that was released to the atmosphere, it is reasonable to assume that the predominant if not sole exposure faced by those who encountered the gas cloud was MIC.

C.1.3. Individual Studies

A total of ten reports were identified that provide information relevant to developmental effects in humans exposed to MIC. The studies are described chronologically in order of publication. Eight studies address pregnancy outcome and neonatal mortality (Shilotri *et al*., 1986; Varma, 1987; Kanhere *et al*., 1987; Bhandari *et al*., 1990; Kapoor, 1991; Varma, 1991; ICMR, 2001; Dhara and Dhara, 2002). Two studies (Ranjan *et al*., 2003; Mishra *et al*., 2009a) examine effects on growth and immunological function, respectively, related to *in utero* exposure.

Shilotri et al. (1986).

Shilotri *et al*. (1986) describe the gynecologic and obstetric findings of a medical survey carried out 105 days after the disaster in Bhopal. Details of the methods for conducting the survey are described in Naik *et al*. (1986). The survey was conducted in camps set up in two different areas, with subjects registered and then directed to a specific station based on age and gender. Uniform details were collected from each individual, including information on duration of possible exposure to the leaked gas, and based on symptoms they were directed to specialists.

Shilotri *et al.*,collected detailed information on gynecological symptoms and any recent obstetric loss from two groups of subjects, all females of child-bearing

age. Group I subjects lived near the site of the disaster, either (a) within 0.5 km distance from the Union Carbide factory (Shakti Nagar, Kaichi Chhola, Risaldar Colony, J. P. Nagar), or (b) between 0.5 to 2 km distance from the factory (Ram Nagar, Rajgarh Colony, Subhash Nagar. Phuta Maqbara, Quazi Camp and Railway Colony). Group II subjects lived in areas beyond 8 km from the factory (Ambedkar Colon, T. T. Nagar and Seva Sadan). Group II subjects, whose participation was voluntary, were preselected to be similar with regard to socio-economic status and the types of houses in which they lived, to serve as controls. However, Naik *et al*. (1986) noted that out of the full group (men, women and children) surveyed, "…42% of Group II subjects had eye symptoms and 22.3% had respiratory symptoms; it is therefore clear that even Group II subjects were affected [by the MIC release] and hence were not true controls...". Women with gynecological complaints in either group were included in the study: from Group I, 88 out of 160 women; from Group II, 12 out of 38 women.

The authors describe the results from obstetric examination of the subjects and reported that seven of the pregnant women from Group I had sub-optimal uterine height, a measure of fetal growth. This condition in the mothers was not accompanied by evidence of explanatory causes of intra-uterine growth retardation (such as severe anemia, toxemia or gross malnutrition). Out of 38 women known to be pregnant at the time of the disaster, 29 women reported first and second trimester spontaneous abortions soon after the disaster. The date of onset of these abortions was not available in many cases, the authors report, because the pregnancy loss followed a period of unconsciousness.

Two women exposed to MIC during 10 to 12 weeks gestation had delivered pre-term babies with birth defects (spina bifida, meningomyeleocele, limb deformities and signs of heart disease) and organ damage who died soon after birth. The authors note: "On autopsy, both babies showed lungs that were enlarged, solid, heavy and showed fibronodular deposit, bilaterally. They also showed liver and brain damage."

Varma (1987).

During early September 1985, a little more than nine months after the disaster, a survey was conducted with regard to pregnancy outcome of 3270 families who lived near the Union Carbide plant. The same families' history of pregnancy outcomes in the two years prior to the disaster served as the comparison, to minimize the effect of socio-economic status and other variables that could affect reporting. The actual conduct of the survey was carried out by high school graduates familiar with the area who used a printed list of questions written in the local language (Hindi).

Of subjects (n=865) who were pregnant at the time of the accident, 379 (43.8%) did not deliver a live baby; this includes "spontaneous abortions, stillbirths, and

possibly intentional abortions." The author reports that the normal incidence of pregnancy loss in Bhopal is 6% to 10%, as estimated by the Indian Council of Medical Research. The survey itself was not able to ascertain the rate of unsuccessful pregnancy in the two years prior to the accident. The rate of unsuccessful pregnancies appeared to be higher for women who were in their first trimester during the accident, as compared to those who were in their second or third trimester. Of the 486 live births, 14.2% of infants died within the first 30 days. The infant death rate in the families surveyed, for the two years prior to the accident, was 2.6% to 3%. Issues with this survey are that it relied entirely on self-reported information by the woman or a senior member of the family. Also, comparison of unsuccessful pregnancies for this area to all of Bhopal may be less accurate than comparing to the same families or another group with similar socio-economic status. The author noted the need for a more controlled epidemiological study to be undertaken.

Kanhere et al. (1987).

Researchers from the Gandhi Medical College in Bhopal, Kanhere *et al*. (1987), studied 134 placentae, including 110 from women who were exposed to the gas released by the Union Carbide plant in Bhopal. The study was conducted during the period from December 3, 1984 through July 31, 1985. Selection criteria are not described, nor is information provided on the demographics (e.g., age) of the women in the study. The control group of placentae were obtained from women who lived in Govindpura, Habibganj and South TT Nagar, presumably areas that were unexposed to the gas. Most of the placentae were from full term deliveries (89 exposed, 15 control). A small number (9 exposed, 2 control) were derived from premature births (defined by the authors as birth before 37 weeks of gestation). A final set were those obtained from medical termination of pregnancy during the first trimester (12 exposed, 7 control), most (93%) of whom terminated pregnancy to limit family size.

The mean weight of placentae from full-term gas-exposed women was significantly lower than that from full-term control women ($p<0.05$). The authors also reported that fetal weight was lower in the gas-exposed group, but do not provide any details of these results. Although the severity of the women's gas exposure was described with respect to the numbers in each group (severe, 28; moderate 57; mild, 25), none of the results were reported with regard to these exposure differences.

With regard to histological changes seen in the placentae, significantly greater ($p<0.05$) numbers of exposed compared to control placentae for both full term and medically terminated pregnancies had hydropic degeneration (full term: 35.4% exposed vs. 17.7% control; medically terminated: 75% exposed vs. 36.7% control). Other histological changes that varied by exposure status include calcification (full term: 56% exposed vs. 46.6% control; medically terminated:

25% exposed vs. 14.2% control), and chorioamnionitis (full term: 28% exposed versus 20% control; medically terminated: 39.3% vs. 34%), although no results of statistical analyses are reported for these endpoints. Due to the small number of premature deliveries in both exposed and control groups, the authors indicate that no comparisons could be made for those placentae.

Bhandari et al. (1990).

A survey was conducted in April – May 1985 to identify women in "10 severely affected areas of Bhopal city" who were pregnant at the time of the Union Carbide disaster (n=2566). The study focus was on adverse pregnancy outcomes. Controls (n=1218), women who were pregnant as of December 3, 1985 (a year after the accident), were drawn from an area chosen due to its similar socio-economic status in a part of Bhopal unexposed to the gas. In the affected area, retrospective reports were taken from those who had suffered a spontaneous abortion or had delivered a baby during the time between the accident and the beginning of the survey, and those who were still pregnant were followed prospectively. All those in the control area were followed prospectively. The socio-economic status of those in the affected area was somewhat less as measured by mean per capita income than in the control area (Rupees (Rs.) 96 ± 87 [time period not specified] vs. Rs.122 ± 69, respectively). However, the literacy rate was slightly higher in the affected area (39.4% vs. 32.5%). In addition, the affected area had a higher proportion Muslim population (40.3%) compared to the control area (14.4%). Mean age of women was similar in both areas (24.4 years, affected vs. 24.9 years, control), although a significantly greater proportion of women included in the study from the affected area were aged 30 years or more (21.6% vs. 15.7%). Average parity of women from the affected area was greater than that of the controls (2.8 total births vs. 2.1 total births). There were differences in the distribution of women according to the point of gestation on December 3rd of the year under study, with fewer pregnancies of gestation week 28 or more from the affected area; the authors speculated that women in the affected areas who were nearing term at the time of the accident went elsewhere for their delivery and were thus not present at the time of the survey.

Table 1 summarizes the results of adverse pregnancy outcomes for the women in the study. Rates of spontaneous abortion (loss before 20 weeks gestation) were significantly higher in women from the affected area. Women from the affected area aged 30 years and over had especially high rates of spontaneous abortion (32.5%), while no case was reported in women over age 30 from the control area. The authors "standardiz[ed] for age according to the control area" and found the spontaneous abortion rate in the affected area (23.6%) continued to be highly elevated relative to controls (5.6%). Rates of intermediate (21 – 27 weeks gestation) fetal deaths, stillbirths and congenital malformations were slightly higher in women from the affected area, but not significantly different.

As shown in Table 1, rates of perinatal (death within seven days of birth) and neonatal mortality (death between 8 – 28 days of birth) were significantly greater in the affected area.

Bhandari *et al*. noted that the stress and trauma of the accident may account for a proportion of the increase in spontaneous abortions. The authors also suggested that effects of MIC on a pregnant woman's respiratory system could have impacted the fetus.

Table 1: Pregnancy Outcome in Women Exposed to MIC in Severely Affected Areas During Bhopal Disaster (data from Bhandari *et al*., 1990)

Outcome measure	Affected area		Control area
	Actual findings	**Age standardized[1] findings**	
Total pregnancies	2566	--	1218
Spontaneous abortion rate (<20 weeks gestation)	24.2%*	23.6%*	5.6%
Intermediate pregnancy loss rate (21-27 weeks gestation)	1.2%	Not reported	0.7%
Still birth (28+ weeks gestation) (per 1000 deliveries)	26.0	Not reported	22.9
Congenital malformations (per 100 births)	14.2	Not reported	12.6
Perinatal death (per 1000 live births)	69.5*	60.6*	50.5
Neonatal death (per 1000 live births)	60.9*	Not reported	44.8

*p<0.001

[1.] Results for women from the affected areas were standardized to the age distribution of women in the control area. The authors report only those standardized results shown in the table.

Kapoor (1991).

This study was carried out in 1985 to determine rates of fetal loss among women exposed to the accidental release of MIC compared to rates among women from an area not affected by the release. These areas were based on an identification made by the Indian Council of Medical Research. Women (n=136) from a severely affected area, Jaiprakash Nagar, were compared to 139 women from control areas (Annanagar, Ishwar Nagar, Gautam Nagar, and Vikas Nagar).

Eligible women were aged 20 to 44 years and had at least one pregnancy during the previous five years. Equal proportions of Hindus and Muslims were selected from each group to ensure comparability. The author compared the groups with regard to literacy, educational attainment, occupation and marital status and found the difference was not statistically significant. However, with respect to the standard of living, the gas-affected households had somewhat better conditions, such as drinking water and toilet facilities; the author indicates that these were a recent change in conditions related to compensation received by those affected by the gas.

Fetal loss was defined as loss occurring up to 28 weeks gestation. Pregnancy status at the time of the accident and in the year that followed was self-reported in response to interviewer questions. Women in the affected area who were pregnant at the time of the accident (n=75) had spontaneous abortions more frequently (26.7%, 20 women) compared to those women (n=60) living in the control area (10%, 6 women). In subsequent years, women in the affected area continued to experience much greater pregnancy loss than those in the control area; out of 255 pregnancies in the affected area, 26.3% were lost compared to 7.8% lost to women in the control area.

When the losses were examined by age of the mother (see Table 2), fetal losses in the control area followed what the author calls a "normal pattern", while for those in the affected area, fetal deaths were higher in all age categories. The author notes the limitation of small study size. The study appears to include multiple pregnancies in the same woman without any control or adjustment for the lack of independence of these events. The author notes that repeated pregnancies and fetal losses may have "weakened women in the affected area leading to continued high fetal losses."

