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Ovarian toxicity of 4-phenylcyclohexene in female B6C3F1 mice

Parola, Lisa Contardi, M.S.
The University of Arizona, 1991
OVARIAN TOXICITY OF 4-PHENYLCYCLOHEXENE
IN FEMALE B6C3F1 MICE

by

Lisa Contardi Parola

A Thesis Submitted to the Faculty of the
DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY
In Partial Fulfillment of the Requirements
For the Degree of
MASTERS OF SCIENCE
WITH A MAJOR IN TOXICOLOGY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1991
STATEMENT BY AUTHOR

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APPROVAL BY THESIS DIRECTOR

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Pharmacology and Toxicology

Date
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ABSTRACT

4-Phenylcyclohexene (4PC), a contaminant released from latex adhesives used to produce carpet, is structurally similar to the ovarian toxicant, 4-vinylcyclohexene (VCH). VCH causes depletion of primary ovarian follicles in mice. Our aim was to determine if the related compound, 4PC, also causes ovarian damage. Groups of B6C3F1 mice were given either: sesame seed oil (vehicle control), 3 mmole/kg 4PC (4PC-3), 6 mmole/kg 4PC (4PC-6), or 6 mmole/kg VCH (positive control), ip, daily for 30 days. Vaginal smears were done daily to determine the stage of estrus. On day 31, the mice were killed and the ovaries were fixed and stained. The numbers of primary and secondary follicles were significantly reduced in the VCH treated mice, but not in 4PC treatment groups. The number of estrous cycles/30 days was significantly reduced in VCH and 4PC-6 treated groups. This study suggests that although the substitution of a phenyl group (4PC) for a vinyl substituaut (VCH) on the cyclohexene ring appears to eliminate the follicular loss, monitoring of the estrous cycles may be an early indication of an adverse reproductive effect for 4PC.
INTRODUCTION

Origin of 4-Phenylcyclohexene

4-Phenylcyclohexene (4PC) is a colorless liquid which has an odor that resembles that of new carpeting. Its physical properties are presented in Table I. The origin of 4PC in homes and offices has been identified by Crabb (1984) to be the latex adhesives employed in carpet backing. One such latex adhesive is carboxylated-styrene-butadiene (XSBR) polymer latex. This product is formed by the polymerization reaction of styrene and 1,3-butadiene and represents the primary latex (95%) used in tufted carpeting today (Meier, Monsanto Pamphlet). Koningsberger and Salomon (1946) investigated the polymerization of styrene and 1,3-butadiene and discovered that they form cyclic dimers as side products in addition to the latex. The dimers identified were 4-vinylcyclohexene (VCH), derived from the reaction of butadiene with itself, and 4PC, derived from the reaction of styrene and butadiene (Figure 1).

Table I. 4-Phenylcyclohexene: Physical Properties

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
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<tbody>
<tr>
<td>Molecular weight</td>
<td>158.3</td>
</tr>
<tr>
<td>Physical State</td>
<td>colorless liquid</td>
</tr>
<tr>
<td>Boiling Point</td>
<td>235°C</td>
</tr>
<tr>
<td>Specific Gravity</td>
<td>0.99 g/ml</td>
</tr>
<tr>
<td>Vapor Pressure</td>
<td>0.66 mmHg (Charkrabriti 1989)</td>
</tr>
</tbody>
</table>
Figure 1. Cyclic dimers, 4-phenylcyclohexene and 4-vinylcyclohexene, formed as a side product in latex manufacture.
Carpet Related Health Problems and Emissions

Initial interest in 4PC began at the University of Arizona, when Crabb (1984) investigated several health complaints which had been filed with various Arizona state health agencies. These reports demonstrated common ill effects among home owners and office workers after installation of new carpeting. Among the most prevalent complaints were eye, nose and throat irritation, headache, fatigue, and occasionally, nausea. In some cases, patients were unable to inhabit their homes or offices until the carpeting was removed.

Since 4PC was suspected to be the agent which had caused the reported symptoms, studies were done in both homes and apartments where new carpeting had been installed. Monitoring of indoor environments revealed air concentrations of 4PC in the range of 0.3 to 20 parts per billion (ppb) (Walsh, 1986). Subsequent studies were performed to examine why there was such a variation in the concentrations reported. These studies revealed that the indoor air concentration of 4PC decayed exponentially over time when installed in a home. Non-detectable levels of 4PC preceded carpet installation, whereas readily detectable concentrations appeared shortly after installation. The average concentration peaked 3 days after installation at 26 ppb, followed by 6 ppb levels after 30 days, and 1 ppb after 10 months (Vogelmann et al., 1988).
More recently, Hirzy and Morison (1989) reviewed an incident at the Waterside Mall facilities of the Environmental Protection Agency (EPA), Washington, D.C. after installation of new carpeting. The installation began in October of 1987 and by January 1988 several employees had complained of ill health with symptoms such as eye, nose and throat irritation, dizziness, fatigue, and nausea. The EPA then hired Dr. Mark Bradley, an occupational physician specializing in pulmonary and immune system disorders, to investigate employee health complaints. He concluded that "there was a strong indication that many employees had hypersensitivity pneumonitis, occupational asthma and irritant-intoxication syndromes". Monitoring of the indoor environment which began approximately 4 months after the installation of the carpeting demonstrated levels of 4PC in the low part per billion range. Hirzy and Morison's report did not conclude that 4PC was unequivocally the cause of the observed health effects. However, they did suggest that indoor air standards for 4PC should be established and that testing be required on finished latex and carpeting products in order to establish compliance with set standards.

Although 4PC has not been shown to exist as an occupational contaminant for carpet manufacture workers, it certainly has been shown to be a contaminant in the indoor environment where new carpeting has been installed. It has
been theorized that 4PC, a high boiling compound (235°C) (Buckingham, 1986) would tend to remain in the latex, while VCH, a low boiling compound (129.5°C), would readily volatilize and be eliminated from the latex during the carpet manufacturing process (Walsh, 1986). Therefore, carpet manufactured with styrene-butadiene latex would be contaminated with 4PC, which would subsequently be emitted into the environment upon installation of the new carpet.

Toxicology of 4-Phenylcyclohexene

The toxicity of 4PC was investigated in male Sprague-Dawley rats by Walsh (1986). Acute oral 24 hour studies revealed extensive gastrointestinal damage and body weight loss within 24 hours of the administration of a single 5000 mg/kg dose of 4PC. Those dosed with 500 mg/kg showed no adverse reactions. Additional studies done by Walsh (1986) included an eye irritation test in male New Zealand albino rabbits and a skin irritation test done in male Hartley guinea pigs. No toxic effects of 4PC were observed in either test by the methods and guidelines set forth in the Principles and Procedures for Evaluating the Toxicity of Household Substances (Committee for the Revision of National Academy of Sciences (NAS) Publication 1138, 1977).

