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REPRODUCTIVE TOXICITY OF 3,3',4,4'-
TETRACHLOROBIPHENYL IN MICE

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Jaime Rodriguez

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REPRODUCTIVE TOXICITY OF 3,3',4,4'-TETRACHLOROBIPHENYL IN MICE

By

Jaime Rodriguez

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ABSTRACT

REPRODUCTIVE TOXICOLOGY OF 3,3',4,4'-TETRACHLOROBIPHENYL IN MICE

By

Jaime Rodriguez

There are a possible 209 polychlorinated biphenyls (PCBs) with physicochemical properties ranging from inflammability to lipophilicity. These properties have made them prime candidates for industrial use. However, as a result of their use, improper disposal practices, and accidents, PCBs have found their way into the environment. Ironically, the same properties have made them an environmental pollutant. 3,3',4,4'-Tetrachlorobiphenyl (TCB) is one of the most toxic PCBs. In this study, female C57BL/6J mice were gavaged with either 7, 14, or 21 mg/kg of TCB during days 1-5, 6-10 or 11-15 of gestation (day 1 = vaginal plug presence). No maternal or pup parameter studied was significant. *In vitro* fertilization trials revealed a significant effect of TCB on *in vitro* fertilization, the number of degenerative ova and the number of abnormal 2-cell embryos.

To

Spark of Revolutions

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Introduction

Polychlorinated biphenyls (PCBs) were first produced in the United States in the late 1920s with peak production in the mid 1970s. Since that time they have been banned for use in the United States. Polybrominated biphenyls (PBBs) were first produced in the early 1970s and their use is regulated by the United States government. These halogenated aromatic chemical mixtures are highly stable, practically insoluble in water, chemically inert and emit toxic fumes when heated to decomposition.

Before 1972, it was common to use PCBs in transformer cooling systems, heat transfer and hydraulic fluids, lubricants, plasticizers, sealants and copy paper. Since 1974, PCBs have been confined to closed systems such as electrical capacitors and transformers, vacuum pumps and gas transmission turbines. On the other hand PBBs were used as flame retardant additives in synthetic fibers and molded plastics, polyesters, polystyrenes and polyoletins, though presently PBBs are not used in consumer products.

Because of their persistent nature, both PCBs and PBBs, are long lasting environmental contaminants. PCBs have been detected in mammalian and non-mammalian species in both the industrialized and non-industrialized areas of the world such as the Arctic and Antarctic. As environmental contaminants, PBBs are similarly widespread. PCBs and PBBs are retained in body fat and are excreted in feces, eggs and milk.

Exposure to these chemicals does not only occur through environmental contamination. Accidental exposure to PCBs in Japan and Taiwan and to PBBs in Michigan have occurred and it was this that brought these chemicals into the limelight. Subsequent studies have consistently shown these chemicals to have negative effects on those exposed. The causative agents of the accidental human exposure in Japan and Taiwan had been considered to be the co-contaminants of PCBs such as polychlorinated dibenzofurans that were formed after heating the PCBs in rice oil. However, recent studies have demonstrated the presence of extremely toxic PCB congeners in commercial mixtures and tissue samples. Thus, a re-evaluation of the current status of PCBs and their congeners is needed.

One of the most toxic PCB congeners is 3,3',4,4'-tetrachlorobiphenyl, TCB. A review of the existing literature on TCB establishes this compound's teratogenicity, and embryo toxic effects, but there is no consensus on whether the parent compound or a metabolite is the active compound. *In vivo* and *in vitro* methods for testing toxicity differ, which can lead to conflicting results. Furthermore, TCB studies also show toxicity to vary with the method of administration--injection, ingestion, or inhalation.

Reproduction is considered as one of the most viable means of testing a compound's toxicity. Although previous studies have demonstrated TCB's toxic effects, a more thorough analysis is needed. The present work examines the toxic effects of TCB throughout the entire murine gestation period by oral ingestion (gavage) for five consecutive days beginning on either day one for the period of implantation, day five for the period of organogenesis or day 11 for the period of embryogenesis. A vaginal plug presence the day after the female mouse is placed with the male is day 1.

LITERATURE REVIEW

Mouse Reproduction and Development

The mouse estrous cycle is four to five days long. To maintain a regular cycling, a day-night cycle should be kept constant. In natural matings, insemination, ovulation, and fertilization occur during the dark period, usually between 10:00 p. m. and 2:00 a.m. Thus, twenty-five percent of natural matings in any one night are usually successful. The presence of a vaginal plug, coagulated ejaculum, the next morning permits the recognition of a successfully mated female and such a presence is denoted as Day 1 of pregnancy. A gestational period of 19 to 21 days follows. Females experience a post partum estrus (Theiler, 1989).

The ovulated ova are in the metaphase stage of the second meiotic division until fertilization by spermatozoa has occurred. Fertilization normally occurs in the ampulla of the oviduct. By 24 hours, the zygote(s) or embryo(s) will have moved into the first and second loop of the oviduct and will have completed the first cleavage division, yielding two blastomeres of about equal size.

Three days later, the presence of 16 cell embryos (morulae) is evident. Blastomeres are of unequal size and dividing mitotically. Compaction of the embryo is readily apparent after the 8 cell stage.

By the third or fourth day, embryos will have moved into the uterus and the blastocoele forms. The embryo is now called a blastocyst. During

this time, the distance between the uterine blastocysts increases and the corpora lutea become profoundly vascularized.

Most blastocytes will have arrived in the uterus by the fourth day clearly spaced and free within the lumen. The zona pellucida will have disappeared and the blastocyst clearly separated into an inner cell mass and trophoblast tissue. At 4 1/2 to 5 days, some embryos will have begun implantation by invading the epithelial lining. Implantation is hormonally influenced, but once established, embryo growth rate increases as a result of the glycogen rich decidua.

Development between days 5 and 10 is referred as the period of organogenesis. It is marked by rapid development of the basic body systems. Paired somites appear along the anterior-posterior axis after day 7 leading to a total of 65 pairs with the chronological and linear of appearance of somites being an indicator of embryonic age. Tissues derived from the inner cell mass will develop into the rudimentary excretory, circulatory, digestive and nervous systems. They will also develop into the limb and tail buds. By day 10, the sense organ primordia are noticeable, the primordial germ cells are migrating toward the gonads, and the heart is removing metabolic waste while delivering nutrients to the rest of the embryo. The remaining days of development are a period of embryogenesis. During this time organ systems are finalized and chondrification occurs (Rugh, 1967).

Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) are a class of halogenated aromatic compounds. They were first synthesized in 1881 by Schimdt & Schultz. In the United States, they were first produced in 1929 by the

Monsanto Company (Tanabe, 1988). Theoretically, there are 209 possible PCBs. Commercially, they are sold under the trade name of Aroclor and each mixture is distinguished by the four numbers (Ballschmiter et al., 1989, Hutzinger et al., 1974). The first two digits, 12, denote polychlorinated biphenyls and the last two digits denote the percent of chlorine in the mixture. PCB production reached its peak in the early 1970s. Gross estimates from 1970 indicate that 20% of sales were in service while the remaining 80% was believe to have been discharged into the environment (Nisbet & Sarofim, 1972). This could have occurred through a variety of means such as improper waste disposal (Shcimdt et al., 1971) and leaching from dumps (Lidgett & Vodden, 1970).

Environmental contamination by PCBs was first detected while screening environmental samples for DDT and related compounds (Jensen, 1966). Since then, PCBs have been detected in almost every component of the various global environments. They have been detected in the North Pacific and Indian Oceans (Tanabe & Tatsukawa, 1980) and the Great Lakes in the United States (Mackay et al., 1983). PCBs have been detected in mammalian and non-mammalian species (Jensen et al., 1977, Bennington et al., 1975). Accidental exposure of humans to PCBs has occurred in Japan (Higuchi, 1976) and Taiwan (Chen et al., 1981). In both cases, the poisoning was a result of cooking rice in PCB contaminated bran oil. It was believed that the symptoms derived from the poisoning were caused by co-contaminants of PCBs such as polychlorinated dibenzofurans (Masuda & Yoshimura, 1984). Recent studies however, have since detected the presence of 3',4'-tetrachlorobiphenyl, an extremely toxic PCB congener, as a

component of the PCB mixture (Kannan et al., 1987, Tanabe et al., 1987, Yoshimura & Yamamoto, 1974).