Table 2: Rate of fetal loss[1] by [mother's] age at conception for women exposed to the Bhopal disaster (adapted from Kapoor, 1991)

Maternal age group	Rate in affected area	Rate in control area
15 – 19 years	38.1%	10.0%
20 – 29 years	24.0%	5.7%
30+ years	18.6%	8.4%

[1] Defined as loss up to 28 weeks gestation

Varma (1991).

Varma (1991) provided details on a survey carried out in August 1986 that

included 2622 households all located within a radius of 1 km of the Union Carbide plant using a similar methodology to that used by the same author in an earlier survey (Varma, 1987). Questions were asked by high school graduates familiar with the area who used a printed list written in the local language (Hindi). Participants were informed that information provided was in no way associated with compensation claims. All of the households included were apparently also part of the earlier survey (Varma, 1987). Information was collected regarding women who were pregnant at the time of the accident (n=638), the outcome of the pregnancy, the health of any children born alive, and the time of death for any children who had died. The author also followed up with a second survey in July 1990 with households to whom a live child had been born (n=273), to determine the health status of the child. As in the previous study (Varma, 1987), Varma (1991) collected information from these women regarding live births and infant mortality in the two years preceding the accident as a comparison.

A large proportion (49.4%) of those who reported being pregnant at the time of the accident did not give birth to a live baby. Varma (1991) notes that the average pregnancy loss for urban India was 7%-11% based on two reports during the mid to late 1980s. Pregnancy loss in the women affected by the accident was significantly higher in women in their first trimester at the time of the accident compared to those in the second or third trimester (1^{st} trimester, 58.8%; 2^{nd}, 42.1%; 3^{rd}, 40.1%; $p<0.001$). Of the 323 live births, 39 infants (12.1%) died in the first 30 days. Varma (1991) compared this to the neonatal death of 2.7% - 3.3% of babies born previously (12 of 441 in 1984; 19 of 584 in 1983) in the families included in the survey. The author noted the higher rates of loss found in this study compared to reports by the Indian Council for Medical Research (ICMR) (24.2% pregnancy loss). He suggested that the women in this study, all of whom had ICMR numbers (and thus were included in ICMR reporting), resided in areas exposed to especially high concentrations of MIC, which he estimated to range from 26 to 100 ppm.

Indian Council of Medical Research (ICMR, 2001).

The Indian Council of Medical Research (ICMR, 2001) initiated its “Population Based Long Term Epidemiological Studies on the Health Effects of the Toxic Gas” on January 15, 1985 and completed them in May 1994. These studies were carried out to examine the impact of the exposure experienced by the residential population in Bhopal who were present at the time of the disaster. Individuals from the affected areas (n= 80,021) were registered for these studies, as were individuals from an area considered “exposed but unaffected” (n= 15,931), as a comparison group. Demographic characteristics of the affected population were generally similar to those in the control area.

The affected areas were divided into three exposure categories (based on death rates within the area and symptoms of the survivors): “severely affected”,

"moderately affected" and "mildly affected." Each household was provided with an identification number, for the purpose of long term follow-up. For the first few months following the disaster, and during some other time periods as well, surveys on morbidity and mortality were conducted each 14 days. All households were revisited in early 1987 to update the cohort registration, and from 1987 onward, data were collected through yearly follow-up on the registered households.

As part of this study, pregnancies and their outcome were included, for the period beginning in late 1984 through 1989. The pregnancy status of all married women age 15 – 49 years along with pregnancy outcome was recorded during surveys of the population. However, the size of the population from which pregnancy data were reported is unclear; the number of pregnancies included in the report's summary of pregnancy losses during the study period appears to be smaller than would be expected in the entire registered population, and also varies widely from year to year. For example, in the area "severely affected", 1261 total pregnancies were reported in 1985, and only 125 pregnancies in 1987. Whether this variation is the result of incomplete reporting of the registered population or more complete reporting of some unspecified subset that varied in size over time could not be determined from the data provided. This report does not define the periods of loss covered by the authors' use of the terms spontaneous abortion, stillbirth, neonatal death or perinatal death, nor do the authors describe which congenital malformations were included in their study.

According to the authors, the rate of spontaneous abortion in the severely affected area immediately after the disaster was 523 per 1000 (i.e., >50%), and showed a decreasing trend from the severely to the mildly affected areas for this time period. In subsequent years, the rate of spontaneous abortion declined somewhat but remained elevated in the affected areas relative to the control area (see Table 3A). For stillbirths, no clear pattern was observed (see Table 3B). No data were reported on neonatal or perinatal mortality, although the authors briefly note that the "perinatal mortality rate seemed to be within the limits reported from other parts of the country."

The authors provide a narrative report on the results of a study of congenital malformations in infants born to mothers who were in the first trimester of pregnancy at the time of the MIC gas leak. They note that thirty infants (out of an unspecified number of live births) were born with congenital malformations, and conclude that the study did not reveal any increase in the incidence of congenital malformations.

Table 3A: Rates of Spontaneous Abortion in Exposed and Control Areas of Bhopal from Late 1984 through 1989 (adapted from ICMR, 2001)

Year	Areas Based on Level of Affect from Bhopal Disaster			
	Severely (SA / total preg)	**Moderately (SA / total preg)**	**Mildly (SA / total preg)**	**Control (SA / total preg)**
Late 1984	(102 / 195) 52%	(62 / 160) 39%	(6 / 30) 20%	(3 / 36) 8%
1985	(169 / 1261) 13%	(158 / 1257) 13%	(65 / 437) 15%	(8 / 359) 2%
1986	(122 / 949) 13%	(72 / 478) 15%	(19 / 319) 6%	(13 / 329) 4%
1987	(18 / 125) 14%	(30 / 222) 14%	(8 / 98) 8%	(8 / 167) 5%
1988	(19 / 277) 7%	(8 / 253) 3%	(9 / 104) 9%	(1 / 137) <1%
1989	(35 / 451) 8%	(23 / 325) 7%	(13 / 138) 9%	(5 / 205) 2%

Abbreviation: SA – spontaneous abortions

Table 3B: Rates of Stillbirth in Exposed and Control Areas of Bhopal From Late 1984 through 1989 (adapted from ICMR, 2001)

Year	Areas Based on Level of Affect from Bhopal Disaster			
	Severely (SB / total preg)	**Moderately (SB / total preg)**	**Mildly (SB / total preg)**	**Control (SB / total preg)**
Late 1984	(4 / 195) 2%	(1 / 160) <1%	(1 / 30) 3%	(1 / 36) 3%
1985	(21 / 1261) 2%	(32 / 1257) 3%	(12 / 437) 3%	(8 / 359) 2%
1986	(24 / 949) 3%	(22 / 478) 5%	(10 / 319) 3%	(3 / 329) <1%
1987	(0 / 125) UD	(2 / 222) <1%	(1 / 98) 1%	(4 / 167) 2%
1988	(2 / 277) <1%	(3 / 253) 1%	(3 / 104) 3%	(2 / 137) 1%
1989	(5 / 451) 1%	(7 / 325) 2%	(1 / 138) <1%	(3 / 205) 1%

Abbreviation: SB – stillbirth, UD – undefined

Dhara and Dhara (2002).

This review article includes a report of a study not available through other channels, conducted by a group cited in Dhara and Dhara (2002) as "Medico Friend Circle, October 1990." The entire report of the study is presented verbatim below:

Medico Friend Circle conducted a pregnancy outcome survey 9 months after the accident in 3 gas-exposed areas of Bhopal. A total population of 8,165 in 1,632 households was surveyed by random sampling. Information on reproductive history and menstrual cycles was collected for the 1 year time period that preceded the gas leak and served as the 'historic control'. A 4-fold increase in overall spontaneous abortion rate for the period after the gas leak was reported. Approximately 24% of women had altered menstrual cycle durations (i.e., 14% of the cycles decreased by ≥7days, 6% were irregular, and 4% increased by ≤7 days). The authors concluded that these effects could be gas-related."

Ranjan et al. (2003).

Ranjan *et al*. (2003) measured physical growth variables in adolescent males and females exposed to the Bhopal disaster as toddlers, *in utero*, or those who were conceived after the disaster by exposed parents. They compared them to age- and sex-matched unexposed individuals from nearby areas (one 15 km southwest, the other 4 km northwest of the Union Carbide plant) that were considered to have been unaffected by the accidental release based on distance from the plant and the wind direction at the time of the release. Households were selected randomly and the survey team was from a different town and "had no prior knowledge of the health of the adolescents." In addition to anthropometric measurements of children, the parents' height, weight and socio-economic status were also recorded and included as potential covariates in the analyses. Mean body weights and heights of exposed and unexposed mothers were similar, as were the same variables for exposed and unexposed fathers.

Data were analyzed separately for girls (n=68) and boys (n=73) from 104 families in the study. Only a small number of individuals were available for analysis regarding *in utero* exposure (exposed: 3 girls, 3 boys; unexposed: 4 girls, 5 boys). Analysis of covariance was performed using SAS. The authors evaluated four groups, including those unexposed and three exposure groups: postnatal exposure, *in utero* exposure, and those born after the incident to exposed parents. Their model also included six potential covariates: age, mother's height and weight, father's height and weight, and socioeconomic status. For each outcome variable for each sex, only the covariates that were statistically significant at $p<0.05$ were retained in the final model. Multiple regression was performed with the three types of exposure forced into the model.

For girls, there was no significant effect of any kind of exposure, including *in utero*. The report does not include the results for girls *in utero* exposure. Significant decreases in most measures, however, were seen in the exposed boys, with the most pronounced effect seen in those exposed *in utero.* Table 4 summarizes these results in boys, omitting the results for those borne prior to the disaster ("postnatal exposure" in the article). Comparisons are based on 36 unexposed boys. The authors note that the "values reported are regression coefficients, equal to the difference between exposed and unexposed boys… after adjustment for age, mother's weight, mother's height, father's height, and socioeconomic status." For example, the effect of *in utero* exposure on height is -13.5 cm, meaning that boys exposed *in utero* were 13.5 cm shorter than unexposed boys of the same age and with the same parental weight and height. The authors indicate that repeating the analyses with only one child per family did not alter the results.

In considering the differential effect seen in exposed boys as compared to girls, the authors note that one of the degradation products of MIC, trimethylamine "has been reported to produce selective growth retardation of male progeny of mice, associated with a decrease in serum testosterone." The authors comment that the finding of nearly identical results in the girls from both exposed and unexposed areas "suggests that the exposed and unexposed groups were well matched." Another potential explanation of the differences observed between exposed and unexposed boys could be other shared exposures faced by these children that may have contributed to their growth deficits, though the lack of an effect in girls in the study makes such an explanation appear less likely. For example, relatively recent reports (Crabb, 2004; Sharma, 2005) indicate that the areas near and including the now defunct Union Carbide plant are still contaminated, including drinking water drawn from ground water in this area. The current locations of residences of the exposed children are not mentioned by Ranjan *et al*.

The small number of children in the exposed groups limits the conclusions that can be drawn from the results; however, the effect appears to be consistent across the measures, with only the triceps skinfold and body mass index lacking a significant deficit. The decreases in height, weight and head circumference for boys with *in utero* exposure compared to unexposed boys are all highly statistically significant.

Table 4: Effect in Boys of Exposure to Gases from Union Carbide Plant Disaster in Bhopal, 1984 (adapted from Ranjan *et al*., 2003)

Variable	**Exposed in utero** (n=3) Effect[1] (95% CI), P value[2]	**Conceived after disaster** (n=6) Effect[1] (95% CI), P value[2]
Weight (kg)	-10.0 (-16.0 to -3.0) p=0.003	-7.9 (-13.0 to -2.0) p=0.007
Height (cm)	-13.5 (-21.0 to -6.0) p=0.001	-8.4 (-15.0 to -1.0) p=0.02
Mid-arm circumference (cm)	-2.7 (-4.6 to -0.8) p=0.005	-2.0 (-3.5 to -0.5) p=0.01
Head circumference (cm)	-3.2 (-4.8 to -1.5) p<0.001	-1.6 (-2.8 to -0.4) p=0.009
Triceps skinfold (mm)	1.9 (-0.3 to 4.0) p=0.09	0.3 (-1.3 to 1.9) p=0.73
Body mass index [weight in grams /(height in meters)^2]	-1.3 (-2.9 to 0.3) p=0.11	-1.4 (-2.6 to -0.2) p=0.03

Abbreviation: CI, confidence interval

[1] Values reported are regression coefficients, equal to the difference between exposed and unexposed boys after adjustment for age, mother's weight, mother's height, father's height, and socioeconomic status.