Studies on 4PC supported by the Styrene-Butadiene Latex Manufacturers Council (SBLMC) have produced conclusions
similar to those previously mentioned. Dermal sensitization studies done in male, Hartley albino guinea pigs at concentrations of 25% or greater only produced a slight reddening of the skin with no allergic reactions (Mitzell, 1989). Further studies designed to investigate whether 4PC would irritate or injure the respiratory tract or other tissues was performed in an unspecified strain of rat. Both single (6 hours) and repeated exposure (9 exposures, 6 hours each, over a two week period) inhalation studies were performed using concentrations between 0 and 60 parts per million 4PC. No compound-related pathological changes were found in the single exposure study. Analysis of blood and urine, as well as microscopic examination of tissues from the repeated exposure study, suggested that 4PC did not cause any apparent injury in rats (SBLMC, 1989).

As mentioned earlier, 4-phenylcyclohexene and 4-vinylcyclohexene (VCH) are both side products of styrene-butadiene latex manufacture. Interestingly, 4PC and VCH are structurally very similar (Figure 1). These compounds share a common cyclohexene base and differ only by a vinyl group at the 4 position of VCH and a phenyl group at the 4 position of 4PC. Therefore, it is of interest to determine if these compounds also have similar toxicologic reactions in animals.
Toxicology of 4-Vinylcyclohexene

4-Vinylcyclohexene is a colorless liquid with a very pungent odor. It is commercially produced by the dimerization of 1,3-butadiene and is used primarily as an intermediate in the production of vinylcyclohexene diepoxide, a reactive diluent in epoxy resin manufacturing (IARC, 1976). In addition, VCH is present as a gaseous discharge during the production of synthetic rubber, especially as a result of curing rubber in tire manufacturing (Rappaport and Fraser, 1976). Workers are exposed by inhalation to VCH both in the rubber industry (Rappaport and Fraser, 1977) and in the production of epoxy resin (IARC, 1986).

The toxicity of VCH was investigated in both male and female B6C3F1 mice and Fisher 344 rats by the National Toxicology Program (NTP) (Collins and Manus, 1987; Collins et al., 1987). Acute (14 day) oral studies revealed no compound-related gross changes or histopathologic effects. However, most of the mice and all of the rats died when the dose was greater than 1250 mg/kg. Histopathologic effects observed in 13-week oral studies revealed hyaline droplet degeneration of the proximal tubule in male rats and a reduction in the number of primary follicles and mature follicles in the ovaries of female mice receiving 1,200 mg/kg of VCH. Although neoplastic and nonneoplastic lesions were observed in both rats and mice, the most significant finding in the two-year oral
carcinogenicity studies was the development of rare ovarian neoplasms in female mice receiving 200 or 400 mg/kg VCH. Furthermore, despite poor survival in dosed female rats, those surviving the entire two years had no apparent ovarian lesions (NTP, 1986).

Studies undertaken by Smith et al., (1990a) were done to determine if a species difference existed in the disposition of VCH between the rat and mice. Female Sprague-Dawley rats and B6C3F1 mice were treated with VCH at 800 mg/kg intraperitoneally. Analysis of blood for VCH metabolites demonstrated the appearance of VCH-1,2-expoxide in mice but not in rats. *In vitro* studies performed in rat and murine hepatic microsomes demonstrated that VCH was metabolized to VCH-1,2-epoxide, VCH-7,8-epoxide, and VCH diepoxide (VCD). These studies also demonstrated that VCH is more rapidly oxidized to VCH-1,2-epoxide in mice than in rats (Watabe et al., 1981, Gervasi et al., 1980 and Smith et al., 1990a). This led to the conclusion that the enhanced ability of the mouse to bioactivate VCH may be an important factor in the susceptibility of this species to VCH-induced ovarian tumors. Subsequent studies done by Smith et al., (1990b) demonstrate that the species difference in the incidence of VCH-induced ovarian tumors may be related to a species difference in the ability of VCH to destroy oocytes. In this study both VCH and VCH epoxides were considered since the potential for epoxide
metabolites were suspected to be greater than for the parent compound. To test this hypothesis, female Sprague-Dawley rats and B6C3F1 mice were treated with 7.4, 3.7 or 0.9 mmole/kg of VCH; 2.74, 1.37, or 0.34 mmole/kg VCH-1,2-epoxide; and 0.57, 0.29, or 0.07 mmole/kg of VCD for 30 days. 4-Vinylcyclohexene treatment destroyed primary follicles of mice with dose dependency, but produced no apparent changes in the oocyte numbers in the ovaries of the rats. However, in both species, the mono and diepoxide metabolites of VCH caused a reduction in the number of primary follicles. This work supports the hypothesis that VCH causes oocyte destruction and induces ovarian tumors in mice but not rats because of a difference in hepatic metabolism of VCH.

Female Reproduction

In females, normal reproductive function involves the appropriate interaction of the central nervous system, ovaries, fallopian tubes, uterus, and cervix. The ovary plays a pivotal role in this system because it performs two important functions; production of mature ova for fertilization and procreation of the species and production and secretion of ovarian hormones.

Follicular changes in the ovary

In humans, as well as in rodents, formation and proliferation of the oogonia, i.e., the ovarian germ cells,
occur during the fetal period. At the time of birth, the oogonia, containing the diploid numbers of chromosomes, divide mitotically and begin meiotic divisions, but become arrested at the diplotene stage. The resulting follicles are a resting pool of cells consisting of a small oocyte and several granulosa cells surrounded by a basement membrane. Following birth the follicles remain arrested in meiosis as primary follicles until puberty when a number of follicles start to grow during each ovarian cycle. As maturation occurs, the anterior pituitary gland begins to produce a gonadotropin, follicle stimulating hormone (FSH) and low levels of luteinizing hormone (LH). These hormones are essential for further follicular development and ultimately stimulate the secretion of estrogen by the growing follicle.

During each cycle, the growing follicle enlarges by the proliferation of granulosa cells and by the recruitment and proliferation of ovarian interstitial cells, which become thecal cells. The follicle is now considered a secondary or growing follicle. With continued proliferation of the granulosa cells there is accumulation of an acidic fluid which ultimately forces the granulosa cells apart to form a central, noncellular cavity, the antrum. The follicle has now become a preovulatory Graafian follicle. At this point many of the follicles regress and only a few complete their maturation and eventually ovulate (Jull, 1973).
Once ovulation (release of the oocyte) has occurred, the follicle becomes luteinized; i.e., the basement membrane breaks down and the granulosa cells differentiate into large luteal cells which form the corpus luteum (yellow body). In humans, the absence of conception and implantation cause the corpus luteum to atrophy and signals the end of a cycle. These changes which involve the pituitary-ovarian-uterine axis are collectively known as the menstrual cycle in the mature human female.

