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CARBON TETRACHLORIDE EFFECTS ON FERTILIZATION AND PREIMPLANTATION DEVELOPMENT IN MICE

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ABSTRACT

CARBON TETRACHLORIDE EFFECTS ON FERTILIZATION AND PREIMPLANTATION DEVELOPMENT IN MICE

By

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Carbon tetrachloride (CCl_4) is a ubiquitous environmental contaminant. Estimates state that as many as 10 million people are occupationally exposed to CCl_4 every day. There is a prodigious amount of information on the hepatotoxicity and bioactivation of CCl_4, yet little datum exist on the reproductive hazard.

In this study female B6D2F1 mice were gavaged with 1/10 LD_{50} and 1/100 LD_{50} of CCl_4 in corn oil once a day from day 1 through 5 of pregnancy (day 1 = vaginal plug). No biologically significant effect on any maternal or neonatal parameters studied throughout gestation and twenty-two days postpartum were seen.

Further studies investigated the effect of CCl_4 on in vitro fertilization. Emulphor was found to be too toxic for use as a vehicle. Concentrations of CCl_4 in 1% t-butanol of 1 - 10 mM caused a significant decrease in fertilization and a significant increase in abnormal ova forms.

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INTRODUCTION

Carbon tetrachloride is commonly referred to by its chemical structure CCl_4. Technical and chemically pure grades of this colorless, highly volatile, dense liquid are produced by six companies in America. CCl_4 is a halogenated hydrocarbon of low molecular weight and is included in the group of environmental contaminants referred to as volatile organic compounds (VOC's).

CCl_4 has been widely used as an industrial solvent, a component of fire extinguisher solutions and a fumigant, however the major use of CCl_4 is in the manufacture of fluorocarbons. In 1980 it was estimated that 3.22×10^8 kg of CCl_4 were produced in the United States and only a moderate 1% decline in CCl_4 production was expected during the following decade (EPA, 1987).

CCl_4 has been detected in ground and municipal water, rivers and oceans, aquatic organisms, soil, the atmosphere, human breath and human tissue. A recent study in Italy (Gilli et al., 1990) and a TEAM (Total Exposure Assessment Methodology) study conducted in North Carolina, New Jersey and North Dakota (Wallace et al., 1987) discovered that indoor concentrations of CCl_4 were greater than outdoor concentrations.

Average adult exposure to CCl_4 has been estimated at 26 μg/day (EPA, 1987). Absorption of CCl_4 occurs rapidly across the intestinal mucosa and the epithelial lining of the lung and more slowly through the skin. The major route of excretion of both oral and inhaled doses is exhalation in the breath. Estimates of the amount of CCl_4 exhaled range from 30 - 60%. The major site of metabolism of CCl_4 is in the liver where reductive dehalogenation, catalyzed by cytochrome P_{450} leads to the production of electrophilic chlorine ($CL^{-}\cdot$) and the trichloromethyl free radical ($\cdot CCl_3$). Ahad et al. (1987) detected formation of free radicals after CCl_4 exposure in liver, kidney, heart, lung, testis, brain and blood. Cytochrome P_{450} catalyzed bioactivation of CCl_4 results in cell death, and has been extensively studied but the exact mechanism is still unclear.

Evidence for a genotoxic or mutagenic action of CCl_4 has been largely negative, although CCl_4 adducts of DNA have been reported in mammalian cell culture systems and positive mutagen tests have been reported with yeast (International Agency for Research on Cancer (IARC), 1987a). CCl_4 has been classified as a class 2-B mutagen with a carcinogenic effect verified in three animal species (rat, mouse, hamster, IARC, 1987b).

The possibility of accidental exposure to CCl_4 has been dramatically demonstrated (Deng et al., 1987) and results in acute and often severe responses. However it is the presence

of the embryo, susceptible to low exposure rates of industrial chemicals and environmental contaminants, that necessitates the assessment of reproductive hazards. Recent reviews (Iannaccone et al., 1987; Spielmann et al., 1989) suggest that possible teratogenic, as well as embryotoxic and embryolethal effects of chemicals on preimplantation embryos need investigation.

Assessments of reproductive effects of CCl_4 in the past have tended to use inappropriate exposure routes and/or very high doses resulting in maternal toxicity and conflicting results. Little if any emphasis has been placed on determining the effects of exposure during the preimplantation period. Current evidence suggests that CCl_4 at high doses is a significant embryolethal threat and an embryotoxic threat at lower doses. The male reproductive system appears to be more susceptible to CCl_4 toxicity than the female reproductive system.

This study tests the effects of orally ingested (gavage) CCl_4 at two dose levels during the preimplantation period in the mouse, The doses of 1/100 and 1/10 of the LD_{50} are given in corn oil once a day on days 1 - 5 of pregnancy to female mice fasted for 2 hours. Corn oil is the traditional and most commonly used vehicle for delivery of water insoluble compounds and has been chosen in this study, although some recent reports suggest that emulphor may be the vehicle of choice for oral administration of CCl_4 (Kim et al., 1990a, 1990b). These authors reported that corn oil

modified the pharmacokinetics of absorption of CCl_4 resulting in a moderation of the hepatotoxic action of CCl_4, however Bull et al. (1988) reported an enhancement in the hepatotoxic effect of a closely related chemical, chloroform ($CHCl_3$) when orally administered in corn oil as oppposed to emulphor. It appears that more work is required to determine which vehicle is most appropriate for oral delivery of VOCs.

Additional experiments are proposed to study the effects of CCl_4 on the mouse in vitro fertilization (IVF) system. Initially the toxicity of emulphor in the IVF system is assessed. Emulphor is capable of maintaining constant concentrations of VOCs in solution over long periods of time, however it has been reported to be too toxic to use as a vehicle in suspended hepatocyte cultures (O'Hara et al., 1989). Finally, the effect of CCl_4 on IVF will be determined by recording the percent of two cell embryos obtained in the presence of different concentrations of CCl_4.

LITERATURE REVIEW

Overview of Carbon Tetrachloride Toxicity

Carbon tetrachloride, commonly abbreviated as CCl_4 is a colorless, volatile organic compound (VOC) of molecular weight 153.8 daltons (physiochemical characteristics in Table 1). The Chemical Abstract series has registered CCl_4 (registration number 56-23-5) as tetrachloromethane and the International Union of Pure and Applied Chemistry (IUPAC) refers to CCl_4 as 1,1,1,1 tetrachloromethane. Common names for CCl_4 include carbona, carbon chloride, tetrachlorocarbon, perchloromethane and methane tetrachloride. The International Agency for Research on Cancer (IARC, 1987b) classified CCl_4 as a class 2B carcinogen stating that evidence for carcinogenicity in humans is inadequate although some studies showed an increase in carcinogenesis of occupationally exposed workers but the increase was not statistically significant. In animals however, the evidence of carcinogenicity is sufficient. Liver neoplasms are commonly seen in rodents after acute and chronic exposure to CCl_4 although some authors suggest that carcinogenesis may be more directly related to the cirrhosis that is initiated by CCl_4 (Louria and Bogden, 1980).

Table 1. Physiochemical characteristics of CCl_4

Description:	colorless liquid
Boiling-point:	76.7^0C
Freezing-point:	-23.0^0C
Density:	1.585 (d25/4)
Refractive index:	1.4607 (n20/D)
Solubility:	miscible with ethanol, diethyl ether, chloroform, benzene, solvent naptha and most fixed and volatile oils
Volatility:	vapor pressure is 91.3 mm at 20^0C
Stability:	decomposes to phosgene in presence of limited quantity of water at 250^0C; noncombustible
Reactivity:	when dry, nonreactive with commonly used construction metals (iron and nickel); reacts, sometimes explosively with aluminum and its alloys; reduced to chloroform with zinc and acid; forms telomers with ethylene and vinyl compunds under pressure in presence of a peroxide initiator
Conversion factor:	1 ppm in air is equivalent to $6.3\ mg/m^3$

IARC, 1979

The genotoxic and mutagenic action of CCl_4 is undefined. Diaz Gomez and Castro (1980) found adducts of ^{14}C-CCl_4 irreversibly bound to liver DNA in vivo. The IARC monographs (1987a) reported that there is no data on genotoxicity of CCl_4 in humans and that animal in vivo and in vitro data was negative while studies in *Saccharomyces cerevisiae* and *Aspergillus* yielded positive results.

Regnault produced CCl_4 in 1839 by chlorinating chloroform ($CHCL_3$) and shortly afterwards Dumas produced CCl_4 by the chlorination of methane (CH_4). In 1843 Kolbe reacted chlorine with carbon disulfide (CS_2) to form CCl_4 (IARC, 1979). Currently CCl_4 is commercially produced by 6 companies in the U.S.A. by three processes 1) as a coproduct with tetrachloroethylene, usually by the chlorination of propane or ethane propylene 2) the chlorination of carbon disulfide 3) the chlorination of methane (IARC, 1979; EPA, 1987). Large scale production of CCl_4 began in the U.S.A. in 1907 and by 1914 it was estimated that 4.5×10^6 kg were produced annually. Reduced demand for CCl_4 led to a 3.5% decrease in production from 1970-1980, however the 1980 production rate was 3.22×10^8 kg. It was estimated that a further 1% drop in production would occur annually through 1985 giving an estimated production of 3.06×10^8 kg of CCl_4 in 1985 (EPA, 1987).

The half life of hydrolytic decomposition of CCl_4 has been estimated at 70,000 years, while the evaporative half life is only 29 minutes (EPA, 1980). Thus, small spills of

CCl_4 rapidly volatize into the atmosphere, while larger spills have the potential to settle under a water layer due to the high specific gravity, low water solubility and high chemical stability of CCl_4. CCl_4 is primarily of anthropogenic origin, however small amounts of CCl_4 are believed to be produced in the troposphere by solar-induced photochemical reactions of chlorinated alkenes, primarily from tetrachloroethylene (IARC, 1979).

The major use of CCl_4 is in the production of fluorocarbons, other applications include a component of fire extinguisher solutions, a grain and industrial fumigant, formulation of petrol additives and as a component of pesticide mixtures. Before a November 11th 1970 ban by the United States Food and Drug Administration (U.S.F.D.A.) CCl_4 was used as a domestic solvent and spot remover and is still used for this purpose in industry, notably the dry cleaning industry although it has been largely been replaced by perchloroethylene (IARC, 1979; EPA, 1980). Traditionally CCl_4 had been used as a treatment for hookworm (1920's), a component of anesthetics and even in shampoos. Its use in the treatment of hookworm is one of the few documented studies of orally ingested CCl_4 except in suicide cases (Louria and Bogden, 1980; EPA, 1980).

Absorption of CCl_4 in mammals occurs readily in the lungs and gastrointestinal tract and at a slower rate through the skin. A typical average exposure for an adult male has been estimated at 13 μg/day from inhalation, 9

μg/day from fluid intake and 4 μg/day from food intake (EPA, 1987). A recently published study in Italy of volatile halogenated hydrocarbons (Gilli et al., 1990) found that atmospheric concentrations of CCl_4 were correlated with intensity of industry, commercialization and demographic density. Disturbingly the concentration of CCl_4 indoors was consistently greater than that outdoors especially in winter. A Total Exposure Assessment Methodology (TEAM) study was carried out in New Jersey, North Carolina, and North Dakota between 1979 and 1988. More than 20 VOC's were measured in personal and outdoor air, drinking water and in the expired air of participants. CCl_4 was identified as one of the ten most common VOC's. As in the Italian study, the concentrations of CCl_4 tended to be greater in indoor air samples than outdoor air samples and breath concentrations were consistently higher than outdoor concentrations (Wallace et al., 1987).

