ABSTRACT

KRISTA L. JOHNSON. Assessing Structure Activity Relationships of Polyhalogenated Aromatic Hydrocarbons With Endometriosis as an Endpoint. (Under The Direction of Dr. LINDA S. BIRNBAUM)

Previous studies have shown that exposure to the environmental contaminant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) enhances the development of endometriotic lesions. In this study, we assessed the structure activity relationships (SARs) and mechanism(s) of endometriotic proliferation by 2,3,7,8-TCDD and related polyhalogenated aromatic hydrocarbons with ligands with varying degrees of affinity for the Ah receptor.

B6C3F1 female mice were treated with 0, 1, 3, or 10 ug 2,3,7,8-tetrachlorodibenzo-*p*-dioxin/kg of body weight (bw); 3 or 30 mg 2, 4, 5, 2', 4', 5'-hexachlorobiphenyl (PCB 153)/kg bw; 100, 300, or 1000 ug 3, 4, 5, 3', 4'-pentachlorobiphenyl (PCB 126)/kg of bw; 10, 30, or 100 ug 2, 3, 4, 7, 8-pentachlorodibenzofuran (4-PeCDF)/kg of bw; or 2 or 20 mg 1, 3, 6, 8-tetrachlorodibenzo-*p*-dioxin (1,3,6,8-TCDD)/kg of bw at 10 ml/kg. The animals were dosed by oral gavage a total of five times with three weeks between each dosing and terminated three weeks after the last dose. At the conclusion of sixteen weeks, endometriotic lesion diameters and weights were measured. In addition, ovaries, uterine horn, and thymus were removed from each

animal. Lesions, uterine horns, and ovaries were fixed for histopathology. Livers were also excised for enzymatic analysis.

Analysis of lesion diameters with the Dunnett's test revealed statistically significant results for animals treated with 1 or 3 ug 2,3,7,8-TCDD/kg bw or 100 ug 4-PeCDF/kg bw. However, animals treated with 10 ug 2,3,7,8-TCDD per kg bw did not have significantly larger lesion diameters than control animals possibly due to ovarian atrophy. Animals treated with PCB 126 showed a trend of lesions with larger mean diameters than control animals, but due to variability the increases were not statistically significant. No effect on lesion diameter was apparent in animals dosed with PCB 153 or 1,3,6,8-TCDD.

Statistically significant increases in lesion diameters of animals dosed with 2,3,7,8-TCDD supports previous work stating exposure to 2,3,7,8-TCDD induces proliferation of endometriotic lesions. Because neither of the "non-dioxin-like" chemicals induced increased proliferation and both "dioxin-like" chemicals caused increased lesion diameters, the data are consistent with the hypothesis that the mechanism of increased proliferation of endometriotic lesions is Ah receptor-mediated.

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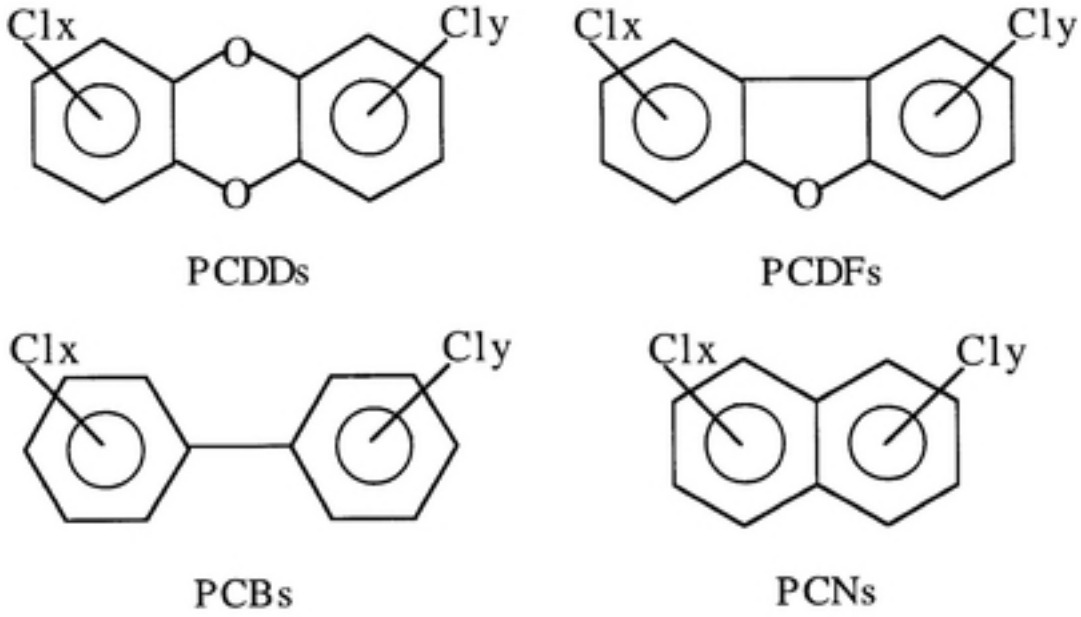
CHAPTER I

INTRODUCTION

1.1 Halogenated Aromatic Hydrocarbons

Halogenated aromatic hydrocarbons (HAHs) are a group of persistent chemical compounds which have been identified in nearly every component of the global ecosystem (Safe, 1990). HAHs include polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated diphenylethers (PCDEs), polychlorinated biphenyls (PCBs), and polychlorinated naphthalenes (PCNs) (Safe, 1990). The basic chemical structures of these groups of HAHs are shown in Figure 1.1. Several chemical and physical properties are common among members of this family of chemicals. For example, most halogenated aromatic hydrocarbons are lipophilic with the level of lipophilicity increasing with the degree of ring chlorination (Safe, 1990). Also, the stability of many members of this family is due to resistance to chemical degradation by acids, bases, heat, oxidation, reduction, and hydrolysis (Safe and Hutzinger, 1990). 2,3,7,8-Tetrachlorodibenzo-p-dioxin has been identified as the most potent HAH and is often used as a marker of comparison to assess the toxicity of other halogenated aromatics (Safe and Hutzinger, 1990).

Figure 1.1 **Structures of Prototypical HAHs**



1.1.1 Congeners

Each group of chemicals within the class of HAHs has many possible congeners. The number and position of halogenation determines whether the congener is dioxin-like or non-dioxin-like in behavior. The most potent chemicals, such as 2,3,7,8-TCDD, are halogenated in all four lateral positions. Decreased potency correlates with either the loss of lateral chlorines or the addition of non-lateral chlorines. Only 7 of the possible 75 PCDD congeners, 10 of the 135 possible PCDF congeners, and 11 of the 209 possible PCB congeners possess significant dioxin-like toxicity (Safe, 1986).

1.1.2 Sources

PCBs were first produced as industrial products, prepared by the chlorination of biphenyls (Safe, 1991). Products of this chemical process were used as industrial fluids, dielectric fluids, heat transfer fluids, organic diluents, flame retardants, plasticizers, sealants, and surface coatings (Safe, 1991). Soon after PCBs were identified as environmental contaminants in the late 1960's, the open use and production of these chemicals was banned in the United States (Safe, 1991). Therefore, the volume of PCBs in the environment today is a function of the production of these chemical almost 20 years ago, as well as international production which continued until 1990.

Unlike PCBs, PCDDs and PCDFs serve no commercial use. The source of these chemicals through the 1970's was primarily as by-products in the synthesis and use of chlorinated phenols, chlorinated aromatic hydrocarbons, and associated derivatives. However, the current primary source of production of these chemicals is

combustion (Dickson and Buzik, 1993). Certain conditions enhance the production of PCDDs and PCDFs as contaminants, such as alkaline conditions, high temperatures, ultraviolet irradiation, and other radical initiators (Safe and Hutzinger, 1990).

In addition to the formation of PCDDs and PCDFs during synthesis of chlorinated phenols, these chemicals have been identified as products from paper and pulp mills (Safe, 1991). Production of PCDDs and PCDFs from pulp and paper mills results from the process of chlorine bleaching of naturally-occurring phenolic compounds, such as lignin, which can result in the production of PCDD and PCDF precursors, like chlorophenols and chlorinated phenoxyphenols. Resultant PCDD and PCDF concentrations have been detected in both paper products and municipal waste (Safe and Hutzinger, 1990). PCDDs and PCDFs can also arise from thermal sources, including natural forest fires and volcanic eruptions (Crummett and Townsend, 1984). Other thermal processes such as wood/fossil fuel combustion and waste incineration can also result in the formation of PCDDs and PCDFs (Bumb *et al.*, 1980). Even though production of these chemicals currently occurs during combustion and incineration processes, the concentration of PCDDs and PCDFs in the environment derives primarily from industrial sources (Czuczwa and Hites, 1984; Czuczwa and Hites, 1986).

1.1.3 Environmental Fate

Because PCBs, PCDDs, and PCDFs are resistant to breakdown by acids, bases, heat, hydrolysis, and biological degradation, the stability

of these chemicals enables their persistence in the environment for long periods of time (Safe and Hutzinger, 1990). These compounds have been detected throughout the world in a wide array of environmental media, including air, air particulates, sediment, soil, and water (Vanden Heuvel and Lucier, 1993). Concentrations of these chemicals are higher in ambient air near industrial and urban sites (Safe, 1991). Accumulation of these compounds also has been detected on the outer waxy surfaces of vegetation, and in marine and aquatic sediments (Safe, 1991). A major health concern due to the stability and lipophilicity of HAHs is the detection of PCBs, PCDDs, and PCDFs in all levels of the food chain (Vanden Heuvel and Lucier, 1993).

1.1.4 Human Exposure

Human exposure to HAHs can occur by environmental, accidental, and occupational exposures. The background levels of exposure of humans to HAHs is much less than for individuals who have been exposed accidentally or occupationally. Human exposure routes include inhalation, ingestion, and dermal contact (Rappe and Kjeller, 1987). Although HAHs have been detected in water, air, sediment, and sludge (Paustenbach *et al.*, 1992), the central source of human intake of PCDDs, PCDFs, and PCBs is contaminated food ingestion, primarily dairy products, meat, and fish (Schecter *et al.*, 1994). Entrance of these chemicals into the food chain causes great environmental concern because of the extreme toxicity of certain congeners. The possibility of high exposure to HAHs through consumption of food is a greater threat to populations who consume

large quantities of contaminated foods from areas with HAH levels above background. For example, Native American populations who consume large quantities of fish are at an elevated risk of high dioxin exposure if primary fishing locations are contaminated waters.

Unfortunately, accidental exposures also provide a means of human exposure to toxic HAHs. One example is the accidental release of approximately 165 g of 2,3,7,8-TCDD from the ICMESA 2,4,5-T herbicide processing plant in Seveso, Italy on July 10, 1976, exposing 30,000 individuals (Hay, 1976; Mocarelli *et al.*, 1991). However, analysis from the exposed population showed little correlation between levels of 2,3,7,8-TCDD exposure and cancer incidence between zones A, B, and R because habitat location was often not a consistent indicator of dose due to people crossing zones for work or various other reasons. (Bertazzi *et al.*, 1993). The results from this data are inconclusive because the population size is small and the time of data collection post-exposure was relatively short (10 years).

An accidental exposure also occurred in 1968 in a food supply of rice in Japan. Approximately 1000 people were exposed to rice contaminated with PCDDs and PCDFs from heating coil leaks used to process the rice oil (Urabe *et al.*, 1979). Reported toxic effects of adult exposure included chloracne, headaches, visual impairments, and decreased peripheral nerve-condition movements. Increased mortality rates due to cancer in this population have been reported; however, the small sampling size was insufficient to draw definitive conclusions about the correlation between PCDD and PCDF exposure and cancer (Urabe *et al.*, 1979).

Another incident of accidental exposure occurred in Taiwan in 1978, where 2,000 central Taiwanese residents were exposed to PCBs in contaminated rice. The PCBs, contaminated with PCDFs, were leaked into the food supply by a machine used for clarifying rice (Raloff, 1995). Children born to women who consumed the contaminated rice oil exhibited small size, dermal abnormalities, lower IQ performance, delayed psychomotor skills (Rogan *et al.*, 1988) and a wide range of physical, neurological, and developmental abnormalities (Raloff, 1995).

An accidental exposure occurring in the United States was the use of 200,000 gallons of industrial waste oil containing PCDDs and PCDFs for dust maintenance. In 1971, this oil was sprayed onto roads and horse arenas in Verona, Missouri (Kimbrough *et al.*, 1984). Approximately 60 horses died and many exposed individuals suffered from nausea, headaches, fatigue, and mild chloracne (Patterson *et al.*, 1986). However, follow-up medical examinations reported no consistent abnormal conditions related to dioxin exposure (Webb *et al.*, 1984), possibly due to the small number of exposed individuals or the relatively low level of exposure as measured by serum levels.

Occupational exposure to PCDDs, PCDFs, and PCBs occurs primarily during the manufacturing of chlorinated phenol-derived products. Symptoms of exposure often include chloracne, hepatocellular necrosis, and vomiting (Kimbrough, 1980). An epidemiologic study, involving over 5,000 people employed at plants across the United States which produced chemicals contaminated by 2,3,7,8-TCDD, found no significant correlation between cancer

mortality and exposure to 2,3,7,8-TCDD (Fingerhut *et al.*, 1991). However, mortality from soft-tissue sarcoma was increased in this study, although not significantly. Limitations in this study include small numbers, misclassifications of death certificates, and confounding effects.

Another study examined the correlation between exposure to PCDDs and PCDFs and mortality due to cancer or ischemic heart diseases (Flesch-Janys *et al.*, 1995). Analysis of data from a cohort of 1,189 male workers from a chemical plant in Hamburg, Federal Republic of Germany, which produced herbicides and insecticides contaminated with PCDDs and PCDFs, showed a strong dose-dependent relationship between mortality due to cancer or ischemic heart diseases and PCDD/PCDF exposure.

Other epidemiologic work on occupationally exposed men has demonstrated long-term immunosuppressive effects of 2,3,7,8-TCDD on T-helper cell function (Tonn *et al.*, 1996), and dioxin-related alterations in male reproductive hormone levels (Egeland *et al.*, 1994).

1.2 Toxic and Biochemical Responses

Halogenated aromatic hydrocarbons evoke a broad range of toxic responses at all levels of biological organization: the cell, tissue, organ system, and whole body. Responses include lethality, wasting, immunotoxicity, chloracne, hepatotoxicity, porphyria, carcinogenicity, induction of phase I and phase II drug-metabolizing enzymes,

teratogenicity/developmental toxicity, and reproductive toxicity (Safe, 1990; DeVito and Birnbaum, 1994). Although species differences exist in the toxic responses caused by exposure to HAHs, all animals studied have elicited adverse effects, many of which are consistent across species (DeVito and Birnbaum, 1994).

1.2.1 Lethality

Lethality from HAHs is characteristically delayed, requiring weeks to progress (Pohjanvirta and Tuomisto, 1994). Dose ranges of HAHs evoking lethality vary with species, strain, sex, age, and route of administration. HAHs can evoke lethality in essentially all vertebrates studied, although LD_{50} values vary across species (DeVito and Birnbaum, 1994). LD_{50} values for 2,3,7,8-TCDD vary 5000-fold between the most sensitive species, guinea pigs, (LD_{50} = 0.6-2.0 ug/kg) and the most resistant species, hamster, (LD_{50} = 1157-5000 ug/kg) (Kociba and Schwertz, 1982; Safe, 1990). The mouse experiences moderate sensitivity to 2,3,7,8-TCDD with an LD_{50} value of 114 to 284 ug/kg. In addition to species differences in susceptibility, different strains of the same species may also exhibit variability in sensitivity. For example, an approximate 1000-fold variability in 2,3,7,8-TCDD LD_{50} values exists between the most resistant rat strain (Han/Wistar) and the most susceptible rat strain (Long Evans) (Pohjanvirta and Tuomisto, 1994). Sensitivity to HAHs can also be a function of animal age and sex, with developing animals and *in utero* fetuses exhibiting a greater sensitivity than adult animals, while gender-related susceptibility varies with strain (Pohjanvirta and Tuomisto, 1994).