**Hormonal changes**

As mentioned, cellular changes in the ovary do not occur in the absence of gonadotropins and steroid hormones. Under the influence of follicle stimulating hormone (FSH), a follicle grows and begins to secrete estrogen. This produces a negative feedback effect on the hypothalamus and pituitary gland, decreasing FSH secretion. The continued increase of estrogen also stimulates the proliferation of the endometrium (lining of the uterus) and the release of luteinizing hormone (LH) which is followed by ovulation. It is at this point that the newly formed corpus luteum begins to secrete progesterone. Increasing levels of progesterone stimulate the endometrium to vascularize in preparation for implantation of a fertilized ovum. If implantation does not occur, the corpus luteum atrophies, progesterone levels decrease and the endometrial lining is sloughed off (Takizawa and Mattison, 1983).
Vaginal cytology

Clinically, an essentially non-invasive way to monitor the changes in the menstrual cycle is to sample the vaginal exfoliate cytology (vaginal smears). The vaginal cytology can be correlated with the ovarian cycle, and can permit recognition of follicular activity during normal menstrual phases, the effects of estrogenic and other therapies, or detection of regional pathologic or malignant conditions (Di Fiore, 1989).

In contrast to the human menstrual cycle, other mammalian species experience ovarian cycling. The rodent cycle, known as the estrous cycle, lasts approximately 4-6 days and can be correlated with circulating levels of ovarian steroid hormones and gonadotropin. It is characterized by a lack of functional corpus luteum and is divided into four distinct phases, proestrus, estrus, metestrus, and diestrus (Allen, 1922). Each of these phases, as in the human female, can be correlated with vaginal cytology (Whitten and Champlin, 1978).

The rodent proestrus often lasts less than a day and is associated with nucleated epithelial cells. These cells are similar to skin epithelial cells, however, they are under hormonal control. Estrus lasts one to two days and exhibits cornified epithelial cells. Cornified epithelial cells are irregularly shaped and no longer have nuclei. Metestrus lasts one to two days and consists of two phases; phase one
demonstrates fields of cornified epithelial cells which are aggregated, phase two consists of fields of cornified epithelial cells and leukocytes (primarily neutrophils). Diestrus lasts approximately one day and consists of a few nucleated epithelial cells, cornified epithelial cells, and a variable number of leukocytes (primarily neutrophils) (Allen, 1922) (Figure 2).

Reproductive Toxicology

Over the past decade, there has been increasing concern over the growing number of human reproductive disruptions, such as germ cell death and sterility. A great deal of the focus has been exposures to xenobiotic compounds such as drugs; biologic, occupational, and environmental contaminants; and physical exposures such as radiation. Unfortunately, all of these are by-products of the age in which we live. In humans, it is estimated that one in five couples are involuntarily sterile; over one third of early embryos die, and about fifteen percent of recognized pregnancies abort spontaneously (Dixon, 1986).

Reproduction is a complex process and as such the biological mechanisms underlying reproductive toxicity are complex as well. The mechanisms of reproductive toxicity have been divided into two categories, direct and indirect. The
Figure 2. **Vaginal cytology of the rodent estrous cycle.**
Each box represents the various phases of the mouse estrous cycle. The vaginal smears were stained with hematoxylin and eosin and examined by light microscopy, 396X.

a: Proestrus  
b: Estrus  
c: Metestrus  
d: Diestrus
direct compounds act either by virtue of structural similarity to an endogenous compound or because of chemical reactivity. Indirect toxicants require biologic modification i.e., enzyme induction or inhabition (Mattison and Thomford, 1989). Some of the variables which affect reproductive toxicity include species differences, gender differences, and/or the time frame during which toxicity occurs (Mattison and Thomford, 1989), in addition to the absorption, distribution, metabolism and excretion of the toxicant.

A growing number of women are exposed to environmental and/or occupational chemicals (Table II). These women have become targets of reproductive dysfunctions. One of the most popular human exposures to an ovotoxic agent is cigarette smoking (Mattison and Thorgeirsson, 1978). A dose-response relationship has been identified between the number of cigarettes smoked and the early onset of menopause (Jick et al., 1977). Among the components of cigarette smoke are a group of chemicals called the polycyclic aromatic hydrocarbons (PAHs). Several investigators have demonstrated that selected PAHs destroy oocytes, produce ovarian tumors, and decrease fertility (Mattison et al., 1983). Specifically, benz(a)pyrene, one of the most common PAHs, causes destruction of primordial oocytes in the mouse ovary (Mattison, 1980). Dobson and Felton (1983) have studied primordial oocyte destruction in mice by a wide variety
Table II. Environmental chemical exposure associated with reproductive dysfunction in women

<table>
<thead>
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<th>Chemicals</th>
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<tr>
<td>Anesthetic gases (operating room personnel)</td>
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<tr>
<td>Aniline</td>
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<tr>
<td>Benzene</td>
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<tr>
<td>Carbon disulfide</td>
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<tr>
<td>Chloroprene</td>
</tr>
<tr>
<td>Ethanol consumption</td>
</tr>
<tr>
<td>Ethylene oxide</td>
</tr>
<tr>
<td>Glycol ethers</td>
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<tr>
<td>Inorganic lead and other smelter emissions</td>
</tr>
<tr>
<td>Organic lead</td>
</tr>
<tr>
<td>Methylmercury</td>
</tr>
<tr>
<td>Pesticides (occupational exposure)</td>
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<tr>
<td>Phthalic acid esters (PAEs)</td>
</tr>
<tr>
<td>Polychlorinated biphenyls (PCBs)</td>
</tr>
<tr>
<td>Styrene</td>
</tr>
<tr>
<td>Tobacco smoking</td>
</tr>
<tr>
<td>Toluene</td>
</tr>
<tr>
<td>Vinyl chloride</td>
</tr>
</tbody>
</table>

* Taken from Dixon (1986).

of chemicals. Of the 77 chemicals tested for ovarian toxicity, 21 caused significant oocyte loss. An interesting finding in their study was that the 21 chemicals which were ovotoxic were also known mutagens or carcinogens (Table III).

The stage of development at which the oocyte is destroyed determines the impact the toxicant will have on reproduction. Chemicals which destroy only the growing or antral follicles will only temporarily interrupt reproduction, since these
Table III. Primordial oocyte killing in juvenile mice by chemicals of various classes

<table>
<thead>
<tr>
<th>Class of compound</th>
<th>Tested</th>
<th>Kill oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polycyclic aromatic hydrocarbons</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>Aromatic amines</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Alkyl halides</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Aryl halides</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Esters, epoxides, and carbamates</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Nitrrosamines</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Fungal toxins and antibiotics</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Metals</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Steroids, alcohols, and aldehydes</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Heterocyclics and nitrogen compounds</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Miscellaneous compounds</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>77</strong></td>
<td><strong>21</strong></td>
</tr>
</tbody>
</table>

*Data taken from Dobson and Felton (1983).*

Follicles can be replaced from the pool of primordial or resting follicles. However, of particular concern are chemicals which destroy the primary follicle, since humans as well as other mammalian species are born with a limited number of primary follicles in each ovary. After birth, many of these follicles die (atresia) and those which survive become reduced in number. It is important to recognize the fact that any agent which damages the small oocyte will accelerate the depletion of the oocyte pool and can lead to infertility in females.
Oocyte destruction by ovarian carcinogens

The mechanism(s) by which chemicals induce ovarian cancers are unknown. However, it has been speculated that the sequence of events is similar to that of other chemically induced cancers, i.e., initiation-promotion-progression (Williams and Weisburger, 1986). The destruction of oocytes by chemicals occurs early and is probably part of the initiation phase. The initiated cells are stimulated by pituitary secretions (promotion) and ultimately a tumor is formed which can progress to a neoplasia (Jull, 1973).