[2] The p-value shown, according to the report, is "for comparison of effects with zero".

Mishra et al. (2009a).

Mishra *et al*. (2009a) carried out a study of a cohort of individuals exposed to MIC *in utero* during the first trimester of pregnancy, examining their immune system function 24 years later. Limited details are provided in the short published report. The investigators carried out an "extensive verification of the birth records of subjects" and limited the exposed group (n=50, Group III) to those whose mothers were physically present within an area of 2.5 km from the Union Carbide plant in Bhopal on the night of the accident in 1984. Comparison groups included one set of "non-exposed healthy" individuals (n=50, Group I) who came from areas of Bhopal more than 25 km from the plant, as well as a second set of "non-exposed healthy" individuals (n=50, Group II) who came from cities outside Bhopal, more than 200 km from the plant. With regard to these comparison groups, no additional information is provided on their demographic

characteristics (e.g., age, gender) to establish comparability with the exposed group.

A range of blood parameters were examined. While absolute lymphocyte counts were not different between groups, there were significantly higher B lymphocytes ($p<0.001$) in the exposed group. Other parameters were also significantly higher in the exposed group, including levels of interleukin (IL) 2 ($p<0.001$), interferon gamma (IFN- γ) ($p<0.001$), IL-4 ($p<0.001$) and IL-10 ($p<0.001$). Immunoglobulin (Ig) levels were also significantly higher in the exposed group (IgA, $p<0.001$; IgG, $p<0.001$; IgM, $p<0.001$; IgE, $p<0.001$). Mishra *et al*. (2009a) note the plausibility of the effect, as demonstrated in "[a]nimals exposed *in utero* to tobacco smoke (which contains MIC) [that] have hyper-immune responsiveness." The authors conclude that *in utero* MIC exposure during the first trimester "has caused a persistently hyper-responsive cellular and humoral immune state in affected individuals." They intend to follow exposed individuals to identify clinical implications, if any, of this immune hyper-responsiveness.

C.1.4. Summary of Developmental Effects Seen in Human Studies

Although the studies examining pregnancy outcome for those exposed during the Bhopal disaster have some limitations, consistent findings across studies of elevated rates of pregnancy loss in the first trimester (spontaneous abortion) and neonatal mortality provide strong evidence of an effect of exposure to MIC. (see Summary of Available Studies, Table 5). No effect on congenital malformations in relation to exposure was reported in the two studies (Bhandari *et al.*, 1990; ICMR, 2001) that examined this endpoint. More recent studies of those exposed to MIC *in utero* provide indications that such exposure may have lasting health implications.

Table 5: Summary of Available Studies[1] of Spontaneous Abortion[2] Rate in Women Exposed to the 1984 Gas Leak in Bhopal While Pregnant

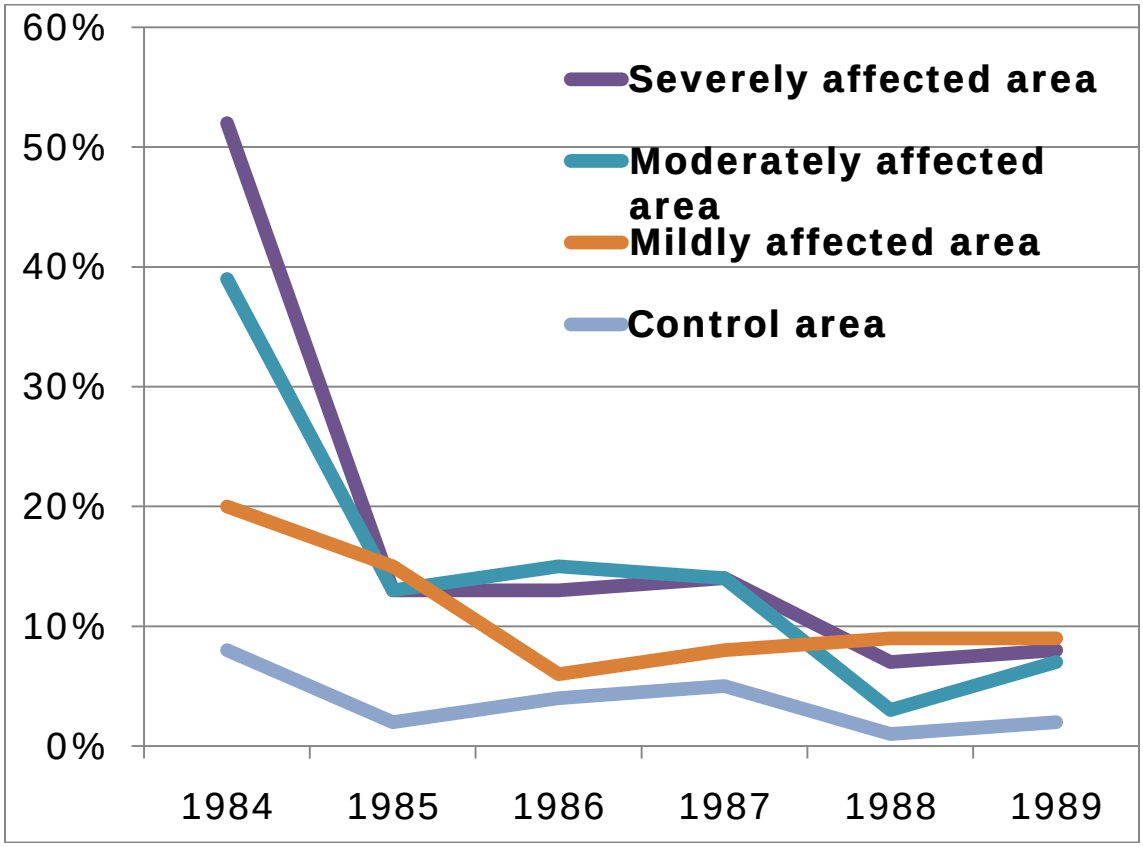
Study Authors (Year)	Population (# Pregnant Women)	Spontaneous Abortion Rate	Comments
Bhandari *et al*. (1990)	Severely affected areas (n=2566) Controls (n=1218)	24% 6%	Controls were drawn from area with similar demographics to exposed area. Control pregnancies occurred in 1985.
Kapoor (1991)	Severely affected area (n=75) Controls (n=60)	27% 10%	Severely affected area based on ICMR identification of areas, according to author.
Varma (1991)	Residence located within 1 km radius of Union Carbide plant (n=638)	59%	The author noted that these women lived in areas exposed to especially high concentrations of MIC.
ICMR (2001)	Area of residence: Severely affected (n=195) Moderately affected (n=160) Mildly affected (n=30) Control (n=36)	 52% 39% 20% 8%	Demographic characteristics of the affected population were generally similar to those in the control area.

1. Although additional studies provided information on pregnancy losses, only the studies shown here presented specific rates for early pregancy loss or spontaneous abortion.
2. The pregnancy losses summarized here include early pregnancy losses as defined in various ways across these studies. Bhandari *et al*. defined spontaneous abortion as loss before 20 weeks gestation; Kapoor reported 'fetal loss' as losses up to 28 weeks gestation; Varma reported losses in the first trimester; the ICMR uses the term 'abortion' without defining it.

In the largest and best controlled study of pregnancy outcome, with more than 2500 exposed pregnancies, Bhandari *et al*. (1990) found significantly higher rates of spontaneous abortion ($p<0.001$) in women from the affected area who were pregnant at the time of the gas exposure. Perinatal and neonatal deaths were also significantly elevated ($p<0.001$) in those exposed to MIC (Bhandari *et al*., 1990). Varma (1987; 1991) also reported that the rate of pregnancy loss in the first trimester and infant deaths occurred much more frequently for pregnancies exposed to the accidental release, though these results are somewhat limited by

potential recall bias for the comparison pregnancies (in the same women two years earlier). In a smaller study, which also lacked a robust control group, Shilotri *et al*. (1986) found both first and second trimester losses were elevated in those exposed while pregnant. The ICMR (2001) study, a large study with limited details on methods and the population studied, reported high rates of spontaneous abortion (>50%) in those from the most severely affected area who were exposed while pregnant. In the ICMR (2001) study, rates of spontaneous abortion were also elevated in women in the moderately and mildly affected areas compared to the control area, with increasing rates with increasing level of exposure as defined by area of residence. In a study with only limited details available (reported by Dhara and Dhara, 2002), large increases in the rate of spontaneous abortion were seen in those exposed while pregnant.

Figure 2. Rate of Spontaneous Abortion over Five Year Period in Women in Bhopal Affected by Gas Leak (Data from ICMR, 2001)



Kapoor (1991) found that women in the affected area not only had higher losses immediately following the accident, but continued in subsequent years to experience higher rates of pregnancy loss than women in a control area. This is consistent with the findings of the larger study conducted by ICMR (2001), which

found that women in the areas affected by the gas continued to experience higher rates of spontaneous abortion in subsequent years than did women with similar demographic characteristics who lived in the control area (see Figure 2).

Data presented by Kanhere *et al*. (1987) also indicate MIC exposure impacted pregnancies. These investigators found that the placenta from full-term pregnancies in gas-exposed women had significantly lower mean weight than those from unexposed women. Kanhere *et al*. also reported a higher percentage of negative histological changes (hydropic degeneration; calcification) in the placenta of exposed women, including both those with full-term pregnancies as well as for medically terminated pregnancies.

In a well controlled study (Ranjan *et al*., 2003), physical size measurements during adolescence for those exposed to MIC *in utero* compared to unexposed children indicate an effect on growth in exposed boys, as measured by significantly decreased height, weight, head circumference, arm circumference, and body mass index. Additional studies that examine these effects are needed to verify these results, which were based on a very small number of boys (n=3) exposed *in utero* in this study.

Mishra *et al*. (2009a) followed up children exposed to MIC *in utero* during the first trimester and examined immunological functioning 24 years after the exposure. They reported significantly elevated blood parameters (B lymphocytes, IL-2, IFN-γ, IL-4, IL-10; immunoglobulins A, G, M and E) that they conclude indicate an immune hyper-responsiveness in these individuals. With regard to clinical implications of these findings, the authors note their expectation that "[w]hether this immune hyper-responsiveness has any clinical implications will be [made] clear by ongoing follow-up of the exposed individuals".

C.2. Animal Studies

C.2.1. Overview

Six animal studies of MIC reporting reproductive or developmental toxicity were identified, as well as two animal studies that did not report reproductive or developmental toxicity. Additional related studies have also been included.

The experimental data available on the toxicity of MIC primarily aid in understanding the effect of MIC as the major component in the chemical cloud released at Bhopal, India, in 1984. There are some critical differences between the exposure of animals to MIC in these studies and the accident involving MIC in Bhopal. Some of the people in Bhopal were undoubtedly exposed to much higher concentrations of MIC than were used in these studies. Also, while there were no deaths among the adult mice exposed to MIC at the concentrations in these studies, the slope of the dose-response curve for MIC-induced toxicity is

quite steep and exposures of mice to MIC at concentrations slightly higher than 3 ppm were fatal. Hence, the 3 ppm for 6 hours selected for these studies closely approaches the lethal level in mice. Another difference between the exposure in Bhopal and that in the animal experiments is that the animals were exposed to pure MIC vapors while the people in Bhopal were exposed to MIC along with other reaction mixtures from the explosion. It is possible that the different reaction products from the Bhopal accident could produce a profile of toxicity different from that associated with MIC alone. The findings from the various animal studies are presented below.