3,3',4,4'-Tetrachlorobiphenyl (TCB)

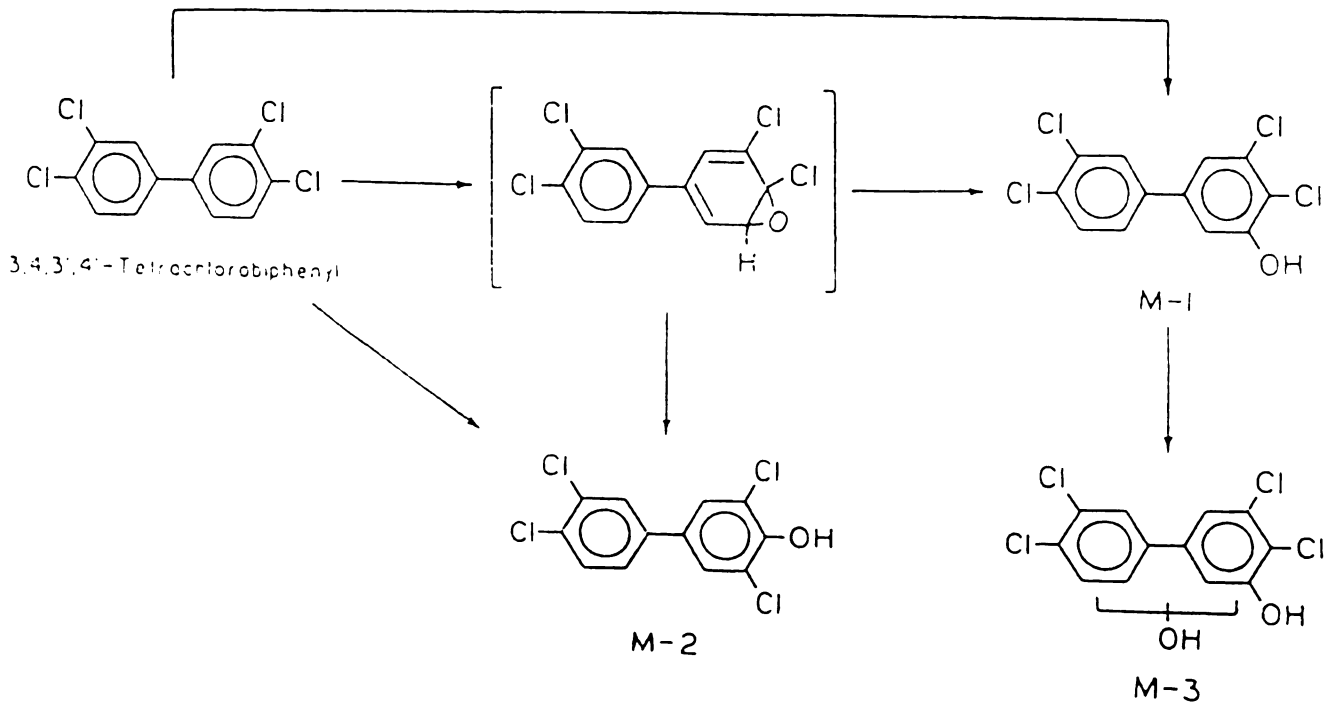
TCB attains coplanarity due to its non-ortho chlorine substitution in the biphenyl rings and because it is an approximate isostereomer of 2,3,7,8-tetrachlorodibenzo-p-dioxin (T_4CDD), it elicits similar toxic and biological responses of dioxins and furans (Safe, 1984). Detection of TCB in PCB mixtures was virtually impossible until recently when a simple and sensitive analytical method was developed to determine the amount of TCB in PCB residue(Tanabe et al., 1987). Studies on TCB indicate that exposure to TCB can result in teratogenicity, carcinogenity and reproductive toxicity (Cornwall et al., 1984; Sargent et al., 1991; Agrawal et al., 1981; Wehler et al., 1990, Lucier et al., 1977).

Metabolism

PCB mixtures will vary with respect to chlorine content and their relative distribution of individual congeners. PCBs are metabolized directly or via arene oxide intermediates into phenolic metabolites, that can be hydroxylated or conjugated to form catechols and phenolic conjugates (Safe, 1994). Unlike the other coplanar and mono-ortho coplanar, TCB is readily metabolized.

Yoshimura and Yamamoto (1973)reported that rats given TCB at an oral dose of 25 mg/kg every third day produced three fecal metabolites in addition to the unchanged TCB. No metabolite was detected in the urine. A study of 2,5,2',5'-tetracholorbiphenyl by Van Miller et al. (1975) had different

Figure1. Proposed metabolism of TCB. (Yoshimura et al, 1987)



results. Tritiated TCB was administered to rats. The rats were sacrificed and their tissues examined for the presence of TCB and its metabolites. The results indicated that 66% of TCB and its metabolites was present in the feces and that 10% was present in urine within three days. Yoshimura and his co-workers (Yoshimura et al., 1987) took an extensive look at the metabolic fate of TCB (Figure 1). The PCB congeners are categorized according to their ability to induce liver enzymes. TCB is categorized as an 3-methylcholamthrene (MC-type) and as such is acutely more toxic than phenobarbital (PB-type) inducers (Yoshimura et al., 1979). However, it had been reported that the 5-hydroxy-metabolite of 2,4,3'4'-TCB was more toxic that its parent compound, TCB (Yamamoto and Yoshimura, 1973). The 3,4-epoxy- and 4-hydroxy-metabolite of 2,5,2',5'-TCB have been reported to have similar metabolic activations (Stadnicki and Allen, 1979). Similarly, the metabolites of TCB in a chick embryotoxicity study were more toxic than the parent compound (Klasson-Wehler et al., 1990). These studies suggesting then that the metabolites may contribute to the toxic effects of TCB. The results of Yoshimura et al. (1987) do not corroborate what has been previously reported. Instead, they found the parent compound to be more toxic than both monohydroxy-metabolites. Furthermore, their results suggest that metabolic activation by hydroxylation occur only in PB-type congeners of PCB, but not in MC-type.

PCB metabolites can have other biological activities. They can act as uncouplers of mitochondrial activity (Ebner et al., 1987), inhibit P450 dependent enzyme activity (Schmoldt et al., 1977), and can bind prealbumin (Rickenbacher et al., 1986). It has also been reported that 3,3',4',5-tetrachloro-4-biphenylol, a TCB metabolite, binds with high affinity to

transthyretin (TTR), a thyroxin transport protein (Brouwer et al., 1990; Brouwer and Van den Berg, 1986). Thus, hydroxylated TCB metabolites are biologically active, but the significance these activities has not been determined.

Carcinogenicty

Studies with a variety of PCBs have reported that after exposure to the chemical, laboratory rodents will develop an increased incidence of liver lesions, including neoplastic nodules and hepatocellular carcinomas (Kimbrough et al., 1975; Shaeffer et al., 1984). Studies have been carried out using a variety of protocols and both long and short term assays to determine the incidence of tumors and preneoplastic lesions such as nodules and papillomas. After exposure, PCBs promoted hepatocellular carcinomas and neoplastic nodules in the rat (Graham et al., 1988). Similar effects were observed in the mouse skin and lung (Anderson et al., 1991; Poland et al., 1982).

When PCBs are given after an initiator, an agent that causes one or more initial basic changes that lead to early pathogenesis, there is convincing evidence that they have a tumor promoting effect (Pereira et al., 1982). Rats fed 3'-methyl-4-dimethylaminoazobenzene for 2 months follow by Kanechlor 400 for 6 months developed a high incidence of hepatocellular carcinomas (Kimura et al., 1976). This assumption was based on the fact that the administration of the initiator by itself, before or concurrently, did not result in tumors. Some PCBs promote effects more in the liver of female than in male rats (Deml & Oesterle, 1982).

PCB mixtures will sometimes contain planar and non-planar congeners. Planar congeners bind to Ah receptors, induce cytochrome P450c and cause effects in the liver and immune cells (Safe et al., 1985; Parkison et al., 1981). Osterele & Deml (1981) found the planar congers to elicit growth of preneoplastic foci. The non-planar congeners are less toxic. They are weak promoters, but do cause hepatic enlargement (Preston et al., 1985). Sargent et al.(1991) studied the effects of planar and non-planar congeners in liver and lymphocytes. Rats were exposed to TCB or 2,5,2',5'-tetrachlorobiphenyl by diet for one year. The results of their study demonstrated that the interaction of both TCBs cause a greater incidence toxicity and mutagenicity in peripheral lymphocytes and hepatocytes than with each TCB alone.

Studies suggest that when studying the carcinogenic effect of PCBs, the time of administration is important. PCBs are inducers of hepatic microsomal enzymes and these enzymes can metabolize carcinogens to less potent metabolites. Thus, administration of a hepatocarcinogen such as diethylnitrosamine can inhibit carcinogenesis (Berry et al., 1979; Makiura et al., 1974).

Because of their lipophilic nature and persistence in the body, Kimbrough (1979) suggested that prolonged administration is not necessary. For example, a single dose of 1 g/kg over a short period caused a high incidence of hepatocellular carcinomas in rats (Kimbrough et al., 1981). This is in contrast to classic tumor promotors such as phenobarbital that has to be administered over an extended period (Pitot et al., 1982).

Reproductive Toxicology

The effects of PCBs on reproduction were first studied in the mink (Ringer et al., 1972). But since then the reproductive toxicity of many commercial and PCB congeners have been assessed. Golub et al. (1991) reported the relationship between dose and the lowest-observable-adverse-effect levels, LOAELs, for various reproductive and developmental end points. The results demonstrate increased malformations at 66.0 mg/kg, reduced litter size at 10 mg/kg and decreased reproductive organ weights, lower birth weights, and decreased postnatal weight gain at 1.0 mg/kg. Some studies, however, fail to demonstrate teratogenicity after mice and rats have been exposed to PCBs during embryogenesis (Villeneuve et al., 1971; Shiota, 1976).

Ronnback (1991)found no observable effect of 1.5 mg/kg and 15.0 mg/kg TCB administered as a single dose to pregnant mice on day 13 of gestation. Lucier et al. (1977) had reported earlier that no observable effect appears in animals administered less than 16 mg/kg/day.

Transplacental movement of PCBs has been detected in a number of species such as primates (Allen et al., 1974) and chicken eggs (Platonow and Reinhart, 1973). The treated female rhesus monkeys were fed 250 mg/kg in their diet for two months. Of the five, 2 conceived and aborted, 2 never conceived and the fifth conceived and delivered. The infant, however, weighed less than the average rhesus infant.

Studies have evaluated the effects of TCB on reproduction, but none have studied its effects throughout the entire mouse gestation period. This study was undertaken to evaluate the effects of TCB on the following stages of mouse development: implantation, organogenesis and embryogenesis.