Spontaneous ovarian tumors in rodents are rare (NTP, 1986). However, ovarian tumors can be induced by several methods including genetic selection, neonatal thymectomy, transplantation of the ovaries to the spleen, exposure to bright illumination, ionizing radiation, and chemical carcinogens (Marchant, 1987). A consistent finding among all treatments in these studies has been the rapid loss of primary oocytes (Murphy and Beamer, 1973; Jull, 1973). Studies by Jull (1973) have shown that destruction of oocytes precedes the formation of tumors. In rodents devoid of oocytes, gonadotropin secretion is increased because the ovary no longer produces the steroids necessary to inhibit its secretion. Therefore, continuous exposure of the ovary to elevated levels of circulating gonadotropins (specifically FSH) is thought to promote formation of ovarian tumors.
(Marchant, 1961; Murphy and Beamer, 1973). This is supported by several studies which have utilized physical or chemical carcinogen exposure to induce ovarian tumors.

It was not until 1936 that the ovarian neoplastic potential of irradiation of the whole mouse was discovered (Furth and Butterworth, 1936). Later studies have shown that irradiation of the ovary alone was all that was required to induce ovarian tumors. Lick et al., (1949) showed that if the ovary was withdrawn from the peritoneal cavity and irradiated while the animals body was protected by lead shielding, ovarian tumors were induced. Conversely, when the ovaries were shielded and the remainder of the animal's body was irradiated, no ovarian tumors were produced (Deringer et al., 1955).

In 1954, the tumorigenic potential of the polycyclic aromatic hydrocarbon 7,12-dimethylbenzanthracene (DMBA) was reported by Marchant et al. and Howell et al. These investigators found that repeated applications of DMBA to the skin resulted in ovarian granulosa cell tumors. Subsequent studies had shown that administration of DMBA by oral (Biancifiori et al., 1961; Jull et al., 1966; Krarup, 1967), intraperitoneal (Krarup, 1967), or subcutaneous routes (Shisa and Nishizuka, 1968) caused ovarian tumors in mice. Other chemicals known to effect ovarian tumor induction include 3-methylcholanthrene and benz(a)pyrene (Biancifiori et al.,
More recently, Maronpot (1987) reported that ovarian toxicity and/or carcinogenicity have been observed for eight chemicals in current NTP toxicity and/or carcinogenicity studies. Three of these chemicals are closely related in structure; 1,3-butadiene, 4-vinylcyclohexene, and vinylcyclohexene diepoxide. These compounds are either chemically reactive compounds such as diepoxide, or compounds that can be bioactivated to reactive chemicals by the intact animal. All three compounds were reported to cause follicular loss, and ultimately neoplasms in the ovaries of mice in the studies performed for 60 weeks or 2 years. Consequently, Maronpot suggested that in order to better understand these ovarian changes observed in treated animals, additional study design considerations should be implemented. These would include increasing the number of female treatment groups, performance of periodic terminations, vaginal cytology, and hormone measurements.

Statement of Problem

Previous studies have demonstrated the vulnerability of women to ovarian damage and the ability of animal studies to serve as models for effects which are or may be observed in humans. However, there is a need to study in more detail the structural features of the chemicals which cause oocyte
destruction. This knowledge would enhance our ability to predict compounds that may be ovotoxic. Humans come into daily contact with numerous compounds containing vinyl subsituents. 4-Vinylcyclohexene (VCH) is one such example. VCH is a compound which is used in industry, it has been shown to be an ovarian toxicant in animals, and induces ovarian neoplasia in B6C3F1 mice. VCH is also structurally very similar to 4-phenylcyclohexene (4PC), a compound which is a common environmental contaminant. However, nothing is known about the effect of 4PC on the female reproductive system. 4PC only differs from VCH by a phenyl substiuent instead of a vinyl group. Therefore, testing its ovotoxic potential can enhance our knowledge of specific structural moieties that participate in oocyte destruction.

Objective

The objective of this research is to determine if 4PC causes ovotoxicity. This effect will be determined by a bioassay which will use histologic examination of the ovaries to confirm toxicity. The positive controls which will be used in this study are VCH, a structural analog of 4PC which causes primary oocyte depletion (Smith et al., 1990b), and benz(a)pyrene, a well studied ovotoxicant (Mattison, 1980). The specific aims of this thesis are to determine if 4PC causes depletion of primary and/or secondary follicles in
mice, and to correlate possible changes in oocyte number with a decrease in the number of estrous cycles within the 30 day treatment period.
MATERIALS AND METHODS

Animals. Female, Balb C mice (20-30g) were provided by Dr. Wayburn Jeter from the Department of Pharmacology and Toxicology at the University of Arizona. Female, 21 day old, B6C3F1 mice were obtained from Harlan Inc. (Indianapolis, IN). The animals were housed 5 per cage in sawdust bedding and had free access to food (Teklad®, Harlan Spraque Dawley Inc., Madison, WI) and water. Animals were maintained on a 12 hour light/dark cycle and acclimated to this environment for at least 7 days before use in toxicity studies.

Chemicals. 4-Phenylcyclohexene (4PC) was contributed by Dr. Mark Van Ert of the University of Arizona (Health Related Professions) and was also purchased from Wiley Organics (Coshocton, Ohio). The purity of 4PC was 98% as determined by gas-liquid chromatography (see Appendix B). 4-Vinylcyclohexene (VCH) containing t-butylcatechol as an antioxidant was purchased from Aldrich Chemical Co. (Milwaukee, WI). Benzo-a-pyrene and sesame seed oil were purchased from Sigma Chemical Co. (St. Louis, MO).

Range Finding Studies: Approximate lethal dose in female Balb C mice. Mature female Balb C mice were given one intraperitoneal (ip) treatment of the test compound dissolved
in sesame seed oil (5.0 ml/kg) and their behavior was noted at 2 and 24 hours post-injection. Doses of 4.0, 3.5, 3.0, 2.0 and 1.0 g/kg (25.3, 22.1, 19.0, 12.6 and 6.3 mmole/kg) were selected for 4PC. A range was selected since there was no prior information on 4PC treatment in mice. Doses of 2.4, 1.6, 1.2, 0.8 and 0.4 g/kg (22.2, 14.8, 11.1, 7.4 and 3.7 mmole/kg) were selected for VCH. VCH had not previously been tested in Balb C mice, therefore a range of doses was selected. This range of doses was chosen based on toxicity reported in a previous NTP study (National Toxicology Program, 1986). The VCH was tested at the same time as 4PC since it served as the positive control. All animals that did not survive were necropsied.