In humans, symptoms of acute exposure include narcosis, gastric irritation, nausea, vomiting, belching, flatulence, drowsiness, headache, dizziness, fatigue, visual field constriction, and moderate to severe damage to the lungs, kidneys and liver (Bogden and Louria, 1980; EPA, 1987). Many cases of acute exposure to CCl_4 have been described (1900's-1950's) although the validity of many early studies is doubtful as the hepatic disorders seen from CCl_4 exposure are similar to effects of hepatitis B and other viruses that were not (or could not be) screened for.

Some recent outbreaks of CCl_4 poisioning have been well documented. An outbreak of CCl_4 poisoning was reported in an isopropyl alcohol packaging plant affecting 14 workers of which four developed renal failure or hepatitis (Folland et al., 1976). Most victims experienced some of the following symptoms: nausea, vomiting, headache, weakness or abdominal pain. Another outbreak of CCl_4 poisioning occurred in a Taiwanese color printing factory, again poisioning was attributed to the combined use of isopropyl alcohol and CCl_4 as solvents (Deng et al., 1987). Recent interest in CCl_4 toxicity has stemmed from the identification of CCl_4 as one of many chemicals found in toxic waste dumps and land fill sites. Liver dysfunction was reported in residents exposed to water sources containing leachate from a toxic dump site in Tennessee (Meyer, 1983). CCl_4 was the major contaminant detected in private wells with concentrations ranging from 61 - 18,700 ug/l. CCl_4 has also been detected at the infamous Love Canal dump site at Niagara Falls, New York (Silkworth et al., 1984).

Animal models have been used to test the toxicity of complex waste mixtures obtained from these dump sites in which it is virtually impossible to assign a particular chemical as responsible for any given effect. Researchers have discovered that serum battery enzyme assays performed in vivo or in vitro provide better predictions of hepatotoxicity than single serum enzyme assays. It is usually thought that CCl_4 is the most likely hepatotoxin

especially considering the ability of other chemicals to potentiate the effect of CCl_4 (Simmons et al., 1988; DeMarini et al., 1989). Other groups have investigated the use of in vitro hepatocyte cultures as models of in vivo hepatotoxicity in experimental animals. Long and Moore (1988) compared the inhibition of the endoplasmic reticulum Ca^{2+} pump, stimulation of phosphorylase a and inhibition of glucose - 6 - phosphatase due to CCl_4 exposure in vivo and in hepatocyte cultures. These researchers found the sequence of events to be the same in both systems, with the onset of every event occurring significantly earlier in vitro. In vitro cytotoxicity of selected VOC's (including CCl_4) was found to be poorly correlated to the in vivo hepatoxicity (Dahlstrom-King et al., 1990). The researchers stated that assessment of release of alanine transaminase (ALT) could not accurately select known hepatotoxic chlorinated hydrocarbons in in vitro culture systems.

The action of CCl_4 as a hepatotoxicant and to a lesser extent a kidney and lung toxicant has been demonstrated in humans and others since the 1920's. Recently some researchers have used the hepatotoxic action of CCl_4 to severely compromise liver function in laboratory animals with the aim of studying the effects of liver dysfunction on other physiological systems. For example the effect of liver dysfunction induced by CCl_4 on the development of hepatic encephalopathy (Yamamoto, 1990a) and blood coagulation variations (Vazquez et al., 1990) have been studied. It is

hoped that direct effects of CCl_4 are considered in the assessment of physiological problems induced by liver dysfunction as these studies could help in the assessment of effects of CCl_4 on other systems such as the reproductive system. Kaminski et al. (1989) reported a direct effect of CCl_4 on humoral and cell-mediated immune responses in the mouse, with T lymphocytes and macrophages exhibiting a greater sensitivity to CCl_4 exposure. A dose-dependent suppression of T cell function was observed at concentrations of CCl_4 that had no effect on liver, kidney, lung or body weight. This is an important report as it suggests that CCl_4 can specifically affect specialized cellular functions apart from the more common damage seen in liver, kidney and lung, at concentrations where no overt signs of toxicity are observable.

Bioactivation and Cytotoxic Mechanism of Carbon Tetrachloride

Since the discovery of the hepatotoxic action of CCl_4, research has centered on determining its mechanism of action. A diverse range of experimental systems such as, perfused isolated liver, cultured or suspended hepatocytes, microsomal preparations and in vivo analysis have been used, yet the exact nature of CCl_4-induced cytotoxicity is still unclear. Although we have presently investigated the effects of CCl_4 on early reproductive events, the ultimate result

of CCl_4 exposure is hepatocyte destruction. Thus it is important to understand the mechanism of action.

The metabolism of xenobiotics is generally considered to be a two step process, occurring primarily in the liver. The first step involves the addition of an electrophilic substituent to the xenobiotic as catalyzed by monooxygenases, reductases or hydrolases termed phase 1 reactions. The second step involves a conjugation reaction with a variety of possible endogenous substances that increase the water solubility of the metabolite in the phase 2 reactions (Neal, 1980). Butler (1961) described metabolism of CCl_4 to chloroform and postulated the intermediate formation of a free radical produced by the homolytic cleavage of a carbon-chlorine bond forming a chlorine ion and trichloromethyl radical ($^{\cdot}CCl_3$). It is now generally accepted that CCl_4 is bioactivated by the cytochrome P_{450} monooxygenase system forming highly reactive free radical intermediates (Recknagel and Glende, 1973; Recknagel et al., 1989). However the identification of the trichloromethyl radical in vivo, and to some extent in vitro, by the spin trapping technique is still controversial (Rosen and Rauckman, 1982). The key, one electron reductive dehalogenation has been shown to be catalyzed by a specific isozyme of cytochrome P_{450} (Noguchi et al., 1982). Ekstrom et al. (1989) described the participation of a human ethanol inducible cytochrome P_{450} in the metabolism of CCl_4. Endoplasmic reticulum is the major source of hepatocyte

cytochrome P_{450} (Wolf et al., 1980). More recently Tomasi et al. (1987) demonstrated that $\cdot CCl_3$ is generated by mitochondrial metabolism of CCl_4 which may explain early destruction of some mitochondrial function. One of the major effects of CCl_4 on mitochondria is the reversible uncoupling of oxidative phosphorylation.

Highly reactive radical species such as $\cdot CCl_3$ and $\cdot OOCCl_3$ have extremely limited range of action due to the quantity of electrophilic targets at their molecular site of origin. Despite this, eventually all parts of the hepatocyte, in particular the plasma membrane, are pathologically affected and it is these effects that are the endpoint of CCl_4 toxicity. Thus metabolism of CCl_4 in the endoplasmic reticulum must set into motion a set of secondary mechanisms which ultimately cause cytotoxicity (Ungemach, 1987). With regard to the nature of the secondary mechanism two major hypotheses exist: 1) long lived toxic products of lipid peroxidation diffuse from the endoplasmic reticulum to other parts of the cell and, 2) a toxogenic increase in hepatocyte cytosolic Ca^{2+} concentration upon CCl_4 metabolism results in cell death. These two hypotheses are discussed below.

Hypothesis 1. Lipid peroxidation and generation of toxogenic substances:

Lipid peroxidation is the metabolism of lipids through pathways involving intermediate formation of lipid peroxides, hydroperoxides and endoperoxides (McCay et al.,

1984). The formation of $\cdot CCl_3$ from CCl_4 as catalyzed by P_{450} occurs at a maximal rate in the absence of oxygen while the formation of the trichloromethylperoxy radical ($\cdot OOCCl_3$) is exquisitively sensitive to oxygen concentration (Keiczka and Kappus, 1980). The $\cdot OOCCl_3$ has been suggested to be the reactive metabolite that initiates lipid peroxidation (Albano et al., 1982). Conner et al. (1986) suggested that $\cdot OOCCl_3$ is the starting point of the oxidative pathway that generates the carbon dioxide anion free radical ($CO^{-}\cdot_2$) and the eventual conversion to respiratory CO_2.

The double allylic hydrogen atom of polyenoic fatty acids are highly susceptible to abstraction by free radicals. Hydrogen abstraction by $\cdot CCl_3$ results in the formation of a lipid free radical (L$\cdot$) and chloroform ($CHCl_3$) while $\cdot OOCCl_3$ results in L$\cdot$ and trichlorocarboxylic acid (Cl_3COOH) formation (Recknagel et al., 1989). Lipid radicals (L$\cdot$) react rapidly with oxygen to form lipid peroxy radicals (LOO$\cdot$) which abstract a hydrogen from adjacent fatty acids to form LOOH and perpetuate L$\cdot$ formation. Also important in the propagation of L$\cdot$ is the regeneration of peroxy (LOO$\cdot$) and alkoxy (LO$\cdot$) lipid radicals from the interaction of LOOH with either Fe (111) or Fe (11) (Simic and Taylor, 1987) which can then initiate new chains of peroxidation. De Groot et al. (1988) utilized an oxystat system which was capable of maintaining steady state partial pressure of oxygen at low levels with a constant amount of respiring biomasss for extended periods of time. The

researchers reported that lipid peroxidation induced by CCl_4 occurred in two stages exhibiting different O_2 requirements. A direct relationship between lipid peroxidation and loss of cell viability was established while conditions where production of $\cdot CCl_3$ were maximal but peroxidation was minimal caused little loss of cell viability.

CCl_4 induced lipid peroxidation has been shown to inactivate both cytosolic and membrane bound enzymes such as cytochrome P_{450}, glucose - 6 - phosphatase and UDP - glucoronyltransferase. The inactivation of these enzymes has been clearly demonstrated in vitro but usually at high concentrations of CCl_4. The extent of in vivo inactivation at relevant exposure levels is not as clear (Horton and Fairhurst, 1987). Recently it has been suggested that CCl_4-induced lipid peroxidation also causes the inactivation of glycerol - 3 - phosphate acyltransferase (GPAT) which is believed to be involved in the rate limiting step of glycerolipid synthesis (Thomas and Poznasky, 1990). These workers proposed that inactivation of GPAT by lipid peroxidation could inhibit membrane lipid repair and thus accelerate membrane disintegration initiated by CCl_4 bioactivation and promote cytotoxicity.

It is not clear to what extent CCl_4-initiated in vivo lipid peroxidation proceeds. Propagation of lipid peroxidation can be terminated through a variety of mechanisms, such as condensation-reactions involving radical-radical interactions (Simic and Taylor, 1987). These

condensation reactions result in cross linking of fatty acyl side chains of phospholipids and highly abnormally branched chains of fatty acids (Link et al., 1984). In addition to physio-chemical termination there are many physiological mechanisms to cope with oxidative stress. Á-Tocopherol (vitamin E) is a well known free radical scavenger. It reacts with lipid hydroperoxy radicals at a much faster rate than do saturated fatty acid bonds. However the concentration of Á-tocopherol is very low in microsomal membranes and for this to be an effective peroxidation chain breaker other mechanisms must exist to regenerate the active form of Á-tocopherol (Recknagel et al., 1989). It has been suggested that ascorbic acid (McCay, 1985) and glutathione may play a role in the regeneration of Á-tocopherol. Urisin and Bindoli (1987) described a phospholipid hydroperoxide glutathione peroxidase that directly reduces phospholipid hydroperoxides to unreactive alcohol derivatives. Miyazawa et al. (1990) demonstrated the formation and accumulation of phospholipid hydroperoxides, especially phosphatidylcholine hydroperoxide in the livers of CCl_4-exposed rats, suggesting that phosphatidylcholine is the most susceptible lipid class to lipid peroxidation in vivo. These workers demonstrated that dietary Á-tocopherol inhibits phosphatidylcholine hydroperoxidation suggesting a normal physiological role of Á-tocopherol in coping with oxidative threat.