Although accidental or environmental exposure to levels of TCB tested in this experiment are highly unlikely, it is believed that the dose levels are made valid because the lipophilic nature of PCBs allows for bioaccumulation and biomagnification in a variety of species, including mammals. Since PCBs have been detected in water sources administration of TCB by oral gavage is similar to this natural method of exposure. Other studies have not examined the entire mouse gestation period or have selected to test TCB with a protocol that does not mimic natural exposure in the environment. Furthermore, this study also examines the effects of TCB on the mouse in vitro fertilization system. Because of PCBs' lipophilic nature and because PCBs have been detected in human follicular fluid (Trapp et al., 1984), TCB could have detrimental effects on the oocyte. By studying the effects of TCB in vitro, information can also be obtained on its ability to affect fertilization and preimplantion. In vitro examination of TCB allows for further testing to determine the mechanisms of toxicity as well as it decreases the cost of whole animal in vivo testing. Therefore, this study is an attempt to determine as well as clarify the reproductive effects of TCB both in vivo and in vitro.

MATERIALS AND METHODS

IN VIVO FERTILIZATION TRIAL

Animals

Male DBA/2J and female C57BL/6J mice were used. These mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and aged 2-3 months and 7-9 weeks, respectively. The B6D2F1 hybrids were produced by mating DBA/2J males with C57BL/6J females at the Endocrine Research Center of Michigan State University. All animals were housed under a 12 hour light/dark photoperiod and maintained in an air conditioned room at 24 ± 2°C. Feed (Mouse Chow® #5015, Purina Mills, Inc.) and water were available *ad libitum* except where mentioned.

Chemicals

3,3',4,4'-tetrachlorobiphenyl, 99% pure by gas chromatography and flame ionization detector, GC/FID, was purchased in neat form, catalog number C-077N, from AccuStandard, Inc. (New Haven, CT). The chemical formula for 3,3',4,4'-tetrachlorobiphenyl is C_6-H_3-Cl_2-C_6-H_3-Cl_2. Lot numbers used were #70075, #10162 , and #011893. The physical properties of TCB are listed on Table 1.

Dosage

Each dose administered was based on the lowest lethal published dose (TD_{LO}) of 7 mg/kg of body weight (b.w.) in mice according to the

Table 1. Identification and physical properties of 3,3',4,4'-tetrachlorobiphenyl (TCB).

CAS Number:	32598-13-3
RTECS Number:	DV8650000
Component:	3,3',4,4'-tetrachloro-1,1'-biphenyl
Chemical family:	halogen compound, aromatic
Description:	colorless crystals with a mild aromatic odor
Trade names/Synonyms	3,3',4,4'-PCB, PCB, tetrachlorobiphenyl, biphenyl, TCB, 4-CB, OHS57117
Molecular formula:	C6-H3-CL2-C6-H3-CL2
Molecular weight:	291.99
Melting point:	351-354 F (177-179 C)
Solubility in water:	practically insoluble
Solvent solubility:	soluble in acetone, ethanol, methylene chloride, oils and organic solvents
Reactivity:	stable under normal temperatures and pressures; exothermic reaction with liquid chlorine; fire and explosive hazard with oxidizers; attacks plastics, rubber and coatings
Decompostion:	Thermal decompostion products may include toxic fumes of phosgene, toxic and corrosive fumes of chlorides, oxides of carbon

(Occupational Health Services, Inc., 1992)

Sax's Dangerous Properties of Industrial Chemicals (1992). Four doses of 3,3',4,4'-tetrachlorobiphenyl were tested. To each 25 mg vial of 3,3'4,4'-tetrachlorobiphenyl, 1 ml of ethyl alcohol, 100%, was added to dissolve the chemical. The volume of liquid administered to each animal was 0.1 ml and doses were reconstituted in 100% pure extra virgin sesame oil (Loriva® Supreme™ Foods, Inc., Hauppauge, NY) and dissolved 3,3'4,4'-tetrachlorobiphenyl to contain the proper concentrations of the test chemical. Control doses consisted of sesame oil and ethyl alcohol..

Doses were prepared in glass bottles that had been wrapped in aluminum foil and autoclaved. Prior to each dosing, the control and treatment solutions were mixed using a Deluxe Mixer (McGaw Park, IL) to assure a homogenous mixture.

Doses per treatment were as follows:

Low dose = 7 mg/kg (TD_{LO})

Medium dose = 14 mg/kg

High dose = 21 mg/kg

Administration

Each pregnant mouse received five consecutive oral administrations of either the control or treatment solutions beginning on their assigned day: Day 1, Day 6 or Day 11. Gavage was accomplished using an 18 gauge, 3.8 cm curved gavage needle (Perfectum, New Hyde Park, NY) attached to a graded 1 ml glass syringe. The gavage needle was inserted into the esophagus and the test solutions expelled into the stomach. Food was withheld for a period of 2-3 hours before each gavage, and each gavage was administered between 1250 and 1350 hours.

Mating

On their day of arrival at the laboratory, female mice were grouped four to a Plexiglass cage 7" x 11 1/2" x 5" (Allentown Caging, Allentown, NJ) and allowed a full week of recuperation. Males were housed individually on their day of arrival. Following this adaptation period female mice were put into a male's cage at a 1:1 or 2:1 ratio late in the afternoon. Females were observed for the presence of a vaginal plug the following morning. The presence of a vaginal plug denoted Day 1 of pregnancy. If no plug was observed the female was removed from the male's cage and they were placed together again late in the afternoon. Mated females were caged together and randomly divided into groups on the first day of treatment. A minimum of 15 females were used per group, three replicate groups of 5 per treatment.

Data Collection

Maternal: Dosed female C57BL/6J mice were weighed on days 1, 8, 15 of pregnancy and on days 1, 8, 15 and 22 following parturition. The gestation length and litter size were noted for each female. In addition, the total weight of the litter was recorded along with the number of males and females delivered. On day 22 postpartum, each female was sacrificed by cervical dislocation. The liver and kidneys were examined for abnormalities, excised and weights recorded. The spleen was also observed, excised and its length measured and recorded. The liver and kidneys, one cut in a sagittal plane and the other in a frontal plane, were preserved in a 10 % buffered formalin solution and catalogued for future studies.

Pups: On Day 1, each pup was visually inspected for gross abnormalities. Also on Day 1, each litter was reduced to 6 pups, whenever possible on a 1:1 sex ratio. Pup weight and crown-rump length were recorded on days 1, 8, 15 and 22. The day of lower incisor eruption, upper incisor eruption and eye opening was recorded. On day 22, each litter was weaned, reduced to 2:2 and allowed to develop until day 43. On day 43, the pups were weighed and sacrificed. The ovaries and testes were excised, their weights recorded, preserved in Bouin's fixative solution and catalogued for future studies.

Statistical Analysis

Analysis of variance (ANOVA) for split-plot design was used to test for differences of treatment means with significance at the 5% level. Day 8 data was not analyzed because one group had been treated, one was under treatment and one had not been treated (Gill, personal communication). Sex ratio data was analyzed by a binomial probability test.

***IN VITRO* FERTILIZATION TRIAL**

Animals

Female and male B6D2F1 mice were bred at the Endocrine Research Center by mating C57BL/6J females with DBA/2J males. The parental strains were obtained from The Jackson Laboratory (Bar Harbor, ME).

Chemical

3,3',4,4'-Tetrachlorbiphenyl was purchased from Accustandard, Inc. (New Haven, CT) in neat form and dissolved in ethyl alcohol. The solution

was then suspended in culture medium to obtain the desired doses with a maximal alcohol content of 0.01% (V/V). Control dishes were loaded with 1 ml of medium containing 0.01, 0.1, 1.0, or 10.0 μg of the desired compound or control medium.

Culture Medium

Brinster's medium for oocyte culture, BMOC-3 with 0.4% BSA (GIBCO, Grand Island, NY) was used for the *in vitro* fertilization trials. A similar medium (BMOC-without BSA) was used in the outer well of Falcon organ tissue culture dishes (Becton-Dickson and Co., No. 3037, Cockeysville, MD). BMOC-3 (1.0 ml) and BMOC (3.0 ml) were placed in the inner and outer wells of each culture dish respectively. The culture dishes, when loaded, were equilibrated overnight in a humidified incubator at 5% CO_2 + 95% air at 37°C. The components of the two culture media are listed on Table 2.

Superovulation

Female mice were superovulated by injecting (ip) 10 IU pregnant mares serum gonadotropin and 10 IU human chorionic gonadotropin (hCG, Sigma Chemical Co., St. Louis, MO) 46-48 hours later.

Gamete Recovery and IVF

Twelve to 15 hours following hCG administration, adult male (3 to 5 months old) mice were sacrificed by cervical dislocation. The cauda epididymides were excised and placed in the inner well of an organ tissue culture dish containing 1.0 ml of BMOC-3. They were then repeatedly

Table 2. Components of the two culture media used in the TCB in vitro fertilization trials.

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

[a]Brinster's medium for oocyte culture (Brinster, 1971).

punctured with a 25 gauge needle to release sperm. The sperm suspension thus obtained was incubated for 1.5 hours. Thirty minutes after incubation, motility was assessed and samples showing >60% motility were used for insemination. Approximately 45 minutes after sperm collection, 5 to 7 superovulated mice were killed by cervical dislocation for oocyte recovery. The ovaries and oviducts with part of the uterus were excised and kept in the inner well of the culture dishes containing BMOC-3. The dilated ampullae of the oviducts were carefully pulled apart with fine forceps to release the cumulus masses containing the oocytes. The oocytes were washed once in BMOC-3 and transferred to the control or the treatment organ culture dishes. The cumulus masses were randomly placed in culture dishes. Fifty μl of sperm suspension was added directly to the inner well containing the cumulus mass. All the dishes were replaced in the incubator for 20-24 hours.