Fourteen day study in female Balb C mice. A 14 day study was conducted to evaluate the cumulative toxic effects of repeated daily administration of 4PC and VCH. All animals who survived the initial 24 hour dose in the previous study were treated for an additional 13 days. The treatment groups consisted of a 1.0 g/kg (6.3 mmole/kg) treatment of 4PC, and 1.0 and 0.8 g/kg (9.2 and 7.4 mmole/kg) treatment of VCH. On day 15 the animals who survived were killed by CO₂ inhalation. Their ovaries were removed and fixed in Bouin's solution for 24 hours, transferred to 70% ethanol, and processed for sectioning. Step sections were prepared at 6-8 um and the
sections were then stained with hematoxylin and eosin. The oocytes were identified by the method of Pedersen and Peters (1968) and counted in every 20th section in the right ovary. The oocyte counts from individual sections were summed to calculate the total oocyte count for each ovary.

**Ovarian Toxicity Studies: Effect of 4-phenylcyclohexene treatment.** Since the Balb C mouse was used in the previous studies to estimate a dosage range, it was necessary to do preliminary studies in the strain of choice, the B6C3F1. Twenty-eight day old female mice were given daily intraperitoneal (ip) treatments of the test compounds dissolved in sesame seed oil (2.5 ml/kg) for 30 days. Doses of 7.4, 6.0, 5.0 and 3.0 mmole/kg were selected for 4PC based on the range finding studies done in the Balb C mice. Doses of 7.4 and 6.0 mmole/kg of VCH were used as a positive control based on ovarian toxicity observed by Smith et al. (1990b). In addition, 0.32 mmole/kg of benz(a)pyrene was given by ip administration, two doses one week apart as a positive control for ovarian toxicity. The dose chosen was based on toxicity observed by Madison et al. (1980). The control group received sesame seed oil at 2.5 ml/kg. All mice were examined for the stage of estrous cycle by vaginal smear and were weighed daily. Starting on day 31 all mice were killed by CO₂ inhalation, their ovaries were removed, processed, and
the oocytes were counted as described in the Balb C mice study.

Vaginal smear for determination of the stage of estrous cycle. Each mouse was picked up by the base of its tail and a fire-polished tip of a Pasteur pipet was inserted into the vagina. One drop of tap water was gently expelled into the vagina. The water was then collected with an inoculating loop and transferred to a glass slide. The smear was immediately examined by light microscopy and if necessary, stained using a 1% solution of toluidine blue. The stage of estrous cycle was classified by the method of Allen (1922). An estrous cycle was defined as a sequence beginning with a period of nucleated and/or cornified epithelium (proestrus and or estrus), followed by a period of aggregated cornified epithelium and/or leukocytes (metestrus), and at last a period of mostly nucleated epithelium, a few cornified epithelial cells, and a few neutrophils (diestrus).

Data Analysis. All data was analyzed using Number Cruncher Statistical System 5.0 (1988), (Kaysville, Utah). A students t-test was used to determine significant differences between two group means. Multiple comparisons were made using one-way analysis of variance (ANOVA). When significant differences were detected within the ANOVA individual groups were compared
using a Newman-Kuel's range test. The weight data required the use of a repeated measures ANOVA in order to compare daily weight means. The level of significance for all tests was $p < 0.05$. 
RESULTS

Range Finding Study in Female Balb C Mice

Approximate lethal dose of 4-phenylcyclohexene and 4-vinylcyclohexene in female Balb C mice after a single treatment. Mice dosed with 4-phenylcyclohexene (4PC) at 4.0 and 3.5 g/kg (25.3 and 22.1 mmole/kg) were severely depressed, recumbent, and were dyspneic two hours post-injection. The animals dosed with 3.0 g/kg (19.0 mmole/kg) were severely depressed. Those dosed with 2.0 and 1.0 g/kg (12.6 and 6.3 mmole/kg) appeared normal at two hours post-dosing. By 24 hours all mice dosed with 4.0, 3.5, and 3.0 g/kg were found dead or were euthanized in a morbid condition. Those dosed with 2.0 g/kg were mildly depressed and recumbent, and those dosed at 1.0 g/kg appeared normal (Table IV). No significant lesions were noted at necropsy in any of the mice.

Included in this study are data for the positive control 4-vinylcyclohexene (VCH). Mice dosed with VCH at 2.4 g/kg (22.2 mmole/kg) exhibited severe depression, dyspnea, and died within 24 hours. Mice dosed with 1.6 g/kg (14.8 mmole/kg) exhibited mild incoordination at 2 hours and appeared normal at 24 hours. Those dosed between 0.4 to 1.2 g/kg (3.7 and 11.1 mmole/kg) appeared normal (Table IV). No significant lesions were noted at necropsy in any of the mice.

Approximate lethal doses of 1-phenylcyclohexene,
vinylcyclohexane, and cyclohexene were also determined since these compounds are all structurally related to the ovotoxicant VCH. The results can be found in Appendix G.

Table IV. Lethality of a single treatment of 4-phenylcyclohexene or 4-vinylcyclohexene in female Balb C mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (g/kg)</th>
<th>Dose (mmole/kg)</th>
<th>Survival*</th>
</tr>
</thead>
<tbody>
<tr>
<td>4PC</td>
<td>4.0</td>
<td>25.3</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>22.1</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>19.0</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>12.6</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>6.3</td>
<td>2/2</td>
</tr>
<tr>
<td>VCH</td>
<td>2.4</td>
<td>22.2</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>14.8</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>11.1</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>7.4</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>3.7</td>
<td>2/2</td>
</tr>
</tbody>
</table>

* Survival 24 hours post-dosing.

Non-lethal dose of 4-phenylcyclohexene and 4-vinylcyclohexene in female Balb C mice after treatment for 14 consecutive days. All mice who survived a single dose without any adverse clinical signs were dosed daily for an additional 13 days. Based on the mortality observed in this study (Table V) 4PC could be administered repeatedly at a dose of 1.0 g/kg (6.3 mmole/kg) and VCH at 0.8 g/kg (7.4 mmole/kg).
Table V. Survival of female Balb C mice after treatment with 4-phenylcyclohexene or 4-vinylcyclohexene for 14 consecutive days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose^a</th>
<th>Dose^b</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>4PC</td>
<td>1.0</td>
<td>6.3</td>
<td>2/2</td>
</tr>
<tr>
<td>VCH</td>
<td>0.8</td>
<td>7.4</td>
<td>2/2</td>
</tr>
<tr>
<td>VCH</td>
<td>1.2</td>
<td>11.1</td>
<td>0/2^c</td>
</tr>
</tbody>
</table>

^a g/kg/day; ip.
^b mmole/kg/day; ip.
^c Death occurred within 7 days of treatment.

Effect of 4-phenylcyclohexene and 4-vinylcyclohexene administration on primary follicle counts in female Balb C mice after treatment for 14 consecutive days. All treated mice had a decrease in the number of primary follicles when compared to an untreated control (Table VI). The control mice had an average count of 256.2 (n=2), the mice dosed with 4PC had counts of 162.5 (n=2) and the mice dosed with VCH had counts of 106.0 (n=2) (Figure 3). These average counts represent a 37% decrease in oocytes in 4PC treated animals and
59% decrease in VCH treated animals when compared to naive controls.

Results for the effect of 1-phenylcyclohexene, 4-vinylcyclohexane and cyclohexene on the primary follicle counts are presented in Appendix G.