1.2.2 Wasting Syndrome

Treatment of animals with acute lethal doses of HAHs, particularly 2,3,7,8-TCDD, evokes a toxic response preceding lethality identified as the wasting syndrome (DeVito and Birnbaum, 1994). The wasting syndrome is characterized primarily by loss of body weight or reduced weight gain resulting from a significant reduction in adipose tissue (Peterson *et al.*, 1984) and muscle (Max and Silbergeld, 1987), as observed at autopsy. This toxic effect is characteristic of most species treated with acute lethal doses of 2,3,7,8-TCDD. Without a definitive cause established for the mechanism of wasting syndrome, most evidence suggests an alteration in the "set point" for body weight in the hypothalamus by dioxin, causing hypophagia or decreased food intake (Peterson *et al.*, 1984; DeVito and Birnbaum, 1994). Other suggested mechanisms are: 1) inhibition of glucose transport in adipose tissue, pancreas, and brain resulting in elevated blood glucose levels (Enan *et al.*, 1992), and 2) decreased phosphoenolpyruvate carboxykinase (PEPCK) levels in the liver which block glucose synthesis and decrease feeding sensations (Stahl *et al.*, 1993).

1.2.3 Immunotoxicity and Thymic Involution

The immune system plays a key role in body homeostasis. Alterations in immune functioning can increase sensitivity, incidence, and severity of infectious diseases; increase cancer incidence; and enhance development of autoimmune diseases (DeVito and Birnbaum, 1994). HAHs, especially 2,3,7,8-TCDD, evoke toxic effects

on the immune system. 2,3,7,8-TCDD can cause lymphoid tissue loss in thymus, spleen, and lymph nodes (Luster *et al.*, 1987), and can suppress both humoral immunity and cell-mediated immunity (Lundberg *et al.*, 1992). Thymus size may severely decrease following exposure to 2,3,7,8-TCDD and is characterized by a depletion of small immature cortical thymocytes (Gupta *et al.*, 1973; Pohjanvirta and Tuomisto, 1994). Possible mechanisms of thymic atrophy include: inability of thymus cells to support the maturation of T-lymphocyte precursors (Greenlee *et al.*, 1985; Pohjanvirta and Tuomisto, 1994), enhanced apoptosis (McConkey *et al.*, 1988; Pohjanvirta and Tuomisto, 1994), blocked or delayed thymocyte maturation (Holladay *et al.*, 1991; Pohjanvirta and Tuomisto, 1994), impaired thymic seeding by prothymocytes (Fine *et al.*, 1990; Pohjanvirta and Tuomisto, 1994), and the stimulation of protein-tyrosine kinase activities (Bombick *et al.*, 1988; Pohjanvirta and Tuomisto, 1994).

1.2.4 Chloracne and Related Dermal Lesions

Chloracne and dermal lesions are primary indicators of both dermal and systemic HAH exposure observed in humans, monkeys, rabbits, and hairless mice (McConnell and Moore, 1979). The lesions involve hyperplasia, hyperkeratosis, and pigmentation variations (Kimbrough, 1984). The occurrence of chloracne lesions is a function of high dose HAH exposure and is often accompanied by thymic atrophy and wasting in species other than humans (DeVito and Birnbaum, 1994). Although the definitive mechanism of chloracne formation is unknown, one suggestion is decreased concentration of

acidic type I keratin, resulting in alterations in epidermal development, keratinocyte hyperproliferation and skin irritations (Molloy and Laskin, 1992).

1.2.5 Hepatotoxicity and Porphyria

Hepatotoxic effects produced by HAHs are species dependent. For example, rats and rabbits exhibit extensive hepatotoxicity following exposure to HAHs, while guinea pigs and hamsters exhibit few overt hepatotoxic effects (DeVito and Birnbaum, 1994). The primary characteristics of hepatotoxicity are hepatomegaly (enlarged liver), hepatocellular hypertrophy, multinucleate hepatocytes, steatosis, and inflammatory cell infiltration (Greig *et al.*, 1973; Pohjanvirta and Tuomisto, 1994). Hepatotoxic alterations may also include proliferation of smooth endoplasmic reticulum and microsomal enzyme induction (DeVito and Birnbaum, 1994).

Another characteristic of HAH exposure is porphyria, a condition occurring from disrupted heme synthesis and increased levels of 7- and 8-carboxy porphyrins in several tissues, including the liver (Vos *et al.*, 1974). Porphyria caused by 2,3,7,8-TCDD is analogous to the human condition Porphyria Cutanea Tarda (PCT). Characteristics of this disease include blistering and fragility of the skin, photosensitivity, pigment changes, and hirsutism (Casarett and Doull, 1991). One possible mechanism for the development of porphyria may involve the induction of cytochrome P-450 enzymes and delta-aminolevulinic acid, and the inhibition of uroporphyrinogen decarboxylase (Goldstein and Safe, 1989).

1.2.6 Carcinogenicity

As with most environmental chemicals, cancer resulting from human exposure to HAHs is a primary health concern. 2,3,7,8-TCDD was shown to be a potent carcinogen in four species of animals: mice and rats (NTP, 1982), hamsters (Rao *et al.*, 1988), and fish (Johnson *et al.*, 1992). Although 2,3,7,8-TCDD is a complete carcinogen as defined for the two-year bioassay, 2,3,7,8-TCDD is not considered a direct genotoxicant, but is considered to be a nongenotoxic carcinogen acting as a promoter (DeVito and Birnbaum, 1994).

1.2.7 Enzyme Induction

HAHs have been identified as microsomal monooxygenase inducers (Whitlock, 1990). The mixed-function oxidase (MFO) system, specifically cytochrome P450 isozymes, is frequently induced by 2,3,7,8-TCDD (Poland and Glover, 1974). The mixed-function oxidases are composed of hemoproteins located in the microsomal endoplasmic reticulum of many tissues, especially liver. The primary function of these enzymes is metabolism of endogenous compounds and detoxification or activation of exogenous chemicals, through oxidation and reduction reactions. The cytochrome P450 system has a broad range of specificity and is highly inducible (Casarett and Doull, 1991). Induction of the MFO system is an extremely sensitive marker of 2,3,7,8-TCDD exposure (Kitchin and Woods, 1979).

CYP450 enzyme induction is frequently measured by aryl hydrocarbon hydroxylase (AHH) activity or ethoxyresorufin *O*-deethylase (EROD) activity, which coincide with increased CYP1A1 gene expression (Goldstein *et al.*, 1984). 2,3,7,8-TCDD is the most

potent MFO inducer. However, HAHs also affect a broad range of other enzyme systems involved in conjugation, biotransformation, and detoxication of chemical compounds (Pohjanvirta and Tuomisto, 1994).

1.2.8 Teratogenicity and Developmental Toxicity

Many species exhibit teratogenic effects after exposure to HAHs. However, mice display the greatest sensitivity (Couture *et al.*, 1990). Low dose levels, which do not produce maternal or fetal toxicity, can induce teratogenic effects in mice, such as cleft palate and hydronephrosis (DeVito and Birnbaum, 1994). Because the mechanisms of action for dioxin are comparable to the mechanisms of action of steroid hormones, the developing fetus may be at greater risk for toxic effects than the adult mother (DeVito and Birnbaum, 1994). The mechanism of dioxin-induced teratogenicity may involve alterations in growth factor activities (Abbott *et al.*, 1987).

1.2.9 Reproductive Toxicity

Dioxin exposure can cause reduced fertility, decreased litter size, diminished uterine weights, and altered ovarian functioning in several species, including mice, rats, and primates (Kociba *et al.*, 1976; Barsotti *et al.*, 1979; Umbreit *et al.*, 1987). A common response elicited by dioxin in many species is the inhibition of estrogen actions. These antiestrogenic effects may be mediated through different pathways. For example, dioxins may result in either a decrease in the concentration of circulating estrogens or a decrease in the levels of estrogen receptors (DeVito and Birnbaum, 1994). In

immature rodents, antiestrogenic effects can often be seen at low doses which do not evoke lethality or wasting (Umbreit *et al.*, 1987).

Other toxic reproductive effects include delayed vaginal opening, cleft phallus/clitoris, and abnormal development of the urethra (hypospadia) in female rats exposed perinatally (Gray *et al.*, 1995). Alterations in male reproductivity following postnatal exposure to HAHs include changes in testicular morphology, losses of germ cells, the presence of deteriorating spermatocytes and mature spermatozoa in the seminiferous tubule lumen, a reduced abundance of tubules with mature spermatozoa, and decreased sperm counts (McConnell and Moore, 1979). These toxic responses are produced at high dose levels which may also induce wasting (DeVito and Birnbaum, 1994).

Finally, recent studies show an effect on the female reproductive disorder known as endometriosis in several species following exposure to HAHs. 2,3,7,8-TCDD induced increased proliferation of endometriotic sites in rhesus monkeys (Rier, *et al.*, 1993), rats (Cummings *et al.*, 1996) and mice (Cummings *et al.*, 1996), following subchronic exposure to 2,3,7,8-TCDD. However, most studies on endometriosis performed using exposure to HAHs focus entirely on the effects of 2,3,7,8-TCDD. Due to exposure of human females to a broad range of environmental contaminants, further studies need to focus on the effects of additional HAHs on the proliferation of endometriosis.

1.3 Aryl Hydrocarbon Receptor

Most of the toxic and biochemical effects of HAHs are mediated through the aryl hydrocarbon receptor (AhR) (Okey *et al.*, 1994). The Ah receptor is an intracellular protein, similar to steroid hormone receptors, which binds with ligands, such as HAHs, to induce gene transcription (Birnbaum, 1994; DeVito and Birnbaum, 1994).

1.3.1 Structure Activity Relationships

The concept of receptor-mediated activity for HAHs was first proposed following early studies on inbred strains of mice. This work showed exposure to 3-methylcholanthrene (MC) induced hepatic microsomal aryl hydrocarbon hydroxylase (AHH) in responsive mice strains (C57BL/6J) and did not induce AHH in nonresponsive mice strains (DBA/2J) (Thomas *et al.*, 1972; Safe, 1988). However, inbred mouse strains, both responsive and nonresponsive strains, exposed to 2,3,7,8-TCDD showed induction of AHH, although 10-fold higher doses of 2,3,7,8-TCDD were required to induce the same effects in nonresponsive strains as in responsive strains (Poland and Glover, 1975). Following these studies, the hypothesis was made that induced AHH activity involved binding of a ligand, such as 2,3,7,8-TCDD, to a receptor protein and that genetic differences in responsiveness to ligands were a function of decreased ligand affinity for this receptor (Poland and Glover, 1975; Safe, 1988). The receptor is now recognized as the aryl hydrocarbon (Ah) receptor and is encoded by the Ah gene locus (Thomas *et al.*, 1972).

The Ah receptor protein is believed to have developed about 450 million years ago (Hankninson, 1995), possibly for detoxification of natural products of combustion, such as benzo[*a*]pyrene (Landers and Bunce, 1991). Thus, the binding of 2,3,7,8-TCDD to the Ah receptor may be coincidental. However, all known effects of 2,3,7,8-TCDD exposure are probably mediated through the Ah receptor (Landers and Bunce, 1991).

Structure-activity relationships (SARs) describe Ah receptor-mediated inducible enzyme activity based on ligand structural configurations. Enzyme induction is highly influenced by halogen-substituted patterns, specifically by chlorines. The most active chemicals are chlorinated in all four lateral positions, such as 2,3,7,8-TCDD. Decreases in enzyme induction and induced toxic effects correlate with both the loss of lateral chlorines and the addition of non-lateral chlorines (Safe, 1986). SARs are also applicable to other classes of HAHs. PCBs, such as 3, 3', 4, 4', 5-penta, which have substituted chlorines in both *para* positions and at least two *meta* positions, resemble 2,3,7,8-TCDD in configuration and induce the greatest degree of enzyme induction of the PCBs (Bandiera *et al.*, 1982).

1.3.2 Structural Binding Relationships

The degree of HAH potency correlates with ligand affinity for the Ah receptor. Usually, Ah receptor binding is stereoselective and saturable. Competitive binding of the Ah receptor occurs among chemicals with various spatial configurations. PCDDs and PCDFs with chlorine substitutions in the 2, 3, 7, and 8 positions exhibit the

greatest binding affinities for the Ah receptor. The addition of nonlateral chlorines decreases binding affinities of these chemicals proportionally. PCB congeners substituted at both *para* positions and at least two *meta* positions, referred to as "coplanar PCBs," are the most competitive ligands for Ah receptor binding of the PCB family. In contrast, "monoortho coplanar PCBs" exhibit decreased competitive binding affinities (Safe, 1988)].

As the most toxic member of the HAHs, 2,3,7,8-TCDD can be used as a measure of comparison for levels of toxicity of other HAHs with similar structural conformations, mechanisms of action, and spectra of responses. Toxic equivalency factors (TEFs) are assigned to other HAHs with these characteristics as a means of estimating their degree of toxicity as a fraction of 2,3,7,8-TCDD toxicity. Toxic equivalency values for mixtures of HAHs (TEQs), are also assigned based on a sum of potencies of all mixture constituents. Induction of toxic responses and enzyme activities correlate with TEF and TEQ values (Birnbaum, 1994; Birnbaum and DeVito, 1995).

1.3.3 Species and Tissue Specificity

The Ah receptor protein has been found in both mammalian and non-mammalian species. Using radiolabelled ligands and either sucrose-density gradient centrifugation or the hydroxyapatite adsorption assay, high levels of Ah receptor were identified in several species, including rodents, rabbits, ground hogs, sheep, cats, ferrets, certain birds, and primates (Gasiewicz and Neal, 1982; Denison *et al.*, 1986). The Ah receptor protein exhibits tissue specificity like steroid hormones. The receptor has been identified in

the rat in the thymus, lung, liver, brain, kidney, testis, and skeletal muscle (Gasiewicz and Rucci, 1984), and in the lung, liver, kidney, placenta, and tonsils of humans (Manchester *et al.*, 1987; Safe, 1988; Safe *et al.*, 1990; Lorenzen and Okey, 1991).

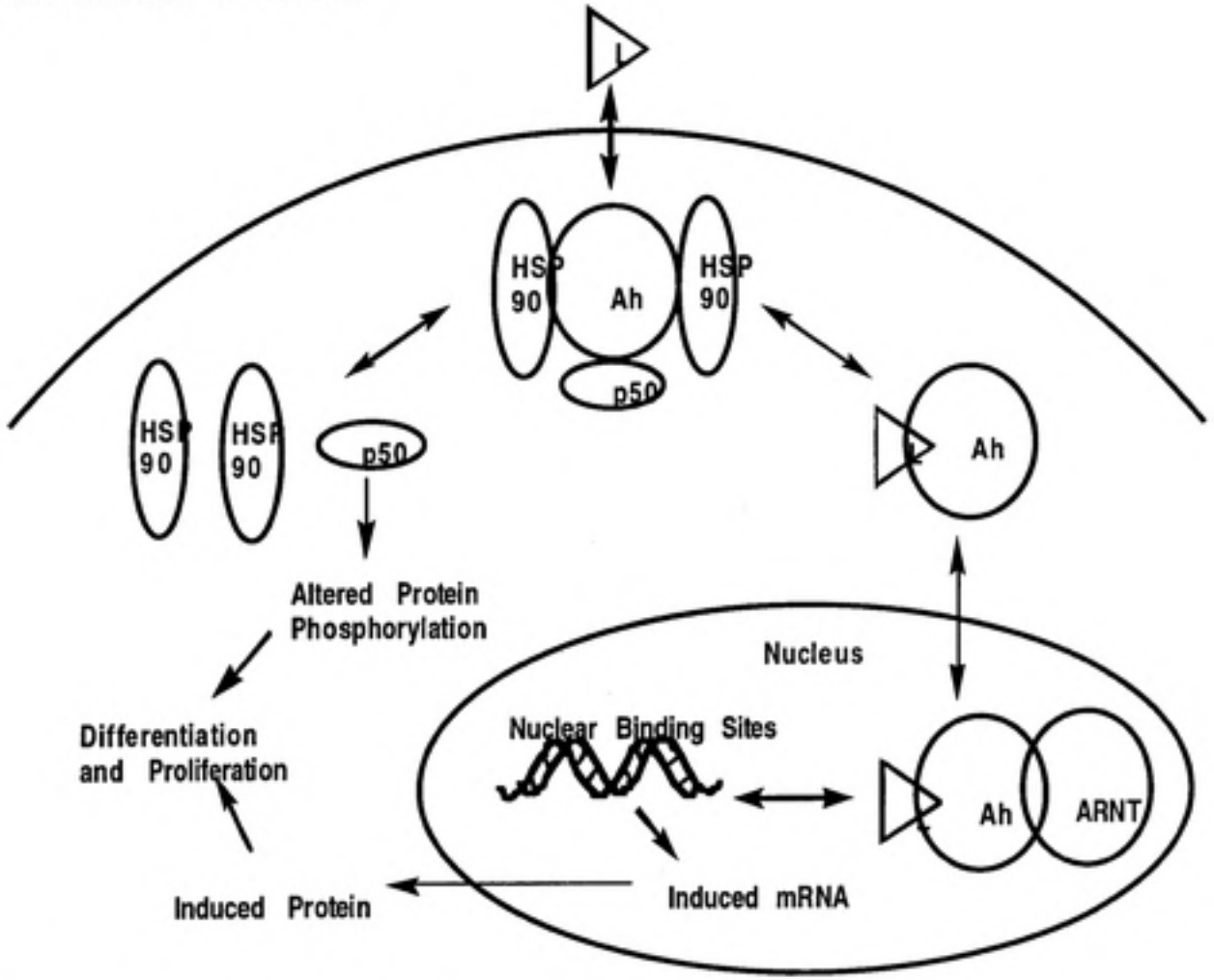
1.3.4 Ah Receptor Mediated Gene Expression