C.2.2. Developmental Toxicity Studies

Schwetz et al. (1987).

This study was conducted to evaluate the effects of sublethal concentrations of inhaled MIC on reproduction and development in mice exposed during late gestation or prior to mating. The experimental design focused on a) Mating trials, b) Perinatal Toxicity, and c) Dominant Lethal Study. Findings from the Dominant Lethal studies are presented in the Male Reproductive Toxicity section of this document.

For the Mating Trials, groups of 30 male and female Swiss (CD-1) mice per dose group were used for mating trials following 4 consecutive days of exposure, 6 hours per day, at concentrations of 0, 1, or 3 ppm MIC. The authors noted that concentrations slightly higher than 6 ppm had caused significant lethality in mice. Mating trials were conducted during weeks 1, 8, and 17 following exposure. Treated males and treated females from the same exposure group were cohabited for 10 consecutive days. During cohabitation, the female mice were checked daily for presence of vaginal plugs as evidence of mating and cohabitation continued for 10 days or until evidence of mating occurred. The day on which a vaginal plug was found was considered day 0 of pregnancy. The females were permitted to deliver their litters, and the pups were observed until 21 days of age. No significant adverse effects were observed in mating trials conducted on male and female mice exposed to MIC vapors. There was no effect on body weight, demeanor, fertility, or litter size. All pairs of animals were mated for a third time during week 17 after exposure and for this mating, there was no adverse effect on fertility at either exposure concentration of MIC, but fertility in all groups, including controls, was slightly lower than during the two previous mating periods. The litter size at birth was not affected by parental exposure to MIC. Also survival of neonates throughout lactation was not compromised in any of the three sets of matings. Representative members of the F1a litters which were selected for a subsequent mating trial at maturity (75 days of age) showed no adverse effects on fertility or litter size at birth of the F2a generation (not presented in Table 6 below).

Table 6: Fertility of MIC-exposed male and female CD-1 mice and live litter size at birth. [a,b,c]

		Exposure group, ppm		
	Litter	0	1	3
No. of pairs		30	30	30
% pregnant (no.)	F_{1a}	90 (27)	97 (29)	97 (29)
	F_{1b}	93 (28)	97 (29)	83 (25)
	F_{1c}	83 (25)	83 (25)	80 (24)
Live litter size at birth[c]	F_{1a}	11.6 ± 1.6	10.9 ± 1.8	11.4 ± 1.3
	F_{1b}	12.5 ± 2.3	12.1 ± 2.1	12.2 ± 2.1
	F_{1c}	10.8 ± 3.7	11.3 ± 3.4	12.4 ± 2.6

[a] Male and female mice were exposed 6 hrs daily for 4 consecutive days; F_{1a}, F_{1b}, F_{1c} litters resulted from matings during weeks 1, 8, and 17, respectively, after exposure.
[b] Values from the treated groups did not differ significantly from the control groups, $p < 0.05$.
[c] Mean ± SD

In the Perinatal Toxicity study design, groups of 39-44 Swiss (CD-1) mice were exposed to inhaled vapors of MIC at concentrations of 0, 1, or 3 ppm, 6 hours per day during days 14 through 17 of gestation. The concentrations of MIC were within approximately 10% of the target concentrations throughout the exposure period. The mated females were weighed on days 14 and 18 of gestation and on day 21 of lactation. The offspring were weighed, counted, and sexed on the first day after delivery and again on days 4, 7, 14, and 21. Litters were culled to eight pups by random selection on day 4, maintaining equal numbers of each sex when possible. No effect on maternal survival, body weight, demeanor, or the length of gestation was observed. All pregnant females delivered litters with one or more live pups. Compared to controls, there was a significant increase in the number of dead pups observed at birth at both 1 and 3 ppm MIC. There was increased mortality among the neonates for these dose groups throughout lactation as presented in Table 7 below.

Table 7: Fetal and neonatal deaths among litters of MIC exposed female CD-1 mice.[a]

	% Dead		
	Exposure group, ppm		
Time of deaths	0	1	3
At birth	0.4 (2/461)[b]	3.3(9/373)[c]	6.4 (22/341)[e]
Days 0–4	2.0 (9/459)[c]	0.8 (3/364)	11.3 (36/319)[e]
Days 5–21	0.3 (1/340)[d]	0.4 (1/271)	2.9 (7/239)[e]

[a] Mice were exposed 6 hr daily on gestation days 14 through 17.
[b] % (no. dead/no. live or dead).
[c] % (no. dead/no. live on day 0).
[d] % (no. dead/no. live on day 4 after culling).
[e] Significantly different from control by Fisher's exact probability test, $p<0.05$.

The increase in neonatal mortality was observed as a significant decrease in live litter size (see Table 8 below), and was particularly evident prior to the time of standardization of litter size to eight pups at 4 days of age. The weights of the neonates at birth and during lactation were no different between the MIC groups and the controls and there was also no effect on demeanor or external abnormalities. The fetal deaths observed in the litters of exposed pregnant females were not accounted for by the complete loss of a few litters, but were distributed among many litters of all sizes. Pup deaths also occurred during lactation. The deaths of pups of MIC-exposed mothers also tended to be distributed among many litters, but there were several litters in which all pups died. The authors reported that it was uncertain whether the fetal and neonatal deaths were from a direct or indirect effect of MIC exposure and thought it possible that the deaths were secondary to acute toxic effects from inhalation of MIC. However, exposure to MIC was only till Day 17 of gestation.

Table 8: Litter Size among MIC-exposed female CD-1 mice

	Average number of pups/litter		
	Exposure group, ppm		
Day	0	1	3
0 (birth)	10.4 ± 2.0[b]	8.7 ± 4.3	8.0 ± 3.3[c]
1	10.3 ± 2.0	8.7 ± 4.3	7.8 ± 3.3[c]
4	10.2 ± 2.1	8.6 ± 4.4	7.1 ± 3.9[c]
4 (culled)	7.7 ± 0.8	6.4 ± 2.9	6.0 ± 2.9
7	7.7 ± 0.8	6.4 ± 2.9	5.8 ± 3.0
21	7.7 ± 0.8	6.4 ± 2.9	5.8 ± 3.0

[a] Mice were exposed 6 hr daily on gestation days 14 through 17.
[b] Mean ± SD of live and dead fetuses combined.
[c] Significantly different from control by a modified Wilcoxon test, $p<0.05$.

The authors summarized that exposure of pregnant Swiss (CD-1) mice to concentrations of 1 or 3 ppm MIC on days 14 through 17 of gestation caused a significant increase in the number of dead fetuses at birth as well as a significant decrease in neonatal survival. No information on presence or persistence of MIC in milk was available.

Varma et al. (1987) and Varma, (1987).

In the study by Varma *et al.* (1987), the effects of MIC vapor on pregnancy and fertility were studied in mice. Timed-pregnant Swiss-Webster mice (53) were exposed to MIC for 3 hours (11 animals to 2 ppm, 12 animals to 6 ppm, 12 animals to 9 ppm and 18 animals to 15 ppm) on day 8 of gestation. Also 8 animals were exposed to MIC on day 14 of gestation (3 to 15 ppm and 5 to 9

ppm). On day 18 the animals were weighed and euthanized and the gravid uteri were exteriorized and fetuses removed by C-section.

MIC vapor resulted in a concentration-dependent decrease in body weights of the pregnant mice and relatively selective fetal toxicity. A statistically significant decrease in fetal body weight at concentrations of 2 ppm and above was noted. Standard teratological examination documented that whole-body exposure of mice to 9 and 15 ppm MIC for 3 hours on day 8 of gestation led to resorption of greater than 80% of implants with some maternal mortality. At these concentrations, in more than 75% of MIC-exposed animals, all implants were lost and external malformations were not observed. However, there was evidence of an increase in visceral abnormalities and a decrease in fetal and placental weights and in fetal skeleton sizes as noted in the Tables 9 and 10 below.

Table 9: Maternal and fetal toxicity of MIC vapor in mice exposed *in utero* on day 8 of gestation.[a]

Treatment	Animals N	Maternal mortality N	Surviving dams losing all embryos %	Fetal body weight g	Placental weight g
None	10	0	0	1.5 ± 0.04	97 ± 3
Air	24	0	0	1.5 ± 0.02	95 ± 2
MIC 2 ppm	11	0	9	1.4 ± 0.02*	88 ± 3*
MIC 6 ppm	12	0	8	1.4 ± 0.03*	85 ± 2*
MIC 9 ppm	12	2	80	1.1 ± 0.02*	80 ± 1*
MIC 15 ppm	18	2	75	1.3 ± 0.04*	74 ± 3*

[a]Animals were exposed for 3 hr to air or MIC on day 8 of gestation and sacrificed on day 18 of gestation.

*Significantly different from the values for the unexposed or air-exposed animals ($p < 0.05$)

MIC also caused a reduction of approximately 20% in the lengths of the mandible and bones of the extremities, as shown in Table 10.

Table 10: Effect of *in utero* exposure on day 8 of gestation to MIC vapor on the skeleton lengths of mouse fetuses examined on day 18 of gestation.

	Exposed for 3 hr on day 8 of gestation to:			
Parameters	Air	MIC 6 ppm	MIC 9 ppm	MIC 15 ppm
Litters examined, N	22	11	2	4
Fetuses examined, N	46	22	7	14
Mandible, mm	5.2 ± 0.08[a]	5 ± 0.13	4.7 ± 0.07*	4.8 ± 0.1*
Humerus, mm	2.9 ± 0.03	2.9 ± 0.02	2.4 ± 0.03*	2.6 ± 0.1*
Radius, mm	3.2 ± 0.05	3.2 ± 0.05	2.6 ± 0.06*	2.7 ± 0.08*
Ulna, mm	2.5 ± 0.04	2.5 ± 0.07	2.0 ± 0.02*	2.1 ± 0.06*
Femur, mm	2.3 ± 0.04	2.3 ± 0.07	1.9 ± 0.02*	2.1 ± 0.07*
Tibia, mm	2.8 ± 0.06	2.9 ± 0.06	2.1 ± 0.02*	2.2 ± 0.06*
Fibula, mm	2.6 ± 0.04	2.5 ± 0.07	2.1 ± 0.01*	2.2 ± 0.07*

[a] Values are mean ± SE.

*Significantly different from the corresponding control values ($p < 0.05$).

The authors also stated that MIC vapor was more toxic to the mother on day 14 of gestation than on day 8 as all 3 animals exposed to 15 ppm for 3 hours on day 14 died within 24 hours compared to 2 of 18 (11%) that were exposed on day 8 of gestation. Also, exposure of 14-day pregnant mice to a lower concentration of MIC (9 ppm) caused higher mortality than exposure on day 8 (2 of 5 vs. 2 of 12) (personal communication).

The findings of these animal experiments along with effects on humans affected in Bhopal were also published separately (Varma, 1987). To simulate the Bhopal incident, the animals were exposed to MIC vapor only once for 3 hours on day 8 of gestation and following standard teratology procedures (Wilson, 1965; Dawson, 1926). Resorptions, live and dead fetuses, and any external, visceral or skeletal abnormalities were recorded. This single exposure of pregnant mice to MIC for 3 hours resulted in a concentration-dependent increase in embryo loss at 9 and 15 ppm MIC. There was complete resorption in more than 75% of the animals. The authors noted that if the animals retained pregnancy, the number of resorptions, as well as the number of dead fetuses, was too small to influence the litter size. At all concentrations, the MIC exposed group demonstrated a decrease in fetal and placental weights.