Table VI. Decrease of primary follicle counts in female Balb C mice after treatment with 4-phenylcyclohexene or 4-vinylcyclohexene for 14 consecutive days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose$^b$</th>
<th>Dose$^c$</th>
<th>Primary follicle Counts$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4PC</td>
<td>1.0</td>
<td>6.3</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td></td>
<td>193</td>
</tr>
<tr>
<td>VCH</td>
<td>0.8</td>
<td>7.4</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td></td>
<td>87</td>
</tr>
<tr>
<td>Control$^a$</td>
<td>--</td>
<td>--</td>
<td>215</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>297</td>
</tr>
</tbody>
</table>

$^a$ Controls represent untreated Balb C mice of the same approximate age as treatment groups.

$^b$ g/kg/day; ip

$^c$ mmole/kg/day; ip

$^d$ Ovaries were serially sectioned at 6-8 um and every 20th section was scored.
Figure 3. Effect of 4-phenylcyclohexene (4PC) or 4-vinylcyclohexene (VCH) administration on primary follicle counts in female Balb C mice after 14 days of consecutive treatment. Each box represents the average of 2 animals. The box labeled control represents the naive control and the box labeled VCH represents the positive control. 4PC or VCH was administered to the mice at a dose of 1.0 g/kg (6.3 mmole/kg), or 0.8 g/kg (7.4 mmole/kg) respectively.

Preliminary Ovarian Toxicity Studies

Dose of 4-phenylcyclohexene in female B6C3F1 mice. A common model used in toxicity testing is the B6C3F1 strain of mouse. Since Balb C mice were used in the previous range finding study, it was necessary to do a preliminary study to find an appropriate dose of 4PC in B6C3F1 mice. An initial experiment (study 1) in the B6C3F1 mice included a 7.4, 6.0, and 5.0 mmole/kg dose of 4PC. This included both a higher and
lower dose than that which was used in the previous Balb C studies. Since unexpected mortality was observed in the mice dosed at 7.4 mmole/kg of 4PC and VCH within the first week the study was terminated and a second experiment (study 2) was planned. This study was performed using doses at which the animals survived in the initial experiment. A summary of the mortality observed in these studies is shown in Table VII. The 6 mmole/Kg dose of 4PC was determined to be the most appropriate for the B6C3F1 strain since this was the highest dose which could be tolerated without overt toxicity.

The positive control, VCH, when dosed at 7.4 mmole/kg exhibited mortality throughout the trials. However, this dose was continued since it had been shown in previous studies to deplete small follicles in the B6C3F1 strain of mouse without causing lethality (Smith et al., 1990b).

Effect of 4-phenylcyclohexene and 4-vinylcyclohexene treatment on weight gain. Body weight measurements made over the entire 30 day period did not indicate any significant difference between the vehicle control and the treatment groups (Figure 4). Both the vehicle control and treated animals gained 4 to 5 grams between day 1 and day 30 of this study.
Table VII. Survival of female B6C3F1 mice after treatment with 4-phenylcyclohexene or 4-vinylcyclohexene

<table>
<thead>
<tr>
<th>Study&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Treatment</th>
<th>Dose&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Survive</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control&lt;sup&gt;b&lt;/sup&gt;</td>
<td>--</td>
<td>5/5</td>
</tr>
<tr>
<td>1</td>
<td>4PC</td>
<td>5</td>
<td>5/5</td>
</tr>
<tr>
<td>1</td>
<td>4PC</td>
<td>6</td>
<td>5/5</td>
</tr>
<tr>
<td>1</td>
<td>4PC</td>
<td>7.4</td>
<td>4/5</td>
</tr>
<tr>
<td>1</td>
<td>VCH</td>
<td>7.4</td>
<td>3/5</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>--</td>
<td>6/6</td>
</tr>
<tr>
<td>2</td>
<td>4PC</td>
<td>5</td>
<td>6/6</td>
</tr>
<tr>
<td>2</td>
<td>4PC</td>
<td>6</td>
<td>4/6</td>
</tr>
<tr>
<td>2</td>
<td>VCH</td>
<td>7.4</td>
<td>3/6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Study 1 represents repeated ip administration for 7 days, study 2 represents repeated ip administration for 30 days.

<sup>b</sup> Sesame seed oil

<sup>c</sup> mmole/kg/day
Figure 4. Preliminary study. Effect of 4-phenylcyclohexene (4PC) or 4-vinylcyclohexene (VCH) treatment on the weight gain in female mice after ip administration. Each point represents the mean (x ± S.D) for 3-6 animals. The treated groups were not significantly different from their respective control.

Effect of 4-phenylcyclohexene and 4-vinylcyclohexene treatment on the number of estrous cycles in female mice. A great deal of variability was exhibited in all groups with respect to the daily examination of the vaginal cytology. The control group as well as the treatment groups did not cycle in a consistent manner until approximately the second week of the study. In spite of this variability, the mice treated with 4PC (6 mmole/kg) and VCH (7.4 mmole/kg) spent more time in diestrus than the control group or mice treated with 4PC (5 mmole/kg). However, only the treatment with VCH (7.4
mmole/kg) had a statistically significant reduction in the total number of estrous cycles during the 30 day period (Figure 5).

![Graph](image)

**Figure 5.** Preliminary study. Effect of 4-phenylcyclohexene (4PC) or 4-vinylcyclohexene (VCH) treatment on the number of estrous cycles in female mice after ip administration for 30 days. Each box represents the mean (x ± S.D.) of 3-6 animals. The asterisk indicates the group which is statistically significant from the control (p<0.05).

**Effect of 4-phenylcyclohexene and 4-vinylcyclohexene treatment on primary and secondary follicle counts.** All mice treated with 4PC and VCH had a decrease in the number of primary follicles. The 4PC treatments caused a 17% and 21% decrease (5 and 6 mmole/kg doses respectively) in small
follicle counts and the positive control, VCH, caused an 89% decrease when compared to controls. However, only the mice dosed with the VCH had a statistically significant reduction in the number of primary follicles (Figure 6).

![Graph showing follicle counts](image)

**Figure 6. Preliminary study. Effect of 4-phenylcyclohexene (4PC) or 4-vinylcyclohexene (VCH) treatment on primary follicle counts in mice after ip administration for 30 days. Each box represents the mean (x ± S.D.) of 3-6 animals. The asterisk indicates the group which is statistically significant from the control (p<0.05).**

Similarly, only the VCH dosed mice demonstrated a significant decrease in the number of secondary follicles (77% decrease). The 4PC treatment caused no change in counts when compared to control values (Figure 7).
Figure 7. Preliminary study. Effect of 4-phenylcyclohexene (4PC) or 4-vinylcyclohexene (VCH) treatment on secondary follicle counts in mice after ip administration for 30 days. Each box represents the mean (x ± S.D.) of 3-6 animals. The asterisk indicates the group which is statistically significant from the control (p<0.05).