Although the Ah receptor is analogous to steroid hormone receptors because both steroid hormone receptors and the Ah receptor bind lipophilic, structurally distinct ligands (DeVito and Birnbaum, 1994), distinct differences exist between these two groups of receptor families (Burbach *et al.*, 1992). In the unbound state, the Ah receptor is a multimeric protein located in the cytosol. Unlike the zinc fingers of steroid receptors, the DNA binding region of the Ah receptor has a protein sequence consisting of a basic/helix-loop-helix region (Birnbaum, 1994; DeVito and Birnbaum, 1994). The unoccupied Ah receptor is a component of a receptor complex which is also composed of two 90-kDa heat shock proteins (Hsp90) and possibly one other 46-kDa protein (Hankninson, 1995) termed p50 (DeVito and Birnbaum, 1994). Hsp90 maintains the Ah receptor ligand binding conformation as well as represses the intrinsic abilities of the Ah receptor to bind DNA (Pongratz *et al.*, 1992). Following dissociation of Hsp90, the ligand-Ah receptor complex binds with the aryl hydrocarbon nuclear transferase (ARNT) protein to form a heterodimer (Birnbaum, 1994). The ARNT protein improves the DNA-binding capacity of the Ah receptor complex, which transforms and translocates into the cell nucleus (DeVito and Birnbaum, 1994). Once inside the nucleus, the complex binds with

high affinity specific genomic sequences, known as dioxin responsive elements (DREs) or xenobiotic responsive elements (XREs). Activated gene transcription can then occur, causing induction of CYP450 1A1 (Birnbaum, 1994; DeVito and Birnbaum, 1994).

The mechanism of induction of CYP1A1 gene transcription by 2,3,7,8-TCDD is shown in Figure 1.2 (Birnbaum and DeVito, 1995).

Figure 1.2 **Mechanism of Action**



* Adapted from Birnbaum and DeVito (1995)

1.4 Endometriosis

1.4.1 Definition

Endometriosis is usually described as the growth of endometrial tissue outside the uterus. During formation of this disease, endometrial glands and stroma often proliferate in the peritoneal cavity, causing infertility and a high degree of pain (Olive and Schwartz, 1993). A major characteristic of endometriosis is the presence of lesions and hemorrhagic cysts in the peritoneum (Nezhat *et al.*, 1995). Cysts are often bluish-gray in color due to encapsulation of menstrual blood by fibrotic tissue, but can range in color from clear to white or red to brown. Endometriotic lesions also vary in size, ranging from several millimeters to 2 centimeters in diameter (Olive and Schwartz, 1993). Lesions may be classified by appearance and color into 1 of 20 categories and may also be described as superficial and deep, infiltrated and invasive, and intra-, sub-, and retroperitoneal (Nezhat *et al.*, 1995).

Two different types of endometrial lesions exist: free-growing and enclosed (Martin, 1993). Free-growing lesions react like superficial endometrium, while enclosed lesions react like basal endometrium. Free-growing lesions have a surface epithelium and exhibit necrosis of the vessels and hemorrhaging at the end of the menstrual cycle. In contrast, enclosed lesions exhibit vasoconstriction and no bleeding at the end of the menstrual cycle.

Enclosed lesions may also show muscular metaplasia, fibromuscular tissue, and deep localization (Martin, 1993).

The development of endometrial lesions can be divided into four stages: microscopic, early active, advanced active (classical), and healed (Nezhat *et al.*, 1995). Microscopic lesions can be further divided into two types: 1) intraperitoneal lesions; in which the mesothelium is replaced by tall epithelium and ciliated cells, and 2) lesions consisting of glands and stroma; which are covered by normal mesothelium. Early active lesions are recognized as cystic glands or polyps resulting from the emergence of glandular tissue under the mesothelium. These lesions are characterized by degree of proliferation and although highly vascularized, are not fibrotic. Early active lesions arise as blisters filled with serous, pink, or hemorrhagic fluid. Advanced active lesions are most easily identified as endometriosis by the presence of inflammation, fibrosis, hemorrhage, and pigmentation. These classic lesions exhibit a decreased hormonal response during the menstrual cycle due to the increased degree of fibrosis. Finally, healed lesions are calcified, scarred remains of endometrial glands surrounded by fibrotic tissue and are often white in color. Although no endometrial activity is suspected in healed lesions, histology is necessary to confirm this suggestion. (Nezhat *et al.*, 1995)

1.4.2 Cause

Currently, three theories of endometrial histogenesis are supported (Olive and Schwartz, 1993). The first theory suggests cells lining the pelvic peritoneum transform metaplastically and develop

endometriosis. Support for this theory stems from the facts that endometrial and peritoneal cells both originate from coelomic-wall epithelium and that adult tissue differentiation can occur. However, skeptics of this theory argue that no evidence exists for the possibility of further differentiation of already differentiated peritoneal cells. Also, if peritoneal cells can transform metaplastically, the transformation should occur readily in men. However, occurrence in men is very rare and coincides with the presence of prostatic carcinomas and treatment with high doses of estrogens. Another problem with this theory is the presence of endometriosis primarily in the pelvic peritoneum, even though the coelomic membrane also covers the abdominal and thoracic cavities. A final problem is the fact that endometriosis usually affects women of reproductive age while the incidence of other metaplastic processes increases with age. Although the target age of endometriosis can be explained by estrogen-induced metaplasia, an inconsistency exists with the low rate of endometriosis in anovulatory women who have consistently elevated estrogen levels (Olive and Schwartz, 1993).

The second theory of endometrial histogenesis suggests the transplantation of endometrial tissue to ectopic locations occurs via such routes as lymphatic system, vascular system, iatrogenic dissemination, and retrograde menstruation. A core characteristic of this theory, the idea that endometrial cells which are shed in the uterus remain viable and capable of implantation, has been supported by *in vitro* growth of shed endometrial cells and the identification of viable endometrial cells in fallopian tubes and

peritoneal fluid. However, direct evidence of implantation resulting in endometriosis is not present (Olive and Schwartz, 1993).

The third theory is a blend of the first two theories and is usually referred to as the induction theory. This theory suggests the release of certain unknown substances by shed endometrial tissue which induce undifferentiated mesenchyme cells to differentiate into endometrium. Some evidence exists to support this theory, such as the identification of glands resembling endometriosis following the subcutaneous deposition of endometrium or the implantation of endometrium in the peritoneum of rabbits. However, no induction of endometrial stroma was identified in this work (Olive and Schwartz, 1993).

Even though many parallels and contradictions exist among these theories of endometrial histogenesis, retrograde menstruation is regularly accepted as a process which coincides with the development of endometrial lesions (Olive and Schwartz, 1993). During the menstrual cycle, pressure in the uterus increases as rhythmic uterine contractions occur to force the expulsion of menses (Haney, 1990). Release of menses can occur through either the cervical canal or the fallopian tubes. The rate of release is a function of route caliber and flow resistance (Haney, 1990). Little resistance present in the oviducts promotes retrograde menstruation (Haney, 1990). In epidemiologic data, an increased incidence of endometriosis correlates with a prolonged regular, spontaneous menstruation, while a decreased incidence of endometriosis coincides with extended use of oral contraceptives, increased number of deliveries, later menarche, and irregular menstrual cycles (Olive and

Schwartz, 1993). Most evidence suggests that transplantation of shed endometrial cells from the uterus to ectopic locations is the primary means for the development of endometriosis and that most transplantation occurs via retrograde menstruation through the oviducts with infrequent transport via lymphatic, iatrogenic, and hemotogenous dissemination (Haney, 1990).

Another factor which may possibly influence the proliferation of endometriosis is the immune system (Dmowski, 1991). Decreases in cell-mediated and humoral immunity may increase the rate of endometriotic proliferation (Dmowski, 1991). Increased incidence of endometriosis and of more aggressive, invasive lesions were observed in rhesus monkeys after the administration of immunosuppressors, such as systemic radiation, and immunotoxicants, such as PCBs (Dmowski, 1991). Because the exact mechanism of influence by the immune system on endometroisis is unknown at present, more research is required to better understand the influences of the immune system on endometriotic growth. Also, the effects of the uterine transplant procedure on the immune system and on the degree of proliferation of endometriotic lesions should be investigated.

1.4.3 Prevalence

The prevalence of endometriosis in the general population is extremely difficult to ascertain because of the difficulties in diagnosis. Laparoscopy or surgery are necessary to confirm

diagnosis (Olive and Schwartz, 1993). Therefore, estimates of prevalence may vary with symptoms, procedure, and surgeon (Olive and Schwartz, 1993). Estimates of the prevalence of endometriosis range from 50% of all menstruating women (Williams and Pratt, 1977) to 1% of fertile women (Candiani *et al.*, 1991). Often, a 10% prevalence rate in the general population is accepted (Olive and Schwartz, 1993). Many early studies concluding higher incidence rates and racial differences in prevalence rates failed to control for the effects of confounders, such as availability of health care, access to contraception, cultural differences affecting childbearing, attitudes toward menses and pain, and the incidence of sexually transmitted diseases (Olive and Schwartz, 1993). However, all estimates of prevalence are for symptomatic women with endometriosis of a degree of severity great enough to warrant surgical treatment (Nezhat *et al.*, 1995).

Some researchers believe the prevalence of endometriosis is increasing in the general population. Data from a report by the National Center for Health Statistics (NCHS) on hysterectomies performed in the United States between 1965 and 1984 provides information of the changing prevalence rates of endometriosis (Statistics, 1987). The data from this study suggested an increase of 121% in the prevalence of hysterectomies due to endometriosis performed during this time frame. Although NCHS researchers suggested changes in medical practices, such as increased awareness due to laparoscopy and increased use of hysterectomies to treat endometriosis, were partially responsible for the increased rate of incidence, other researchers disagree with the suggestion that an

increased rate of hysterectomies would be performed only for women with endometriosis and not for women with other conditions requiring hysterectomies (Nezhat *et al.*, 1995). Also, during this time, laparoscopy not only developed as a means of diagnosis, but also as a method of treatment (Nezhat *et al.*, 1995). Even though demographic changes, such as delayed childbearing and declined use of oral contraceptives, may in part account for increased hysterectomies due to endometriosis (Nezhat *et al.*, 1995), these data support the idea that endometriosis increased in prevalence during this time period and may still be on the rise.

1.4.4 Related Research

Estimations of prevalence of endometriosis in Belgium are as high as 60 - 80% in women with infertility or pain (Koninckx *et al.*, 1991). Some researchers suggest the incidence rate of endometriosis correlates with levels of dioxin pollution (Konickx *et al.*, 1994). This theory is supported by several facts, such as increased blood levels of PCBs discovered in women who suffer from endometriosis. Another argument stems from the reality that women in developed countries have higher prevalence rates of endometriosis than women in developing countries and "career-oriented" women develop endometriosis more often than other women. Also, the increased incidence of endometriosis in developing countries correlates with the increased use of PCBs and other forms of dioxin pollution since World War II. A final point in support of the theory that endometriosis correlates with degree of dioxin pollution is the idea that endometriosis is mediated by the immune system. Dioxins are

known to compromise the immune system (Lundberg *et al.*, 1992), which in turn could facilitate the formation of endometriotic lesions (Konickx *et al.*, 1994).

Some studies have been performed to access the influence of 2,3,7,8-TCDD on the proliferation of endometriosis. In rhesus monkeys, incidence of endometriosis and severity of lesions was directly correlated with exposure to 2,3,7,8-TCDD and administered dose level (Rier *et al.*, 1993). Other work by Cummings *et al.* (1996) demonstrated a positive correlation between dioxin exposure, administered dose level, and incidence and degree of severity of endometriotic lesions in rats and mice. Because rodents have a closed reproductive tract with a bursa enclosed ovary and an estrous cycle instead of a menstrual cycle, they do not develop endometriosis naturally. However, the effects of dioxin on endometriosis in these species can be studied via the induction of endometriosis through surgical methods (Vernon and Wilson, 1985).

The mechanism of action by which dioxin induces increased proliferation of endometriosis has not been determined. Objectives of this research project were to investigate the mechanism of action of HAH-induced endometriosis and to assess the structure activity relationships of polyhalogenated aromatic hydrocarbons, with endometriosis as an endpoint. Therefore, a selection of HAHs with varying degrees of affinity for the Ah receptor and varying TEF values were selected for administration to mice to assess the degrees of increased endometriotic proliferation. In addition to 2,3,7,8-TCDD, four other chemicals were also administered: PCB 126, PCB 153, 1,3,6,8-TCDD, and 4-PeCDF. The hypothesis of this study is that

induction of the endpoint, endometriosis, is Ah receptor-mediated and will correlate with the structure activity relationships of HAHs. 2,3,7,8-TCDD and the two additional "dioxin-like" compounds (PCB 126 and 4-PeCDF) should evoke increased proliferation of endometriotic lesions, while the "nondioxin-like" chemicals (PCB 153 and 1,3,6,8-TCDD) should not induce increased endometriotic proliferation.

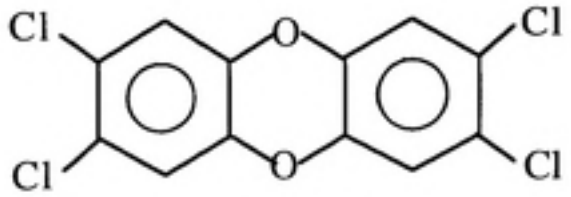
CHAPTER II

MATERIALS AND METHODS

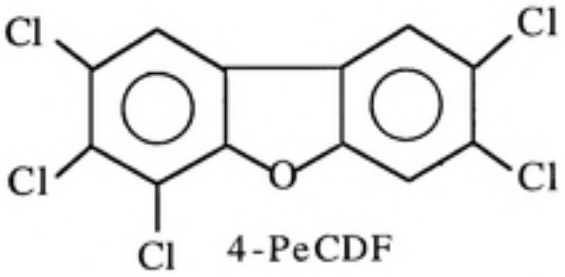
2.1 Chemicals

To assess structure activity relationships of HAHs in this study, both "dioxin-like" and "non-dioxin-like" chemicals were used in addition to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) (DeVito and Birnbaum, 1995). "Dioxin-like" chemicals used were PCB 126 (3,4,5,3',4'-pentachlorobiphenyl) and 4-PeCDF (2,3,4,7,8-pentachlorodibenzofuran), while "non-dioxin-like" chemicals used were PCB 153 (2,4,5,2',4',5'-hexachlorobiphenyl) and 1,3,6,8-TCDD (1,3,6,8-tetrachlorodibenzo-*p*-dioxin.) PCB 126 was chosen for this study because it is most "dioxin-like" in its chemistry, while PCB 153 is a major PCB in human tissue and does not have a strong affinity for the Ah receptor. 4-PeCDF is a major environmental contaminant which comprises a large portion of estimated TEQ concentrations. 1,3,6,8-TCDD was used as a negative control in this study because it does not readily bind the Ah receptor. Structures of chemicals used in this study are diagramed in Figure 2.1.