At the end of the incubation period, each culture dish was scored for the percentage of oocytes fertilized. Oocytes were considered fertilized by the presence of one cell with two pronuclei, one cell with two polar bodies, or 2-cell embryos. Oocytes were considered unfertilized by the presence of a single cell or if degenerative. The abnormal 2 cell embryos were considered as fertilized. The number of degenerative oocytes and abnormal embryos was also recorded.

Statistical Analysis

Results of the IVF trials were analyzed by Chi Square and Bonferroni Chi Square contingency tables. The differences between the groups were also analyzed by individual Chi Square tests.

RESULTS

In Vivo Fertilization Trials

Data from replicate treatment sets was pooled for tests of statistical significance. A total of 300 female C57BL/6J mice were mated with a pool of fertile DBA/2J male mice. Of these, 180 were confirmed mated by the presence of a vaginal plug and were allocated randomly to one of the four groups.

Maternal Parameters: Since Day 8 data was not analyzed, the selected graphic representations are shown as examples of dose response curves. The body weight of the pregnant dams was recorded on days 1, 8 and 15 of pregnancy and was recorded on days 1, 8, 15 and 22 postpartum. Weight means ± SEM for dams treated on days 6 to 10 are listed on Table 3. No effect of dose over treatment period was indicated by analysis of variance for split-plot design. In addition, there was not an interactive effect between the time period of dose administration and the dose administered. There was no effect of gestation period on weight gain on the three period tested. Figures 2, 3 and 4 show the mean body weight for dams treated with 7, 14 and 21 mg/kg TCB, respectively.

The liver and kidneys of sacrificed dams were excised, weighed and preserved. The absolute and relative weights for these organs are listed on Table 4. No dose, time or dose/time interaction effect was found for maternal liver or kidney weights. Figures 5 and 6 present the mean body weight of mice administered TCB by oral gavage on day 8 and 15 of gestation.

Table 3. Maternal body weight of C57BL/6J mice administered TCB by gavage on days 6 to 10 of pregnancy.*

Prepartum	Control	7mg/kg	14mg/kg	21mg/kg
Day 1	19.14±0.2	18.61±0.2	18.47±0.3	18.31±0.2
Day 8	20.63±0.4	19.96±0.2	20.34±0.4	19.75±0.2
Day 15	30.10±0.4	29.59±0.2	29.41±0.3	28.64±0.2
Postpartum				
Day 1	25.39±0.3	25.58±0.4	24.64±0.4	25.48±0.3
Day 8	28.46±0.3	27.77±0.4	28.16±0.4	28.31±0.4
Day 15	30.46±0.4	30.01±0.3	30.20±0.4	30.85±0.5
Day 22	25.69±0.3	25.85±0.4	26.09±0.4	26.22±0.4

*Mean ± SEM in grams.

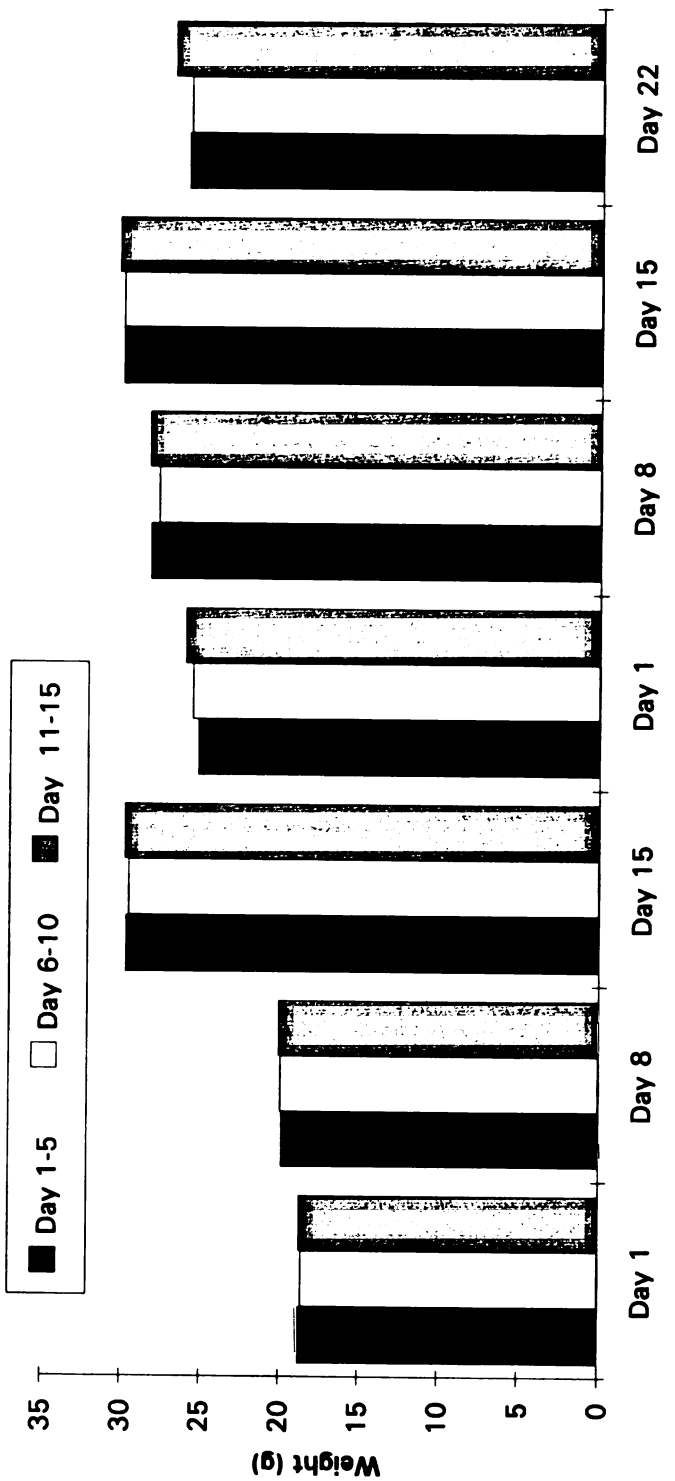


Figure 2. Mean body weight pre- and postpartum of C57BL/6J mice administered 7 mg/kg TCB by gavage.

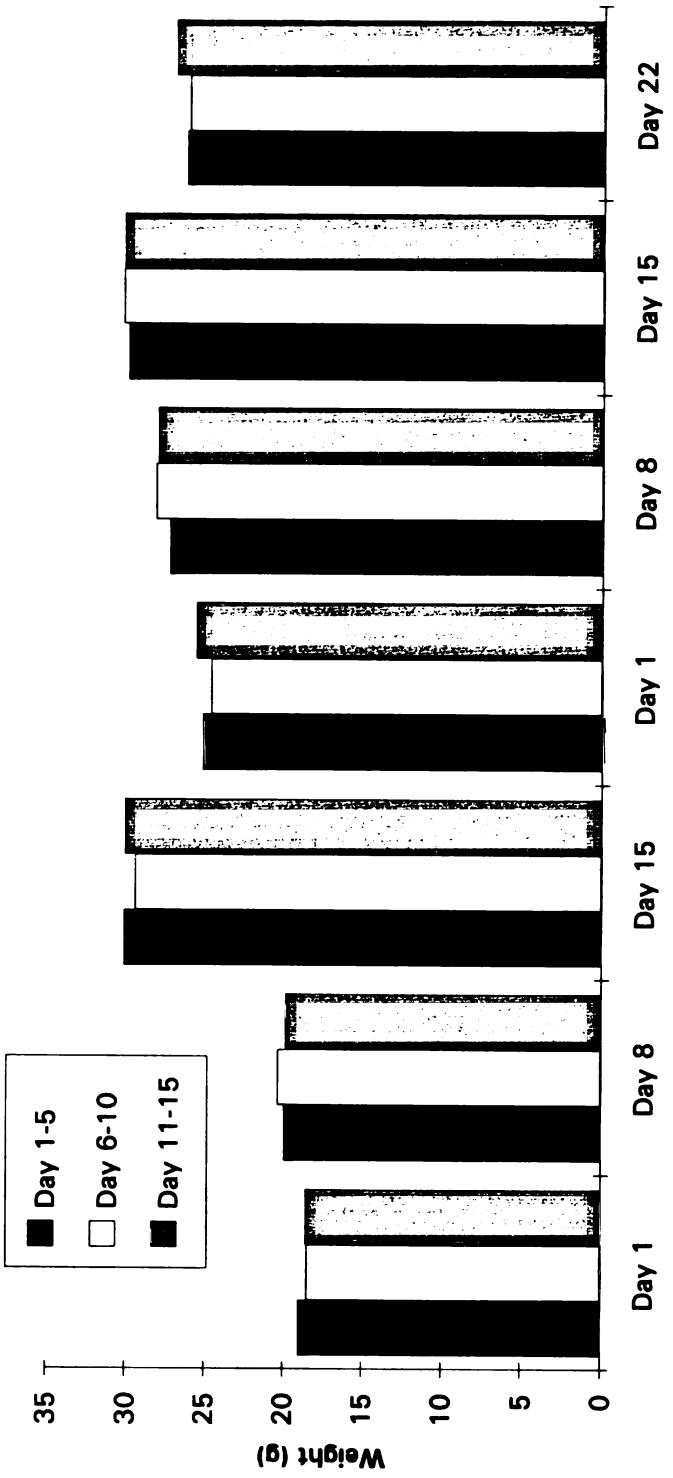


Figure 3. Mean body weight pre- and postpartum of C57BL/6J mice administered 14 mg/kg of TCB by gavage.