Ovarian Toxicity Study

Ovotoxicity of 4-phenylcyclohexene and 4-vinylcyclohexene. The parameters investigated in the preliminary studies were reexamined in this study with the addition of larger numbers of animals per group, and a low dose of 4PC (3 mmole/kg) as well as the high dose (6 mmole/kg). Also, the VCH treatment dose of 7.4 mmole/kg was replaced with a dose of 6 mmole/kg to make it possible to compare equimolar doses of 4PC and VCH and to prevent the mortality which occurred in the VCH treated animals in the preliminary study. In addition, a benz(a)pyrene (BP) treated
group (n=10) was added as an additional positive control for depletion of primary and/or secondary follicles.

Body weight measurements taken over the entire 30 days were compared for each group. The body weight data were similar to those obtained in the preliminary study. Weight gain in all groups was statistically similar with the exception of the group dosed with 4PC (6 mmole/kg) which had a larger weight gain over the 30 days. The mice gained between 2.9 and 5.7 grams. The group treated with BP had the lowest and the 4PC (6 mmole/kg) treated group had the highest (Figure 8).

Figure 8. Effect of 4-phenylcyclohexene (4PC) or 4-vinylcyclohexene (VCH), benz(a)pyrene (BP) treatment on the weight gain in female mice after ip administration. Each point represents the mean (x ± S.D) for 3-6 animals. The asterisk indicates the group which is statistically significant from the control (p<0.05).
Again, the mice did not begin to exhibit regular estrous cycles until approximately the second week of the study. When all groups were compared, the 4PC (6 mmole/kg) and the VCH treated animals had statistically significant lower numbers of cycles/30 days (Figure 9).

Figure 9. Effect of 4-phenylcyclohexene (4PC), 4-vinylcyclohexene (VCH), or benz(a)pyrene (BP) treatment on the number of estrous cycles in mice after ip administration for 30 days. All animals were dosed daily with the exception of the BP treated mice which received two doses one week apart. Each box represents the mean (x ± S.D.) of 10-15 animals. The asterisk indicates the groups which are statistically significant from the control (p<0.05).
When the ovaries for all the treatment groups were examined by light microscopy, only the treatment with VCH or BP, had caused a noticeable reduction in the number of primary and/or secondary follicles. These treatment groups had follicular losses throughout the entire ovary when compared to the control group. These losses were very apparent when examining the hilar region of the ovary. The 4PC treatment groups (3 and 6 mmole/kg) had ovaries which appeared similar to the control group (Figures 10, 11, and 12).

The VCH treatment caused a 92% decrease in primary follicles and a 23% decrease in secondary follicles when compared to the number of follicles presented in the control groups. Benz(a)pyrene treatment caused a 72% decrease in primary follicles and a 19% decrease in secondary follicles. Changes produced by other treatments were not significantly different (Figure 13).
Figure 10. Hilar region of mouse ovary after treatment with sesame seed oil (2.5 ml/kg) or 4-phenylcyclohexene (3 mmole/kg). Mice were treated daily for 30 consecutive days by ip administration. Their ovaries were removed, fixed, sectioned, and stained with hematoxylin and eosin and examined by light microscopy, 396X. Small arrows indicate primary follicles and large arrows indicate secondary follicles.  

a: Sesame seed oil control; this ovary had 296 primary and 110 secondary follicles.  
b: 4-Phenylcyclohexene; this ovary had 342 primary and 112 secondary follicles.
Figure 11. Hilar region of mouse ovary after treatment with 4-phenylcyclohexene (6 mmole/kg) or 4-vinylcyclohexene (6 mmole/kg). Mice were treated daily for 30 consecutive days by ip administration. Their ovaries were removed, fixed, sectioned, and stained with hematoxylin and eosin and examined by light microscopy, 396X. Small arrows indicate primary follicles and large arrows indicate secondary follicles.

a: 4-Phenylcyclohexene; this ovary had 282 primary and 124 secondary follicles.

b: 4-Vinylcyclohexene; this ovary had 26 primary and 15 secondary follicles. However, in this figure there are only small nests of granulosa cells in which no oocyte is present.
Figure 12. Hilar region of mouse ovary after treatment with benz(a)pyrene (0.32 mmole/kg). Mice were treated twice, one week apart by ip administration. Their ovaries were removed, fixed, sectioned, and stained with hematoxylin and eosin and examined by light microscopy, 396X. This figure shows a section of an ovary which had 60 primary and 97 secondary follicles. No follicles are present in this figure.
Figure 13. Effect of 4-phenylcyclohexene (4PC), 4-vinylcyclohexene (VCH), and benz(a)pyrene (BP) treatment on primary and secondary follicle counts in mice after ip administration for 30 days. All animals were treated daily with the exception of the BP mice which received two doses one week apart. Each box represents the mean (x ± S.D.) of 10-15 animals. The asterisk indicates the groups which are statistically significant from the control (p<0.05).
DISCUSSION

There is considerable evidence which shows that physical and chemical agents can result in oocyte destruction (Jull, 1973). Recently, a number of chemicals have been shown to destroy primary follicles in the ovaries of rodents (Dobson and Felton, 1983). 4PC is structurally similar to VCH, a chemical which has been shown to destroy primary follicles in a dose and time dependent fashion (Smith et al., 1990b), and produce ovarian tumors (NTP, 1986) in B6C3F1 mice. Therefore, the primary goal of this research was to determine if administration of 4PC (considered an analog of VCH) reduces the number of ovarian follicles. The parameters investigated included the effect of 4PC treatment on the: primary and secondary follicle counts in female mice, the number of estrous cycles within a 30 day period, and body weight.

Range Finding Study in Female Balb C Mice

Since there are no toxicity data for 4PC in mice and a large number of untreated, female Balb C mice became available to us, they were treated ip, with 4PC in order to determine a dose range that would allow animal survival. It was shown that 4PC could be administered repeatedly at a dose of 1.0 g/kg (6.3 mmole/kg) to female Balb C mice without apparent toxicity.
These data provided an approximate dose at which to start the preliminary study in B6C3F1 female mice, the test strain for comparison of 4PC with VCH. This strain rarely develops ovarian tumors and is the strain used by the NTP for cancer assays. A decrease in primary follicle counts was observed in the two Balb C mice after administration of 4PC (1.0 g/kg) for 14 days. No conclusions can be drawn from these data because of the small number of animals assigned to the preliminary study.

**Preliminary Ovarian Toxicity Studies**

Preliminary studies were performed to determine the sensitivity of female B6C3F1 mice to 4PC under the conditions of the 30 day bioassay. It was revealed that female B6C3F1 mice could be treated daily with 4PC for 30 days at a dose of 6 mmole/kg/day without lethality. In addition, it was discovered that the positive control, VCH, was causing excessive mortality at the dose which was previously reported to only cause ovotoxicity (7.4 mmole/kg/day) in a similar 30 day bioassay (Smith et al., 1990b). Therefore, the dose of VCH in the actual ovarian toxicity study was reduced to 6 mmole/kg/day. This dose should lower mortality as well as represent a dose directly equivalent to 4PC.