This report also included information on intraperitoneal (IP) exposure. In a limited number of experiments, IP injection of liquid MIC was used. A dose of 80 mg/kg into 8-day pregnant mice resulted in sytemic toxicity such as a decrease in spontaneous activity, difficulty in breathing, central nervous system excitation as caused by cholinesterase inhibitors, paralysis, and death. A dose of 10 mg/kg to 13-day pregnant Sprague-Dawley rats resulted in an increase in resorptions, but this caused only a slight decrease in fetal and placental weights. The authors stated that since hypoxia resulted in a different array of abnormalities, the

findings suggest that pulmonary involvement and attendant hypoxia may not be the sole cause of the fetal toxicity of MIC. Moreover, the fact that fetal toxicity of MIC was produced after IP injections indicated that pulmonary irritation was not essential for the toxicity. The involvement of non-pulmonary factors in the fetal toxicity to MIC could be via nonspecific stress. The authors concluded that MIC exerts relatively selective fetal toxicity, although the mechanism of the reproductive toxicity of MIC remains to be identified. The authors noted that in mice there was no evidence of external malformations although there was some increase in visceral anomalies (thinning of myocardium in two fetuses, diaphragmatic hernia in two fetuses). The observed decrease in length of bones noted in fetuses of MIC-exposed mice via inhalation, may be indicative of inadequate skeletal formation and possibly support the clinical complications (spina bifida) associated with infants born to women exposed in Bhopal.

Singh, et al. (1996).

In this study, Charles Foster female rats (5 per group) were exposed to 0 ppm (control: Group I), 0.212 ppm (low: Group II), 0.265 ppm (high: Group III) and 0.353 ppm (highest: Group IV) of MIC vapors for half an hour and then mated with normal males of the same strain. Standard teratology procedures were conducted on day 20 of gestation. No deviation from control value (Group I) was noted in food and water intake and no mortality was observed. Resorptions for Groups I, II, III and IV were 3%, 4%, 6% and 9% respectively. While the authors reported that the rate of resorptions was increased in a dose-dependent manner, they did not note whether the increase was statistically significant. Fetal weight was decreased by 3%, 4% and 7% in the low, high and highest dosed groups. Teratological anomalies such as wrist drop, everted claw, valgus deformity, syndactyly, blood clot formation, liver enlargement, cleft palate formation and unequal ribs were observed in the fetuses. Table 11 below reports the visceral and skeletal abnormalities (% change) observed in the fetuses.

Table 11: Visceral and Skeletal abnormalities (% change) in fetuses of MIC exposed rats

Abnormalities	Group I	Group II	Group III	Group IV
Visceral				
Liver enlargement	0	0	5	7
Cleft palate formation	0	0	7	11
Humerus immovale	0	0	2	5
Skeletal				
i) Partial ossification of skull bone	2	0	5	11
ii) Non-ossification of skull bones	0	8	12	14
iii) Ribs attached to sternum	0	5	10	22
iv) Absence of ribs	4	20	27	32
v) Unequal ribs	2	5	10	17
vi) Ribs bent upward	0	0	8	10
vii) Ribs bent downward	0	4	5	5
viii) Partial ossification of ribs	0	2	8	11

No. of animals used in each group was 5

C.2.3. Developmental Endpoints from Reproductive Toxicity Studies

Kumar and Srivastava (1988).

In this report the authors described fertility studies in Wistar rats (six-seven month old). Female rats with a history of regular estrous cycles were mated with males exposed to MIC 70 days prior to mating. MIC (99% pure) diluted in olive oil was injected at 2/3 the LD_{50}: male- 218 mg/kg; female – 174 mg/kg via subcutaneous injection. Exposed males were mated with unexposed females and unexposed males were mated with exposed females. Parameters examined included periodicity of estrous cycle, receptivity, fertility, gestation length, mating behavior of males, litter size, litter weight and neonatal survival of pups.

Food intake and body weight gain was not affected. Pups born out of mating both exposed males and exposed females exhibited normal reproductive behavior on attaining puberty, and no adverse effects on reproduction were noted for the parameters examined. The effects on reproduction in rats are presented in Table 12 below. No adverse effects on fertility were noted.

Table 12: Effect of MIC on reproduction in rats

Description of groups of animals	Receptiveness in female rats	Virility in male rats	Fertility	Total number of pups born	Litter size (numbers)	Body weight of pups (10-12h old) (g)
Control		+	+			
male (*n = 2*) female (*n = 6*) (1:3)[a]	+		+	39	6.5 ± 0.76	6.01 ± 0.36
Treated males (*n = 6*) mated with untreated females (*n = 6*) (1:1)[a]	+	+	+	46	7.66 ± 0.49 NS	5.12 ± 0.33 NS
Treated females (*n = 6*) mated with untreated males (*n = 2*) (1:3)[a]	+	+	+	41	5.85 ± 0.72 NS	6.53 ± 0.74 NS

Values are mean ± SE; NS = not significant, compared with control group; [a] Mating ratio = male:female; The periodicity of oestrus cycle (6cycles) was normal in all cases. The gestation period was 21–23 days and the neonatal survival was 100%.

C.2.4. Relationship Between Developmental and Maternal Toxicity

Varma et al. (1990).

This study was conducted to study the contribution of hormonal changes and pulmonary damage to the fetal toxicity of MIC in mice and rats. To simulate the accident in Bhopal, pregnant Swiss mice and Sprague-Dawley rats were exposed to vapors of MIC as follows: Inhalation exposure to 9 ppm MIC for 3 hours on day 8 of gestation (mice) and on day 10 of gestation (rats); inhalation exposure to 20 ppm of MIC for 2 hours on day 8 (mice) and day 10 (rats). The embryos were removed for culture immediately after this exposure. Another approach included culturing some control group embryos in the presence of MIC vapor. In addition, a group of mice received IP injections of 6 µmol/kg progesterone in oil from day 9 to day 17 of gestation, subsequent to MIC exposure on day 8. The authors also reported that metabolites of MIC, namely monomethylamine (MMA), dimethylamine (DMA) and trimethylamine (TMA), were injected IP to pregnant mice at dose levels of 0.3, 1 or 3 mmol/kg to simulate exposure to 9 ppm of MIC for 3 hours. Subsequent to this exposure, animals were sacrificed on day 18 and the effects on fetuses studied.

Embryos exposed to MIC vapor both *in utero* or *in vitro* exhibited a concentration-dependent decrease in growth in culture (Tables 13, 14, and 15).

Table 13: Effects of Exposure of Pregnant Mice to MIC Vapor on the Growth of Their Embryos in Culture[a]

Parameters	Air-exposed (controls)	MIC-exposed
Litters (n)	2	4
Embryos (n)	6	15
Yolk sac (mm)	4.2 ± 0.04	3.8 ± 0.1[b]
Crown-rump (mm)	3.6 ± 0.08	3.3 ± 0.06[b]
Head length (mm)	1.9 ± 0.09	1.7 ± 0.1
Embryos dead (%)	0	0
Protein/embryo (µg)	78 ± 7.5	43 ± 8.5[b]

[a]Mice were exposed to air (control) or 20 ppm MIC for 2 h on d 8 of gestation and immediately killed; embryos were removed and cultured under identical conditions.
[b]Significantly ($p < .05$) different from the corresponding control values; data are means ± SE.

Table 14: Effects of Exposure of Pregnant Rats to MIC Vapor (20 ppm for 2 hrs) on the Growth of Their Embryos in Culture[a]

Parameters	Air-exposed (controls)	MIC-exposed
Litters(n)	7	7
Embryos (n)	34	34
Somites (n)	34.4 ± 0.18	31.9 ± 0.49[b]
Yolk sac (mm)	5.4 ± 0.07	4.7 ± 0.09[b]
Crown-rump (mm)	4.9 ± 0.06	4.3 ± 0.09[b]
Head length (mm)	2.5 ± 0.04	2.3 ± 0.05[b]
Embryos dead (%)	3	26
Protein/embryo (µg)	142 ± 22	93 ± 15[b]
DNA/embryo (µg)	30 ± 5.2	24 ± 2.1

[a]Rats on d 10 of pregnancy were exposed to air or 20 ppm MIC for 2 h and then killed. Embryos were removed and cultured under identical conditions.
[b]Significantly ($p < .05$) different from the corresponding value for the embryos from the air-exposed (control) group of rats; data are means ± SE.

Table 15: Growth of Embryos from Untreated 10-Day Pregnant Rats in Culture in the Presence of MIC Vapor

MIC added[a] (ppm)	MIC present[b] (ppm)	n	Score[c] (units)	Embryos Yolk sac (mm)	Embryos Crown-rump (mm)	Embryos Dead (%)
0	0	22	54 ± 0.4	5.3 ± 0.1	4.9 ± 0.1	4.5
714	0	11	45 ± 1.9[d]	4.9 ± 0.1[d]	4.3 ± 0.2[d]	27.2[d]
1072	0	12	39 ± 4.6[d]	4.5 ± 0.3[d]	4.2 ± 0.3[d]	42.3[d]
1429	18 ± 6	19	41 ± 3.2[d]	4.7 ± 0.3[d]	4.2 ± 0.3[d]	58.5[d]
1786	30 ± 10	11	41 ± 4.4[d]	4.7 ± 0.2[d]	4.1 ± 0.3[d]	64.3[d]
2144	24 ± 6	11	35 ± 8.2[d]	4.9 ± 0.2[d]	4.1 ± 0.9[d]	89.2[d]

[a]MIC vapor was injected only once into the sealed bottles at the start of the culture.
[b]Separate bottles were prepared for the assay of MIC.
[c]Morphological score expressed in arbitrary units.
[d]Significantly ($p < .05$) different from the corresponding control values in the top row for untreated embryos; data are means ± SE.

Exposure to MIC significantly decreased maternal plasma progesterone levels in mice that lost, but not in mice that retained, pregnancy. Fetal toxicity of MIC was not related to changes in maternal plasma corticosterone levels. Neither chronic administration of progesterone (6 µmol/kg i.p daily from day 9-17 of gestation) nor the suppression of pulmonary edema with dexamethasone (5µmol/kg i.p, 3 hours before exposure to MIC) decreased fetal toxicity of MIC. An acute dose (3 mmol/kg) of the MIC metabolites (methylamine, dimethylamine, trimethylamine, dimethyl urea (DMU)) did not cause fetal toxicity (Table 16).

Table 16: Fetal Toxicity of MIC Metabolites in Mice

Variables	Saline	MMA	DMA	TMA	DMU
Mice (n)	14	11	12	11	6
Day 12 body weight (g)	24 ± 1	15 ± 1	25 ± 1	24 ± 1	25 ± 1
Day 18 body weight (g)	51 ± 2	48 ± 3	51 ± 1	46 ± 2	48 ± 2
Implants lost (%)	8 ± 3	12 ± 9	20 ± 3	12 ± 9	9 ± 7
Fetal body weight (mg)	1290 ± 40	1260 ± 30	1410 ± 30	1290 ± 20	1320 ± 30
Placental weight (mg)	110 ± 3	110 ± 30	110 ± 40	120 ± 7	110 ± 10
Litter size (n)	10.7 ± 0.6	11.2 ± 0.6	9.8 ± 0.5	10.7 ± 0.5	9.5 ± 1.0

[a]MMA (monomethylamine), DMA (dimethylamine), TMA (trimethylamine), and DMU (dimethylurea) were injected (3 mmol/kg, ip) on d 8 of gestation

These data suggest that the fetal toxicity of MIC is partly independent of maternal toxicity and may result from the transfer of MIC across the placenta and interaction with fetal tissues. The authors also reported that the results from this study suggest that the fetal toxicity of MIC is not exerted through methylamines,

the known metabolites of MIC.

C.3. Developmental Toxicity: Other Relevant Data

C.3.1. Distribution and Metabolism in Pregnant Females and Conceptuses

Administration of ^{14}C-labeled MIC to mice by inhalation demonstrated that radioactivity rapidly distributed to all tissues including the uterus, placenta and fetus (Ferguson *et al*., 1988). Based on this and other findings it has been suggested that placentally-transferred MIC can directly affect the fetus (Varma *et al*., 1990).

C.3.2. Mechanism(s) of Developmental Toxicity.

Guest and Varma (1991).