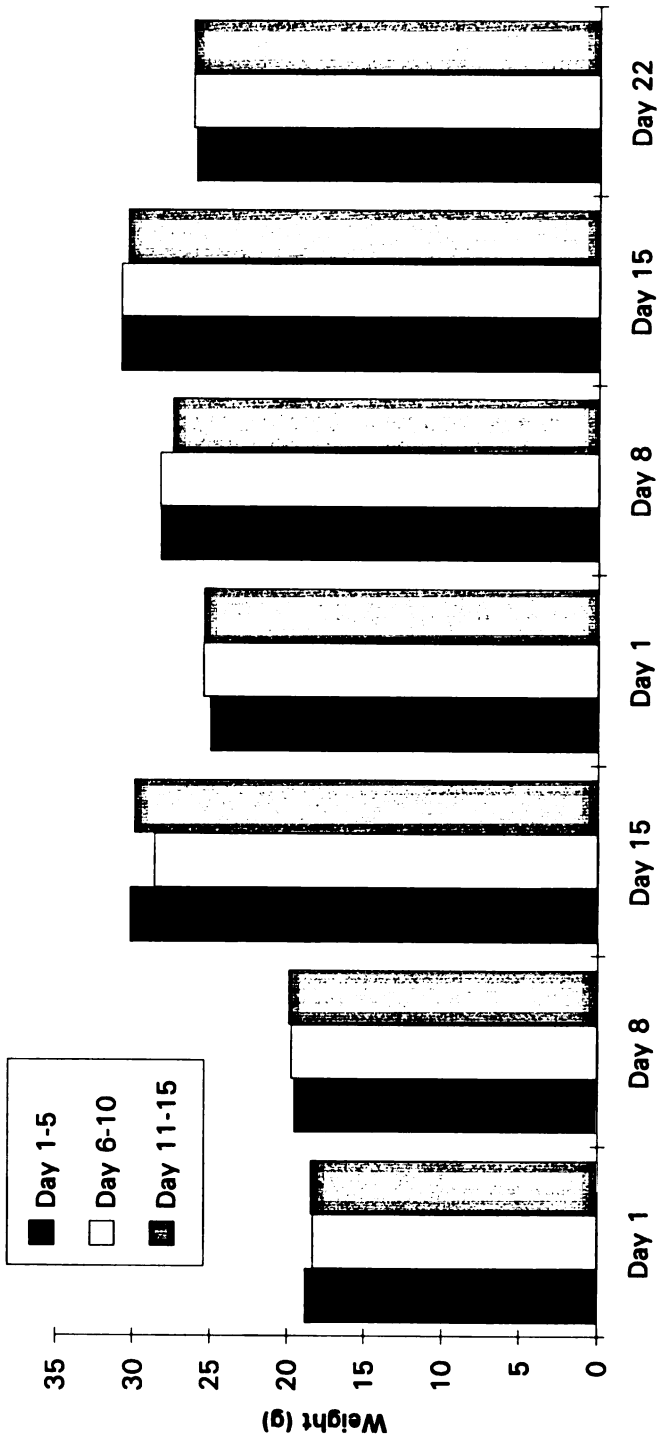


Figure 4. Mean body weight pre- and postpartum of C57BL/6J mice administered 21 mg/kg of TCB by gavage.

Table 4. Liver and kidney weights of female C57BL/6J mice administered TCB by gavage.*

	DAYS 1-5			
	Liver		Kidney	
	Absolute	Relative	Absolute	Relative
Control	1.95±0.67	7.49±0.01	0.31±0.19	1.18±0.03
7 mg/kg	1.93±0.06	7.47±0.01	0.30±0.25	1.14±0.04
14 mg/kg	1.82±0.08	6.96±0.01	0.30±0.31	1.14±0.03
21 mg/kg	1.81±0.08	6.97±0.01	0.31±0.35	1.20±0.03

	DAYS 6-10			
	Liver		Kidney	
	Absolute	Relative	Absolute	Relative
Control	1.89±0.06	7.35±0.01	0.32±0.23	1.26±0.03
7 mg/kg	1.91±0.54	7.42±0.01	0.31±0.23	1.21±0.04
14 mg/kg	1.88±0.07	7.21±0.01	0.32±0.22	1.22±0.03
21 mg/kg	1.89±0.06	7.23±0.01	0.32±0.24	1.22±0.03

	DAYS 11-15			
	Liver		Kidney	
	Absolute	Relative	Absolute	Relative
Control	1.83±0.09	6.94±0.01	0.31±0.28	1.17±0.03
7 mg/kg	1.83±0.04	6.82±0.01	0.30±0.16	1.13±0.03
14 mg/kg	1.85±0.07	6.85±0.01	0.30±0.22	1.13±0.03
21 mg/kg	1.86±0.06	7.11±0.01	0.31±0.23	1.17±0.03

*Absolute liver and kidney weights are mean ± SEM in grams. Relative liver and kidney weights are mean percent body weight in grams ± SEM.

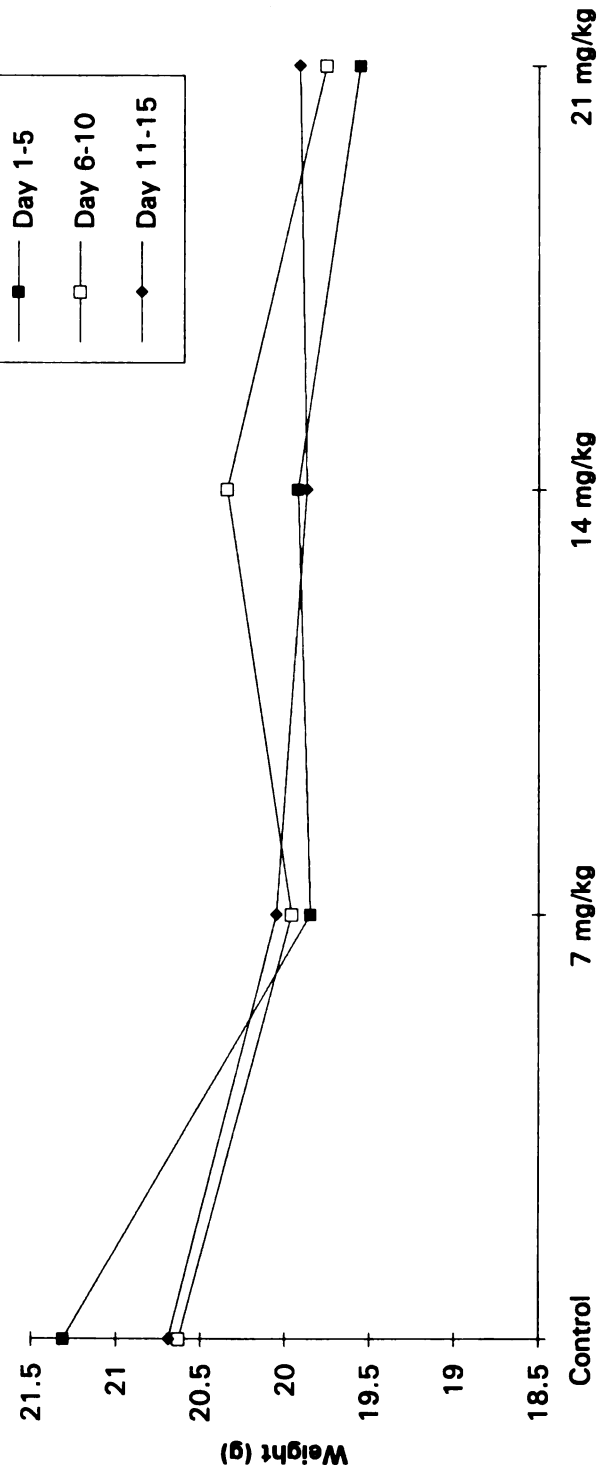


Figure 5. Mean body weight on day 8 prepartum of C57BL/6J mice administered TCB by gavage.