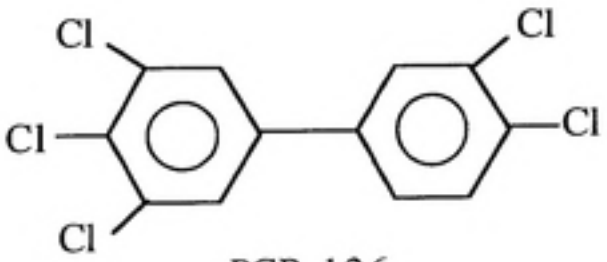
Figure 2.1 **Chemical Structures**



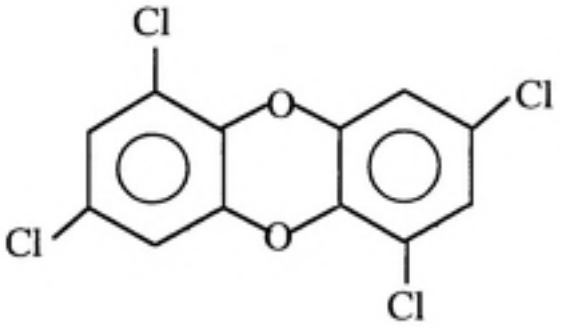
2,3,7,8-TCDD



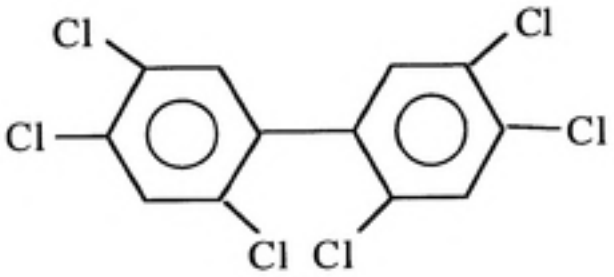
4-PeCDF



PCB 126



1,3,6,8-TCDD



PCB 153

2,3,7,8-TCDD had a stated purity greater than 98% as determined by gas chromotography/mass spectroscopy and originally by Radian Corp. (Austin, TX). PCB 126 was purchased from Ultra Scientific Chemical Co. (North Kingstown, RI). 4-PeCDF, PCB 153, and 1,3,6,8-TCDD were purchased from Accustandard (New Haven, CT). All chemicals had purities greater than 98%. The dosing vehicle, corn oil, was obtained from Sigma Chemical Co. (St Louis, MO).

A stock solution of 1 mg 2,3,7,8-TCDD/10 mL corn oil was made as described (Diliberto *et al.*, 1994) and stored at -20 °C until the time of dosing solution preparation. Stock solutions of the additional four chemicals were prepared approximately one week before administration of the intial dose of each chemical. Stock solutions were prepared with the following concentrations: 8 mg PCB 126/5 mL corn oil, 1.2 mg 4-PeCDF/5 mL corn oil, 92.4 mg PCB 153/5 mL corn oil, and 57 mg 1,3,6,8-TCDD/10 mL corn oil. Stock solutions were prepared by adding 1 mL of acetone to each scintillation vial containing the appropriate amount of chemical. The solution then was vortexed and sonicated for 10 minutes. After dissolution of the chemical in acetone, 5 mL of corn oil were added to the vial. The solution was vortexed again and placed on a Savant speed vac (Savant Instruments, Inc., Farmington, NY) for 2 to 4 hours to evaporate the acetone. Each vial was then weighed to ensure the entire volume of acetone had evaporated. Because of the large volume required for this study and the solubility of 1,3,6,8-TCDD, 5 mL of toluene were added to ensure the chemical dissolved into solution. Five additional mL of corn oil were added to the 1,3,6,8-TCDD solution followed by evaporation of the acetone and toluene.

Dosing solutions were prepared at the concentrations listed in Table 2.1. All animals were dosed with a corn oil dosing vehicle at 10 mL/kg body weight.

TABLE 2.1

Chemical	Dose (ug or mg/kg body weight/10mL dosing volume)
Control I	0 ug
2,3,7,8-TCDD	1 ug
	3 ug
	10 ug
Control II	0 ug
PCB 126	100 ug
	300 ug
	1000 ug
PCB153	3 mg
	30 mg
Control III	0 ug
4-PeCDF	10 ug
	30 ug
	100 ug
1,3,6,8-TCDD	2 mg
	20 mg

2.2 Animals

Female B6C3F1 mice were obtained from Charles-River Breeding Laboratories (Raleigh, NC) at seventy days of age. All animals were housed in an environment with controlled humidity (40-50%), 12-hour light cycle, constant temperature (20-24 °C), and standard food and water maintenance. Animal caretakers changed the cages of all animals regularly except during the week after animals were dosed with 2,3,7,8-TCDD, PCB 126, and 4-PeCDF. Because portions of these hazardous chemicals were present in the animal excreta, animals were placed in disposable cages for two changes (approximately 3-4 days per cage) after dosing. Since the parent compound PCB 153 is not as readily excreted as the other chemicals and 1,3,6,8-TCDD is nontoxic at the dose levels administered, animals dosed with these chemicals remained in regular cages during and following dosing. Initially, precautions for hazardous excretions were taken only for 2,3,7,8-TCDD. However, after reconsideration, animals dosed with PCB 126 and 4-PeCDF were placed in disposable cages for the fifth doses.

2.3 Treatment / Dosing Regimen

As the mice arrived in the animal facility, they were randomly assigned to a dose group and earpunched for identity. Chemicals were chosen based on "dioxin-like" toxicity characteristics and doses were assigned based on comparable TEF values (Birnbaum and DeVito, 1995). Experiment I included doses of 0 (corn oil), 1, 3, and 10 ug 2,3,7,8-TCDD/kg body weight with 10 animals per group. Experiment II included doses of 0, 3, and 30 mg PCB 153/kg body

weight and 100, 300, and 1000 ug PCB 126/kg body weight with 10 - 12 animals per group. Experiment III included doses of 0, 10, 30, and 100 4-PeCDF/kg body weight and 2 and 20 mg 1,3,6,8-TCDD/kg body weight with 10-12 animals per group. Animals were allowed to acclimate one week prior to the first dose. Dosing was administered via oral gavage a total of five times, at three week intervals between each dosing (Cummings *et al.*, 1996). Animal weights were recorded immediately before administration of each dose. Surgeries were performed during the week of the second dose. Therefore, the timeline was as follows:

Week 1	Week 4	Week 7	Week 10	Week 13	Week 16
Weigh Dose 1	Weigh Dose 2 Surgeries	Weigh Dose 3	Weigh Dose 4	Weigh Dose 5	Weigh Sacrifice

2.4 Surgery

Because mice have a closed reproductive tract with a bursa enclosed ovary and an estrous cycle instead of a menstrual cycle, they do not develop endometriosis naturally (Cummings and Metcalf, 1995a). Therefore, endometriosis must be induced in these animals through surgical methods performed during the week of the second dosing.

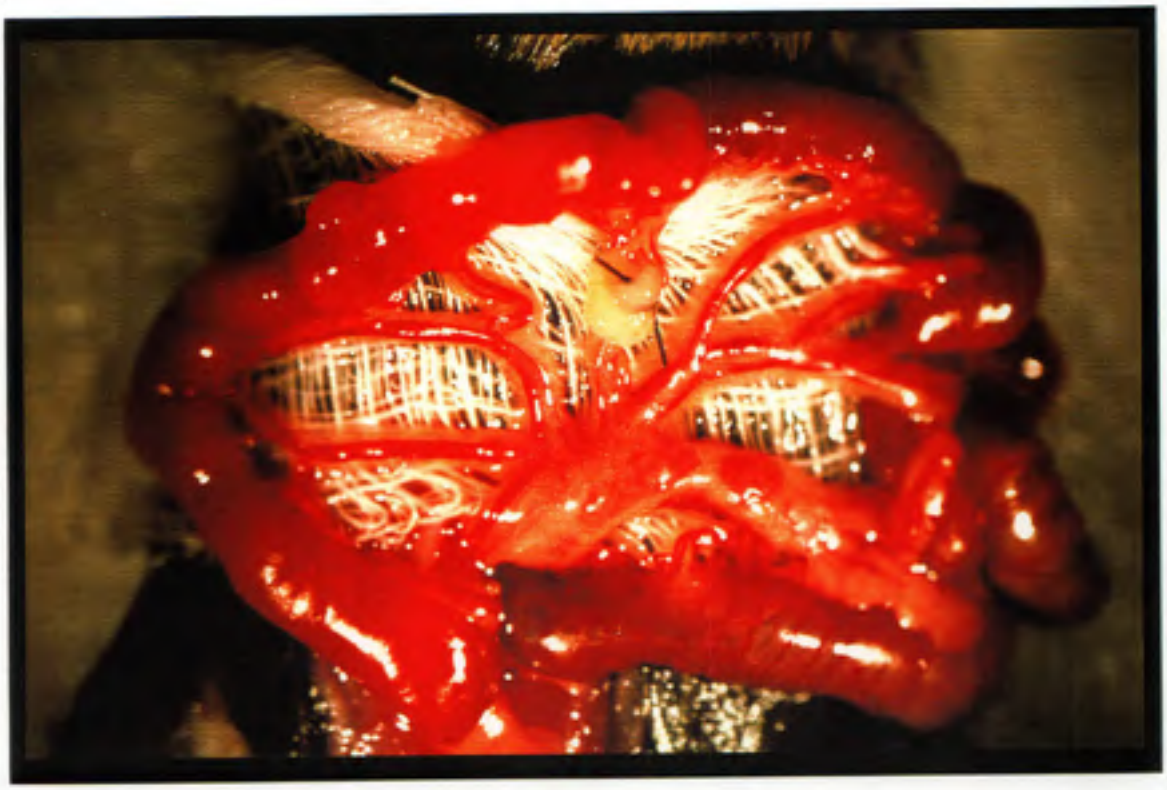
2.4.1 Surgical Equipment and Supplies

Surgical instruments used included two pairs of forceps (mouse-toothed and medium), two pair of scissors (blunt-sharp and fine), wound clips, mechanical wound clipper, gauze, silk sutures, 4-0 nylon suture (Look, Inc.; Norwell, MA), 2-0 absorbable suture (Look, Inc.; Norwell, MA), Betadine (Kerr Drug; Durham, NC), ethanol (McCormick Distilling Co., Inc.; Weston, MO), F-10 Ham's nutrient mixture (Gibco BRL; Gaithersburg, MD) with penicillin-streptomycin (Gibco BRL; Gaithersburg, MD), and anesthesia-grade ether (Mallinckrodt U.S.P.; Chesterfield, MO).

2.4.2 Surgical Techniques

The two major steps of the surgical process as described by Vernon and Wilson (1985) in a rat model and extended to mice by Cummings and Metcalf (1995a) were ablation of the left uterine horn and implantation of uterine segments into the peritoneal cavity. Figure 2.2 shows the surgical result immediately following implantation of a uterine segment onto a mesenteric blood vessel.

Figure 2.2 **Surgical Technique**



(Cummings and Metcalf, 1995)

Seventy percent ethanol was used to sterilize surgical equipment overnight and to swab the surgical platform on the day of surgery. Hams F-10 media with antibiotics (penicillin /streptomycin) was prepared and warmed to 37 °C.

Surgeries were performed in a hood since ether was used as the anesthesia source. The anesthesia jar was filled with anesthesia-grade ether to saturate the towel lining the bottom of the jar. The abdomen of the mouse was clipped before the animal was anesthetized. The mouse was then placed into the anesthesia jar until it was properly anesthetized. Upon removal from the anesthesia jar, a nosecone with an ether soaked pad was used to maintain a proper level of anesthesia.

Approximately 3 mL of media were poured into a 35 mm petri dish for easier application. The mouse was placed in dorsal recumbancy on the surgical table and the clipped abdomen was swabbed with a Betadine mixture (one capful of Betadine in 350 mL water.) A 2-3 cm midline incision was made in the skin beginning approximately 1 cm above the pubic symphysis. Another incision was made through the underlying muscle, paying special attention to the location of the small intestines. Once the peritoneal cavity was exposed, the animal's left uterine horn was located. Color and size of the uterus were recorded to estimate stage of estrus cycle. The uterine horn was then suspended with forceps while another pair of forceps was placed through the underlying mesentery. A 3-0 silk suture was pulled through the mesentery and used to tie off the uterotubal junction, leaving the ovary in place. A second suture was used to tie off the distal end of the uterus. The uterine horn could

then be ablated and placed in the petri dish of media. No bleeding occurred when the uterine vein and artery were both properly tied off with the sutures. Sterile media was placed into the peritoneal cavity and the incision was covered with gauze to prevent any tissues from drying while the ablated uterine horn was bisected. Any fat and mesentery were trimmed from the uterine horn. The segment was cut longitudinally to expose the epithelial lining and then cut into thirds of equal size. All cutting and trimming was performed in the media to prevent the segment from drying.

The second surgical procedure involved implantation of the uterine pieces into the peritoneal cavity. Gauze was wet with media and placed directly above the incision as a pad on which the intestines were placed. A mesenteric blood vessel, at least two blood vessels to the left of the cecum towards the small intestines, was identified. The uterine square was then tied onto the mesenteric blood vessel using a 3-0 suture, with a single tie through the center of the square tied twice. Uterine pieces were tied onto alternating blood vessels, towards the intestines. Approximately one mL of the Hams media was placed into the cavity before closing. The abdomenal muscles were sutured using a continuous interlocking stitch of 2-0 gut suture and the skin was closed with wound clips.

The mice were placed in a warm recovery area after surgery until regaining consciousness. The wounds were examined for blood leakage before the mice were returned to their permanent cages. If blood was discovered at the incision site, the incision was reopened, examined, repaired if necessary, and reclosed.

2.5 Tissue Preparation and Necropsy

At the conclusion of 16 weeks, the animals were euthanized by carbon dioxide asphyxiation followed by exanguination via cardiac puncture. Necropsies were performed in a random order to prevent biases in the measuring of lesion diameters. The peritoneal cavity was opened and the endometriotic lesions exposed. Lesions were removed from the animal and all fat, mesentery, and intestinal segments surrounding the lesion were trimmed away. Lesion diameters were measured using calipers and recorded. Any unusual features or discolorations were also noted. The lesions were weighed and placed in scintillation vials containing 10% formalin. Both ovaries and the remaining uterine horn were removed and weighed. The intact uterine horn and its ovary were also placed into the scintillation vial with the endometriotic lesions. Liver, adrenals, lungs, kidneys, thymus, spleen, and skin were also extracted and weighed. These tissues were frozen via dry ice, and stored at -70 °C for later analysis.

2.6 Analysis of Lesion Diameter and Statistical Analysis of Endometriosis

Primary statistical analysis of endometriotic lesion diameters and secondary analyses of lesion weights, ovarian weights, uterine weights, and thymus weights from all chemical treatment groups were performed by the Dunnett's test. Means with standard deviations were determined for all dose groups for liver weights, body weights, ovarian weights, uterine weights, lesion weights, and lesion diameters. Ratios were determined for lesion diameter to

lesion weight, liver weight to body weight, and ovarian weight to body weight.

2.7 Hepatic Microsomal Preparation

2.7,1 Tissue Homogenization

Livers were rinsed in ice-cold saline (0.9% w/v) the day of necropsy, frozen via dry ice, and stored at -70 °C until time of microsomal preparation and enzyme analysis. Livers were homogenized in a volume of ice-cold buffer (250 mM sucrose, 0.5 mM EDTA, 1 mM dithiothreitol, 25 mM KCl, 10 mM HEPES, and 10% (v/v) glycerol, pH 7.4) at 4 times the weight of the liver with a Teflon drill apparatus.

2.7.2 Preparation of Supernatant

The liver homogenate was centrifuged at 9000 X g at 4 degrees Celsius in a Beckman model J2-21M induction drive centrifuge with a JA-17 rotor (Beckman Instruments, Fullerton, CA) to separate the homogenate into the supernatant (S9) and pellet (P9) fractions. The S9 fraction contains cytosol and microsomes, while the P9 fraction contains nuclei, lysosomes, mitochondria, and plasma membranes.

2.7.3 Preparation of Microsomes

To separate the S9 fraction into the high speed supernatant (S100) and pellet (P100), the S9 fraction was again centrifuged at 105,000 X g for 60 minutes at 4 °C in a Beckman L3-50

ultracentrifuge with a 50.2 Ti rotor. The S100 fraction contains cytosol, while the P100 contains microsomes. The hepatic microsomes were used immediately for enzymatic studies.