A role for methylamine, a metabolite of MIC, in reproductive/developmental toxicity was investigated by Guest and Varma (1991). In a mouse study, pregnant dams were exposed to varying doses (intraperitoneal) of methylamine (as well as the di- and trimethyl compounds). Reproductive toxicity was not observed for methylamines. However, in cultured embryo experiments, decrements in crown-rump length, yolk-sac diameter, head length, and embryo survival were observed.

C.3.2.1. Active Agent

Guest et al. (1992).

S(N-methylcarbamoy1)glutathione (SMG), is a conjugate formed by the reversible reaction between MIC and glutathione. In this study, the toxicity of SMG on mouse embryos was examined. Embryos were explanted on day 8 of gestation and cultured in rat serum for 42 hours. The authors reported that SMG caused concentration-dependent decreases in growth and development over the range 0.1-2 mM, without causing significant mortality. At a concentration of 2 mM, SMG completely arrested embryo development, but heartbeat was absent in only one of nine embryos at 42 hr. At a concentration of 0.25 mM, SMG reduced embryo size to 75% and protein content to 63% of the control; 18% of embryos failed to rotate. At this concentration (0.25 mM), which was selected for all other studies, spinal kinks and somite pair distortion in the region of the forelimb were evident in 38% of embryos; no other abnormalities were noted. DNA content of and thymidine incorporation by embryos and yolk sacs was reduced by SMG, although this was more pronounced in the yolk sac than in embryos. At subtoxic concentrations, the L-cysteine precursor (-)-2-oxo-4-

thiazolidine-carboxylic acid did not, but glutathione GSH did, inhibit embryotoxicity of SMG. The authors concluded that SMG exerted embryotoxic and dysmorphogenic effects and may contribute to systemic toxicity of MIC.

C.3.2.2. Biological Mechanisms of Action

Guest and Varma (1994).

In this study, the authors attempted to determine the effects of an embryotoxic concentration of SMG on mouse yolk sac uptake mechanisms, lysosomal proteolysis and the incorporation of [^{3}H] leucine in mouse embryonic and limb-bud proteins. After *5* h of culture, SMG inhibited the uptake of [^{14}C] sucrose and ^{125}I-labelled bovine serum albumin in isolated day 15 yolk sacs to 62 and 77% of control, respectively. Lysosomal proteolysis was not inhibited, as judged by the release of trichloroacetic acid-soluble radioactivity into the culture media. Uptake and incorporation of [^{3}H] leucine from free [^{3}H] leucine or from [[^{3}H] leucine-labelled protein in SMG-treated day 9 embryos were inhibited, respectively, to 61 and 25 % of the control uptake during a 16-h labelling period. SMG also inhibited the incorporation of free [^{3}H] leucine into limb-bud proteins. SMG-induced inhibition of ^{125}I –labelled bovine serum albumin uptake by yolk sacs was partially prevented by the thiol donors N-acetylcysteine and glutathione but not by acivicin (γ-glutamyl transpeptidase inhibitor) and aminooxyacetic acid (cysteine conjugate β-lyase inhibitor). The authors concluded that SMG suppresses embryonic growth primarily by an inhibition of nutrient uptake by the yolk sac and postulated that this inhibition was due to tissue carbamoylation by MIC released from SMG. These findings also support the conclusion that direct actions of SMG on embryonic tissues as a component of the embryopathological effects cannot be excluded.

C.4. Summary of Developmental Effects Seen in Animal Studies

Exposure of pregnant Swiss (CD-1) mice to concentrations of 1 or 3 ppm MIC on days 14 through 17 of gestation caused a significant increase in the number of dead fetuses at birth as well as a significant decrease in neonatal survival. However, the same concentrations of MIC had no effect in mating trials conducted on exposed male and female mice. Thus, in contrast to the effect when there was exposure during pregnancy, there was no effect on fertility or on fetal or neonatal survival when males or females were exposed to MIC prior to mating, suggesting that exposure of a conceptus *in utero* results in more toxicity than exposure of the gonadal cells prior to mating. Findings from other studies in mice also led authors to conclude that MIC exerts relatively selective fetal toxicity though the mechanism of the reproductive toxicity of MIC remains to be identified. In other studies, the authors noted that in mice there was no evidence of external malformations but some increase in visceral anomalies and a

decrease in length of bones in fetuses. In a study in female rats exposed prior to mating, teratological anomalies were observed in the fetuses along with a dose-dependent increase in resorptions.

Embryos exposed to MIC vapor both *in utero* or *in vitro* exhibited a concentration-dependent decrease in growth in culture. Data suggest that the fetal toxicity of MIC is at least partly independent of maternal toxicity and may result from MIC's transfer across the placenta and interaction with fetal tissues. The authors also reported that the fetal toxicity of MIC is not exerted through methylamines, the known metabolites of MIC, but concluded that SMG, a conjugate of MIC and glutathione, exerted embryotoxic and dysmorphogenic effects and may contribute to systemic toxicity of MIC.

C.5. Integrative Evaluation

There is a striking consistency in the effects of MIC in both humans and animals with regard to survival of the exposed conceptus. Multiple studies of women exposed during the gas leak at Bhopal reported elevated rates of pregnancy loss in the first trimester (spontaneous abortion) and neonatal mortality. The animal data also indicate an effect on fetal loss and neonatal survival subsequent to *in utero* exposure to MIC. In humans, the trend of fetal loss persisted as noted in studies that followed women during the years after the accidental release of MIC in Bhopal. This was demonstrated most strongly in the one study that was able to differentiate groups of women based on the extent of exposure they experienced, which found higher rates of spontaneous abortion in women who resided in areas more severely affected by the gas cloud. The increased rates of spontaneous abortion seen in these women in the years after the exposure occurred, suggests an overall reproductive effect in women in the affected area.

The skeletal effects in the animal studies, a shortening of bones, provides biologic plausibility that supports the findings of the only human study that evaluated postnatal growth of children exposed *in utero*. In that well-controlled study, boys born to mothers who were exposed during pregnancy were found to be significantly shorter in stature.

D. Female Reproductive Toxicity

D.1. Human Female Reproductive Toxicity Atudies

D.1.1. Overview

Two studies provide findings of menstrual dysfunction and other gynecological complaints not related to pregnancy outcome (Shilotri *et al*., 1986; Medico Friend Circle, 1990, reported by Dhara and Dhara, 2002) and are described here. Eight

studies address pregnancy loss (Shilotri *et al*., 1986; Varma, 1987; Kanhere *et al*., 1987; Bhandari *et al*., 1990; Kapoor, 1991; Varma, 1991; ICMR, 2001; Dhara and Dhara, 2002) and are summarized above (under Developmental effects). Pregnancy loss may result from either impacts on the fetus, impacts on the mother, or impacts on both. Thus, the findings of the pregnancy loss studies may be relevant to consideration of female reproductive toxicity. Several recent review articles refer to continuing reproductive health problems in women exposed to the gas (Varma and Varma, 2005; Sharma, 2005; Mishra *et al*., 2009b).

D.1.2. Individual Studies

Shilotri et al. (1986).

As described above, Shilotri *et al*. (1986) carried out a medical survey 105 days after the disaster in Bhopal, including gynecologic findings. Group I subjects lived near the site of the disaster, and Group II subjects lived in areas beyond 8 km from the factory, and were similar with regard to socio-economic status and house type. Although Group II subjects were selected to serve as controls, Naik *et al*. (1986) noted that "…Group II subjects were affected [by the MIC leak] and hence were not true controls...". Women with gynecological complaints in either group were included in the study: from Group I, 88 out of 160 women; from Group II, 12 out of 38 women. Results of the gynecologic exam are shown in Table 17.

Of the women with "Excessive vagina discharge without infection or pelvic factor", women from Group 1 had inflammatory (22 of 39) or dysplastic (2 of 39) cervical smears. Numbers of Group II women similarly affected provided results of cervical smears that were too small for meaningful comparison (inflammatory, 2 of 3; dysplastic, 0 of 3). No statistical analyses of the findings are included in the report. The authors suggest that "The frequent occurrence of inflammatory smear and dysplasia calls for periodic follow up of these patients in view of possibility of development of carcinoma in future."

Table 17: Gynecological complaints and examination findings in women exposed to Bhopal disaster (Adapted from Shilotri *et al*., 1986)

Parameter	Group I (n=88) (Lived near factory) Number affected (%)	Group II (n=12) (Lived >8 km from factory) Number affected (%)
Leucorrhoea	66 (75%)	7 (58%)
Irregular menses	16 (18%)	3 (25%)
Menorrhagia	9 (10%)	2 (17%)
Excessive vagina discharge without infection or pelvic factor	39 (44%)	3 (25%)
Presence of local infection	19 (22%)	4 (33%)
Pelvic factor accountable	8 (9%)	--

Dhara and Dhara (2002).

This review article includes a report of a study not available through other channels, conducted by a group cited in Dhara and Dhara (2002) as "Medico Friend Circle, October 1990". The entire report of the study is presented verbatim below:

> Medico Friend Circle conducted a pregnancy outcome survey 9 months after the accident in 3 gas-exposed areas of Bhopal. A total population of 8,165 in 1,632 households was surveyed by random sampling. Information on reproductive history and menstrual cycles was collected for the 1 year time period that preceded the gas leak and served as the 'historic control'. A 4-fold increase in overall spontaneous abortion rate for the period after the gas leak was reported. Approximately 24% of women had altered menstrual cycle durations (i.e., 14% of the cycles decreased by ≥7days, 6% were irregul ar, and 4% increased by ≥7 days). The authors concluded that these effects could be gas-related.

D.1.3. Summary of Female Reproductive Effects Seen in Human Studies

Shilotri *et al*. (1986) examined gynecological complaints in exposed women soon after the accident, and reported finding cervical inflammation and dysplasia that led them to call for periodic follow-up regarding potential carcinogenesis. Their

comparison group was small and may not have been adequate for examining these effects. The brief report on the Medico Friend Circle study (reported by Dhara and Dhara, 2002) notes alteration in menstrual cycle duration in women exposed to the gas cloud, without comparison to an unexposed population.

As discussed above (under developmental effects), the studies examining pregnancy loss, despite their limitations, show consistent findings across studies of elevated rates of spontaneous abortion following exposure to MIC. The mechanisms of the fetal losses described in these studies are unclear. The two studies (Kapoor, 1991; ICMR, 2001) that continued to follow women exposed to MIC for years after the gas exposure both found that these women continued to experience higher rates of spontaneous abortion. Thus, the spontaneous abortions seen in these studies may reflect female reproductive toxicity as well as, or instead of, direct effects on the fetus. The data presented by Kanhere *et al*. (1987) indicate that MIC exposure had an impact on the placentas of these women, including significantly lower mean weight of placenta from full-term pregnancies in gas-exposed women compared to those from unexposed women. Kanhere *et al*., also reported a higher percentage of negative histological changes in the placenta of exposed women, including both those with full-term pregnancies as well as for medically terminated pregnancies, with statistically significant increases in hydropic degeneration of the placenta of exposed women. Kapoor (1991) reported that women in the affected area not only had higher pregnancy losses immediately following the accident, but continued in subsequent years to experience higher rates of pregnancy loss than women in a control area, possibly an indicator of poor reproductive health in these women.

More recent reports of problems affecting Bhopal residents exposed to the gas suggest that such gynecological problems are continuing and refer to but do not provide data from studies that address this effect. Varma and Varma (2005), in a review prepared on the occasion of the 20th anniversary of the accident, indicate that there are anecdotal reports suggesting "menstrual problems in girls affected by the gas." Mishra *et al*. (2009b), in a review article, note that "Clinicians at Bhopal have observed that now the girls who were exposed during their infancy and those [exposed] in their mother's womb are experiencing 'menstrual chaos'." Sharma (2005) states that those exposed to MIC "continue to suffer from… reproductive [and other] disorders." Mishra *et al*. (2009b) cites "the final technical report of the [Indian Council of Medical Research, ICMR]" as having reported "increased menstrual irregularities and excessive bleeding among gas-exposed inhabitants…. [S]everal of these women had episodes of miscarriages later on, and many could not conceive at all." In OEHHA staff's examination of the ICMR report (2001) which covered research carried out from 1985 to 1994, no such information on menstrual irregularities was found. It is unclear whether there may be an additional report to which Mishra *et al*. (2009b) had access, as they cite the document as ICMR (2004).