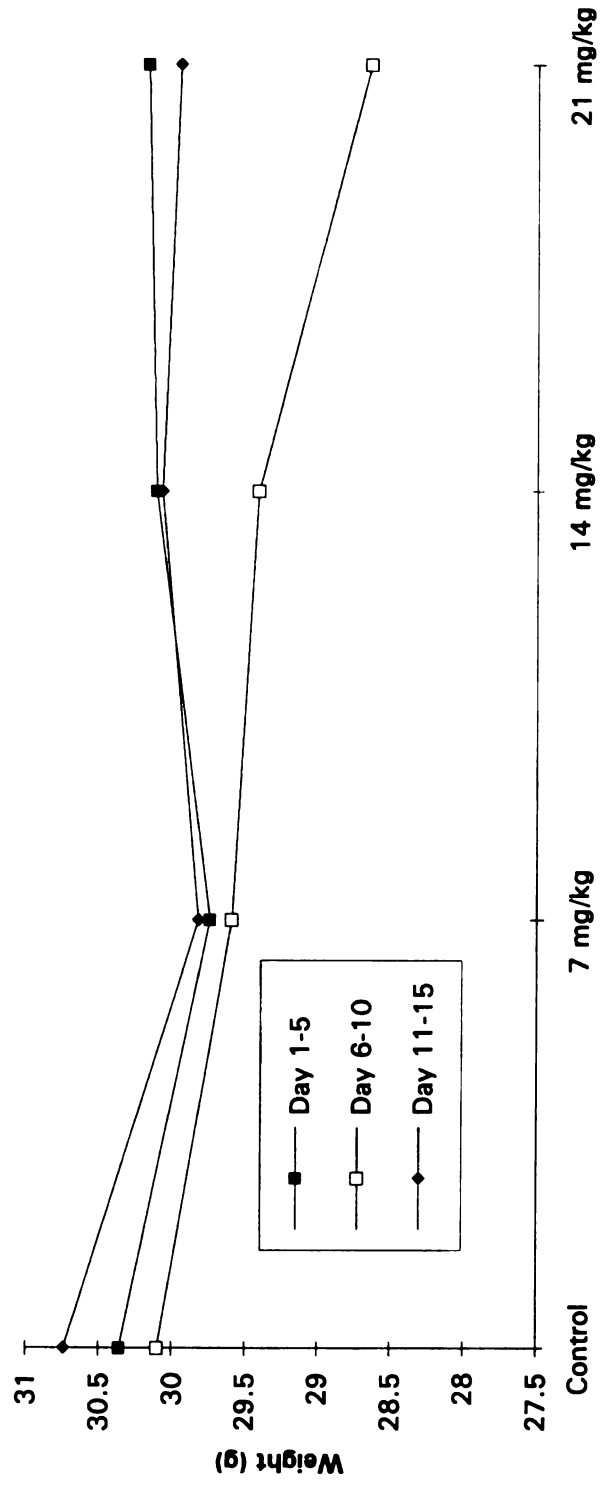


Figure 6. Mean body weight on day 15 prepartum of C57BL/6J mice administered TCB by gavage.

Table 5 lists the mean ± SEM of dam litter size as well as the pooled number of males and females for treatment groups. No significant difference was seen in litter size or sex ratio.

Pup parameters: Pup weight and crown-rump length were recorded on days 1, 8, 15 and 22 postpartum. Figures 7, 8 and 9 reflect the mean pup weight for treatment groups. No dose, time period, or dose/time period interaction effect was noted in either of these parameters. The crown-rump length mean totals are listed on Table 6. No significant difference was noted for day of eye opening or lower and upper incisor eruption. No anomalies were observed in any of the pups. Each of the other parameters studied showed no significant difference between the treatment groups.

In Vitro Fertilization

Table 7 shows the effect of TCB on IVF in B6D2F1 mice. Analysis of variance revealed a significant effect of TCB on IVF. The IVF rate dropped as the TCB dose increased in the culture media. TCB at the levels of 1.0 μg/ml and 10 μg/ml was significantly lower than the control. The IVF rate of the 10.0 μg/ml group was also significantly lower than the 1.0 μg/ml group rate.

The effect of TCB on oocyte degeneration and abnormal embryos is shown on Table 8. The rate of both oocyte degeneration and number of abnormal embryos is significantly higher than the control as revealed by chi square analysis. Furthermore, two treatment groups, 1.0 μg/ml and 10.0 μ g/ml, had a significantly higher rate of degeneration and abnormal embryos from than the control group. The data in Tables 7 and 8 were included in a recent publication (Kholkute et al., 1994).

Table 5. Litter size and total number of male and female mice delivered by C57BL/6J females exposed to TCB by gavage.*

	Day 1-5	Day 6-10	Day 11-15
Control	8.1±0.36	8.0±0.17	8.0±0.32
Males	65	65	60
Females	56	57	52
7 mg/kg	7.7±0.21	8.3±0.21	8.0±0.22
Males	61	63	67
Females	55	61	54
14 mg/kg	7.4±0.40	8.1±0.24	8.3±0.25
Males	56	60	60
Females	55	61	64
21 mg/kg	7.9±0.27	7.5±0.55	8.0±0.26
Males	60	51	58
Femals	56	61	62

*Litter size expressed in mean ± SEM.

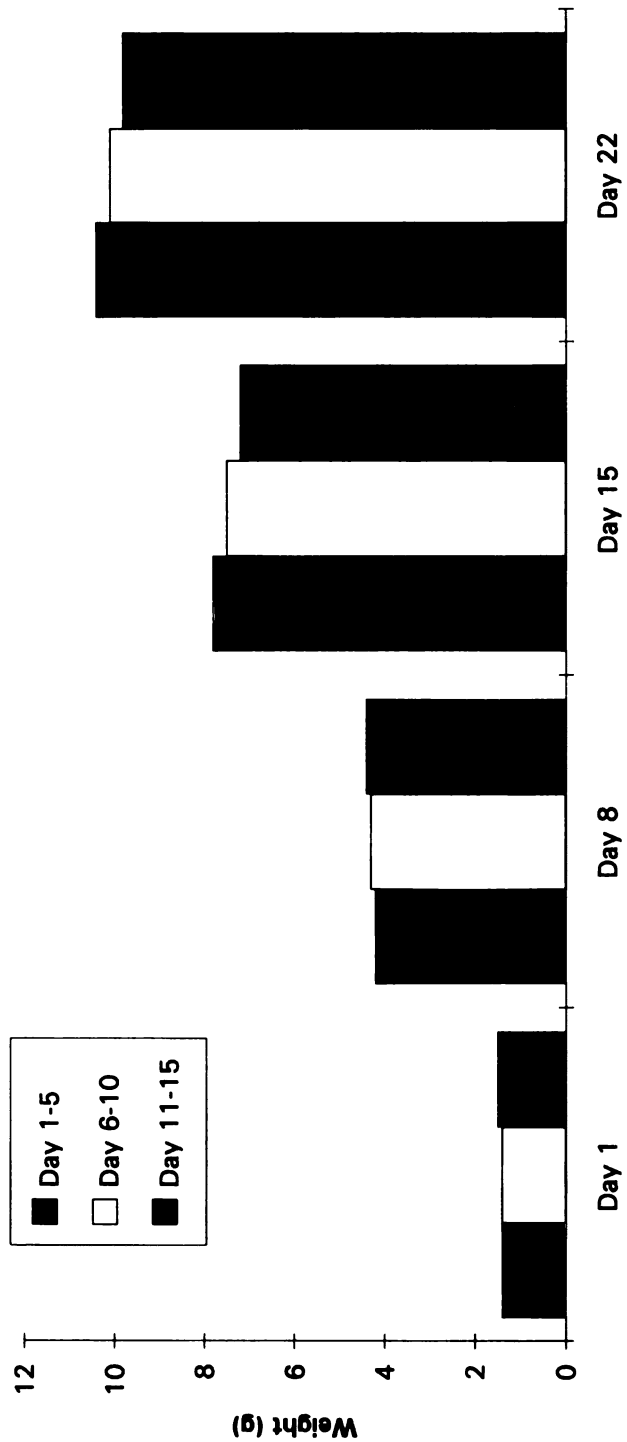


Figure 7. Mean pup weight of B6D2F1 mice for days 1 to 22 after dam was exposed to 7 mg/kg TCB by gavage.

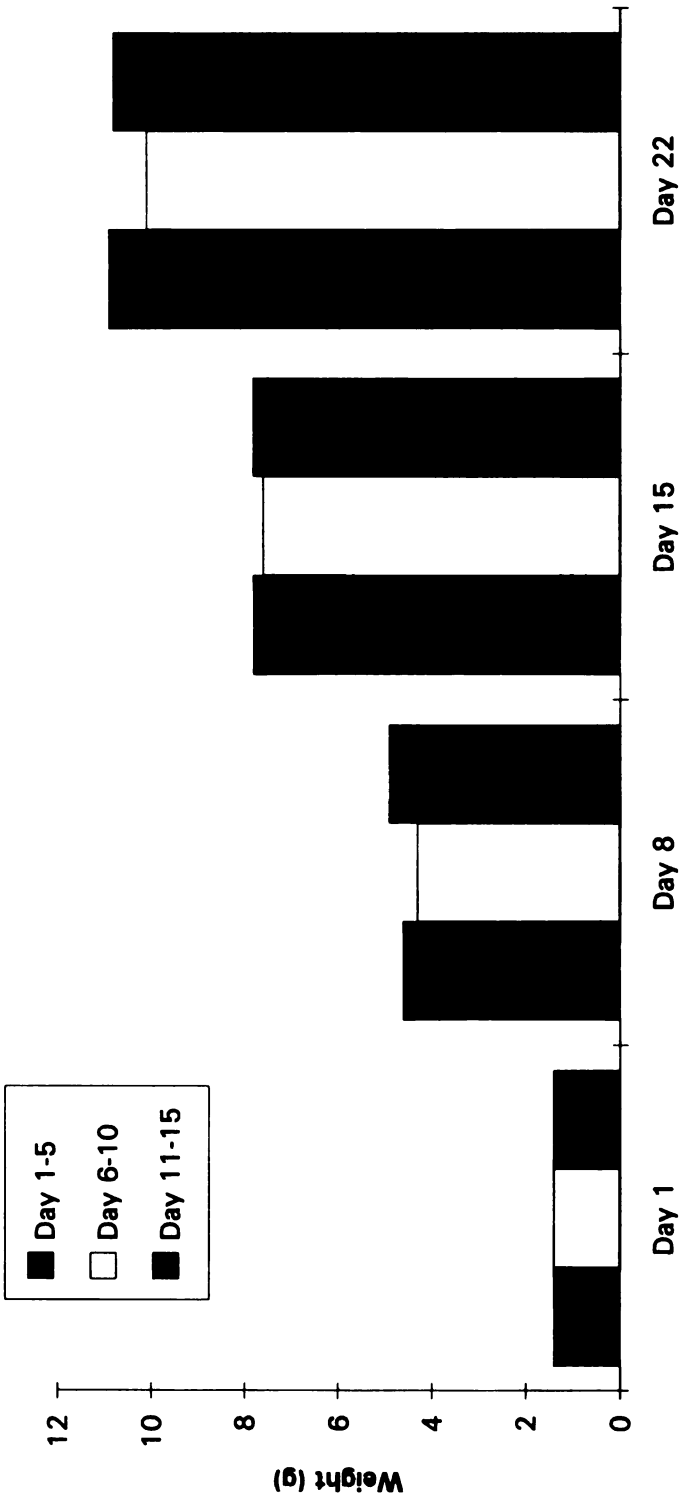


Figure 8. Mean pup weight of B6D2F1 mice for days 1 to 22 after dam was exposed to 14 mg/kg TCB by gavage.