CCl_4-reactive metabolites may also be quenched by covalent binding to cellular molecules. Diaz Gomez and

Castro (1980) found $^{14}CCl_4$ metabolites bound to endoplasmic reticulum proteins and lipids and liver nuclear DNA but not RNA. The process of lipid peroxidation is violently destructive to endoplasmic reticulum. There have been many reports of minor CCl_4 exposure protecting aginst further exposure to larger doses (Lindstrom and Anders, 1977). This is believed to be due to CCl_4-mediated destruction of cytochrome P_{450} in the endoplasmic reticulum membrane. The precise nature of the destruction of cytochrome P_{450} is uncertain. One hypothesis is that the propagation of lipid peroxidation is responsible and another that free radicals generated by cytochrome P_{450} reduction of CCl_4 directly bind to the cytochrome P_{450} molecules.

Lipid peroxidation yields a variety of stable, diffusable endproducts which have been suggested as the second messengers that diffuse from the site of CCl_4 bioactivation to distal cellular sites resulting in cell death (Comporti, 1985). These endproducts, particularly the 4- hydroxyalkenals have been linked to most physiological manifestations of CCl_4 toxicity such as loss of glucose-6-phosphatase activity, inhibition of protein synthesis and increased permeability of the plasma membrane. However increasing evidence is being found that 4-hydroxyalkenals are completely detoxified by the glutathione S-transferase enzyme system and that the concentration of these products in vivo does not correspond to the concentrations required

for toxicological effects in vitro (Recknagel et al., 1989).

A significant increase in arachidonic acid (AA) levels has been observed in hepatocyte culture following exposure to low concentrations of CCl_4 (Chiarpotto et al., 1990). The increase in AA levels is believed to be due to activation of phospholipase A_2 by CCl_4 via a mechanism independent of CCl_4 bioactivation. The relevance of this finding to the actual cause of CCl_4 induced cytotoxicity is unclear as inhibitors which prevent activation of phospholipase A_2 and increases in AA do not protect against cell membrane damage induced by CCl_4.

Hypothesis 2. Ca^{2+} hypothesis:

Alternatively, lipid peroxidation may destroy the ability of the endoplasmic reticulum to sequester Ca^{2+} leading to an increase in the cytosolic free Ca^{2+} level. It has been proposed that this increase in Ca^{2+} acts as the second messenger ultimately responsible for cell death (Recknagel, 1983; Recknagel et al., 1989). Early in vitro studies using concentrations of CCl_4 above 1mM showed very rapid increases in cytosolic Ca^{2+} due to entrance of Ca^{2+} from the culture medium into hepatocytes (Brattin and Waller, 1984). It has been shown that these saturating concentrations of 1 mM and above elicit hepatotoxicity by direct solvent effects resulting in increased permeability of the cell membrane and influx of Ca^{2+} from the medium (Berger and Sozeri, 1987). The relationship between the

solvent effects and other pathological effects of CCl_4 in causing toxicity is still under debate. Ca^{2+}-activated fluorescent labels have given contradictory results in analysis of Ca^{2+} release from intracellular stores in response to CCl_4 challenge (Brattin et al., 1985; Long and Moore, 1987). While it is accepted that general lipid destruction in the endoplasmic reticulum results in loss of Ca^{2+} into the cytosol in vitro (Long and Moore, 1987), there is still controversy over the possibility of loss of Ca^{2+} stores from the mitochondria either due to mitochondrial P_{450} bioactivation of CCl_4 or the uncoupling of oxidative phosphorylation by CCl_4 (Recknagel et al., 1989).

Recently it has been shown that Ca^{2+} levels in rat liver in vivo rise before significant cell membrane damage is apparent. Within 30 min of CCl_4 challenge it was found that cytosolic phosphorylase a activity increased in response to increased cytosolic free Ca^{2+} (Tsokos-Kuhn, 1989). This confirmed that phosphorylase a activity increased and endoplasmic reticulum Ca^{2+} pump activity decreased in isolated hepatocytes exposed to CCl_4 (Long and Moore, 1986). Further in vitro evidence for the Ca^{2+} hypothesis was gained by Srivastava et al. (1990) using hepatic microsomal preparations. These workers used concentrations of CCl_4 (0.5 - 2.5mM) that have been measured in rat plasma following acute dosing and showed a significant decrease in the ATP dependent uptake of Ca^{2+}. It was proposed that $\cdot CCl_3$ directly oxidizes the sulfhydryl and

possibly tryosoyl residues of the Ca^{2+} pump resulting in a decreased uptake of Ca^{2+} by endoplasmic reticulum even in the absence of lipid peroxidation. Parola et al. (1990) reported that in vivo administration of CCl_4 to rats results in a step 2 inhibition of the plasma membrane Ca^{2+}-ATPase. This enzyme has been shown to be a plasma membrane Ca^{2+} pump that only operates by displacing Ca^{2+} from inside the cell to the outside. Although the exact CCl_4 induced mechanism of pump inhibition is not known, the first step of pump damage has been demonstrated to be independent of lipid peroxidation. Damage to the pump is proposed to exacerbate the increase in cytosolic free Ca^{2+} levels as the cells ability to remove excess Ca^{2+} to the exterior is diminished. Controversially other experiments have found that incubation of hepatocytes with 0.172 mM CCl_4 caused a rapid decrease in both mitochondrial and extramitochondrial stores of Ca^{2+} without an associated increase in cytosolic free Ca^{2+} levels (Albano et al., 1989). An increase in mitochondrial Ca^{2+} and endoplasmic reticulum Ca^{2+} was found after rats were treated with 96 mg/kg CCl_4 (Yamamoto, 1990b). This is opposite to other researchers who have found a decrease in Ca^{2+} content in endoplasmic reticulum after CCl_4 exposure. However Yamamoto (1990b) reports that this finding supports the Ca^{2+} hypothesis as an increase in mitochondrial and endoplasmic reticulum Ca^{2+} levels reflects an increase in cytosolic free Ca^{2+} levels.

The Ca^{2+} hypothesis suggests that an initial rise in cytosolic free Ca^{2+} is due to selective inhibition of the Ca^{2+} pump in the endoplasmic reticulum and results in activation of Ca^{2+}-dependant enzymes that lead to increased permeability of the plasma membrane, rapid influx of extracellular Ca^{2+} and cell death. In support of this hypothesis, phospholipase A_2 was found to be stimulated in isolated hepatocytes in parallel with increasing free cytosolic Ca^{2+} concentrations (Glende and Pushpendran, 1986). Phospholipase C has also been shown to be directly activated by CCl_4 in vivo and in vitro and activation corresponded with a loss of membrane phospholipid content resulting in membrane dysfunction (Lamb and Schwertz, 1982; Coleman, 1988). Furthermore it has been demonstrated that oxidative damage similar to that caused by CCl_4 stimulates phospholipases as part of the cellular protection system against oxidative stress (van Kuijk, 1987). Thus it is unclear whether phospholipase stimulation is a protective event or whether CCl_4 and the resultant increase in cytosolic Ca^{2+} could overactivate phospholipases resulting in membrane damage. The rise in cytosolic free Ca^{2+} levels could theoretically activate other enzyme systems resulting in pathological effects. Recent evidence has indicated that the Ca^{2+}-dependent endonucleases are not stimulated in vitro or in vivo in response to CCl_4 exposure (Long et al., 1989), thus endonuclease damage of DNA resulting in initiation of, or contribution to the hepatoxicity of CCl_4 is unlikely.

Another set of Ca^{2+}-dependent enzymes, the protein kinase C system, has been shown to be inhibited in isolated hepatocyte culture exposed to CCl_4 (Poli et al., 1988). The prospective inhibitory compounds are the 4-hydroxyalkenals produced by lipid peroxidation and found in concentrations similar to those required to inhibit protein kinase C activity in isolated experiments (Poli et al., 1988). Other authors contest any toxicological role for the 4-hydroxyalkenals. While this report is contrary to the Ca^{2+} hypothesis the inhibition of protein kinase C could be involved in CCl_4-initiated cell injury due to the impaired formation of phosphoproteins.

In summary a complex cascade of events links CCl_4 metabolism, generation of free radicals, lipid peroxidation, 4-hydroxyalkenal formation, macromolecular and free radical interactions and loss of Ca^{2+} homeostasis. The relative importance of each event in the ultimate development of cell death upon exposure to cytotoxic levels of CCl_4 is still open to conjecture. Future studies utilizing other cell culture systems affected by CCl_4 could be assessed for the endpoint of CCl_4 toxicity, thereby contributing to deciphering the cytotoxic action of CCl_4. These systems could include kidney cortical slices (Smith, 1988) and the in vitro fertilization system used in the present studies.

Essential Aspects of Early Mouse Pregnancy

There have been many detailed reviews of mouse reproduction. Mature female mice exhibit a regular 4-6 day estrous cycle that is characterized by changing structure and function of the sex organs dramatically demonstrated by the continual growth and regression of the uterine and vaginal epithelial lining. Females that are maintained in a regular light cycle tend to ovulate 3-5 hours after the onset of the dark period during the estrous phase (Champlin et al., 1973).

After ejaculation sperm begin to reach the ampulla region of the oviduct within 10 minutes and once capacitation has been achieved (less than one hour), sperm penetrating the cumulus mass surrounding the oocytes come in contact with zona pellucida protein receptors that are believed to trigger the acrosome reaction (Bleil and Wassarman, 1983). Acrosome reacted sperm are capable of fertilizing the oocytes in the ampulla region of the oviduct. Some of the events immediately preceding the acrosome reaction are, penetration of oocyte outer layers resulting in sperm fusion with the oocyte's plasma membrane stimulating ionic changes within oocytes, and initiating the cortical reaction which blocks polyspermy. Pronuclear formation is followed by DNA synthesis, metabolic changes, mitosis and the first embryonic cleavage to form a two cell embryo (Whittingham, 1979). For the next few days the embryo divides slowly with little increase in mass occurring.

During this time the uterine lining is developing and preparing for implantation under ovarian hormone control.

Up to the early 8 cell stage there is good evidence that the blastomeres of the mouse embryo are equipotent (Rossant and Vijh, 1980). During the next stages of development the embryo undergoes compaction and blastulation during which events significant to toxic insult occur e.g. development of gap junctions for cell to cell communication (Lo and Gilula, 1979) and expression of cytochrome P_{450} monooxygenases (Spielmann and Vogel, 1989). During these stages the development of two distinct cell lineages, the trophectoderm and inner cell mass (ICM) begins.

The embryo enters the uterus on day 4 at the blastocyst stage and implantation occurs on d4-5. Implantation is under hormonal control and requires synchronous development of embryonic and uterine tissues. The trophectoderm is responsible for invasion of the maternal uterine tissue and develops into the extraembryonic tissues including the placenta while the ICM develops into the fetus proper (Enders et al., 1981).

Between the fifth and tenth day of gestation there is a dramatic increase in the growth rate of the embryo during which time the basic body systems are established. Pairs of somites are formed along the body axis followed by initiation of the development of the neuronal and respiratory systems and establishment of the limb buds.

During this period of embryogenesis, exposure to toxicants may result in teratogenesis (Hogan et al., 1985).

The duration of gestation in the mouse is strain dependent; in the B6D2F1 strain used in this work it is 19 days. At birth, typical of all mammalian species, the mouse is still relatively underdeveloped and relies completely on maternal care for survival.