2.8 Enzyme Analysis

Microsomes were resuspended in 100 uL of 0.1 M Potassium Phosphate (KPO_4) and added to reaction buffer (0.1 M KPO_4 and 2 mg/mL bovine serum albumin at pH 7.5) containing 1.5 nM ethoxyresorufin (Molecular Probes, Eugene, OR.) The total volume of reaction mixture was 2.3 mL. The samples were incubated at 37 °C for two minutes prior to initiation of the reaction by 100 uL of B-NADPH (5 mg/mL). The reaction was monitored spectrofluorometrically at 37 °C for 2 minutes. Ethoxyresorufin-*O*-deethylase activity was quantified spectrofluorometrically as described (Pohl and Fouts, 1980) and expressed as pmoles/min/mg protein. Protein concentrations were determined by the Bradford reaction using bovine serum albumin as a standard (Bradford, 1976).

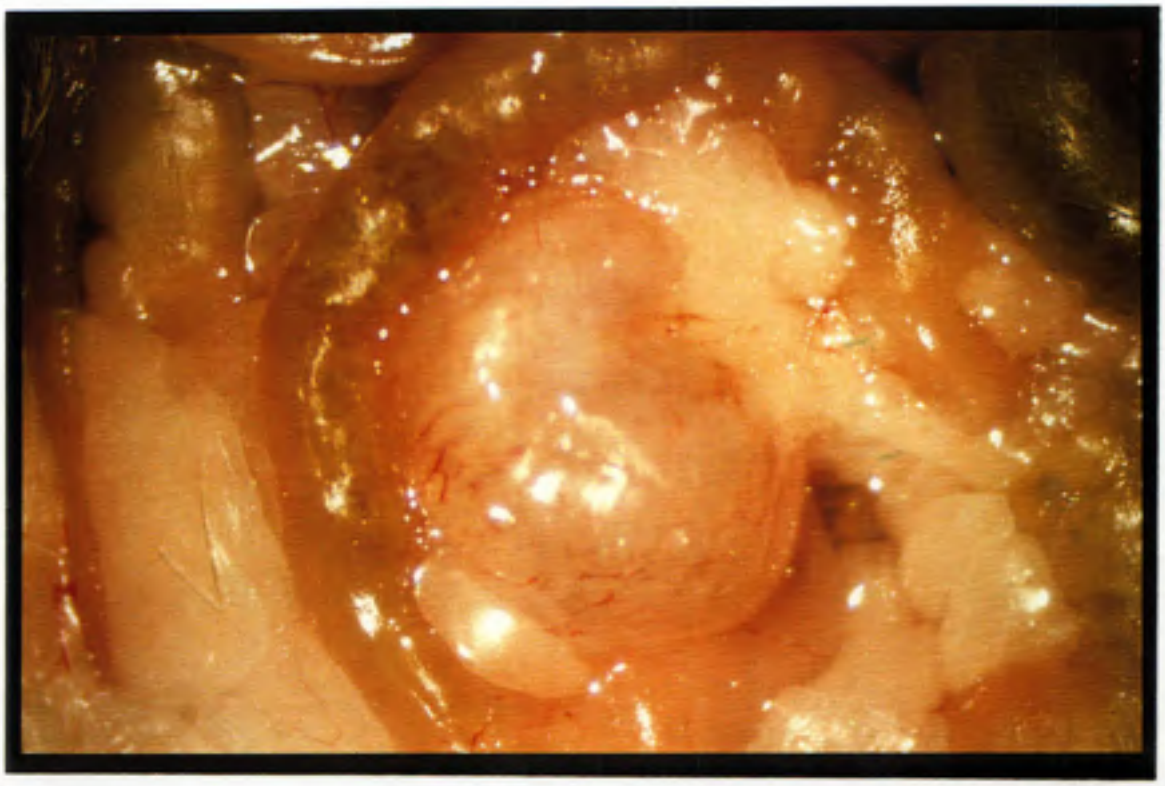
2.9 Histopathological Examination

A microscopic examination of endometriotic lesions and ovarian tissue was performed to characterize histological changes associated with exposure to 2,3,7,8-TCDD, PCB 153, PCB 126, 1,3,6,8,-TCDD, or 4-PeCDF. Animals were selected for histopathology randomly to obtain an unbiased, respresentative sample for review. Samples were divided into three categories: "normal lesion," "abnormal lesion," and ovaries. Lesions were characterized as either normal or abnormal based on color and shape; with clear, symmetrical lesions

characterized as normal and blood or pus-filled, assymetrical lesions characterized as abnormal. Figures 2.3 and 2.4 demonstrate the differences between lesions characterized as normal and those characterized as abnormal. Figure 2.3 demonstrates a "normal" lesion which is clear in color and symmetric in shape. Figure 2.4 shows an "abnormal" lesion which is blood-filled, dark in color, and connected to another abnormal lesion.

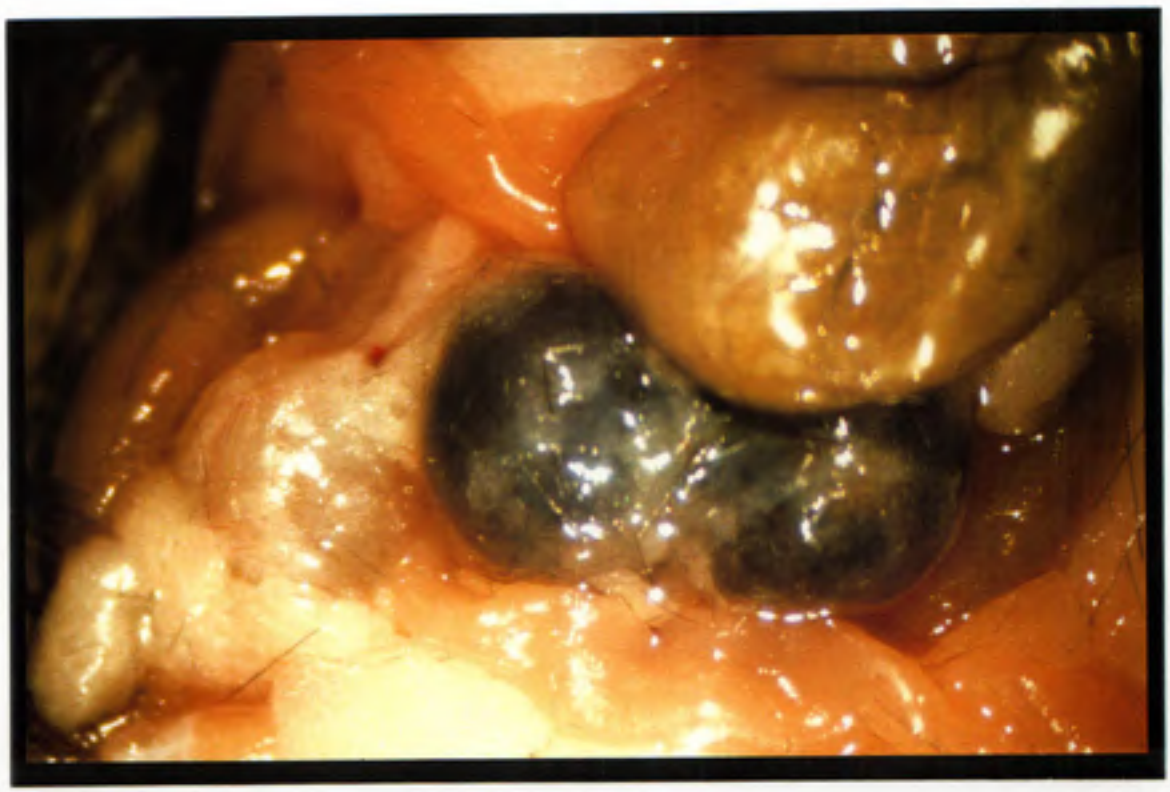
Endometriotic lesions were examined for the presence of inflammation and luminal exudate or transudate. Ovaries were examined for the presence of primary, growing, and antral follicles as well as for the presence of corpora lutea (both active and regressing.) Primary, growing, and antral follicles were not quantitated (counted) because this information would be somewhat meaningless from a single, random histological section which appeared normal in all other aspects. Corpora lutea (active and regressing) were counted in each ovary. Active corpora lutea were those that were judged to represent newly formed corpora lutea to those that became increasingly eosinophilic and foamy appearing. Regressive corpora lutea were characterized by degeneration, necrosis, and fibrous tissue proliferation.

Figure 2.3 **"Normal" Lesion**



* Lesion was observed in an animal treated with 300 ug PCB 126/kg body weight, but is representative of all lesions characterized as "normal".

Figure 2.4 **"Abnormal" Lesion**



* Lesion was observed in an animal treated with 3 mg PCB 153/kg body weight, but is representative of all lesions characterized as "abnormal".

CHAPTER III

RESULTS

3.1 Endometriotic Lesion Diameter

Previous studies in a rodent model for 2,3,7,8-TCDD-induced endometriosis focused on lesion diameter as an indicator of 2,3,7,8-TCDD responses (Cummings *et al.*, 1996). Table 3.1 shows the endometriotic lesion diameter data for female B6C3F1 mice treated with various concentrations of 2,3,7,8-TCDD, PCB 153, PCB 126, 1,3,6,8-TCDD, and 4-PeCDF.

Examination of lesion diameter in three separate control groups indicated similar values with no statistical differences. Treatment of animals with 1 or 3 ug 2,3,7,8-TCDD/kg body weight resulted in a statistically significant increase in lesion diameter. Although the diameter was increased relative to controls at 10 ug 2,3,7,8-TCDD/kg body weight, it was not statistically significant due to variability (Table 3.1). An increase in lesion diameter with dose was observed in animals treated with 4-PeCDF. However, a significant increase in lesion diameter compared to control animals was only observed in animals treated with 100 ug 4-PeCDF/kg body weight (Table 3.1). Animals treated with PCB 126 resulted in an apparent increase in lesion diameter from 100 to 300 ug/kg body weight; however, this

increase was not significant compared to controls (Table 3.1). Analysis of lesion diameter values for animals treated with PCB 153 or 1,3,6,8-TCDD resulted in lesion diameter values similar to control animals in all treatment groups (Table 3.1).

Table 3.1 Endometriotic Lesion Diameter

CHEMICAL	DOSE	MEAN LESION DIAMETER (mm)	STANDARD DEVIATION OF MEAN DIAMETER
	0	5.46	1.27
2,3,7,8-TCDD	1 ug/kg	7.27*	1.42
	3 ug/kg	6.96*	0.77
	10 ug/kg	6.12	0.76
	0	5.71	1.43
PCB 153	3 mg/kg	5.74	0.90
	30 mg/kg	5.84	0.52
PCB 126	100 ug/kg	6.25	1.03
	300 ug/kg	6.83	1.46
	1000 ug/kg	6.61	1.75
	0	6.05	0.86
1,3,6,8-TCDD	2 mg/kg	5.98	0.91
	20 mg/kg	5.96	0.82
4-PeCDF	10 ug/kg	6.05	0.54
	30 ug/kg	6.26	1.27
	100 ug/kg	7.32*	1.29

* Statistically different ($p<0.05$) from control animals as determined by Dunnett's Test

3.2 Endometriotic Lesion Weight

Although endometriotic lesions are usually round in shape, occasionally their symmetry may be slightly uneven. This may be especially true if the lesion has unusual characteristics, such as extensive hemorrhaging or invasive growth. Instances may also arise in which one lesion is double-lobed or two lesions can not be completely separated for analysis. For these reasons, endometriotic lesions were weighed to provide additional information on changes in endometrial size due to HAH administration.

Endometriotic lesion weight was used as an additional marker to examine halogenated aromatic hydrocarbon (HAH)-promoted endometriosis. Table 3.2 illustrates the endometriotic lesion weights in female B6C3F1 mice treated with various concentrations of 2,3,7,8-TCDD, PCB 153, PCB 126, 1,3,6,8-TCDD, and 4-PeCDF. Examination of lesion weights in three separate control groups was highly variable and lesion weights for control animals in experiment I were significantly different from control animals in experiments II and III, which may contribute to the variability in results among dose and chemical groups. Treatment of animals with 1-10 ug 2,3,7,8-TCDD/kg body weight resulted in a dose-dependent decrease in endometriotic lesion weight. However, endometriotic lesion weights in all dose groups of 2,3,7,8-TCDD treated mice were significantly elevated compared to controls (Table 3.2). An apparent, but non-significant dose-dependent increase in lesion weight was observed in animals treated with 10-100 ug 4-PeCDF/kg body weight or 100-300 ug PCB 126/kg body weight. Lesion weight

values for the highest dose of PCB 126, 1000 ug/kg body weight, decreased from 300 ug/kg body weight. Elevated endometriotic lesion weights were not observed in the lowest treatment groups for 4-PeCDF or PCB 126 when compared to controls. Analysis of lesion weights in mice treated with PCB 153 or 1,3,6,8-TCDD resulted in similar endometriotic lesion weights to control animals.

Table 3.2 Endometriotic Lesion Weight

CHEMICAL	DOSE	MEAN LESION WEIGHT (g)	STANDARD DEVIATION OF MEAN LESION WEIGHT
	0	0.0225	0.0130
2,3,7,8-TCDD	1 ug/kg	0.1092*	0.0636
	3 ug/kg	0.0945*	0.0434
	10 ug/kg	0.0617*	0.0267
	0	0.0713	0.0515
PCB 153	3 mg/kg	0.0595	0.0262
	30 mg/kg	0.0641	0.0189
PCB 126	100 ug/kg	0.0657	0.0289
	300 ug/kg	0.1010	0.0558
	1000 ug/kg	0.0919	0.0579
	0	0.0710	0.0317
1,3,6,8-TCDD	2 mg/kg	0.0633	0.0278
	20 mg/kg	0.0610	0.0294
4-PeCDF	10 ug/kg	0.0700	0.0220
	30 ug/kg	0.0842	0.0464
	100 ug/kg	0.1143	0.0585

*Statistically different ($p<0.05$) from control animals as determined by the Dunnett's Test

3.3 Ovarian Weight

Ovarian weights, including attached oviducts, were measured for the ovary attached to the ablated uterine horn (left ovary) and the ovary attached to the intact uterine horn (right ovary). Ovarian weights from female B6C3F1 mice treated with various concentrations of 2,3,7,8-TCDD, PCB 153, PCB 126, 1,3,6,8-TCDD, or 4-PeCDF are shown in Table 3.3. Because body weight values did not vary significantly across chemical classes or doses, similar results were observed in crude ovarian weights (Table 3.3) and ovary/body weight ratios (data not shown). The ovarian weights of ovaries of ablated uterine horns appear slightly lower than the ovarian weights from intact uterine horns (Table 3.3). However, trends in both sets of ovarian weights are consistent within most dose groups. Therefore, the ovarian weight in ovaries with an intact uterine horn was used as a marker to describe the dose-dependent effects of those chemicals on ovarian weights.

Although no significant differences were found in comparisons of treated animals to control animals using the Dunnett's Test, examination of ovarian weights from animals treated with 1 or 3 ug 2,3,7,8-TCDD/kg body weight revealed a trend toward an increase in ovarian weights, that was followed by an apparent decrease in ovarian weight in animals treated with 10 ug 2,3,7,8-TCDD/kg body weight (Table 3.3). Animals treated with PCB 153 exhibited an apparent but non-significant increase in ovarian weight with increasing dose (Table 3.3). In contrast, mice treated with PCB 126 or 4-PeCDF showed an apparent decrease in ovarian weight with

increasing dose (Table 3.3). However, this response was present in both ovaries of animals treated with PCB 126, but only in the right ovary of animals treated with 4-PeCDF. Furthermore, analysis of ovarian weights from mice treated with 1,3,6,8-TCDD showed that at all doses tested ovarian weights of treated animals were similar to controls (Table 3.3).

To better understand the structure-activity relationships of HAH-induced effects on endometriotic lesion diameter and ovarian weights, the ratio of these two parameters was studied (Figures 3.1-3.5). Compared to control animals, mice treated with 1 or 3 ug 2,3,7,8-TCDD/kg body weight showed an elevated lesion diameter/ovarian weight ratio (Figure 3.1), which was further elevated in mice treated with 10 ug 2,3,7,8-TCDD/kg body weight. The lesion diameter/ovarian weight ratios for PCB 153 and 1,3,6,8-TCDD are similar to control values at all doses tested (Figures 3.2 and 3.4). Figure 3.3 shows that lesion diameter/ovarian weight ratios increased with increasing dose of PCB 126. Similarly, the results observed in mice treated with 4-PeCDF suggest a trend toward increasing ratios with increasing dose (Figure 3.5).