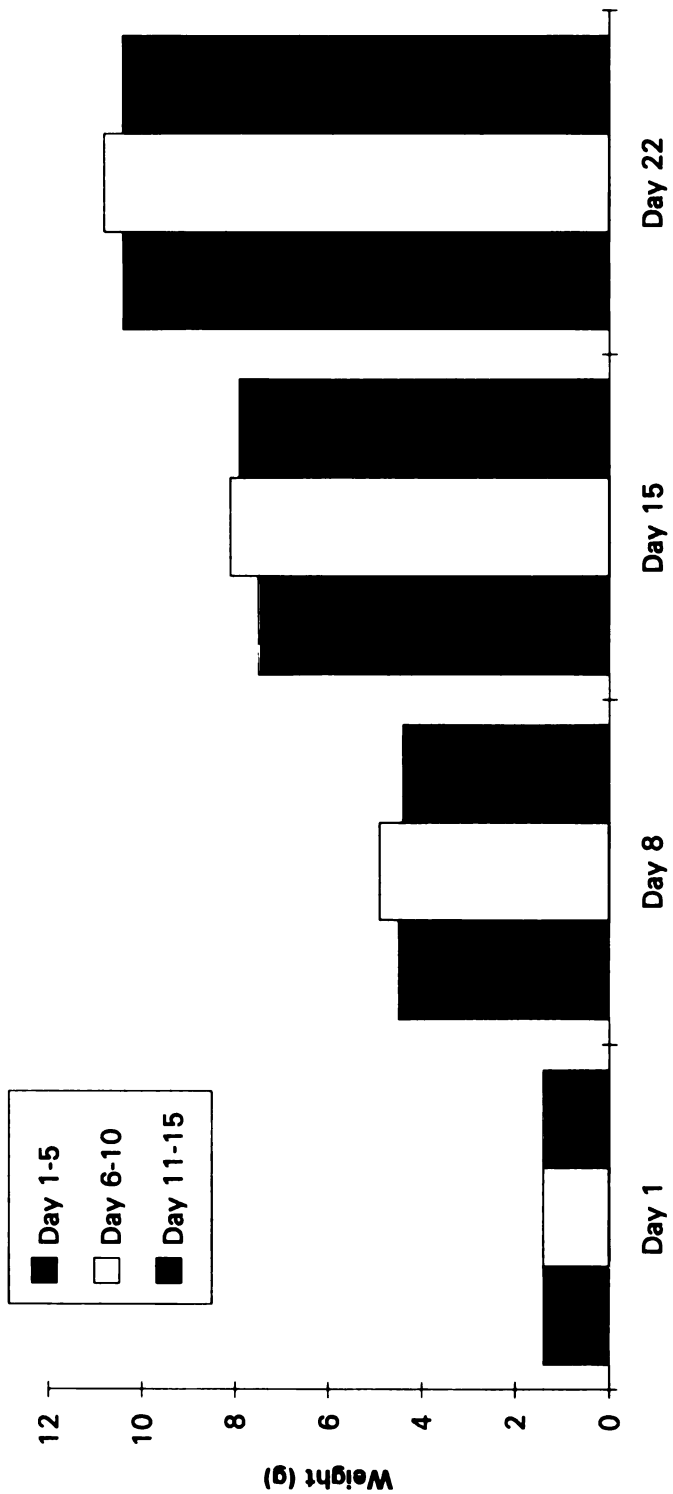


Figure 9. Mean pup weight of B6D2F1 mice for days 1 to 22 after dam was exposed to 21 mg/kg TCB by gavage.

Table 6. Crown-rump length of B6D2F1 mice for days 1 to 22 after dam was exposed to TCB by oral gavage.*

Dam treated Days 1-5				
Postpartum	Day 1	Day 8	Day 15	Day 22
Control	28.6±0.2	38.0±0.8	48.6±0.5	54.8±0.3
7 mg/kg	27.6±0.1	38.8±0.2	48.1±0.3	54.0±0.6
14 mg/kg	27.5±0.1	37.3±0.3	48.9±0.3	54.8±0.5
21 mg/kg	28.8±0.8	37.0±0.3	48.4±0.5	54.9±0.6
Dam treated Days 6-10				
Postpartum	Day 1	Day 8	Day 15	Day 22
Control	27.1±0.3	39.0±0.4	48.5±0.3	56.9±0.6
7 mg/kg	27.2±0.3	38.6±0.2	48.4±0.3	56.2±0.1
14 mg/kg	27.6±0.3	38.8±0.1	48.0±0.3	56.5±0.4
21mg/kg	27.7±0.3	38.9±0.5	48.6±0.9	56.9±0.3
Dam treated Days 11-15				
Postpartum	Day 1	Day 8	Day 15	Day 22
Control	28.4±0.4	39.3±0.6	44.2±0.9	56.3±0.4
7 mg/kg	28.3±0.4	39.3±0.5	44.4±0.6	56.3±0.6
14mg/kg	27.4±0.2	40.3±0.5	45.7±0.7	56.4±0.8
21 mg/kg	27.4±0.2	40.2±0.4	45.1±0.3	57.2±0.8

*Mean ± SEM in grams.

Table 7. Effect of TCB on In Vitro Fertilization in B6D2F1 mice.

Group	Total Ova	%Fertilized (mean ±sem)
Control	111	83.8±0.011
0.01 μg/ml	116	77.6±0.012
0.1 μg/ml	132	72.7±0.008
1.0 μg/ml	109	68.8±0.005[a]
10.0 μg/ml	173	53.7±0.004[a,b]

ANOVA on % fertilized in trial for each group revealed a significant effect of TCB, $p<0.001$.
[a]Significantly different from control, $p<0.01$.
[b]Significantly different from 1 μg/ml, $p<0.01$.

Table 8. Effect of TCB on oocyte degeneration and abnormal embryos.

Group	Total Ova	No. degenerative ova (%)	Total 2-cell embryos	Abnormal 2-cell embryos (%)
Control	111	3 (2.7)	86	2 (2.3)
0.01 μg/ml	116	4 (3.4)	84	3 (3.6)
0.1 μg/ml	132	6 (4.5)	86	4 (4.6)
1.0 μg/ml	109	9 (8.2)	61	6 (9.8)[a]
10.0 μg/ml	173	19 (11)[a]	69	14 (20.3)[a]

Overall chi square significant, $p<0.001$.
[a]Individual chi square significantly different from control, $p<0.05$.

DISCUSSION

3,3',4,4'-Tetrachlorobiphenyl was tested at double, triple and the lowest lethal published dose for mice (Lucier et al.,1978). The results of this study demonstrate that female C57BL/6J mice administered a single dose of TCB for five consecutive days at 7, 14, and 21 mg/kg of body weight by gavage will not experience a significantly different gain in weight for any of the periods of gestation tested. The results of this study did not demonstrate a dose, period or dose/time interaction effect. That is, the doses tested did not affect any of the parameters measured. The time of gestation that the doses were administered was also not a significant factor.

Marks et al. (1989) reported that CD-1 mice exposed to TCB by gavage at doses of 16, 32 and 64 mg/kg of body weight will experience decreased weight gain between days 6 to 10 as the dose increases, but that mice exposed to doses below 16 mg/kg between days 6 to 17 of gestation will not experience a significantly different pattern of weight gain. The data of the present study, are consistent with this study, not having been significantly lower than the control at 7 and 14 mg/kg, however, the present study did not demonstrate a significant difference in weight gain at the 21 mg/kg dose level. Thus, the effect of TCB could possibly be strain dependent. A similar dose dependent pattern of weight gain was reported by d'Argy et al., (1987). On average, C57BL/6 mice administered a single intraperitoneal dose of 6 mg/kg or 16 mg/kg of TCB on day 12 were found to weigh less on

day 18 as the dose increased. Data from both studies, however, were not significant when compared to the controls. This study, however, is different in the route of exposure, having chosen to test TCB by i.p. versus oral gavage.

In the cotton top marmoset monkey, weight loss was significant when exposed to a bi-weekly oral dose of TCB of 0.1 mg/kg, 1.0 mg/kg and 3 mg/kg of body weight (van den Berg et al., 1988). Mc Nulty et al., (1980) described a similar pattern of weight loss when rhesus monkeys were exposed to food containing dissolved TCB at 3 ppm. Thus, the effect of TCB on weight gain could be species dependent.

In rat studies, Chen et al., (1992) found no difference in weight gain when they were exposed to a single intraperitoneal injection of 150 μmol/kg of body weight of TCB. Previously however, Leece et al., (1985) had reported an ED_{50} of 3.3 μmol/kg and an ED_{25} of 2.0 μmol/kg body weight to induce weight loss when exposed to TCB by a single intraperitoneal injection. The data in this study support the hypothesis that exposure to TCB will cause a decrease in the rate of weight gain.