Reproductive Toxicology

Reproductive health hazards are foremost in the minds of many modern day toxicologists. The adverse reproductive effects of many metals such as lead, mercury, cadmium, arsenic and lithium have been described in both humans and experimental animals. Chemicals including insecticides, herbicides, fungicides polybrominated biphenyls (PBBs) and polychlorinated biphenyls (PCBs) have been valuable agricultural or industrial chemicals due to their chemical stability, low volatility and nonflammability, the same characteristics that govern their persistence in the environment and accumulation in the food chain. These compounds are also believed to be potential reproductive hazards. Vinyl halides, widespread in the manufacture of plastics are believed to cause sexual dysfunction in men and increase fetal mortality. Organic solvents, the group to which CCl_4 belongs have a potential exposure to an estimated 10 million workers daily and yet only limited studies have

been conducted to ascertain their reproductive threat (U.S. Congress Office of Tech. Assess., 1985).

Reproductive hazard to the male is usually perceived as a decreased libido or sexual ability due to toxic effects on hormonal balance or effects on the peripheral or central nervous systems. Most commonly a reduction in gamete production, viability or normality can occur from chemical insult. Fertility hazards in the female are similar to those in the male although the time periods at which the gametes are most susceptible to toxic insult differ. Although the male gametes are more susceptible to mutagens the recovery of a normal genetic complement occurs very rapidly. Any mutation of female gametes remains permanently in the limited population of oocytes (Barlow and Sullivan, 1982; Christian, 1983a).

The most perplexing reproductive hazard effects are those on the development of the embryo and fetus. Smith and Costlow (1985) stated that it is the potential, unknown presence of the conceptus in the workplace or other hazardous environment which requires society to provide effective control of exposure to hazardous chemicals. Karrh et al. (1981) defined an embryo-fetotoxin as any chemical which manifests an effect upon the conceptus during any stage of gestation, from fertilization until birth. This includes the induction of the death of the conceptus, any structural malformation or any physiological dysfunction. Also involved is any manifestation of growth retardation,

developmental delay of a physical or psychological nature at birth or the subsequent postnatal period. This definition also fits the term teratogen, however Staples (1975) specifies that a true teratogen must produce adverse effects on the embryo at concentrations below those necessary to produce overt signs of toxicity in the mother. Maternal exposure to chemicals that specifically effect the embryo is possible during the critical periods of embryogenesis (Kretchmer, 1978).

The scope of the problem is astonishing. The human reproductive system is relatively inefficient with only 50% of all conceptions resulting in normal offspring. A third of all conceptuses are believed to be lost before pregnancy is recognized and an approximate 15% of all recognized pregnancies have been estimated to end in spontaneous absorption. A further 8% of births exhibit developmental abnormalities that interfere with survival or quality of life (Smith and Costlow, 1985). It has also been estimated that 2-3% of birth defects can be attributed to known drugs and environmental chemicals (Karrh et al. 1981). Interestingly, various surveys suggest that less than 1% of industrial chemicals have been adequately studied for their potential reproductive hazard (Karrh et al., 1981).

In Vivo and In Vitro Systems for Reproductive Toxicity

The goal in the evaluation of hazardous chemicals is the determination of the toxicological profile using in vivo

and in vitro animal studies to allow assessment of the potential risk to humans. Schardein and Keller (1989) have discussed the four well established manifestations of developmental toxicity in laboratory species. These are:

1. Growth retardation, generally measured by fetal body weight and crown-rump length;
2. Embryolethality, in mammalian species, this is seen as a decrease in implantation sites or increase in resorption or stillbirth. In humans embryolethality most commonly results in miscarriage, spontaneous abortion or stillbirth.
3. Malformation, is sometimes classed as structural malformation or teratogenesis and includes primarily functional and metabolic disorders.
4. Functional disorders, this class of developmental toxicity is not as well defined as the other three and includes dysfunction of any biological system. More specifically Vorhees (1986) states that impaired cognitive, affective, social, arousal, reproductive and sensorimotor behaviors are the major effects in this class.

It is not within the purview of this literature review to discuss the complex field of human risk assessment and the relationship of animal models to human toxicology. Rather it is to highlight the in vivo and in vitro test systems for the analysis of reproductive toxicity.

The in vivo tests for reproductive toxicity can be divided into two main types, single generation and multigenerational studies. In 1966 the U.S.F.D.A. published

guidelines for single generation studies (Christian, 1983b). These consist of three treatment protocols. The segment 1 studies are usually carried out in rodents and are used to assess the toxicants effect on mating behavior and gonadal function, conception rates, maintenance of pregnancy, parturition, lactation and postnatal development. Males are dosed for 60 days before mating to cover one cycle of spermatogenesis and females are dosed for 14 days prior to mating and throughout gestation until sacrifice. Half of the females are sacrificed at day 13 of gestation to ascertain number of corpora lutea, implantation sites and live or resorbing embryos. The remaining females are allowed to deliver and nurse the offspring with evaluation of gestation length, litter size, sex ratio, body weight and normality of pups. Segment 2 studies test the toxicant over the period of teratogenesis. In rats or mice the females mated with unexposed males are exposed to the toxicant during days 6-15 of gestation or more uncommonly, days 6-17 (mouse) or days 6-19 (rat). The females are sacrificed on the day prior to parturition and the fetuses examined for viability, weight, and congenital abnormalities. Soft tissue, and skeletal examination of the fetuses are commonly performed. Segment 3 studies expose the mated female (with unexposed males) during the perinatal and postnatal period. Generally exposure begins around day 15 of gestation and is continued for 21 days postpartum. Litter size, pup weight and normality are evaluated at birth and at selected intervals

until weaning. While these three protocols are generally accepted, some authors suggest that a more segmental approach is just as valid, with exposure of chemicals limited to defined periods of the reproductive process (Barlow and Sullivan, 1982).

Fabro et al. (1982) described a teratogenic test protocol in CD-1 mice with toxicant exposure on days 6 through 10 of gestation. These authors developed a probit model from calculation of adult lethality and teratogenic concentrations of eight test chemicals, enabling the estimate of a tD_{50} (teratogenic dose in 50% of the population). Cummings (1990) suggested the establishment of a dose response curve for exposure to chemicals from days 1 to 8 of pregnancy. Subsequently the author described three test protocols to localize toxic effects on the pre and post implantation period, embryo or uterine physiology and rate of embryo transport relative to uterine preparation for implantation. Kline et al. (1981) discussed the problems in adapting models for reproductive toxicity from pre-established models for carcinogenesis. These workers suggested that a multiple exposure model was better suited to exposure of the conceptus. Contrary to the traditional view that chemical/toxicant effects are all or none (resulting in death or normal offspring) (Austin, 1973), recent studies have demonstrated abnormalities in term fetuses from maternal preimplantation exposure to genotoxic chemicals (Iannaccone et al., 1987; Spielmann and Vogel,

1989). Takeuchi (1984) exposed pregnant mice on days 2, 3 and 4 of gestation to 10 mg/kg of methylnitrosourea (MNU) (maternal LD_{50} is 400 mg/kg). Examination of the fetuses on day 18 revealed malformations including exencephaly, cleft palate and abnormal tails. Bossert and Iannaccone (1985) exposed blastocysts obtained from nonexposed mothers to MNU and subsequently transferred the blastocysts to nonexposed surrogate mothers. Examination of transferred blastocysts on day 12 or 15 revealed a dose dependent decrease in fetal body length and an increase in abnormal tissue formation. Vogel et al. (1989) cultured preimplantation embryos obtained from pregnant mothers exposed to MNU on day 3 of pregnancy. The embryos demonstrated a significant inhibition of development in vitro, a reduction in cell numbers and an increase in structural chromosomal defects suggesting that within hours of intraperitoneal (i.p.) injection MNU is capable of reaching the embryo and inducing the malformations seen at term. When pregnant mice were exposed to a single i.p. injection of 40 mg/kg MNU on day 2 of gestation there was 100% embryolethality but no maternal toxicity. However a single i.p. injection of 20 mg/kg MNU on day 2 induced developmental abnormalities of vertebrae, ribs, long bones and kidneys in 40% of the live fetuses (Spielmann et al., 1989). These experiments with MNU indicate that chemicals can induce teratogenic malformations through exposure of preimplantation embryos, at concentrations that cause no overt maternal toxicity.

Other experiments have demonstrated similar effects with other chemicals e.g. $NiCl_2$ (Storeng and Jonsen, 1981), cyproterone acetate and medroxyprogesterone acetate (Eibs et al., 1982), and methylmethanesulfonate (Fabro et al., 1984). However the preimplantation teratogenic nature of these chemicals is not as clear due to maternal toxicity, inability to directly associate the chemical effect to embryo damage or the long half life of chemicals possibly resulting in initiation of the toxic effect during postimplantation fetal development.

In vivo and in vitro methods for assessing early developmental toxicity have recently been described (Spielmann, 1987; Spielmann and Vogel, 1989). These include in vitro culture of maternally exposed cleavage stage embryos and transplantation into unexposed surrogate mothers. The authors suggest that evaluation of in vivo exposure to chemicals during the preimplantation period should include determination of malformations, growth retardation, and fetal survival as well as the traditional assessment of implantation failure.

Blazak et al. (1985) determined possible indicators of normal sperm production in Fischer 344 rats. These authors suggested that a complete analysis of the normal parameters of male reproduction should include, testicular sperm production, epididymal sperm number, sperm transit time and motility for each species. Their findings indicated that the current practice of determining caudal epididymal sperm

number as a useful measure of toxic effects on spermatogenesis is not accurate based on the wide variation normally found in this parameter. However, in vitro assessment of sperm motility in the presence of the test substance can be a useful measure of toxicity. In addition lactate dehydrogenase isozymes in plasma and testis can be used as markers of testicular damage (Reader et al., 1988). Urinary creatine has also been suggested to be a possible marker of testicular damage, in studies which showed that increasing urinary creatine and reduction in relative testis weight are dose related (Rawcliffe et al., 1989). However other authors have demonstrated that decreased testis weight is not a good measure of toxic effects on sperm production rate (Blazak et al., 1985).

With the growing concern over the number of chemicals with unknown reproductive effect, increasing costs of whole animal in vivo assessment and the desire to uncover mechanisms of reproductive toxicity, more toxicologists are defining in vitro assay systems to test potential reproductive toxicants. The use of human cumulus granulosa cells obtained from in vitro fertilization and embryo transfer procedures can be used for in vitro toxicant screening (Hughes et al., 1990). A recent review edited by Kimmel and Kochhar (1990) evaluates in vitro assessment systems for determining toxicant effects on cell-cell gap junctional communication, cell migration and contractility, embryonic cell and tissue proliferation, cell adhesion,

human placental function, the pharmacokinetics of teratogenicity and approaches to study embryonic cell death.