Table 3.3 Ovarian Weight

CHEMICAL	DOSE	AVERAGE OVARIAN WEIGHT (right ovary of intact uterine horn) (grams)	STANDARD DEVIATION OF RIGHT OVARIAN WEIGHT	AVERAGE OVARIAN WEIGHT (left ovary of ablated uterine horn) (grams)	STANDARD DEVIATION OF LEFT OVARIAN WEIGHT
	0	0.0085	0.0008	0.0075	0.0010
2,3,7,8-TCDD	1	0.0091	0.0022	0.0080	0.0028
(ug/kg)	3	0.0093	0.0014	0.0081	0.0010
	10	0.0070	0.0020	0.0064	0.0017
	0	0.0084	0.0030	0.0073	0.0018
PCB 153	3	0.0102	0.0011	0.0085	0.0016
(mg/kg)	30	0.0135	0.0106	0.0125	0.0078
PCB 126	100	0.0107	0.0020	0.0104	0.0021
(ug/kg)	300	0.0099	0.0016	0.0085	0.0026
	1000	0.0091	0.0022	0.0088	0.0038
	0	0.0108	0.0018	0.0096	0.0028
1,3,6,8-TCDD	2	0.0092	0.0017	0.0081	0.0021
(mg/kg)	20	0.0104	0.0016	0.0092	0.0012
4-PeCDF	10	0.0104	0.0024	0.0090	0.0012
(ug/kg)	30	0.0096	0.0017	0.0095	0.0020
	100	0.0091	0.0011	0.0089	0.0011

Figure 3.1

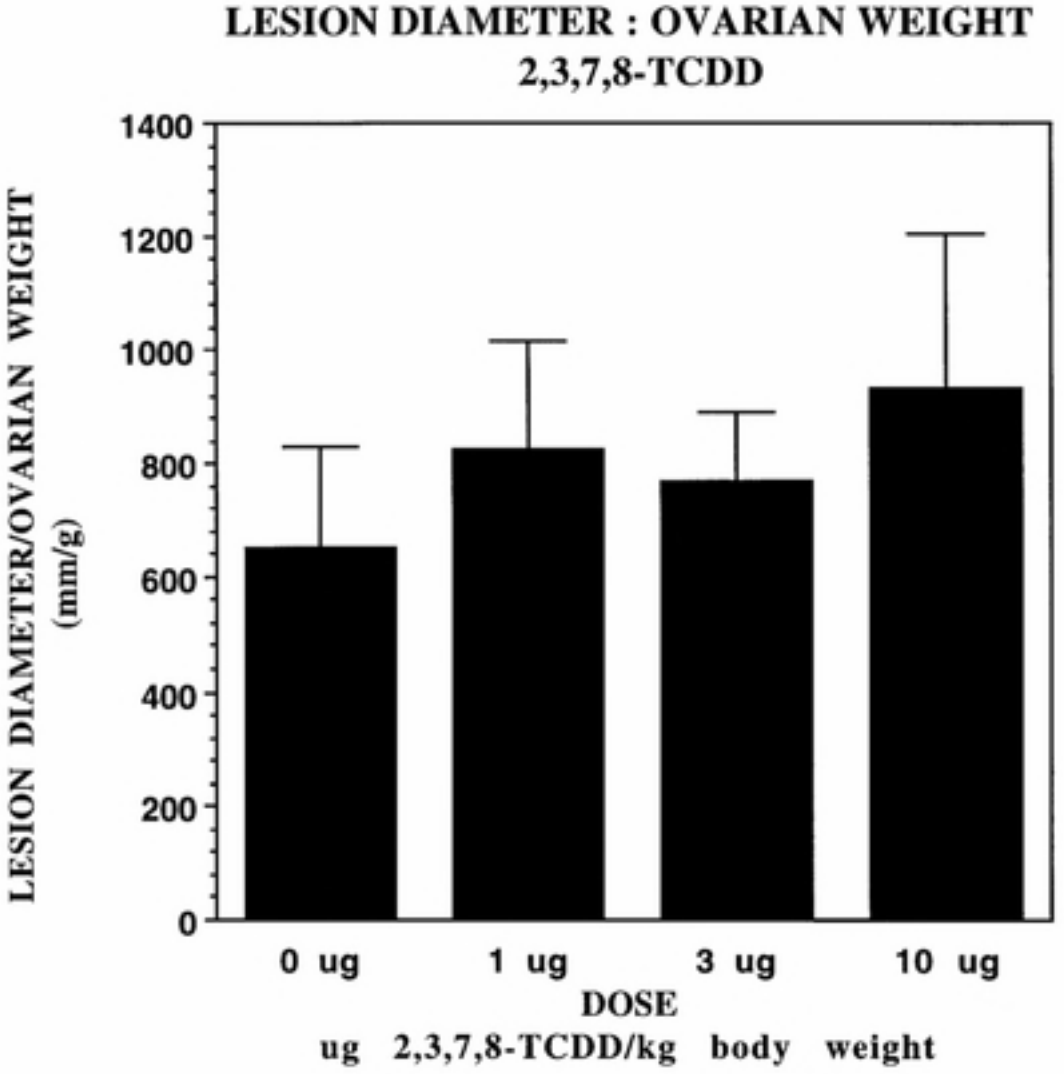

LESION DIAMETER : OVARIAN WEIGHT
2,3,7,8-TCDD
LESION DIAMETER/OVARIAN WEIGHT
(mm/g)
1400
1200
1000
800
600
400
200
0
0 ug
1 ug
3 ug
10 ug
DOSE
ug 2,3,7,8-TCDD/kg body weight

Figure 3.2

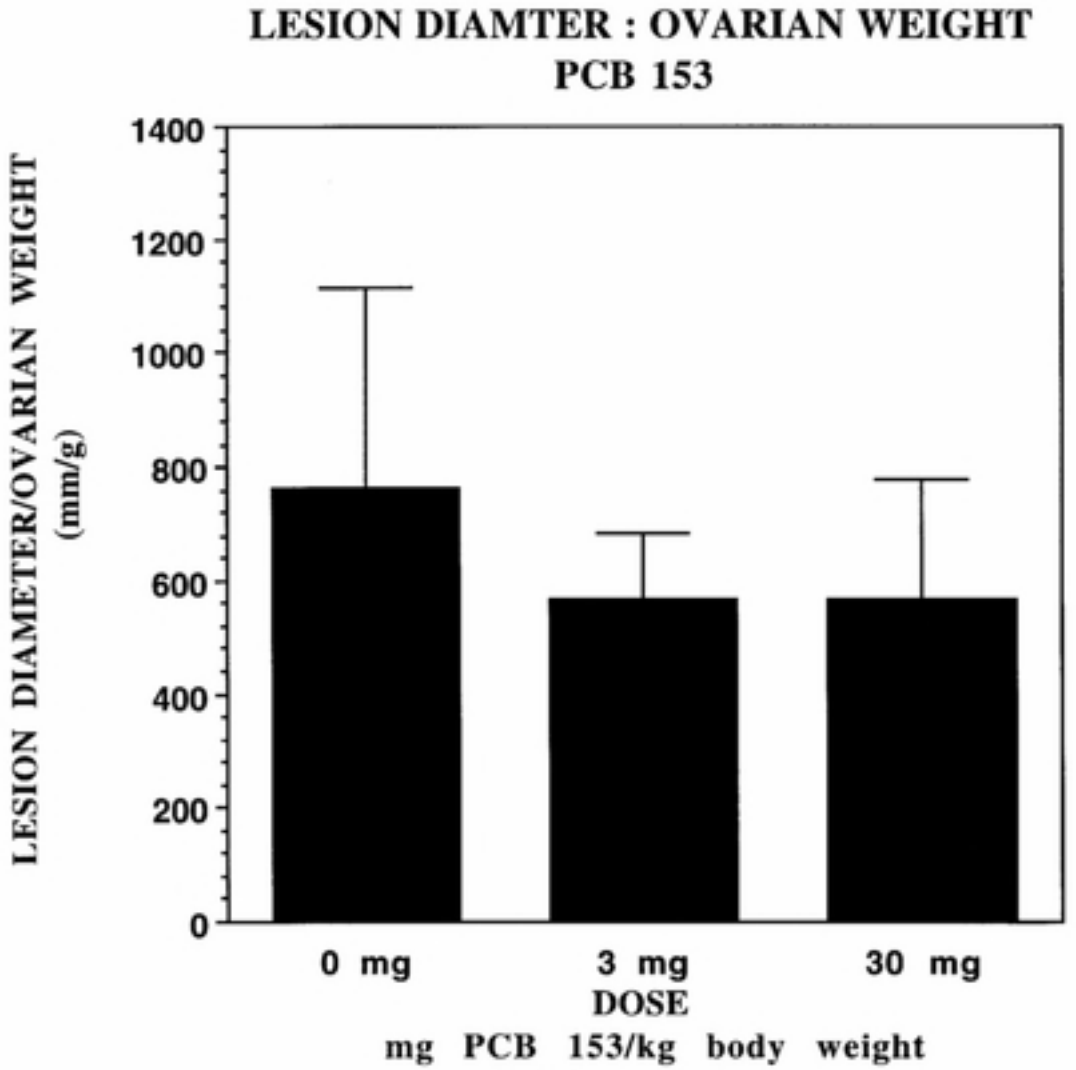

LESION DIAMTER : OVARIAN WEIGHT
PCB 153
LESION DIAMETER/OVARIAN WEIGHT (mm/g)
1400
1200
1000
800
600
400
200
0
0 mg
3 mg
30 mg
DOSE
mg PCB 153/kg body weight

Figure 3.3

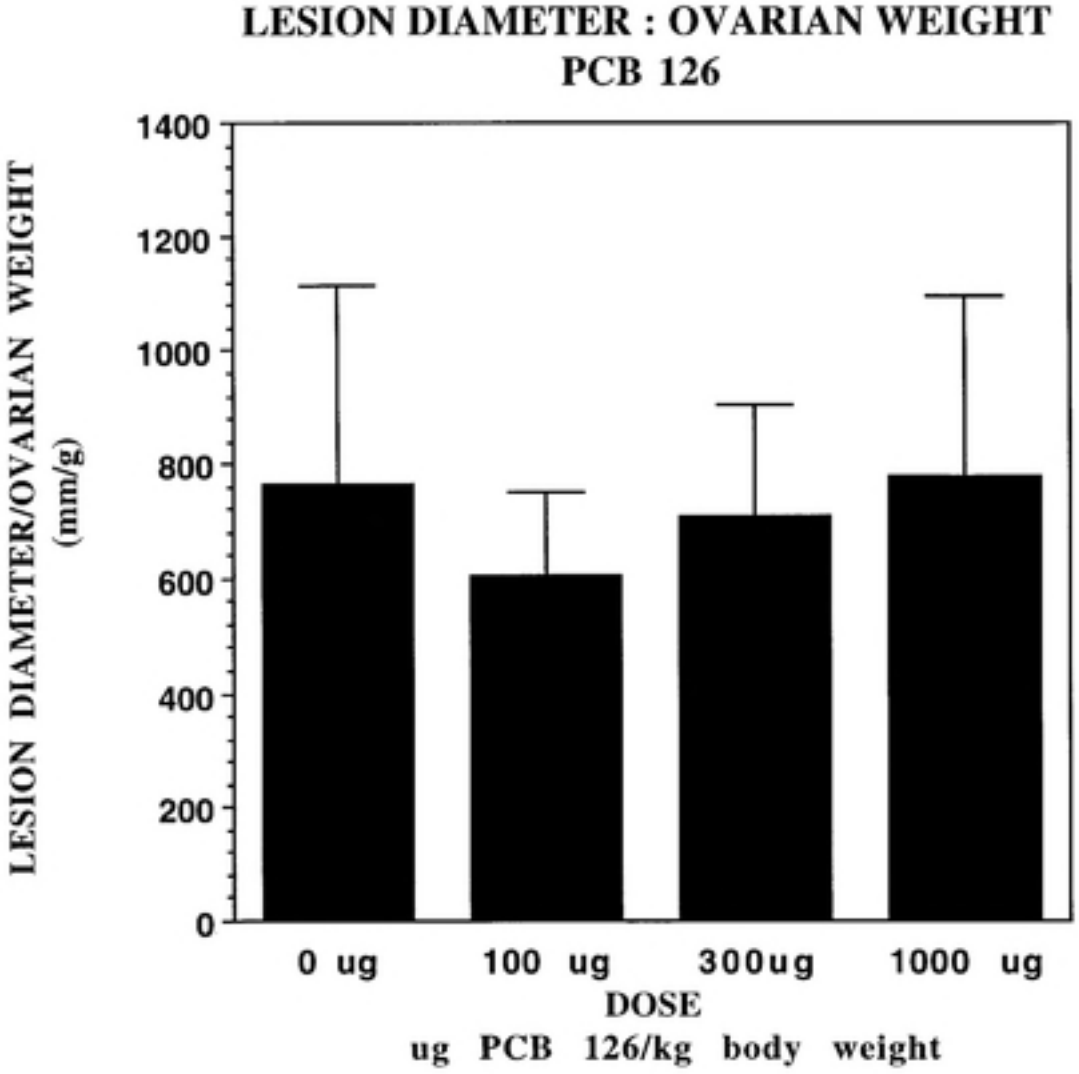

LESION DIAMETER : OVARIAN WEIGHT
PCB 126
LESION DIAMETER/OVARIAN WEIGHT
(mm/g)
1400
1200
1000
800
600
400
200
0
0 ug
100 ug
300ug
1000 ug
DOSE
ug PCB 126/kg body weight