The data in the present study, however, differ from reports in relation to liver and kidney weights. No significant difference in weight for either organ was found between the control and treatment groups in the present study. Mean liver weights were significantly different when marmosets were exposed to 3 mg/kg body weight of TCB, but the authors also found no significant difference in mean kidney weight in the same species (van de Berg et al., 1988). Buchmann et al., (1991) found a significant difference in mean liver weight of TCB treated rats compared to the controls at dose levels of 150 μmol/kg and 15 μmol/kg of body weight after a nine week period.

Results from this study also indicate that litter size and gestation length were not affected by exposure to TCB. In rats however, gestational length increased when treated with 3 mg/kg body weight of TCB. Of the 98% of rats that gave birth between days 20 and 22 of gestation, 80 % gave birth on day 21, the other 18% were divided equally between days 20 and 22. For treated rats, 96% gave birth on days 21, 22 or 23. Of these, 46% gave birth on day 22 or 23 (White et al., 1983).

The results of the present study agree with what was reported by Ronnback (1991) who found no significant difference in litter size after exposing C57/Bl dams to a single intraperitoneal injection of TCB on the 13th day of gestation for any of the doses administered ranging from 1.5 mg/kg to 15.0 mg/kg body weight.

The results of the present study also demonstrate that TCB adversely affects in vitro fertilization in the mouse. Treatment reduced the IVF rate significantly at the 0.1, 1.0 and 10.0 μg/ml dose levels. Furthermore it increased the number of degenerative ova and increased the number of abnormal 2-cell embryos.

While some studies exist on the reproductive toxicity of TCB on mammalian and non-mammalian species, no other information on the in vitro fertilization toxicity of TCB was located in the literature. Therefore, an evaluation of TCB's effects on the oocyte, spermatozoa, fertilization and preimplantion needed to be conducted. Many environmental toxins have been detected in human follicular fluid and because of TCBs lipophilic nature, it could have serious effects on the fertilization ability of the oocytes (Trapp et al., 1984).

But did the TCB actually reach the fetus? This question was thought to be answered through the examination of gross abnormalities and certain growth parameters. Though liver, kidney, ovary and testis samples were preserved, they were not assayed for levels of TCB. By conducting an assay on these tissue samples, one could come to know if TCB actually reached the fetus. A chromosomal analysis on some of the pups could also help determine the toxicity of TCB in mice. Although no anomalies were noted, TCB could have acted mutagenicaly. Hormone levels were also not assayed during the treatment period. By conducting assay of hormone levels during pregnancy, one could possibly start shedding some light on the mechanism of action.

Other possibilities of testing TCB's reproductive toxicity are to administer the toxicant before mating or after delivery. By administering TCB before mating, information on it's effects on sexual receptivity and the estrous cycle could be obtained. Because TCB has been detected in the mammary gland and milk, administering the dose after delivery could ensure that the pups are exposed to TCB. Thus, results from this study are in no way comprehensive in any manner. Further studies that examine the mechanisms behind TCB's toxicity are warranted.

Summary and Conclusions

Polychlorinated biphenyls (PCBs) are persistent environmental contaminants. Tests on animals have revealed these aromatic hydrocarbon compounds to be teratogenic, carcinogenic, and mutagenic. The reproductive effects of PCBs vary depending on the route of administration, amount of administration, time of administration and most importantly, the PCB administered.

Oral administration of 7, 14 and 21 mg/kg b.wt. of TCB to C57BL/6J mice for five consecutive through day 15 of pregnancy resulted in no observable reproductive effects. However, TCB did affect the in vitro fertilization at concentrations of 0.1 μg/ml, 1.0 μg/ml, and 10.0 μg/ml.

The following conclusions are drawn:

1. Oral administration of TCB to pregnant C57BL/6J mice had no effect on maternal liver or kidney weight. TCB did not affect litter size or sex ratio.
2. Maternal weight gain was not affected by TCB during any period of gestation tested.
3. Pup weight and crown-rump length were not affected by TCB. Incisor eruption and eye opening were also not affected.
4. After 43 days of development, pup gonadal weight was not affected by TCB.
5. TCB affected B6D2F1 mouse in vitro fertilization. TCB increased the number of degenerative ova and increased the number of abnormal 2-cell embryos.

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Appendix

APPENDIX A

CURRICULUM VITA

Name: Jaime Rodriguez

Citizenship: USA

Education:

University of Texas at Arlington 1985-1989
Arlington, Texas 76019
Major: Psychology
Honors: Honor Roll

University of Texas - Pan American 1989-1992
Edinburg, Texas 78539
Major: Psychology
Minor: Biology
Degree: Bachelor of Science 1992
Honors: Psi Chi (National Honor Society in Psychology)
National Dean's List

Michigan State University 1992-Present
East Lansing, Michigan 48824
Major: Zoology
Honors: Minority Competitive Doctoral Fellowship, 1992-1996

Professional Positions:

Research Intern, Summer 1991 (Full-time)
Department of Psychology
Michigan State University
East Lansing, Michigan 48824
Assisted in a study conducted on the neuromechanics of ferret reproduction, to include behavioral testing, record keeping, histology, daily care of the ferrets, a computerized atlas of the ferret brain and the application of different protocols.
Supervisor: Cheryl Sisk, Ph.D.

Appendix A (cont'd)

Teaching Assistant, 1991-1992 (Half-time)
Department of Psychology and Anthropology
University of Texas - Pan American
Edinburg, Texas 78539
Duties as assigned; grade and keep record of all student's statistics homework and offer tutorial service to those needing help.
Supervisor: Valerie James-Aldridge, Ph.D.

Graduate Assistant, 1992-Present (Half-time)
Department of Zoology
Michigan State University
East Lansing, Michigan 48824
Duties: Research Assistantship, Summer 1993
B.S. 111-Cell and Molecules T.A., Fall 1993
ZOL 234-Comparative Anatomy T.A., Spring 1994
ISB 204-Applications of Environmental and Organismal Biology T.A., Fall 1994

Memberships in Professional Associations:

Society for the Study of Reproduction, Trainee Membership
Midwest Teratological Society, Member
Sigma Xi, Associate Membership

Professional Activities:

Administrative

Actively recruited minority students to apply for summer research programs at Michigan State University.

Papers Presented:

1993 26th Annual Meeting of the Society for the Study of Reproduction
Abstract: The effects of perchlorinated terphenyls (PCT) and bromobiphenyls (BP) on *in vitro* fertilization in the mouse.

1993 First Annual Research Day Department of Zoology
Abstract: *In vitro* and *in vivo* reproductive effects of 3,3', 4,4'-tertrachlorobiphenyl (4-CB) in the mouse.

Appendix A (cont'd)

1992 National Conference on Undergraduate Research
Abstract: Behavioral and neuroendocrine effects of testosterone implants in the preoptic area and medial basal hypothalamus.

1991 Committee on Institutional Cooperation Conference
Presentation: Behavioral and neuroendocrine effects of testosterone implants in the preoptic area and medial basal hypothalamus.

Papers Published

1. Rodriguez, J. Behavioral and neuroendocrine effects of testosterone implants in the preoptic area and medial basal hypothalamus. Conference Proceedings of the 6th Annual National Conference on Undergraduate Research (1992). (Abstract)

2. Rodriguez, J. *In Vivo* and *in vitro* reproductive effects of 3,3'4,4'-tetrachlorobiphenyl (4-CB) in the mouse. 1st Annual Research Day Department of Zoology Program (1993). (Abstract)

3. Rodriguez, J., Kholkute, S.D., and Dukelow, W.R. The effects of perchlorinated terphenyls (PCT) and bromobiphenyls (BP) on *in vitro* fertilization in the mouse. Conference Proceedings of the 26th Annual Meeting of the Society for the Study of Reproduction (1993). (Abstract)

4. Kholkute, S.D., Rodriguez, J., and Dukelow, W.R. The effects of polychlorinated biphenyls (PCB) on *in vitro* fertilization in the mouse. Conference Proceedings of the 26th Annual Meeting of the Society for the Study of Reproduction (1993). (Abstract)

5. Kholkute, S.D., Rodriguez, J., and Dukelow, W.R. (1993) Effects of a pesticide mixture and two herbicide mixtures on *in vitro* fertilization in the mouse. *In Vitro* Toxicology. **6**: 291-298.

6. Kholkute, S.D., Rodriguez, J., and Dukelow, W.R. (1994) The effects of polybrominated biphenyls and perchlorinated biphenyls on *in vitro* fertilization in the mouse. Archives of Environmental Contamination and Toxicology. **26**: 280-211.

Appendix A (cont'd)

7. Kholkute S.D., Rodriguez, J., and Dukelow, W.R. (1994) Effects of polychlorinated biphenyls (PCBs) on *in vitro* fertilization in the mouse. Reproductive Toxicology. **8**: 69-73.

8. Kholkute, S.D., Rodriguez J., Rawlins, R., and Dukelow, W.R. (1994) Glass wool column filtration: Effects on motility, viability, and fertilization ability of the mouse epididymal spermatozoa. Laboratory Animal Science. **44**: 537-539.

9. Kholkute, S.D., Rodriguez, J., and Dukelow W.R. Reproductive Toxicity of Aroclor-1254: Effects on oocyte, spermatozoa, *in vitro* fertlization and embryo development in the mouse. (Reproductive Toxicology, 1994). (In Press)

10. Kholkute, S.D., Rodriguez, J., and Dukelow, W.R. *In vitro* fertilization and acrosome reaction of the mouse epididymal sperm following exposure to progesterone and 17 α-hydroxyprogestorone. (submitted International Journal of Andrology, 1994).