Reproductive Effects of CCl_4

Chatterjee (1966) administered a single i.p. injection of 1.5 ml/kg of CCl_4 in coconut oil to sexually mature male rats after they had been fasted for 24h. At sacrifice, four days later the testes were noticeably retracted into the abdomen. There was a significant decrease in both testes and seminal vesicle weight. Histological studies showed atrophic changes and definite spermatogenic arrest. Furthermore, CCl_4 administration produced a significant increase in adrenal weights and noticeable fat accumulation in the liver. Treatment of male rats with 1.5 ml/kg of CCl_4 i.p. and phentolamine (Chatterjee, 1967a) prevented any effects of CCl_4 on the gonadal, adrenal or hepatic parameters and treatment with ascorbic acid (Chatterjee, 1967b) prevented any gonadal toxicity (preventitive effects on the liver or adrenal gland were not reported). Adams et al. (1952) reported a chronic inhalation study of CCl_4 in the rat. Moderate to marked degeneration of the germinal elements of the testis was found after exposure to 200 or 400 ppm for 7 h/day, 5 days/week. Severe general toxicity also occurred as 9 of 15 rats died during the 200 ppm treatment and 13 of 15 died during the 400 ppm treatment. Lower exposure rates of 400 ppm for 1 h/day, 5 days/wk for 6 weeks elicited no effects on the testis and limited general toxicity. Adams et

al. (1952) also exposed guinea pigs to 400 ppm for 7 h/day 5 days/wk resulting in the death of most animals within 4 weeks and minor nonspecific pathological changes in the testis.

Male rats were given a single i.p. injection of 1.5 ml/kg of CCl_4 in coconut oil every day for either 10, 15, or 20 days (Kalla and Bansal, 1975). These authors found a decrease in epididymal, seminal vesicle, prostate and testis weight after all three treatment periods. However, the decrease in testis weight was greatest for the 10 day treatment while the decrease in seminal vesicle weight was more pronounced in the animals treated for 15 and 20 days. Histological examination of testicular tissue revealed no abnormalities in the 10 day group, slight reduction in spermatogenic cell number and an increase in lumen size in the 15 day group while the 20 day treatment group showed massive changes in the arrangement and number of germ cells, luminal diameter integrity of basement membrane and interstitial material. No documentation of other toxic effects was presented. Seiler (1987) orally dosed male mice with 2000 mg/kg of CCl_4 and measured the amount of ^3H-thymidine incorporated into testicular DNA. CCl_4 treatment resulted in a 50% reduction in incorporation of label into testicular DNA. The author described this technique as a novel way to detect testicular carcinogenic and mutagenic chemicals but no measure of CCl_4 toxicity to testicular cells was mentioned. de Toranzo et al. (1978) measured a 20-

36% decrease in rat testicular cytochrome P_{450} content 3 or 6 h after i.p. administration of 1 ml/kg of CCl_4. The authors reported that ^{14}C adducts of microsomal lipids were found in both the testis and liver. While significant lipid peroxidation was found in the liver 6 h after the CCl_4 dose, no significant lipid peroxidation was found in the testis indicating that loss of cytochrome P_{450} in the testis is due to direct metabolite attack initiated by CCl_4 and not by lipid peroxidation. Spin-trapping of free radicals by Á-phenyl N-tert-butyl nitrose (PBN) yielded a similar resonance pattern in liver and testicular microsomal preparations 2 h after i.p. injection of 1 ml of a 12% CCl_4 solution in the gerbil (Ahmad et al., 1987). The resonance pattern was characteristic of the $\cdot CCl_3$ adduct indicating that cytochrome P450 in the testis can metabolize CCl_4 to the highly reactive $\cdot CCl_3$ adduct.

Alumot et al. (1976) performed a long term exposure trial with rats fed mash that had been fumigated with CCl_4. They were able to overcome decreasing CCl_4 residues in the feed by using appropriate storage containers and training the rats to feed before significant loss of CCl_4 occurred. After 6 wk the males and females fed mash containing either 80 ppm CCl_4 or 200 ppm CCl_4 were mated and thereafter, while treatment continued the animals were mated every 2 months. No significant difference in litter size or pup weight at birth or 10 days after birth between control and treated groups was found. After five pregnancies the reproductive

efficiency in all groups began to decrease dramatically, with the distribution of sterile males not significantly different between control and treated groups. Higher concentrations of CCl_4 were not used in long term reproductive studies as significant paternal toxicity was seen after a six week preliminary trial period, during which no reproductive assessment was made.

The actual effect of exposure to CCl_4 on testicular function is difficult to assess from the above studies as most of the researchers administered CCl_4 by i.p. injection, a very unlikely exposure route that tends to bypass the first pass effect of the liver (Lukas et al., 1971). Also the majority of testicular damage was seen in experiments where significant toxicity of other organ systems occurred or the majority of test animals died. Fasting has been shown to change the toxic effect of halogenated hydrocarbons including CCl_4 (Harris and Anders, 1980) and some of the above workers fasted animals before exposure to CCl_4. The study by Alumot et al. (1976) used levels of CCl_4 where no reproductive effects in male or female rats were seen, however the precise relationship between CCl_4 bound to food molecules and entry into the body was not considered thus it is possible that the rats were actually exposed to CCl_4 levels well below doses where any effect is likely to be seen. Chatterjee et al. (1966, 1967a, 1967b) and Kalla and Bansal (1975) proposed that the testicular damage in CCl_4 treated males is due to adrenal hypertrophy and disruption

of the pituitary-hypothalamic-gonadal axis but this has yet to be proven. Other reports (Amad et al., 1987; deToranzo et al., 1978) suggest that testicular damage is mediated through cytochrome P_{450} bioactivation of CCl_4.

Toxic effects can interfere with female reproduction either by altering hormone metabolism or directly disrupting one or more of several crucial periods - the estrous cycle leading to ovulation, corpora lutea function, preimplantation embryo development, implantation and postimplantation embryo development.

Only a few studies have investigated the effects of CCl_4 on sex hormone metabolism. Levin et al. (1970) found that as little as 0.06 ml/kg of CCl_4 administered orally decreased the metabolism of estradiol-17ß and estrone by 72% after 24 h in isolated microsomes. Pretreatment with CCl_4 before the administration of ^{3}H-estradiol-17ß or ^{3}H-estrone led to an increase in uterine weight and labelled compounds in the uterus. The authors showed that the increase in tritiated compounds in the uterus increased with increasing doses of CCl_4. Furthermore, pretreatment with CCl_4 was found to inhibit the total body metabolism of estrone. All concentrations of CCl_4 tested caused marked increases in plasma transaminase associated with liver damage. Unfortunately no assessment of CCl_4 inhibition of estrogen metabolism on the normal reproductive states or pregnancy was presented. Hipkin (1969) reported that a single i.p. injection of 0.1 ml CCl_4 inhibited the uterotrophic response

to administration of 0.5 I.U. human chorionic gonadotropin (hCG) or pregnant mares serum gonadotropin (PMSG) in rats. However no effect on the uterotrophic response was seen when 5 I.U. hCG or PMSG was administered after CCl_4 administration. Conversely to Levin et al. (1970), Hipkin (1969) did not see any potentiation of the uterotrophic effect of estrone after CCl_4 administration. This difference is difficult to explain as the exact effect of CCl_4 on pituitary secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) and secretion of estrogen, also the physiological response to the stress caused by CCl_4 toxicity, is not clear.

A recent report (Larroque and Lange, 1987) using liver microsomes isolated from male rats treated with the cytochrome P450 inducer triacetyloleandomycin, demonstrated that progesterone metabolism was altered by the presence of 20 mM CCl_4. The authors did not investigate or discuss the relationship of CCl_4 effects on in vivo progesterone metabolism in male or female rats and normal reproductive function. The use of a P_{450} inducer and very high concentrations of CCl_4 were not related to any in vivo effect.

Inhibition of the estrous cycle, a decrease in uterine weight and a variable effect on ovarian weight was seen in fasted, sexually mature rats after i.p. injection of 1.5 ml/kg of CCl_4 (Chatterjee and Mukherji, 1966; Chatterjee, 1967a, 1967b, 1968). Exposure to CCl_4 during pro-estrus

(determined by vaginal smear) led to persistant vaginal estrus, while exposure during diestrus resulted in the maintenance of a diestrus smear. Persisting corpora lutea and some atretic follicles were seen in rats following CCl_4 injection with significant atrophy of ovarian structures noted in histological examinations. As in the experiments with male rats, the toxic effects of CCl_4 in female rats was alleviated by treatment with phentolamine (Chatterjee, 1967a) and ascorbic acid (Chatterjee, 1967b) with concomitant restoration of normal adrenal function. In contrast to this inhibition of the estrous cycle, Khominska (1974) reported that when normally cycling rats received 3 ml/kg of CCl_4 s.c. four times on one day the mean length of the estrous cycle increased from 4.56 to 5.21 days with the estrous phase prolonged by greater than one day.

A number of workers have investigated the effects of CCl_4 on pregnancy. In a study of preimplantation development, McLaughlin (1964) injected 160, 80, 32 mg of CCl_4 into the yolk sac of fresh fertile chick eggs. Respectively, 0, 40, 80 % of eggs hatched and no report of teratogenesis or birth defects in hatched eggs was given. Poorly reported studies by Heine et al. (1964, cited in Barlow and Sullivan, 1982) and Neumann (1977, cited in Barlow and Sullivan, 1982) stated that no malformations occurred when rabbits were given 50 or 60 mg/kg of CCl_4 orally on day 6 of pregnancy. Preimplantation blastocysts were obtained from rabbits fed 0.6 ml/kg CCl_4 on day 5 or

1.0 ml/kg on days 4 and 5 and examined histologically (Adams et al., 1961). The blastocysts recovered on day 6.5 of pregnancy from rabbits treated with the lower dose were normal while blastocysts recovered from the high dose group exhibited some cellular degeneration of the embryonic disc and some trophoblastic cells contained very large nuclei with prominent nucleoli. No correlation between embryo toxicity and implantation success was studied.

S.c. injection of 0.8 ml or 0.3 ml CCl_4 orally was used in a study to examine the effects of severe liver damage on normal pregnancy (Wilson, 1954). Treatment was given for 2 or 3 days with 2-3 doses per day between days 7 and 11 of pregnancy. Of the 29 rats treated, 59% failed to produce offspring of which 38% lost their entire litter due to early resorption. A further 21% of pregnant mothers died and the remaining mothers, except one, produced normal litters. Little emphasis can be placed on this study as exact dosing schedules were not reported and high concentrations of CCl_4 were used, in fact the study was not designed to determine the effects of CCl_4 on pregnancy but that of severe liver damage.

Roschlau and Rodenkirchen (1969) gave 0.1 ml CCl_4 either s.c. or i.p. to pregnant mice on day 16 or day 18 followed by sacrifice on day 19. The authors reported that only one of approximately 50 mothers died although the normal pattern of liver necrosis was apparent in the rest. There was dramatic fetal mortality in the group injected

i.p. on day 16, while fetal liver damage was most evident in the group injected on day 18. Unfortunately an indirect cause of fetal death cannot be ruled out as all maternal groups displayed disturbed placental circulation and placental necrosis.

Khominska (1974) also investigated the effect of CCl_4 induced hepatotoxicity in pregnant rats. Preimplantation losses were found to increase from 5% in controls to 21% in treated rats and postimplantation losses increased from 7% to 18% after female rats were injected with 3 ml/kg CCl_4 in 4 s.c. injections on one day and mated 7 days later.

A report by Gilman (1971), documented exposure of rats for 8 h/day for 5 days between days 10-15 of pregnancy, to 250 ppm CCl_4. There was no significant effect on skeletal abnormalities, litter size, numbers of resorptions or stillbirths. However a significant increase in pup deaths from day 1-4 and 4-21 postpartum was found.

Dowty et al. (1976) reported a study of 11 cord blood-maternal blood paired samples and found that the concentration of CCl_4 in the cord blood was either equal to, or greater than the concentration in maternal blood. The authors suggested there may be a selective transfer of CCl_4 to the infant. No effect of CCl_4 on maternal status or on the infants was reported. Tsirelnikov and Tsirelnikova (1976) gave 0.15 ml/kg CCl_4 orally once on either day 12, 14, 16 or 18 of pregnancy in the rat and reported reduced glycogen levels and increased division rate of cells of the

chorionic epithelium of the placenta. Administration on days 12 or 14 also led to destruction of the chorionic epithelium of the labyrinthine portion of the placenta. Unfortunately no data on fetal survival or development was given.