D.2. Animal Female Reproductive Toxicity Studies

D.2.1. Fertility

Varma (1987).

As described earlier, timed pregnant Swiss-Webster mice were exposed to MIC for 3 hours (11 animals to 2 ppm, 12 animals to 6 ppm, 12 animals to 9 ppm and 18 animals to 15 ppm) on day 8 of gestation. Also 8 animals were exposed to MIC on day 14 of gestation (3 to 15 ppm and 5 to 9 ppm). The effects of MIC vapor on pregnancy and fertility were studied. MIC vapor resulted in concentration-dependent decrease in body weights of the pregnant mice and relatively selective fetal toxicity with significant increases in the number of implants resorbed in a dose-dependent manner along with all embryos being resorbed in >75% animals exposed to 9 and 15 ppm MIC (Table 18).

Table 18: Maternal and fetal toxicity of methyl isocyanate (MIC) vapor in mice exposed *in utero* on day 8 of gestation.[a]

Treatment	Animals, *N*	Maternal mortality, *N*	Surviving dams losing all embryos, %	Fetal body weight, *g*	Placental weight, *g*
None	10	0	0	1.5 ± 0.04	97 ± 3
Air	24	0	0	1.5 ± 0.02	95 ± 2
MIC 2 ppm	11	0	9	1.4 ± 0.02*	88 ± 3*
MIC 6 ppm	12	0	8	1.4 ± 0.03*	85 ± 2*
MIC 9 ppm	12	2	80	1.1 ± 0.02*	80 ± 1*
MIC 15 ppm	18	2	75	1.3 ± 0.04*	74 ± 3*

[a]Animals were exposed for 3 hr to air or MIC on day 8 of gestation and sacrificed on day 18 of gestation.
*Significantly different from the values for the unexposed or air-exposed animals ($p < 0.05$).

The authors also stated that MIC vapor was more toxic to the mother on day 14 of gestation than on day 8 as all 3 animals exposed to 15 ppm for 3 hours on day 14 died within 24 hours compared to 2 of 18 (11%) that were exposed on day 8 of gestation. Also, exposure of 14-day pregnant mice to a lower concentration of MIC (9 ppm) caused higher mortality than exposure on day 8 (2 of 5 vs. 2 of 12) (personal communication).

MIC toxicity was noted in both pregnant and non-pregnant mice associated with changes in serum hormone levels. Increased serum corticosterone levels were noted in male and non-pregnant mice exposed to MIC, but not in pregnant mice that retained pregnancy. However, when MIC exposure led to resorption of all fetuses, a significant decrease in serum corticosterone was noted (Table 19).

Table 19: Effects of Methyl Isocyanate (MIC) Vapor on Serum Corticosterone and Progesterone Levels in Mice[a]

		Corticosterone		Progesterone	
Animals	MIC (ppm)	n	ng/ml	n	ng/ml
Male	0	5	6 ± 1.7		Not done
Male	9	7	30 ± 8.7[b]		Not done
Non-pregnant	0	14	19 ± 2.3		Not done
Non-pregnant	9	9	88 ± 22[c]		Not done
Pregnancy retained	0	8	560 ± 30	11	3.7 ± 0.69
Pregnancy retained	6	11	577 ± 41	11	3.4 ± 0.15
Pregnancy retained	9		702		Not done
Pregnancy not retained	9	8	198 ± 93[d]	5	1.7 ± 0.37[d]

[a] Pregnant mice were exposed to MIC for 3 h on d 8 and samples were collected on d 18 of gestation. Blood samples from other animals were collected 10-12 d after exposure. Controls (MIC 0 ppm) were exposed to air. Data are means ± SE.
[b] Significant difference compared with control males ($p < 0.05$).
[c] Significant difference compared with control non-pregnant females ($p< 0.05$).
[d] Significant difference compared with values for pregnant animals exposed to 0, 6, or 9 ppm MIC ($F=8.35$, df 27, $p < 0.001$).

Varma et al., (1990).

In the study described previously in section C.2.4., MIC disturbed the estrous cycle and decreased the mating and pregnancy rate of female mice. In general MIC-exposed mice had lower plasma progesterone levels than controls from day 10-18 of gestation. While MIC exerted no significant effects on serum progesterone levels of pregnant mice if the pregnancy was retained, a significant decrease in the serum levels in animals that lost all the implants was noted. Also, daily administration of progesterone (which can prevent pregnancy loss in ovariectomized rats) did not decrease the fetal toxicity in MIC-exposed animals, suggesting that a decrease in plasma progesterone was not the primary event leading to a loss of fetuses. Plasma corticosterone levels of MIC-exposed mice was similar whether the mice retained or lost their pregnancies, with levels significantly higher levels in MIC- exposed animals than those of controls on day 2, 5 and 6 days after exposure but not on other days. According to the authors, since levels of plasma corticosterone were not significantly different between animals that retained and those that lost pregnancy, the hormone does not appear to be an important contributory factor to the fetal toxicity of MIC. However, earlier findings noted MIC toxicity in both pregnant and non-pregnant mice associated with changes in serum hormone levels and when MIC exposure led to resorption of all fetuses, a significant decrease in serum corticosterone was noted (Table 19).

Kumar and Srivastava (1988).

In this report described previously in section C.2.3, the authors described fertility studies in Wistar rats (six-seven month old). Female rats with a history of regular estrous cycles were mated with males exposed to MIC 70 days prior to mating. MIC (99% pure) diluted in olive oil was injected at 2/3 the LD_{50}: male- 218 mg/kg; female – 174 mg/kg via subcutaneous injection. Exposed males were mated with unexposed females and unexposed males were mated with exposed females. Parameters examined included, periodicity of estrous cycle, receptivity, fertility, gestation length, mating behavior of males, litter size, litter weight and neonatal survival of pups.

Food intake and body weight gain were not affected. Pups born out of mating exposed males or exposed females exhibited normal reproductive behavior on attaining puberty. No adverse effects on reproduction were noted for the parameters examined.

D.3. Summary of Female Reproductive Effects Seen in Animal Studies

MIC vapor resulted in concentration-dependent decrease in body weights of pregnant mice and relatively selective fetal toxicity. Significant, dose-dependent increases in the number of implants absorbed were observed. Decreases in placental weight in animals exposed to MIC were also noted. Examining the hormonal effects of MIC in mice, plasma progesterone levels were significantly lower in MIC-exposed mice that did not retain their pregnancy. However, animals that retained their pregnancy did not exhibit a significant decrease in progesterone, and daily administration of progesterone did not decrease fetal loss in MIC-exposed animals. According to the authors this suggests that a decrease in plasma progesterone was not the primary event leading to a loss of fetuses. In general, an increase in plasma corticosterone was noted in MIC-exposed mice. Since levels of plasma corticosterone were not significantly different between animals that retained and those that lost pregnancy, the hormone does not appear to be an important contributory factor to the fetal toxicity of MIC. No adverse effects on reproduction were noted after exposure of female rats to MIC 70 days prior to mating.

D.4. Integrative Evaluation

Adverse female reproductive effects reported in both studies of women exposed to the gas release in Bhopal and in experimental animal studies of MIC include fetal and neonatal mortality that may reflect female reproductive toxicity as well as, or instead of, direct effects on the fetus. Although the animal studies found no adverse effects on reproduction after exposure of female rats to MIC 70 days prior to mating, the two studies in humans that continued to follow women in

Bhopal in the years following the accident found that these women continued to experience higher rates of spontaneous abortion for years after the exposure. This was demonstrated most strongly in the one study that was able to differentiate groups of women based on the extent of exposure they experienced, and found higher rates of spontaneous abortion in women who resided in areas more severely affected by the gas cloud. The increased rates of spontaneous abortion seen in these women in the years after exposure occurred support the possibility that this endpoint is mediated at least in part through female reproductive toxicity. In addition, a study of placentae from women exposed in Bhopal found placentae from full-term pregnancies in those exposed had a lower mean weight, indicative of effects on the female reproductive system. This observation is consistent with the decreases in placental weight in animals exposed to MIC that have been reported.

E. Male Reproductive Toxicity

E.1. Human Male Reproductive Toxicity Studies

E.1.1. Overview

Two studies evaluated possible toxic effects on male reproduction (Daniel *et al*., 1987; Deo *et al*., 1987) from MIC exposure.

E.1.2. Individual Studies

Daniel et al. (1987).

Effects on sperm were examined six months after the Bhopal disaster in 18 men heavily exposed to the gas release compared to 10 men who were not exposed. Sixty exposed men identified from a list compiled by the Indian Council of Medical Research were contacted and invited to participate. Of the 29 who volunteered, only 18 were able to provide a semen sample for analysis. All the exposed men had severe respiratory and ocular symptoms, and 16 had been hospitalized, following exposure to the gas. The control group was drawn from an area without exposure to the gas (Lucknow) and, according to the authors, was matched for age, socio-economic status, history of smoking and alcohol intake. The exposed men were older than the control group (exposed: range 20-45, mean 27.7±7.9; control: range 19-35, mean 26.7±4.8) and had a lower incidence of alcohol use (17% exposed vs. 30% controls).

The researchers had been unable to impose a definite period of sexual abstinence on subjects prior to semen collection. Twenty percent of the controls and six percent of the exposed had a sperm count less than 20 million per milliliter, and were excluded from the comparisons. None of the differences in the parameters compared (semen volume, sperm motility, sperm count, sperm

head shape, F-bodies) were significant. The authors note that given the maturation cycle of spermatogenesis (approximately 64 days), the timing of their study was not soon enough to detect any acute effect of gas exposure that may have occurred.

Deo et al. (1987).

As part of a study looking for potential immunological, mutagenic and genotoxic effects, Deo *et al*. (1987) carried out a small study on sperm count, morphology and motility. Exposed men included in the various portions of the studies reported in Deo *et al*., were comprised of employees of the railroad residing in the East Railway Colony who had been admitted to various railway hospitals, as well as individuals living near the Union Carbide plant who had been admitted to Hamidia hospital in Bhopal. The authors indicate that semen samples (n=19) were collected from railway employees in March-April and June 1985; it's unclear if any of the samples were collected from individuals living near the Union Carbide plant. No information is provided as to how the men included in the study were selected from the larger set of participants in other portions of the study (e.g, 56 adult males were involved in the immunological studies). The authors note that a detailed history was recorded. However, no details other than age of the subject are presented in the table that provides the only specific information on the results of the semen study. For example, no information is available on a subject's smoking status or sexual abstinence prior to semen collection.

Deo *et al*. note that "all parameters appeared to be within normal limits" and reference an article on sperm counts in fertile and infertile men. Their table includes information on individual semen volume, sperm counts, and percent motility, and provides a comparison range for sperm count and motility. One subject had a sperm count below the listed normal range, and four men had motility that was lower than the reference value listed. No specific information on abnormal morphology in the samples is provided. Deo *et al*., note that spermatogenesis duration "is about 74 days" and that their sample collection occurred "between 100 to 120 days after MIC exposure."

E.1.3. Summary of Male Reproductive Effects Seen in Human Studies

No adequate study of male reproductive toxicity in humans was identified. Both of the studies (Daniel *et al*., 1987; Deo *et al*., 1987) that evaluated sperm parameters in men exposed to MIC were carried out too long after exposure (6 months and 100 –120 days, respectively) to have detected an effect on spermatogenesis. Neither study found significant differences in sperm counts or other parameters measured. Other limitations of these studies, such as the subjects' lack of abstinence prior to semen collection and the small numbers of

subjects, would have made finding an effect unlikely, even if the studies had been conducted sooner after the accident.