Figure 3.4

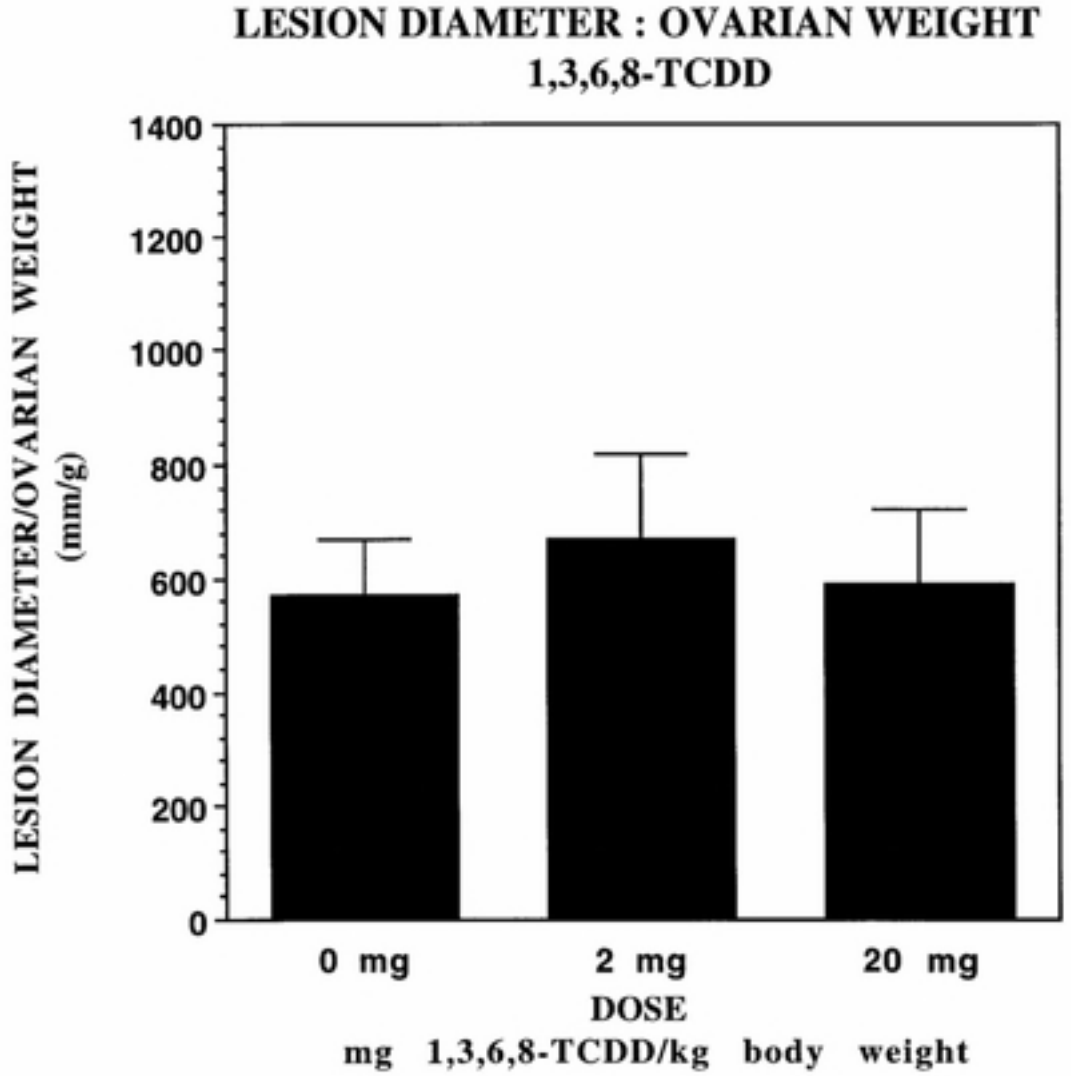
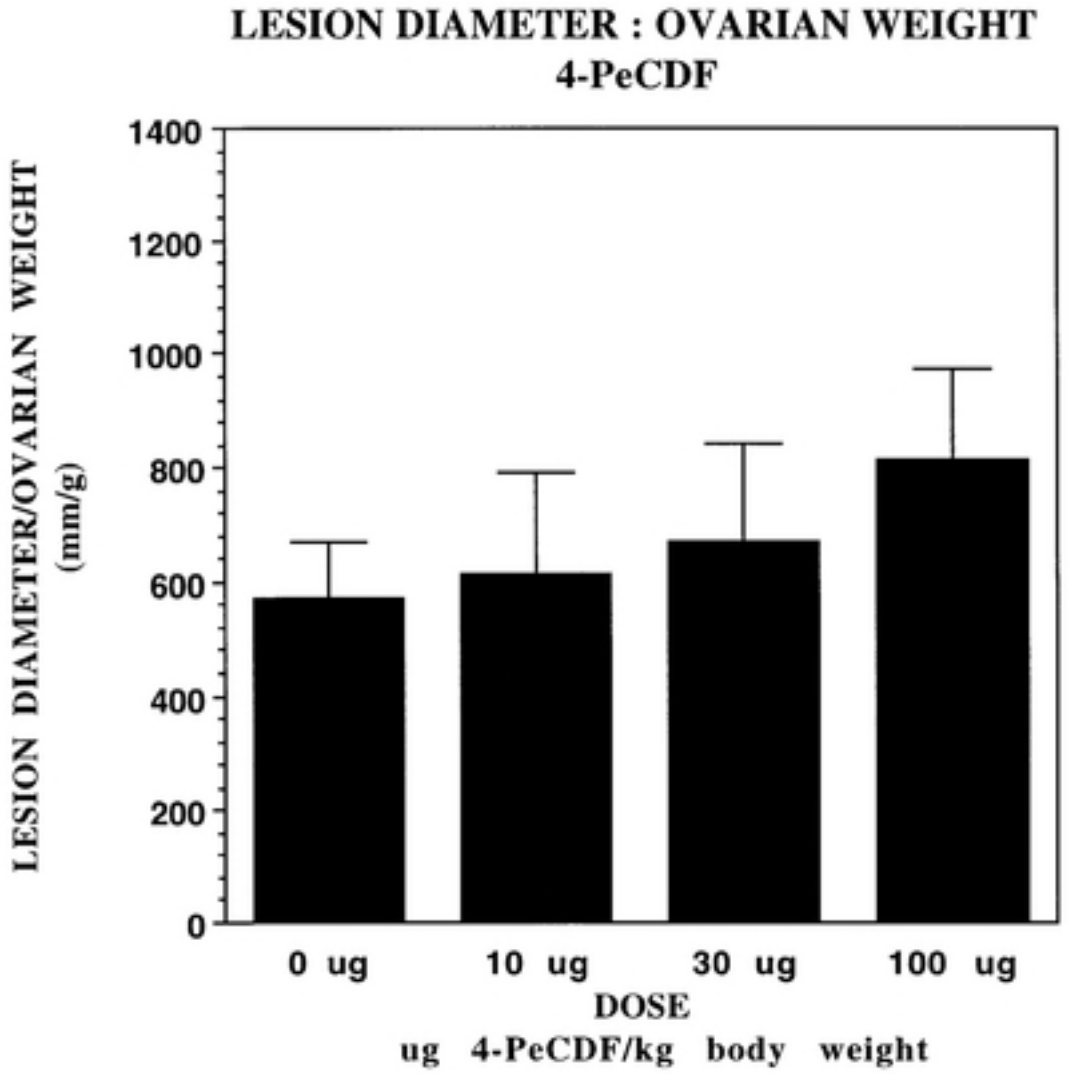

LESION DIAMETER : OVARIAN WEIGHT
1,3,6,8-TCDD
LESION DIAMETER/OVARIAN WEIGHT (mm/g)
1400
1200
1000
800
600
400
200
0
0 mg
2 mg
20 mg
DOSE
mg 1,3,6,8-TCDD/kg body weight

Figure 3.5


LESION DIAMETER : OVARIAN WEIGHT
4-PeCDF
LESION DIAMETER/OVARIAN WEIGHT
(mm/g)
1400
1200
1000
800
600
400
200
0
0 ug
10 ug
30 ug
100 ug
DOSE
ug 4-PeCDF/kg body weight

3.4 Uterine Weight

Intact uterine horns were weighed to analyze the effects of HAHs on uterine weight and to obtain additional information about estrous cycle stage, in the event results were inconsistent across lesion diameter and ovarian weight (Table 3.4). Uterine weights of all treated animals were not statistically different from values of control animals by the Dunnett's Test and analysis of uterine weights for three separate control experiments were similar. There was an apparent increase in uterine weights of female B6C3F1 mice treated with 3 ug 2,3,7,8-TCDD/kg body weight, which was followed by an apparent decrease in uterine weights in the highest dose group, 10 ug 2,3,7,8-TCDD/kg body weight. These effects on uterine weights were similar to those observed in animals treated with PCB 126. A trend towards a decrease in uterine weights with increasing dose of 4-PeCDF was observed (Table 3.4). Treatment of animals with all doses of PCB 153 resulted in a slight, non-significant increase in uterine weights as compared to controls. However, all animals treated with 1,3,6,8-TCDD had similar uterine weights as compared to controls.

Table 3.4 Uterine Weight

CHEMICAL	DOSE	MEAN UTERINE HORN WEIGHT (intact uterine horn) (grams)	STANDARD DEVIATION OF MEAN UTERINE WEIGHT
	0	0.0690	0.0164
2,3,7,8-TCDD	1 ug/kg	0.0602	0.0203
	3 ug/kg	0.0741	0.0292
	10 ug/kg	0.0584	0.0203
	0	0.0500	0.0234
PCB 153	3 mg/kg	0.0574	0.0192
	30 mg/kg	0.0580	0.0199
PCB 126	100 ug/kg	0.0488	0.0075
	300 ug/kg	0.0579	0.0154
	1000 ug/kg	0.0529	0.0105
	0	0.0610	0.0169
1,3,6,8-TCDD	2 mg/kg	0.0528	0.0138
	20 mg/kg	0.0609	0.0155
4-PeCDF	10 ug/kg	0.0630	0.0154
	30 ug/kg	0.0598	0.0285
	100 ug/kg	0.0519	0.0227

3.5 Thymus Weight

Previous studies have shown that the thymus is a target organ for 2,3,7,8-TCDD and related compounds (Birnbaum, 1994). Therefore, the effect of 2,3,7,8-TCDD and related compounds on thymus weights in mice with endometriosis was determined (Table 3.5). Analysis of thymus weights from three separate control groups were similar. Treatment of mice with 1 or 3 ug 2,3,7,8-TCDD/kg body weight resulted in a trend toward increased thymus weights; while animals dosed with 10 ug 2,3,7,8-TCDD/kg body weight had thymus weights no different from controls. Animals treated with PCB 153 or 1,3,6,8-TCDD showed an apparent increase in thymus weight with increasing dose. In contrast, a dose-dependent decrease in thymus weights with increasing dose of 4-PeCDF or PCB 126 was observed (Table 3.4). Thymus weights of animals dosed with 30 or 100 ug 4-PeCDF/kg body weight were significantly less than control thymus weights ($p<0.05$).

Table 3.5 Thymus Weight

CHEMICAL	DOSE	MEAN THYMUS WEIGHT (grams)	STANDARD DEVIATION OF THYMUS WEIGHTS
	0	0.0308	0.0119
2,3,7,8-TCDD	1 ug/kg	0.0345	0.0144
	3 ug/kg	0.0478	0.0223
	10 ug/kg	0.0311	0.0132
	0	0.0331	0.0038
PCB 153	3 mg/kg	0.0348	0.0095
	30 mg/kg	0.0424	0.0113
PCB 126	100 ug/kg	0.0426	0.0116
	300 ug/kg	0.0312	0.0122
	1000 ug/kg	0.0279	0.0081
	0	0.0355	0.0053
1,3,6,8-TCDD	2 mg/kg	0.0386	0.0090
	20 mg/kg	0.0405	0.0067
4-PeCDF	10 ug/kg	0.0364	0.0069
	30 ug/kg	0.0268*	0.0075
	100 ug/kg	0.0260*	0.0055

*Statistically different ($p<0.05$) from control animals as determined by the Dunnett's Test

3.6 Liver Weight

Many studies have used the liver as a target organ to study the toxic and biological effects of 2,3,7,8-TCDD and related congeners (DeVito and Birnbaum, 1994). Therefore, the dose-dependent effects of 2,3,7,8-TCDD and structural analogs on liver weights in female B6C3F1 mice with endometriosis was also addressed in this study. Because body weights varied little among animals, data are expressed as crude liver weights with standard deviations (Table 3.6).

Treatment of animals with 2,3,7,8-TCDD, PCB 126, or 4-PeCDF resulted in an increase in liver weight with increasing dose (Table 3.6). In contrast, exposure of animals with PCB 153 or 1,3,6,8-TCDD resulted in similar liver weight in all dose groups compared to controls.

Table 3.6 Liver Weight

CHEMICAL	DOSE	MEAN LIVER WEIGHTS	STANDARD DEVIATION OF LIVER WEIGHT
	0	1.247	0.107
2,3,7,8-TCDD	1 ug/kg	1.359	0.148
	3 ug/kg	1.323	0.115
	10 ug/kg	1.581	0.159
	0	1.238	0.140
PCB 153	3 mg/kg	1.291	0.166
	30 mg/kg	1.306	0.146
PCB 126	100 ug/kg	1.410	0.160
	300 ug/kg	1.367	0.151
	1000 ug/kg	1.521	0.128
	0	1.329	0.081
1,3,6,8-TCDD	2 mg/kg	1.197	0.087
	20 mg/kg	1.300	0.182
4-PeCDF	10 ug/kg	1.359	0.131
	30 ug/kg	1.440	0.310
	100 ug/kg	1.467	0.211

3.7 Ethoxyresorufin *O*-Deethylase (EROD) Activity

EROD activity is a marker for CYP1A1-dependent enzyme induction by 2,3,7,8-TCDD and related compounds, while CYP1A1 induction is often used as a surrogate for Ah receptor activation (DeVito and Birnbaum, 1995). Therefore, the dose-dependent effect of 2,3,7,8-TCDD and related compounds on EROD activity in the liver of female B6C3F1 mice with endometriosis was measured. Table 3.7 shows activity, expressed as pmoles/min/mg protein, in mice treated with various concentrations of 2,3,7,8-TCDD, PCB 153, PCB 126, 1,3,6,8-TCDD, or 4-PeCDF.

Constitutive EROD activity in control animals for three separate experiments were relatively similar. A dose-dependent increase in EROD activity in mice treated with increasing doses of 2,3,7,8-TCDD, PCB 126, or 4-PeCDF was observed (Table 3.7). The highest EROD activity was observed in animals treated with 10 ug 2,3,7,8-TCDD/kg, 1000 ug PCB 126/kg, or 100 ug 4-PeCDF/kg body weight. Animals treated with 3 ug 2,3,7,8-TCDD/kg body weight exhibited similar EROD activity to animals treated with 10 ug 4-PeCDF/kg body weight. In addition, treatment of mice with 10 ug 2,3,7,8-TCDD/kg body weight resulted in similar EROD activity as mice treated with 100 ug PCB 126/kg body weight. EROD activities were also similar between animals treated with 300 ug PCB 126/kg body weight and those treated with 100 ug 4-PeCDF/kg body weight. In contrast, mice treated with 1,3,6,8-TCDD or PCB 153 had EROD activities similar to control groups at all doses tested.

With a model developed by van Birgelen *et al.* (1996), EROD activity values for animals dosed with 2,3,7,8-TCDD were used to estimate liver concentration in ng/g liver. Control animals had an estimated liver concentration of less than 0.04. Animals treated with 1, 3, or 10 ug 2,3,7,8-TCDD/kg had estimated liver concentrations of 0.09, 0.50, and 0.6 respectively. This model has only been developed for 2,3,7,8-TCDD and can not yet be used accurately to predict liver concentrations of additional chemicals based on EROD data.

Body burdens were also estimated for animals treated with 2,3,7,8-TCDD by the equations: $t_{1/2} = 0.693/K$ and $ab_t = ab_a\, e^{(-kt)}$ (Casarett and Doull, 1991). K represents the elimination rate constant, while ab_t represents the dose remaining at time (t) and ab_a represents the initial dose. Estimated tumor promoting body burdens of animals dosed with 1, 3, or 10 ug/kg body weight were 0.2295, 0.6885, and 2.2951 ug respectively. Body burdens were not estimated for the additional chemicals because established half-life values were not readily available.

Table 3.7 Ethoxyresorufin *O*-Deethylase Activity

CHEMICAL	DOSE	MEAN EROD ACTIVITY pmoles/min/ mg protein	STANDARD DEVIATION OF MEAN EROD ACTIVITY
	0	114.35	40.23
2,3,7,8-TCDD	1 ug/kg	201.15	99.57
	3 ug/kg	657.30	377.93
	10 ug/kg	843.83	386.37
	0	71.48	27.44
PCB 153	3 mg/kg	115.62	51.00
	30 mg/kg	90.90	31.76
PCB 126	100 ug/kg	804.73	408.38
	300 ug/kg	1575.81	420.38
	1000 ug/kg	1826.85	438.79
	0	110.24	33.85
1,3,6,8-TCDD	2 mg/kg	83.77	23.21
	20 mg/kg	159.25	48.94
4-PeCDF	10 ug/kg	675.69	292.92
	30 ug/kg	1302.80	526.85
	100 ug/kg	1515.80	782.06

3.8 Histopathology

A microscopic examination of endometriotic lesions and ovarian tissue was performed to characterize histological changes associated with exposure to 2,3,7,8-TCDD, PCB 153, PCB 126, 1,3,6,8,-TCDD, or 4-PeCDF. In all endometrial lesions examined, endometrial epithelium, endometrial glands with stroma, and the myometrium were present, but these structures varied in thickness and prominence. Significant alterations in these three structures due to exposure to any of the chemicals were not apparent. The presence of luminal exudate or transudate was more severe in lesions characterized as "abnormal" than those characterized as "normal," but no significant dose-dependent distribution of severity was noticed. Incidence rates of abnormal lesions appeared to be consistent across dose groups and chemical classes. Inflammation of the endometriotic uterine segments was consistent across all animals and in most instances appeared acute (associated with polymorphonuclear cells.) Examination of ovarian tissue revealed the absence of active corpora lutea in several animals from the groups which received 10 ug 2,3,7,8-TCDD, 100 ug PCB 126, or 1000 ug PCB 126/kg body weight.

CHAPTER IV

DISCUSSION

4.1 Endometriotic Lesion Diameter and Weight

Previous work involving exposure of rhesus monkeys to 2,3,7,8-TCDD showed a dose-dependent increase in endometriotic incidence and severity (Rier *et al.*, 1993). However, because primate research proves both costly and of extensive duration, rodent models have been employed to assess the influence of 2,3,7,8-TCDD on the promotion of endometriosis (Cummings *et al.*, 1996). In one study, rats and mice were treated with 0, 3, or 10 ug 2,3,7,8-TCDD/kg body weight 21 days prior to induction surgery, at the time of surgery, and at 3, 6, and 9 weeks following surgery. Analysis at 3, 6, 9, and 12 weeks postsurgery revealed a time and dose-dependent increase in endometriotic lesion diameter in rats. Decreased ovarian weight was also observed in animals treated with the highest dose at 9 and 12 weeks, with histological support of ovulatory arrest at 12 weeks. In rats, thymic atrophy, body weight reduction, and liver weight increases were apparent in a dose-dependent fashion.