A number of studies have reported the relative resistance of the fetal liver to CCl_4 induced damage in comparison to the maternal liver (Bhattacharyya, 1965; Roschlau and Rodenkirchen, 1969; Kyutukchiev and Matrova, 1971), even if fetal mortality is very high. Tsirelnikov and Dobrovolskaya (1973) reported that exposure of maternal rats to 3 ml/kg CCl_4 did not induce morphological changes in fetal liver unless exposure was on day 19 of pregnancy, although exposure on earlier days led to decreased fetal liver metabolism and delayed glycogen synthesis. Kretchmer (1978) suggested that cytochrome P_{450} monooxygenase enzyme systems may be active in early fetal life and Spielmann and Vogel (1989) state that the onset of activity of the phase 1 and cytochrome P_{450} monooxygenase systems and phase 2 conjugation enzyme systems coincides with the formation of the blastocyst cavity in the preimplantation mouse embryo. Interestingly Khominska (1974) documented the increased sensitivity of 4 wk old rats, born from rats exposed to CCl_4 before pregnancy, to CCl_4 induced hepatotoxicity, presumably due to delayed induction of cytochrome P_{450}.

Schwetz et al. (1974) evaluated the effects of subanesthetic concentrations of CCl_4 on rat embryonal and fetal development coupled with careful assessment of

maternal toxicity. No significant effect on fetal resorptions, litter size or conception rates in rats exposed to 300 or 1000 ppm CCl_4 on days 6-15 of gestation was reported, although 1 in 23 litters in the 1000 ppm group was completely resorbed while none of 43 litters was resorbed in the control group. At both CCl_4 concentrations significant reduction in fetal body weight and crown-rump length occurred however no gross morphological abnormalities were observed. A significant occurrance of subcutaneous edema in fetuses was noted in the 300 ppm group and a significant increase in sternebral anomalies in fetuses of the 1000 ppm exposed mothers also occurred, however neither effect occurred in both groups clouding the overall significance of this teratogenic effect. Further confounding the assessment of teratogenicity and embryotoxicity, the occurrence of overt maternal toxicity was high. The food consumption of mothers in both dose groups dramatically decreased over the 40 day exposure period and correspondingly there was a significant decrease in maternal body weight. Hepatotoxicity of maternal dosed groups was also seen, as measured by increasing serum enzyme levels during the dose period and gross morphological changes in the liver typical of CCl_4 toxicity.

A more recent study (Clemedson, 1989) investigated the embryotoxicity of CCl_4 in the post-implantation rat embryo culture system and in ovo chick embryos. Embryos recovered from the rat at 9.5 days of gestation were exposed in vitro

to 10, 100, 300, 600 or 1000 mu g/ml of CCl_4 in rat serum with or without the rat S-9 mix liver microsomal activating system. Without the S-9 mix in the system no toxic effects were seen with concentrations of 300 mug/ml or less. Concentrations of 600 mug/ml and above affected the somite number, growth and morphology of embryos and was interpreted as a general toxic effect. Addition of the S-9 mix resulted in the same generalized toxicity evident at concentrations of 300 mug/ml and above. The chick embryos were exposed to CCl_4-vapor at concentrations of 25, 35 and 75 ppm, with 25 ppm and above resulting in a reduction of somite number and a decreased growth rate. The authors concluded that these experiments indicate that there is no specific malformation pattern induced by exposure to CCl_4 or its activated metabolites. Another set of experiments utilizing the in ovo exposure of chick embryos to CCl_4 described the establishment of neuronal and astrocyte cell cultures from exposed chicks (Clemedson et al., 1989b). Exposure of the embryos to 25 ppm CCl_4 on days 5 - 8 did not affect subsequent in vitro development of neuronal cells. However a reversible effect on the growth rate and respiratory activity of cultured astrocytes was found.

As in the male reproductive effects of CCl_4, the effects on female reproduction are unclear. Many studies used inappropriate exposure regimens and high doses resulting in maternal toxicity. The exact effect of CCl_4 on estradiol metabolism and corresponding uterotrophic growth

is unclear as different authors present opposite effects. Opposite effects are also seen in the studies of CCl_4 on estrous cycles, where one report suggests CCl_4 exposure stops the cycle in either estrus or diestrus, while another suggests a small increase in cycle length.

Many studies reported effects of CCl_4 on selective reproductive functions but did not study the effects in relation to prevention of pregnancy or embryo development. It appears that the predominant effects of CCl_4 on the developing embryo are an inhibition in developmental rate, or embryo lethality. However it is not clear whether this is a direct effect or an indirect effect resulting from maternal toxicity such as damage to the placenta. Only one report of significant teratogenesis was found and the result was unclear as the teratogenic effect was not dose dependent. Clearly more decisive investigation of the toxic effect of CCl_4 on reproductive function in both male and females is required.

MATERIALS AND METHODS

Animals

Female B6D2F1 mice were obtained from one of two sources. Six week old females used for in vivo experiments were obtained from The Jackson Laboratories (Bar Harbor, ME). All other mice (B6D2F1) were obtained by mating C57BL/6 females with DBA/2 males at the Endocrine Research Center. All animals were housed in a 12 h light : 12 h dark photoperiod at 27^0C. Food and water was available ad libitum except where mentioned.

In vivo experiments

Mating procedure: On the evening of their arrival, females from The Jackson Laboratories were placed with fertile males in a ratio of 1:1 and checked on three subsequent mornings for the presence of vaginal plugs. Most females with plugs were obtained on the third morning as expected from the Whitten effect of induction of estrus (Whitten, 1971). Females were randomly assigned to one of three groups (control, 1/100 LD_{50}, 1/10 LD_{50}) on day 1 of pregnancy (= day of plug) and housed in groups of 5 until day 15 at which time they were separated. A minimum of four animals per

group per replicate were used for the two replicates giving a total of 31 animals: 10 control, 10 1/100 LD_{50} and 11 1/10 LD_{50}.

CCl_4 solutions: At room temperature, CCl_4 (catalog number CAS 56-23-5, spectrophotometric grade, Mallinckrodt Int., Paris, KY) was dissolved in corn oil on the first day of treatment and stored at room temperature in light protected, sealed bottles. All solutions were stirred using a teflon coated magnetic bar on each day immediately before use. Animals received either 0.2ml corn oil day^{-1} (control), 1/100 LD_{50} (82.63 mg/kg) or 1/10 LD_{50} (826.3 mg/kg) in 0.2ml corn oil. The LD_{50} of 8263 mg/kg was obtained from the Materials Safety Data Sheet OH504310 (Occupational Health Services Inc., New York, New York). All procedures using CCl_4 were performed in a certified fume hood.

Experimental protocol:

Gavage protocol: Females were gavaged by placing a curved gavage needle (size 18, Perfektum, New Hyde Park, NY) attached to a 1ml glass syringe into the stomach through the esophagus. Prior to the actual experiments, 10 animals of a similar size to the experimental groups were gavaged with 0.2ml 2% methylene blue and killed by anesthetic overdose using tribromoethanol while the needle was in place. The position of the needle and localization of the dye to the

stomach was checked to ensure that no organ damage occurred as a result of the procedure.

Gavage took place mid-morning on each of day 1 through 5 of pregnancy after a 2 h period of fasting. Within each group animals were gavaged in random order and the order of administration to the groups was randomized on each day.

Maternal parameters: Maternal weight (g) was determined on days 1, 8 and 15 of pregnancy and the 22nd day postpartum. On that day the females were killed by cervical dislocation, the kidneys and liver removed, blotted dry, and weighed. A portion of the liver and the kidneys (one sliced longitudinally, the other horizontally) were stored in formalin for histological processing.

Neonatal parameters: On the day of birth, litter size was determined and each neonate was weighed. Crown-rump (C-R) length was measured and individuals were sexed and checked for obvious birth abnormalities e.g. extra/missing digits, cleft palate, exencephaly and spina bifida. After recording these parameters, litter size was reduced to 6, where possible leaving equal numbers of males and females.

Neonatal weight and C-R were measured again on day 8, 15 and 22. In addition, lower incisor eruption and eye opening was assessed in all pups on day 11 and 15 respectively. On day 22 neonates were weaned and two of each sex allowed to grow to maturity (6 wk), the others were

killed by CO_2 asphyxiation. At 6 wk the four remaining young were killed by cervical dislocation and the ovaries or testes removed, blotted dry and stored in Bouin's fixative.

Statistical analysis: All in vivo parameters were analyzed by one way ANOVA. When statistical significance between control and treatments were found pairwise analysis of treatment and control was performed using Dunnett's t test (Gill, 1987).

In vitro experiments

Culture Medium: Brinster's medium for oocyte culture (BMOC-3, Gibco, Grand Island, New York) was used for incubation of oocytes and sperm in the center well of Falcon organ tissue culture dishes (Becton-Dickinson and Co., #3037, Cockeysville, MD). Alternatively, BMOC medium was prepared in the laboratory and used in the outer well of the tissue culture dishes. The constituents of BMOC and BMOC-3 are listed in Table 2 (Brinster, 1971).

Table 2. Components of two culture media (BMOC and BMOC-3) used for IVF.

Component	BMOC	BMOC-3
NaCl	119.37 mM	94.90 mM
Na·lactate	---	20.11 mM
Na·pyruvate	1.02 mM	0.51 mM
KCl	4.78 mM	4.78 mM
$CaCl_2 \cdot 2H_2O$	1.71 mM	1.28 mM
KH_2PO_4	1.20 mM	1.20 mM
$MgSO_4 \cdot 7H_2O$	1.19 mM	1.19 mM
$NaHCO_3$	25.07 mM	25.07 mM
Bovine serum albumin (BSA)	---	5 g/L
Glucose	5.55 mM	5.55 mM

Superovulation: Six - eight week old B6D2F1 females were injected s.c. with 8 I.U. pregnant mare serum gonadotropin (PMSG) followed 45 - 49 h later with 8 I.U. human chorionic gonadotropin (hCG) s.c.. The PMSG and hCG were obtained from Sigma Chemical Co. (St. Louis. MO).

Emulphor: Emulphor (Emulphor^R EL-620, Rhone-Poulenc, Princeton, NJ) was dissolved in BMOC-3 to the concentrations of 0.1%, 0.01%, 0.001% and 1 ml was added to the center well of duplicate dishes at the same time that 1 ml of BMOC-3 was added in the center well of duplicate control dishes. 0.5 ml of BMOC-3 was added to the center well of plates designated for addition of the epididymis. BMOC medium (3 ml) was added to the outer well of all dishes. The dishes were then placed

in a 5% CO_2 incubator at 37.5^0 C approximately 16 h before IVF.

CCl_4: The BMOC and BMOC-3 media were added to the culture dishes 16 h before IVF as in the emulphor experiments except that the CCl_4 was not added at this time. CCl_4 was dissolved in t-butanol (Catalog number B 2138, Sigma Chemical Co.) to a concentration 100 times the final concentration in a total volume of 10 μl to be added directly to the 1 ml BMOC-3 medium at the time stated. Final concentrations of CCl_4 in the center well of organ tissue culture dishes were 0.05, 0.5, 1, 2, 5 or 10 mM.