E.2. Animal Male Reproductive Toxicity Studies

Varma et al. (1987).

As described earlier in section C.2.2, timed pregnant Swiss-Webster mice were exposed to MIC for 3 hours (11 animals to 2 ppm, 12 animals to 6 ppm, 12 animals to 9 ppm and 18 animals to 15 ppm) on day 8 of gestation. Also 8 animals were exposed to MIC on day 14 of gestation (3 to 15 ppm and 5 to 9 ppm). The effects of MIC vapor on pregnancy and fertility were studied. The mating performance of MIC-exposed male mice was decreased. Exposure to MIC increased serum corticosterone levels of male and nonpregnant female mice (Table 19).

Kumar and Srivastava (1988).

In this report, described previously in section C.2.3., the authors described fertility studies in Wistar rats (six-seven month old). Female rats with a history of regular estrous cycles were mated with males exposed to MIC 70 days prior to mating. MIC (99% pure) diluted in olive oil was injected at 2/3 the LD_{50}: male-218 mg/kg; female – 174 mg/kg via subcutaneous injection. Exposed males were mated with unexposed females and unexposed males were mated with exposed females. Parameters examined included, periodicity of estrous cycle, receptivity, fertility, gestation length, mating behavior of males, litter size, litter weight and neonatal survival of pups.

Food intake and body weight gain was not affected. Pups born out of mating exposed males and exposed females exhibited normal reproductive behavior on attaining puberty and no adverse effects on reproduction were noted for the parameters examined. The effects on reproduction in rats are presented in Table 20 below.

Table 20: Effect of MIC on reproduction in rats

Description of groups of animals	Receptiveness in female rats	Virility in male rats	Fertility	Total number of pups born	Litter size (numbers)	Body weight of pups (10-12h old) (g)
Control		+	+			
male (*n = 2*) female (*n = 6*) (1:3)[a]	+		+	39	6.5 ± 0.76	6.01 ± 0.36
Treated males (*n = 6*) mated with untreated females (*n = 6*) (1:1)[a]		+	+			
	+		+	46	7.66 ± 0.49 NS	5.12 ± 0.33 NS
Treated females (*n = 6*) mated with untreated males (*n = 2*) (1:3)[a]	+		+	41	5.85 ± 0.72	6.53 ± 0.74
		+	+		NS	NS

Values are mean ± SE; NS = not significant, compared with control group; [a] Mating ratio = male:female; The periodicity of oestrus cycle (6 cycles) was normal in all cases. The gestation period was 21–23 days and the neonatal survival was 100%.

The authors commented that since the overall time of spermatogenesis extends to over 9 weeks in rats, the study extending for 12 weeks after treatment would have revealed possible effects on any stage of spermatogenesis. However, examining parameters 70 days after exposure would only verify the absence of permanent damage to spermatogenesis and would not serve to determine immediate effects.

E.2.1. Reproductive Organ Toxicity

Arora and Vijayaraghavan (1989).

Sexually mature Swiss male mice were exposed to MIC at a concentration of 134 mg/m^3 for 30 min (reported by authors as = 4020 mg/min/m^3) and a control group was maintained in a similar exposure chamber for 30 minutes without MIC. The animals were subsequently sacrificed (six per group) at 1 hour, 24 hour, 3, 8, 15 or 35 days after exposure. Histomorphology of the testis and other organs was examined. A marked loss of body weight (10%) after 24 hours was noted in the exposed group and the mean body weight of the exposed group was significantly less than that of the controls, even 15 days after the exposure. No significant change was observed in relative testicular weight. Spermatozoa in the seminiferous tubules disappeared 3 days post exposure. Primary and secondary spermatocytes were hypertrophied. The authors reported that eight days after exposure, spermatozoa started appearing but the heads of the spermatozoa were rod-shaped instead of hook–shaped. The spermatogonia were not altered. Normalization of spermatid and spermatozoa occurred after 15 days. Photomicrographs demonstrating these effects were presented and the authors

reported that MIC did not cause irreversible changes in testicular histomorphology and commented that this effect is supported by earlier findings by Daniel *et al*. (1987) in humans and Kumar and Srivastava (1988) in the rat.

Agarwal and Bose (1992).

Adult male Wistar rats in a whole body inhalation chamber were exposed to MIC at 3.2 mg/l, single inhalation exposure for 8 minutes or ethyl methanesulphonate (EMS, 150 mg/kg, single ip dose). Sequential matings of treated males with normal females on days 1-7, 8-14 and 15-21 post-exposure were conducted and did not indicate any induction of dominant lethal mutation (increased frequency of preimplantation losses and early fetal deaths) by MIC. However, dominant lethal mutations were significantly increased after exposure to EMS compared to respective controls. Males, necropsied after 21 days of exposure, showed no effect of MIC on epididymal sperm density and morphology. EMS also had no effect on sperm density, but it significantly induced morphological abnormalities in sperm as compared to untreated controls. An acute and transitional reduction in reproductive performance (10%-21% fewer vaginal plugs in the cohabitated females) of MIC-exposed males during days 1-14 post-exposure followed by recovery to the normal level during days 15-21 post-exposure was reported. The progeny of MIC-exposed males were also normal in terms of litter size, litter weight, neonatal survival and body weight gain in litters up to 10 days post-partum. The authors concluded that MIC did not cause germ cell mutagenicity, probably because of its poor biodistribution to the target site(s). This may be because of the conjugation of MIC with biological thiols and non-specific proteins in the lung, the primary organ of exposure, resulting in the restriction of its biodistribution to the testis. According to the authors, the transient reduction in the reproductive performance of MIC-exposed males is a result of general stress and unsuccessful copulation as noted by fewer vaginal plugs in the cohabitated females.

Bose et al. (1994).

This study was conducted to identify the target cell type responsible for the decreased fertility observed in earlier studies, to analyze the action of MIC on spermatogenesis during one cycle of seminiferous epithelium and also evaluate whether dietary protein deficiency influences the toxic potentials of MIC on testicular tissues. Four groups of male albino rats (24/group) were given either 20% or 8% protein in an otherwise isocaloric diet. Half the animals from each dietary group were exposed individually to a single dose of 1.60mg MIC/L of air for 8 min in a whole-body inhalation chamber. The remaining animals were exposed to fresh air to serve as control groups. Animals from both the dietary treated groups and respective controls were sacrificed immediately after exposure and on days 2, 5, 8, 10 and 12 post-exposure. Testes were collected and processed for histopathology. Qualitative analysis of spermatogenesis was

done at different stage groups viz., stages I-IV, V-VI, VII-VIII, IX-XII and XIII-XIV of the seminiferous epithelial cycle.

The growth rate of animals maintained on 8% protein diet was comparatively slow. The animals of the 20% dietary group reached 150g body weight when they were around 3 weeks younger than the 8% protein dietary group. MIC exposure effect on body weight was noted up till day 5 in both dietary groups. While spermatogenesis in the 8% protein control group was unaffected and testes apparently looked normal, leptotene and pachytene spermatocytes contents were lesser as compared to the 20% dietary protein control group. Also, the authors reported that MIC exposure led to accumulation of fluid in the interstitium in rats on the 8% protein diet on day 5 and progressive degenerative changes including disintegration of spermatogenic cell nuclei, cytoplasmic blebbing, vacuolation of some spermatid nuclei and cell loss were observed till day 8. The authors concluded that MIC might possibly alter the integrity of extra and intra tubular blood testis barrier, by binding with specific membrane proteins and cause reversible testicular damage. Further, deficiency of protein may potentiate the effect of MIC within the frame work of these experiments.

E.2.2. Dominant Lethal and Sperm Effects

Schwetz et al. (1987).

In addition to the sperm parameters examined in the two previous studies, dominant lethal effects were evaluated in one part of this study. Thirty male Swiss (CD-1) mice per dose group were mated with untreated females for 8 weeks (different females each week) following the last of 4 consecutive days of exposure, 6 hr per day, to 0, 1, or 3 ppm MIC. Male mice were cohabited with female mice on a one to-one basis and the animals were observed daily for signs of toxicity and twice daily for any moribund or dead mice. The female mice were replaced with new, untreated female mice of the same age at 7-day intervals. Male mice were weighed on the day before the first exposure, the first day after the fourth exposure, and at weekly intervals when the female mice were replaced. Following removal from the exposed males, the female mice were housed separately and sacrificed 11 days after removal from the males. At the time of sacrifice, the uterine contents were examined for pregnancy and the number of live and dead implants to determine the percent of resorptions relative to the number of implantation sites.

There was no evidence of a dominant lethal effect in exposed male mice. The fertility of these male mice mated with unexposed females during 8 weekly intervals following exposure was comparable to control values. There was no effect on the incidence or distribution of resorptions in the pregnant females mated to the treated males (Table 21).

Table 21: Dominant lethal test in MIC-exposed male CD-1 mice.[a]

	Week	Exposure group, ppm		
		0	1	3
No. of males		30	30	30
% pregnant females (no.)	1	93 (28)	93 (28)	97 (29)
	2	93 (28)	93 (28)	83 (28)
	3	97 (29)	83 (25)	97 (29)
	4	100 (30)	93 (28)	100 (30)
	5	100 (30)	97 (29)	100 (30)
	6	93 (28)	93 (28)	100 (30)
	7	93 (28)	87 (26)	100 (30)
	8	97 (29)	93 (28)	100 (30)
% Resorptions,	1	5.6 ± 8.3	4.9 ± 6.2	4.3 ± 6.4
mean ± SD	2	5.3 ± 8.8	4.7 ± 6.5	4.1 ± 5.4
	3	6.8 ±11.5	5.2 ± 7.0	5.5 ± 7.0
	4	2.6 ± 4.9	2.3 ± 4.3	4.3 ± 6.9
	5	5.5 ± 6.3	6.7 ±18.6	4.5 ± 5.7
	6	5.2 ± 8.2	5.3 ± 10.6	6.1 ± 9.4
	7	6.6 ± 6.7	3.7 ± 5.2	3.5 ± 6.2
	8	7.7 ± 8.3	6.2 ± 6.8	5.1 ± 7.9

[a]Values from the treated groups did not differ significantly from the control groups. $p<0.05$.

E.3. Summary of Male Reproductive Effects Seen in Animal Studies

The mating performance of MIC-exposed male mice was decreased. Exposure to MIC increased serum corticosterone levels of male and non-pregnant female mice. A fertility study in Wistar rats demonstrated normal reproductive behavior on attaining puberty and no adverse effects on reproduction were noted. The effects noted in another study in rats have been interpreted by the authors to be transient with the reduction in the reproductive performance of MIC-exposed males being a result of general stress. In other studies in rats, authors concluded that MIC might possibly alter the integrity of extra and intra tubular blood testis barrier, by binding with specific membrane proteins and cause reversible testicular damage. Further, deficiency of protein may potentiate the effect of MIC within the frame work of these experiments. There was no evidence of a dominant lethal effect in exposed male mice. The fertility of these male mice mated with unexposed females during 8 weekly intervals following exposure was comparable to control values. There was no effect on the incidence or distribution of resorptions in the pregnant females mated to the treated males.

E. 4. Integrative Evaluation

The data in animals suggest that exposure to MIC resulted in disappearance of spermatozoa in seminiferous tubules 3 days post exposure with recovery at 8 days and normalization at 15 days. Additionally, some researchers have concluded that MIC might possibly alter the integrity of extra and intra tubular blood testis barrier, by binding with specific membrane proteins and cause reversible testicular damage with protein deficiency potentiating the effect of MIC. However, no dominant lethal effects were noted in the animal studies.

The human data did not include any adequate study of male reproductive toxicity. Both of the studies (Daniel *et al*., 1987; Deo *et al*., 1987) that evaluated sperm parameters in men exposed to MIC were carried out too long after exposure (6 months and 100 –120 days, respectively) to have detected an effect on spermatogenesis and were essentially inconclusive for sperm counts or other parameters measured.

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