Also in this previous study, mice treated with 2,3,7,8-TCDD also exhibited an increase in lesion diameter at 9 and 12 weeks (Cummings *et al.*, 1996). Decreases in ovarian weight and body

weight were not observed. However, thymic atrophy and liver weight increases occurred related to time and dose (Cummings *et al.*, 1996). Because mice showed a greater degree of endometriotic severity, appeared to lack overt endocrine disruption, and demonstrate greater immunosensitivity (Hanson and Smialowicz, 1994) following exposure to 2,3,7,8-TCDD, the mouse model was chosen for future work assessing the effects of HAHs on endometriotic proliferation.

In this study, the effects of 2,3,7,8-TCDD, PCB 126, PCB 153, 1,3,6,8-TCDD, and 4-PeCDF on endometriotic lesion diameter were assessed (Table 3.1). Exposure to 2,3,7,8-TCDD, PCB 126, or 4-PeCDF caused an increase in lesion diameter, while exposure to 1,3,6,8-TCDD or PCB 153 caused no apparent change in lesion size. The mechanism by which chemicals, such as 2,3,7,8-TCDD, increase lesion diameter is unknown, but may require binding to the Ah receptor (Table 4.1), as for all other well-studies effects induced by 2,3,7,8-TCDD. Structure binding relationships (SBRs) are based on affinity of ligand binding for the Ah receptor and are often indirectly assessed by induction of AHH, EROD, and other enzyme activities, which are described by structure activity relationships (SARs). SBRs for HAHs are associated with three dimensional structure, planarity, size, and lateral halogenation. Previous studies have shown a correlation between endpoints, such as CYP1A1 induction, and affinity for the Ah receptor, expressed through SARs (Safe, 1986). For example, in this study, 2,3,7,8-TCDD, PCB 126, and 4-PeCDF, the congeners which caused increased lesion diameters, have the strongest binding affinities for the Ah receptor (Table 4.1). In contrast, compounds,

such as 1,3,6,8-TCDD and PCB 153, which did not induce increases in lesion diameter, have a weak affinity towards the Ah receptor.

Endometriotic lesion weights were also measured for secondary analysis of changes in endometriotic lesion sizes due to HAH exposure (Table 3.2). Increases in lesion weight correlate with the increases in lesion diameter. Again, 2,3,7,8-TCDD, PCB 126, and 4-PeCDF caused increases in lesion weights, while PCB 153 and 1,3,6,8-TCDD caused no apparent changes when compared to control animals. Because increases in lesion weight occur in animals exposed to chemicals with the highest affinities for the Ah receptor and not in animals exposed to chemicals with significantly lower binding affinities, promotion of endometriosis by HAHs appears to be Ah receptor-mediated.

Table 4.1 Binding Affinities to the Ah Receptor

Compound	EC_{50} (nM)
2,3,7,8-TCDD	10[a]
PCB 126	120[b]
PCB 153	79000[c]
1,3,6,8-TCDD	1800[d]
4-PeCDF	15[b]

a) (Mason *et al.*, 1986)

b) (Safe, 1990)

c) (Kafafi *et al.*, 1992)

d) (Bandiera *et al.*, 1983)

4.2 Ovarian Weight

Ovarian atrophy was identified in Sprague-Dawley rats in studies involving the effects of 2,3,7,8-TCDD on estrous cyclicity and ovulation (Li *et al.*, 1995). Previous studies assessing the influence of 2,3,7,8-TCDD on endometriosis also identified changes in ovarian weights in Sprague-Dawley rats treated with 3 or 10 ug 2,3,7,8-TCDD/kg, but not in B6C3F1 mice (Cummings *et al.*, 1996). The hypothesis of ovarian atrophy in 45% of treated rats at 9 and 12 weeks was supported by observed decreases in ovarian weights, persistent vaginal estrus, and histological evaluation. However, subsequent evaluations did not reveal ovulatory arrest in treated mice.

In contrast, this study in mice revealed changes in ovarian weight and differences in ovarian histopathological evaluation based on chemical and dose (Table 3.3). Decreases in ovarian weight with increasing dose were apparent in animals exposed to 2,3,7,8-TCDD, PCB 126, or 4-PeCDF. Animals exposed to PCB 153 exhibited an increase in ovarian weight, while animals exposed to 1,3,6,8-TCDD had ovarian weights comparable to controls. As with other endpoints, chemicals with greater binding affinities for the Ah receptor evoke greater toxic responses, such as decreases in ovarian weights.

Histological examination of animals treated with 1 or 3 ug 2,3,7,8-TCDD/kg body weight, PCB 153, 1,3,6,8-TCDD, or 10 or 30 ug 4-PeCDF/kg body weight was consistent with ovarian weights for these animals, indicating an absence of ovarian atrophy. The absence

of corpora lutea in animals treated with 10 ug 2,3,7,8-TCDD, and 100 or 1000 ug PCB 126/kg body weight, observed during histopathological examination, supports the hypothesis that ovarian atrophy occurred in animals treated with high doses of chemicals with strong affinities for the Ah receptor. The absence of detection of ovarian atrophy by histopathological examination in animals dosed with the middle dose administered of PCB 126 (300 ug/kg body weight) or the highest dose of 4-PeCDF administered (100 ug/kg body weight) may be due to limitations in histological procedures which provide a single cross-sectional view of the ovary for analysis, not a stepwise, progressive view of the entire ovary. For these reasons, conclusions about ovarian atrophy based entirely on histopathological evaluation are difficult to uphold. However, these data are sufficient for support of conclusions about ovarian activity based on ovarian weights. Another possible explanation for the lack of ovarian atrophy observed in the 4-PeCDF treated animals may be due to the low concentration of 4-PeCDF available to extrahepatic tissues due to its sequestration in the liver (Brewster, 1987).

Previous work showed antiestrogenic effects of 2,3,7,8-TCDD in rats (Safe *et al.*, 1991) and immature mice (DeVito *et al.*, 1992), possibly via downregulation of the estrogen receptor (Safe *et al.*, 1991). An ensuing effect of this estrogen receptor downregulation and interrupted nuclear binding of estradiol may be desensitization of the hypothalamic-pituitary axis to endogenous estrogen causing disruption of cyclic luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion (Cummings *et al.*, 1996). Observable results from this chain of effects may be (1) ovulatory arrest, (2) decreased

ovarian weight, (3) imbalance in serum estradiol and progesterone and (4) persistent vaginal estrus. Serum estradiol and progesterone levels were not measured in this study and vaginal estrus can not be routinely measured in mice as the procedure often results in the induction of pseudopregnancy. Therefore, histopathological evidence of ovulatory arrest and decreased ovarian weights were used as indicators of this phenomenon.

The hypothesis of the occurrence of ovarian atrophy at high doses of 2,3,7,8-TCDD and PCB 126 is consistent with the results of decreased lesion diameters and weights observed in animals administered high doses of these compounds compared to the resulting lesion diameters and weights in animals administered the middle doses. Potential antiestrogenic effects of HAHs administered at high doses could cause ovarian atrophy and resulting decreased endometriosis because ovarian estrogens may support lesion growth (Cummings and Metcalf, 1995b). Thus, Ah receptor binding may correlate with ovarian atrophy, lesion diameter and lesion weight. However, if ovarian atrophy varied only with Ah binding affinity, a greater antiestrogenic effect should be apparent at 100 ug 4-PeCDF/kg body weight. In this study, a slight decrease in ovarian weight with increasing dose is observed only in the right ovary and not the left for animals dosed with 4-PeCDF. This variability may account for the discrepancy between ovarian atrophy and Ah receptor binding affinity. Also, 4-PeCDF may not be as available to extrahepatic tissues as the other chemicals used in this study due to its sequestration in the liver.

4.3 Uterine, Thymus, and Liver Weights

HAHs, such as 2,3,7,8-TCDD, cause a wide array of toxic and biochemical effects in laboratory animals and mammalian cultured cells (Birnbaum, 1994). Therefore, several additional endpoints were measured in this study to assess the effects of HAH exposure on reproductive toxicity, immunotoxicity, and hepatotoxicity.

One observed response of HAH exposure is an antiestrogenic effect, possibly mediated by either a decrease in the concentration of circulating estrogens or a decrease in the levels of estrogen receptors (DeVito and Birnbaum, 1994). One potential indicator of antiestrogenic responses in rats and mice may be decreases in uterine weights following exposure to HAHs. Such effects may involve decreases in uterine estrogen receptors (DeVito *et al.*, 1992). Analysis of uterine weights in this study revealed a correlation between changes in uterine weights and binding affinities for the Ah receptor. Chemicals which bind strongly to the Ah receptor (2,3,7,8-TCDD, PCB 126, and 4-PeCDF) caused decreases in uterine weights at high doses, while chemicals which fail to bind the Ah receptor (PCB 153 and 1,3,6,8-TCDD) evoked no apparent decreases in uterine weights when compared to controls (Table 3.4). Therefore, a relationship appears to exist between structure activity and binding relationships and antiestrogenic effects, such as decreases in uterine weights.

Another significant effect often associated with 2,3,7,8-TCDD exposure is thymic atrophy. This result is associated with lymphocyte depletion in the thymic cortex (Pohjanvirta and

Tuomisto, 1994), and also poses a threat to immunocompetence primarily in developing rodents, not in adults (Vos *et al.*, 1978). However, immunomodulation can occur at doses below those causing thymic atrophy. Previous studies have shown that thymic atrophy induced by 2,3,7,8-TCDD and related compounds is Ah receptor-mediated (Safe, 1986). Analysis of thymic atrophy in this study revealed decreases in thymus weights at high doses of chemicals with high binding affinities for the Ah receptor (2,3,7,8-TCDD, PCB 126, and 4-PeCDF) and increases in thymus weights in animals dosed with chemicals which do not bind the Ah receptor readily (PCB 153 and 1,3,6,8-TCDD) (Table 3.5). However, for animals dosed with the highest dose of 2,3,7,8-TCDD, 10 ug/kg body weight, the thymus weights decreased from the middle dose but were comparable to control values. Therefore, the mechanism of thymic atrophy correlates with structure binding relationships of HAHs observed in mice with endometriosis.

Hepatotoxicity is a common response in several animals species following exposure to HAHs. Symptoms of hepatotoxicity often involve hypertrophy and hyperplasia of parenchymal cells which may cause hepatomegaly (DeVito and Birnbaum, 1994). Increased liver weights are often indicative of hepatotoxicity (Pohjanvirta and Tuomisto, 1994), and therefore were measured in this study. Increased liver weights with increasing dose were apparent in animals dosed with 2,3,7,8-TCDD, PCB 126, and 4-PeCDF, the chemicals which bind with great affinity to the Ah receptor. However, animals dosed with PCB 153 and 1,3,6,8-TCDD, the chemicals which do not readily bind the Ah receptor, showed no

increase in liver weights at any dose used when compared to controls (Table 3.6). Therefore, increases in liver weight and hepatotoxicity correlate with binding affinities for the Ah receptor. These data suggest that structure-activity relationships for Ah receptor-mediated responses are apparent in mice with HAH-induced endometriosis.

4.4 Ethoxyresorufin *O*-Deethylase Activity

HAHs have been identified as microsomal monooxygenase inducers (Whitlock, 1990) and frequently induce cytochrome P450 isozymes (Poland and Glover, 1974). CYP 450 enzyme induction is often measured by AHH activity or EROD activity in the laboratory. Increased enzyme activity coincides with increased gene expression, such as CYP1A1 gene expression, and may correlate with HAH exposure (Goldstein *et al.*, 1984). Therefore, EROD activity was measured in this study as an indicator of HAH-induced effects (Table 3.7).

EROD activity can also be used as a basis for estimation of relative chemical potencies. Toxic Equivalency Factors (TEFs) are estimates of the relative chemical potencies for compounds for which little toxicity and mechanistic data are available, as compared to chemicals for which the degree and mechanism of toxicity is well-defined, such as 2,3,7,8-TCDD (Eadon *et al.*, 1986). 2,3,7,8-TCDD is used as the prototype for dioxins and is often assigned a TEF value of 1.0 (Birnbaum and DeVito, 1995). The TEF values assigned to the other chemicals used in this study are presented in Table 4.2.

Table 4.2 Toxic Equivalency Factors

Congener	TEF Values
2,3,7,8-TCDD	1.0[a]
PCB 153	0.00002[b]
PCB 126	0.1[a]
1,3,6,8-TCDD	0.0006[c]
4-PeCDF	0.5[a]

a) (Birnbaum and DeVito, 1995)

b) (Safe, 1990)

c) estimated by Ah binding affinities (Kafafi *et al.*, 1992)

Analysis of EROD activity in this study showed similar enzyme induction in animals dosed with 3 ug 2,3,7,8-TCDD/kg body weight and animals dosed with 10 ug 4-PeCDF/kg body weight (Table 3.7). Based on this observation, the relative potencies for 2,3,7,8-TCDD and 4-PeCDF would be 1:3.33, values which closely correlate with the assigned TEFs (Table 4.2). Similarities in enzyme activity were also apparent between animals dosed with 10 ug 2,3,7,8-TCDD/kg body weight and animals dosed with 100 ug PCB 126/kg body weight (Table 3.7). Relative potencies for 2,3,7,8-TCDD and PCB 126 based on EROD activity would be 1:10. These estimates directly reflect the assigned TEF values for these chemicals (Table 4.2). A final similarity was evident in animals dosed with 300 ug PCB 126/kg body weight and animals dosed with 100 ug 4-PeCDF/kg body weight. Relative potencies obtained from this observation would be 3:1 and closely correlate with assigned TEF values. Finally, the greatest induction of EROD activity occurred in animals dosed with 1000 ug PCB 126/kg body weight (Table 3.7). This value is not comparable with enzyme activities of any dose groups of any other chemicals. Based on the assigned TEF values (Table 4.2), a dose of 1000 ug PCB 126/kg body weight would induce enzyme activity similar to doses of 100 ug 2,3,7,8-TCDD/kg body weight or 200 ug 4-PeCDF, doses which were not administered in this study. Because relative potencies obtained from EROD activities in this study correlated with relative potencies based on accepted TEF values, the TEFs used to set the administered dose levels in this study in turn appeared to be reasonable predicters of endometriotic response.

4.5 Summary and Future Research

This study was an analysis of the influence of structure activity relationships of polyhalogenated aromatic hydrocarbons on the proliferation of surgically-induced endometriotic lesions in B6C3F1 female mice. Analysis of all the parameters measured, especially lesion diameter, suggests the mechanism of HAH-induced endometriosis is Ah receptor mediated with structure activity and structure binding relationships influencing the degree of endometriotic proliferation. EROD activity from this study allowed calculations of relative potencies which further supported the assigned TEF values. Therefore, this unusual dosing regimen was consistent with estimates of enzyme induction based on previously assigned TEF values.

Unlike enzyme activity, other parameters measured in this study, such as endometriotic lesion diameter, did not correlate exactly in a linear dose-response relationship because of influences of additional responses such as hormonal interactions and antiestrogenic effects. Chemicals exerting antiestrogenic effects at high doses induce ovarian atrophy, which in turn decrease circulating estrogen levels and proliferation of endometriosis. In general, the responses in lesion diameter, lesion weight, ovarian weight, uterine weight, and thymus weights correlate with structure binding relationships of the administered HAHs. Specifically, analysis of the primary endpoint measured, endometriotic lesion diameter, demonstrates a dose-dependent increase in size following administration of chemicals with strong binding affinities for the Ah

receptor, when the effects of ovarian atrophy on lesion diameter are controlled. Therefore, structure binding relationships obtained from this study correlate with previously assigned TEF values and determine the degree of proliferation of HAH-induced endometriosis.

Future projects should investigate the dose-response relationships with more doses per chemical, especially at dose levels in the low dose region, due to the presence of U-shape dose-response curves observed in these studies and the need to investigate the antiestrogenic effects on ovaries at continuous dose levels. Another project should determine the role of the Ah receptor in HAH-induced endometriosis in the Ah receptor knock-out mouse as a model (Fernandez-Salguero *et al.*, 1995). In addition, future projects should use larger animal numbers to decrease the variability observed in the parameters measured in these studies and to increase statistical power. Possible antiestrogenic effects of 2,3,7,8-TCDD and related compounds on HAH-induced endometriosis should also be studied by measuring serum estradiol and progesterone as a measure of antiestrogenicity. Finally, further work could investigate the individual effects of the immune system, chemical mixtures, prenatal exposure, and steady-state dosing on endometriosis.

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