Gamete recovery and IVF: Twelve hours after females received hCG, 2 or 3 males of 6 - 10 months of age were sacrificed by cervical dislocation for each IVF experiment and a single epididymus was placed directly into the center well of a organ dish containing 0.5 ml BMOC-3. The epididymus was then repeatedly punctured (approximately 30 times) with a 25 G needle before the dish was returned to the incubator for 1 - 1.5 h.

Approximately 45 min after sperm collection the superovulated females were sacrificed by cervical dislocation for collection of the cumulus mass containing the oocytes. One side of each reproductive tract (ovary, oviduct and uterine horn) was placed in the outer well of the organ dishes containing 3 ml of BMOC. The cumulus mass

was removed from the oviduct under a dissecting microscope, quickly washed and placed in the center well of a treatment or control organ dish. The cumulus masses were randomly placed in either treatment or control across replicates to avoid the same female donating to the same treatment group.

For the emulphor experiments the cumulus mass was added to dishes already containing the desired concentrations of emulphor. For the CCl_4 experiments, the organ dishes were placed in the incubator after cumulus mass addition while the CCl_4 solutions were prepared. CCl_4 solutions were added to the organ dish containing the cumulus mass, with care to avoid directly adding the 10 μl CCl_4 or 10 μl t-butanol (control) on top of the cumulus mass.

Immediately after the addition of the cumulus mass (for emulphor experiments) or the CCl_4/t-butanol, 50μl of sperm suspension was added directly to the center well of the dishes taking care that each replicate was inseminated from a different, randomly selected male. All treatment and control dishes were replaced in the incubator for 26 h before assessment of 2 cell oocytes (which represent fertilized eggs) was conducted.

Statistical analysis: All statistical analysis of IVF experiments used Chi square and Bonferronni Chi square contingency tables(Gill,1987).

RESULTS

In vivo experiments

Maternal Parameters: Replicate treatment data sets were pooled for tests of statistical significance. A total of 60 female B6D2F1 mice were mated with a pool of fertile male B6D2F1 mice. Thirty-five females were designated as pregnant due to the presence of a plug, of these 31 were assigned to experimental groups. All 31 treatment females maintained pregnancy to term and a total of 294 pups were delivered.

Body weight (mean values ± SEM) of dams recorded on days 1, 8 and 15 of pregnancy are listed in Table 3. No significant difference in maternal body weight across treatments on the days measured were seen. Dams were sacrificed on day 22 postpartum (day 1 = day of parturition) and liver and kidney weights were recorded, body weight was also recorded to enable calculation of relative organ weights presented as means ± SEM in Table 4. Relative and absolute liver and kidney weights and maternal weights were not significantly different across treatment groups. Tissue samples of liver and kidney were prepared and preserved in formalin for possible histopathological examination at a later date.

Table 3. Maternal body weight[1] of B6D2F1 mice during pregnancy after oral exposure to CCl_4 on days 1 - 5 of pregnancy.

Day	Control	1/100 LD_{50}	1/10 LD_{50}
1	21.3 ± 0.19	21.0 ± 0.22	21.2 ± 0.19
8	22.4 ± 0.29	22.0 ± 0.35	21.5 ± 0.25
15	32.1 ± 0.60	32.3 ± 0.66	31.4 ± 0.35

[1] Expressed as means ± SEM in g; N = 10-11.

Table 4. Maternal body, liver and kidney weights[1] of B6D2F1 mice on day 22 postpartum after oral exposure to CCl_4 on days 1 - 5 of pregnancy.

	Control	1/100 LD_{50}	1/10 LD_{50}
Body	30.1 ± 0.73	30.3 ± 0.86	28.3 ± 1.02
Liver			
absolute	2.0 ± 0.1	2.1 ± 0.1	2.0 ± 0.1
relative	6.8 ± 0.2	6.9 ± 0.2	6.9 ± 0.1
Kidney			
absolute	0.43 ± 0.01	0.43 ± 0.02	0.42 ± 0.02
relative	1.44 ± 0.20	1.42 ± 0.02	1.47 ± 0.02

[1] Maternal body weights are mean ± SEM in g; absolute liver and kidney weights are mean ± SEM in g; relative weights are mean percent body weight in g ± SEM, N = 10 - 11.

Litter size and the percent male pups measured on the day of birth are expressed as means ± SEM and presented in Table 5. No significant differences were seen in litter size or the percent of male pups across treatment groups.

Table 5. Litter size and percent of males in litters exposed to CCl_4 by gavage to the dam on days 1 - 5 of pregnancy.

	Control	1/100 LD_{50}	1/10 LD_{50}
Litter size	9.6 ± 0.4	9.5 ± 0.5	9.4 ± 0.2
Percent male	52.9 ± 5.1	50.4 ± 5.4	46.3 ± 4.1

Results are mean ± SEM, N = 10 - 11.

Neonatal parameters: Pup weight and crown rump length were measured on days 1, 8, 15 and 22 postpartum and are presented as means ± SEM in Table 6 and 7 respectively. A significant difference between control and treated groups was seen on day 15 postpartum. No other significant differences were seen across treatment groups in pup weight or crown rump length at any other time.

No malformations were detected in any pup on day 1 postpartum (observations not presented). The normal development of pups was assessed from observation of incisor eruption on day 11 and eye opening on day 14. The results are presented as means ± SEM in Table 8.

Table 6. Mean pup weight[1] of B6D2F1 mice on days 1 - 22 postpartum after exposure to CCl_4 by gavage to the dam on days 1 - 5 of pregnancy.

Day	Control	1/100 LD_{50}	1/10 LD_{50}
1	1.29 ± 0.02	1.31 ± 0.04	1.32 ± 0.02
8	4.71 ± 0.12	4.89 ± 0.08	4.81 ± 0.07
15	9.28 ± 0.19	9.29 ± 0.16	8.77 ± 0.10[a]
22	12.09 ± 0.25	12.32 ± 0.36	12.55 ± 0.17

[1] Expressed as the mean ± SEM of the mean per dam in g, N = 10 -11

[a] significantly different from control, Dunnett's t test, $p < 0.05$

Table 7. Crown-rump length[1] of pups on days 1 - 22 postpartum after exposure to CCl_4 by gavage to the dam on days 1-5 of pregnancy.

Day	Control	1/100 LD_{50}	1/10 LD_{50}
1	24.4 ± 0.2	24.4 ± 0.2	24.2 ± 0.1
8	35.6 ± 0.4	35.9 ± 0.3	35.6 ± 0.2
15	46.1 ± 0.9	45.9 ± 0.3	45.9 ± 0.2
22	56.0 ± 0.4	56.3 ± 0.4	56.3 ± 0.1

[1] Expressed as the mean ± SEM of the mean per dam in mm, N = 10 - 11

Table 8. Percentage of pups exposed to 1/100 and 1/10 LD_{50} CCl_4 by gavage to the dam on days 1 - 5 of pregnancy showing incisor eruption on day 11 and eye opening on day 14.

	Control	1/100 LD_{50}	1/10 LD_{50}
Incisor eruption	75.0 ± 2.2	72.2 ± 2.2	78.5 ± 2.2
Eye opening	83.3 ± 1.4	83.3 ± 1.4	84.9 ± 2.1

Results are mean ± SEM of the mean per dam, N = 10 - 11

In vitro experiments

Emulphor: In vitro fertilization trials for emulphor and CCl_4 were pooled for statistical analysis. Emulphor at 0.1%, 0.01% and 0.001% was added to organ tissue culture dishes the night before IVF trials. Table 9 shows the number of two cell embryos, total number of ova and the percent fertilization for all emulphor trials. The fertilization rate for all emulphor concentrations tabulated was significantly different from the control.

CCl_4: CCl_4 was added directly to the center well of organ culture dishes, containing the cumulus before addition of sperm, to the concentrations of 0.05 mM, 0.5 mM, 1 mM, 2 mM, 5 mM and 10 mM. The total number of two cell embryos, total

number of ova and percent fertilization for all CCl_4 trials are shown in Table 10.

Table 9. Effect of emulphor[1] on mouse in vitro fertilization

Dose	2 cell	total ova	% fertilized
control	85	93	91.4
0.1%	23	100	23.0[a]
0.01%	32	65	49.2[a]
0.001%	58	90	64.4[b]

[1] g/100ml
[a] significantly different from control, $p < 0.001$
[b] significantly different from control, $p < 0.01$

Table 10. Effects of CCl_4 (mM) and t-butanol (v/v) on mouse in vitro fertilization.

Dose	2 cell	total ova	% fertilization
control	298	321	92.8
1% t-butanol	215	240	89.6
0.05	179	197	90.9
0.5	169	186	90.9
1.0	49	109	45.0[a]
2.0	29	94	30.9a
5.0	23	75	21.9a
10.0	9	75	12.0a

[a] significantly different from t-butanol, $p < 0.001$

The vehicle for delivery of CCl_4 was t-butanol and the percent fertilization in 1% t-butanol was not significantly different from the control. Thus t-butanol was used as the control for further statistical analysis. CCl_4 at concentrations of 0.05 mM and 0.5 mM had no significant effect on fertilization while 1 mM, 2 mM, 5 mM and 10 mM CCl_4 caused a significant decrease in fertilization. A significant linear relationship ($p < 0.001$) between 1 mM, 2 mM, 5 mM and 10 mM CCl_4 and decrease in fertilization was found.

In the presence of 1mM, 2 mM, 5 mM and 10 mM CCl_4 abnormal ovum forms were observed. The number of abnormal forms, total number of ovum and percent abnormal forms are listed in Table 11. All doses of CCl_4 resulted in a significant increase in abnormal forms and a significant linear relationship ($p < 0.001$) was found to exist between dose and percent of abnormal forms.

A pair of typical two cell embryos can be seen in Figure 1a to be compared to the abnormal forms seen at CCl_4 concentrations above 1 mM demonstrated in Figure 1b. The abnormal forms are characterized by a compact dense cellular region surrounded by pycnotic structures. These represent degenerating oocytes.

Table 11. Effect of CCl_4 (mM) on ovum morphology in mouse in vitro fertilization.

Dose	abnormal	total ova	% abnormal
1% t-butanol	0	175	0
1.0	9	109	8.0[a]
2.0	15	94	16.0[a]
5.0	69	105	65.7[b]
10.0	53	100	53.0[b]

[a] significantly different to t-butanol, $p < 0.001$
[b] significantly different to t-butanol, $p < 0.01$

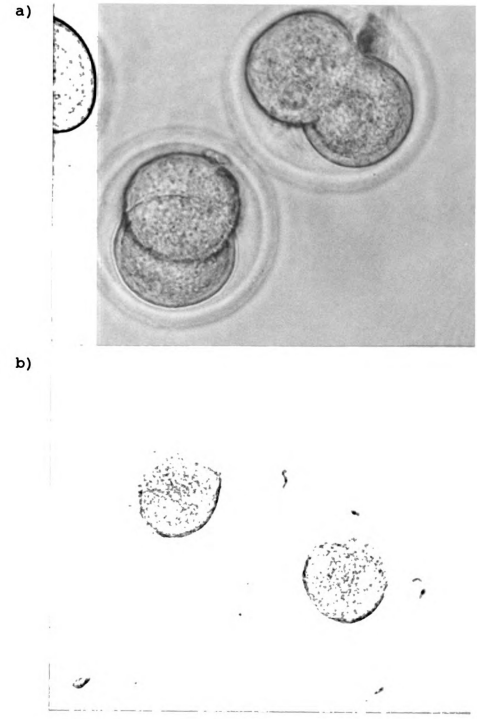

Figure 1. Appearance of normal two cell embryos (a), and abnormal forms seen at CCl_4 concentrations above 1 mM (b).

DISCUSSION

CCl_4 is a common industrial chemical and a ubiquitous environmental contaminant. Disturbingly, levels of CCl_4 are frequently greater in indoor air than outdoor air samples (Wallace et al., 1987; Gilli et al., 1990). The effects of acute exposure to CCl_4 are well documented and include centrilobular necrosis and hemorrhage of the liver followed by regeneration, proximal followed by distal tubule damage in the kidney, peripheral and central neurological disturbances, a host of overt symptoms such as vomiting, disorientation, nausea and headache (Louria and Bogden, 1980). Interestingly, 50,000 persons infected with hookworm were reported to have been treated with 3 to 4 ml of CCl_4 orally during the 1920's with few reports of any toxic side effects. The most severe cases of CCl_4 acute toxicity are often associated with dual exposure to chemicals such as ethanol, isopropyl alcohol and chlordecone (Folland et al., 1986; Mehendale, 1989). CCl_4 has been designated as a class 2B carcinogen (IARC, 1987b) which states that evidence for human carcinogenicity is insufficient, although Blair et al. (1979) reported that workers exposed to CCl_4 over long periods in the dry-cleaning industry showed higher than expected numbers of cancer related deaths for each race-sex group but this was not statistically significant.

Although a moderate amount of research has been reported on the reproductive effects of CCl_4 in experimental animals the results are unclear. Many of the reports have contradictory findings and often overt signs of maternal toxicity are seen. The routes of exposure are usually not biologically relevant as most studies exposed animals via i.p. or s.c. injections. Very little research has been reported on CCl_4's effect during the preimplantation stages of pregnancy.

This work was designed to investigate the effects of 1/10 LD_{50} and 1/100 LD_{50} of CCl_4 administered by oral gavage in corn oil once a day on days 1 - 5 of gestation (day 1 = day of plug identification). These dose levels did not cause any overt signs of maternal toxicity although the average maternal weight in the high dose groups was consistently lower than the other groups on day 8 and 15 of gestation and day 22 postpartum, these differences were not statistically significant. The average maternal liver and kidney weight of the high dose group measured on day 22 postpartum was also less than the other two groups but not statistically significantly. No overt morphological damage of maternal liver or kidney was seen on day 22 postpartum however it should be noted that this examination was 36 days after the last CCl_4 exposure.

Hayes et al. (1986) reported that gavage of mice for 14 days with 625 mg/kg of CCl_4 in corn oil led to measurable liver damage. Kaminski et al. (1990) found that 500 mg/kg

delivered orally to mice once a day for seven days resulted in a specific effect on the immune system. These authors also reported a decrease in total hepatic microsomal protein, aniline hydroxylase activity and aryl hydrocarbon hydroxylase activity following treatment with CCl_4 for seven days with doses as low as 5 mg/kg. Additionally Kim et al. (1990b) reported that even though corn oil reduced the hepatoxicity of CCl_4, a hepatotoxic effect was still found when rats were gavaged once with 25 mg/kg of CCl_4 in corn oil.

Although it is not possible to predict the exact extent of liver toxicity expected to be seen in this study from the literature, it appears highly likely that some loss of liver function will occur at both doses of CCl_4 used in this study. Manifestation of liver toxicity depends upon the route of exposure, administration protocol, administration vehicle, species and strain of animal and even the weight of the animal. The important point is that no signs of overt maternal toxicity were seen in this study that would confound the assessment of any reproductive effect.

Exposure of pregnant mice to 1/10 LD_{50} and 1/100 LD_{50} (which is equivalent to 826.3 mg/kg and 82.63 mg/kg or 0.52 ml/kg and 0.05 ml/kg respectively) over the first five days of gestation has had no effect on fetal mortality and development or postnatal abnormality and development. Average litter size of all groups was not statistically significant and no still births were observed indicating

that CCl_4 had no effect on implantation, resorption or induction of any embryo lethal effects. Average crown-rump length of pups measured on days 1, 8, 15 and 22 postpartum was not significantly different across treatments. No significant difference in average pup weight was seen across treatments except on day 15 when only the high dose was significantly different from the control. This difference is not interpreted as biologically significant as no other difference in pup weight was seen and the day 15 pup weights within litters were more dispersed than at other times. Also, crown-rump length is a more sensitive measure of neonatal growth and no differences were seen in this parameter.

Recent reviews (Iannaccone et al., 1987; Spielmann et al., 1989) have reported that exposure to chemicals during the preimplantation period can lead to teratogenesis. MNU has been shown to act on preimplantation embryos resulting in teratogenesis visualized at birth (Takeuchi, 1984). This study provides evidence that maternal exposure during the preimplantation period to CCl_4 at 1/10 LD_{50} or 1/100 LD_{50} does not result in teratogenesis in mice.

The genotoxic potential of CCl_4 is unclear, it acts as a mutagen in some yeast culture systems but not in bacterial systems, CCl_4 metabolites bind mammalian DNA in vivo and in vitro but no mutagenic effect has been reported. Spielmann et al. (1989) suggests that teratogenesis induced by chemicals during the preimplantation period is due to a

mutagenic event. The fact that no teratogenic effect of CCl_4 was found in this study provides more evidence that CCl_4 is not genotoxic in mammalian systems. CCl_4 exposure in this study did not result in delayed development assessed by observing incisor eruption and eye opening.

In conclusion this study clearly demonstrates that exposure of maternal mice by oral gavage to 1/10 LD_{50} and the 1/100 LD_{50} of CCl_4 once a day from day 1 through 5 of gestation has no embryotoxic, embryolethal or teratogenic effects.

These results agree quite well with the literature in that CCl_4 induction of embryotoxic and or embryolethal effects occur at concentrations where significant overt maternal toxicity is seen. However this report is significant in that no other studies were found where the effects of CCl_4 were determined during the preimplantation period and most previous studies demonstrated high levels of maternal toxicity.

In the above experiments CCl_4 was administered in vivo approximately 12 hours after the expected time of fertilization. Thus an additional set of experiments examined the effect of CCl_4 on fertilization utilizing the well established mouse in vitro fertilization system.

Initially the use of emulphor as a vehicle for delivery of CCl_4 in vitro was assessed. Emulphor proved to be highly toxic at all concentrations tested. A linear relationship between inhibition of fertilization and emulphor was

established. This result agrees with those of O'Hara et al. (1989) who reported that emulphor was too toxic to be used as a vehicle for the study of CCl_4 and $CHCl_3$ in isolated hepatocytes. It appears that emulphors' ability to maintain relatively constant levels of VOC's in solution will not be useful in in vitro systems due to a high level of toxicity. Toxicity testing was undertaken using t-butanol as a vehicle to solubilize the CCl_4 before addition to the IVF system. A comparison between t-butanol and no vehicle demonstrated that t-butanol had no effect on the IVF system at the concentration used to deliver CCl_4. The ability of CCl_4 to elicit a toxic effect in vitro has been repeatedly demonstrated in hepatocyte culture systems at concentrations ranging from 0.02 mM - 10 mM.

In these experiments CCl_4 at 0.05 mM and 0.5 mM was found to have no statistically significant effect on the percent of fertilization. While the concentrations of 1 mM through 10 mM had a markedly significant effect on fertilization. A linear relationship between dose and inhibition of fertilization was established with the lowest effective dose of 1 mM inhibiting fertilization by approximately 50%. Interestingly the inhibition of fertilization was not just marked by a reduction in 2 cell embryos and an increase in one cell ova. An ovum exhibiting a very dense constricted cellular/nuclear region and a dust like circle of degraded cell constituents surrounding the dense body, was observed. The appearance of these forms was

statistically significant at all doses of CCl_4 from 1 mM to 10 mM and a dose related increase in these forms was established.

Recently some authors have reported that rapid halogenated hydrocarbon toxicity in isolated hepatocytes resulting in morphological alterations is due to a direct solvent effect (Berger et al., 1987; Berger and Sozeri, 1987). Interestingly, Kaminski et al. (1989, 1990) have reported that CCl_4 suppression of the immune system exhibits a very flat dose response curve suggesting that bioactivation of CCl_4 is required for this effect. However direct solvent toxicity appears to be very dose dependent.

The results of this IVF study clearly demonstrate a dose dependent effect of CCl_4 indicating that the effect seen is a direct solvent effect and not a specific one. However Dahlstrom-King et al. (1990) studied eight chlorinated hydrocarbons in the isolated rat hepatocyte system and found that toxicity did not relate to physiochemical properties (notably the oil/water partition coefficient) of the chemicals. In fact the authors found that the in vitro toxicity did not correlate well with the in vivo toxicity of the eight chemicals. The appearance of the abnormal ovum in this study corresponds well with morphological changes in hepatocytes reported to be due to solvent action (Berger et al., 1987). However it is interesting to note that at 1 mM CCl_4 a 50% reduction in fertilization occurs with only a small highly variable

percent of abnormal forms seen, while at 10 mM CCl_4 there is an approximate 80% reduction in fertilization with a more consistent 58% appearance of abnormal forms. This could indicate that at the lower effective levels of 1 mM and 2 mM CCl_4 there are other mechanisms apart from the solvent effect in operation resulting in the inhibition of fertilization.

One of these mechanisms could be a disruption of Ca^{2+} homeostasis which is critical to fertilization and proposed as a mechanism of CCl_4 induced hepatotoxicity. It should be pointed out that at 0.5 mM CCl_4 only a two-fold reduction from 1 mM CCl_4 (where a significant 50% reduction is seen) there is no effect on the fertilization. This could be interpreted in favor of either direct solvent effects of CCl_4 or some other mechanism. Obviously more research is required to determine the effect of CCl_4 on the IVF system and perhaps some insight into the general mechanism of CCl_4 cytotoxicity may be gained.

Finally Kim et al. (1990a) reported that a single oral dose of 25 mg/kg CCl_4 in the rat can result in a plasma concentration of 0.3 mM and Srivastava et al. (1990) have stated that concentrations of 0.5 - 2.5 mM CCl_4 can be found in the blood of intoxicated animals. The results from this IVF study suggest that a severe decrease in fertilization rate would occur if the ovum were exposed to levels of CCl_4 above 1 mM.

SUMMARY AND CONCLUSIONS

CCl_4 is a halogenated hydrocarbon primarily of anthropogenic origin. The major source of CCl_4 comes from the manufacture of fluorocarbons. CCl_4 is one of the ten most common VOC's found in dump sites, water supplies, urban and industrial air samples. The IARC has classified CCl_4 as a class 2B carcinogen. Most reports of CCl_4 effects on reproduction are clouded by high maternal toxicity, inappropriate exposure routes or conflicting findings.

Administration of 826 mg/kg and 82.6 mg/kg of CCl_4 in corn oil by gavage in B6D2F1 mice during the first five days of pregnancy resulted in no embryotoxic, teratogenic or embryolethal effects. At concentrations above 1 mM, CCl_4 had a significant effect on the mouse in vitro fertilization system.

In conclusion:

1. Oral administration of CCl_4 to B6D2F1 mice had no effect on maternal liver, kidney or body weight. No effect on litter size, sex ratio, pup weight, crown-rump length or normal postpartum development was seen.
2. Emulphor is too toxic to be used as a vehicle for delivery of volatile/insoluble chemicals in the mouse IVF system.

3. CCl_4 has a significant effect on in vitro fertilization resulting in a decrease in the percent fertilization and an increase in abnormal ovum form.

4. Abnormal ovum forms are most likely due to a direct solvent effect which is seen in hepatocyte cultures at concentrations of CCl_4 above 1 mM.

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