In this preliminary study only the mice who survived VCH treatment (7.4 mmole/kg/day) demonstrated a significant
reduction in the number of primary and secondary follicles when compared to the vehicle controls. The number of estrous cycles per 30 days was significantly reduced in the VCH and slightly reduced in 4PC treatment groups. The results are interpreted as a possible effect of 4PC on the reproductive system.

Ovarian Toxicity Study

Although 4PC and VCH are structurally similar, they demonstrated an obvious difference in their ability to destroy ovarian follicles. When 4PC was administered ip to mice daily for 30 days, the number of primary and secondary follicles were not significantly reduced in either the high dose (6 mmole/kg) or low dose (3 mmole/kg) treatment groups. The mice treated with the positive control, VCH, at an equimolar dose (6 mmole/kg) sustained a 92% and 23% decrease in primary and secondary follicles, respectively. These data agree with those of Smith et al., (1990b). In their studies VCH depleted primary follicles. The studies reported here demonstrate a VCH-induced reduction in the number of secondary follicles, an effect not previously examined at this dose (6 mmole/kg). In addition, BP, a model ovotoxicant (Mattison, 1980), also caused a significant decrease in primary and secondary follicles (72% and 19% respectively).

Smith et al., (1990a) demonstrated that treatment of
Fisher 344 rats and B6C3F1 mice with VCH caused oocyte destruction in mice, but not in the rats. In studies in which VCH was incubated with murine and rat hepatic microsomes, VCH-1,2-epoxide, VCH-7,8-epoxide, and VCH-diepoxide were detected as metabolites (Smith et al., 1990a; Watabe et al., 1981; Gervasi et al., 1980). Based on studies with BP, which showed epoxide metabolites to be more potent than BP at causing ovarian toxicity, Smith et al., (1990a) suggested that these metabolites of VCH may actually be responsible for VCH-induced oocyte loss. This suggestion was reinforced by the data of Smith et al., (1990a) that showed a difference between the two species in their ability to metabolize VCH. Studies with microsomes showed the mouse had a much higher capacity to metabolize VCH. In vivo studies supported these findings, VCH-1,2-epoxide was observed circulating only in the blood of mice.

In order to determine if epoxidation of VCH is required for oocyte destruction, Smith et al., (1990b) administrated the epoxide metabolites of VCH (VCH-1,2-epoxide, VCH-7,8-epoxide, and VCH-diepoxide) to mice and rats. In mice, the epoxides were more potent in destroying oocytes. In rats, the administration of the epoxides elicited ovarian damage, even though treatment related changes were not present following VCH dosing. Such findings supported the argument that bioactivation of VCH is required for this compound to be
ovotoxic. Smith et al., (1990b) also demonstrated that pretreatment of mice with chloramphenicol, a inhibitor of xenobiotic metabolism, reduced the concentration of VCH-1,2-epoxide found in the blood after VCH treatment. This inhibition of VCH epoxidation was partially effective in protecting the mice from VCH-induced ovarian injury. Complete protection from toxicity was not expected since the blood concentration of VCH-1,2-epoxide in chloramphenicol-pretreated mice was not reduced to subtoxic levels. Although the exact metabolite which causes the ovotoxicity in the mouse is unknown, it is apparent that bioactivation of VCH is necessary.

Whether or not 4PC is converted to epoxides has not been established. If 4PC is not metabolized to these products, then one would not expect 4PC to be ovotoxic. Alternatively if 4PC is being metabolized, then it is conceivable that it is readily being detoxified. VCH epoxides are known to be substrates for epoxide hydrolase and probably glutathione-S-transferases (Sipes and Gandolfi, 1986).

It is possible that the phenyl substituent at the 4 position of 4PC, being less reactive than the vinyl substituent in VCH, could possibly be interfering with the bioactivation of the cyclohexene base and/or other vinyl moieties. Another possibility is the fact that the phenyl group, being a large substituent, may be sterically hindering a
binding site at the ovary which is critical to ovarian toxicity. Finally another possibility is that the ovary is not a target of 4PC's suspected toxicity.

All of the treatment groups (controls, 4PC, VCH and BP) exhibited variability (lack of regular progression) in vaginal cytology for approximately the first two weeks of the study. It is well established that housing female mice in groups can cause some disruption of normal (4-5 day) cycles (Lamond, 1959; Champlin, 1971). The fact that the mice display suppression of estrus if they are exposed to new social environments has lead to the suggestion that pheromones may play a role in reestablishing continuity of the rodent cycle. Since all of the mice were exposed to the same housing conditions and they all began to exhibit cornified vaginal cytology (an indication of estrogen stimulation) at similar intervals the variability was not considered treatment related.

Examination of vaginal cytology has proven to be a useful tool in detecting possible ovarian abnormalities. Krarup (1969) demonstrated that administration of 9,10-dimethyl-1,2-benzanthracene, an ovotoxicant and ovarian carcinogen, causes irregular estrous cycles with prolonged periods of diestrus in mice. In the study reported here a significant decrease in the number of estrous cycles was observed in the mice treated with 4PC (6 mmole/kg) and VCH. Although there was not a
significant decrease in the 4PC treated animals in the preliminary study, there was a statistically significant decrease in the number of estrous cycles in the major study. Apparently, the increase in the number of animals per group allowed the data to become statistically significant.

These data suggest that monitoring of estrous cycles may be a useful indicator of the toxic effects of 4PC on the mouse reproductive system. It is possible that these findings could prove to be an early indication of ovotoxicity if 4PC treatment continued for a period longer than 30 days.

It can not be denied that the stress of daily dosing may have played a part in the number of estrous cycles observed. However, it is unlikely that only 4PC and VCH treatment would have resulted in a decrease in the number of estrous cycles. Another consideration is that the BP treated mice showed a similar degree of oocyte destruction as the VCH treatment. Nevertheless, the BP group did not demonstrate lengthening of the estrous cycle when compared to the control group. Only two doses of BP were administered to mice over 30 days, as opposed to 30 doses of VCH or 4PC. Thus, the estrous cycle may be sensitive to the stresses of daily dosing with high concentrations of the compounds such as 4PC (6 mmole/kg) and VCH. Another possibility is that the effect of 4PC and VCH on follicle counts is unrelated to oocyte destruction or that these compounds are acting directly on the vaginal epithelium.
Administration of daily doses of toxicant can lead to alterations or reductions in weight gain in rodents, and is sometimes an indication of stress which can have toxicological consequences. Therefore, body weight measurements were made daily for each animal. The weight data for all groups studied was statistically similar over the 30 day period with the exception of a group dosed with 6 mmole/kg 4PC. This group had a slightly higher weight gain over the 30 days. This is not believed to be treatment related, since, even though the mice were randomly grouped, this group of mice happened to have a lower body weight at the start of the study, therefore, they gained more weight during the 30 day period.

In conclusion, 4PC does not appear to cause oocyte destruction in B6C3F1 mice at the dose regimen chosen for this study. However, 4PC did cause a decrease in the number of estrous cycles expected in a 30 day period. Although the specific mechanism by which 4PC causes the effect on the mouse estrous cycle is unknown, it is possible that this finding may be an early indication of toxicity to the reproductive system.

The findings from this research raise a number of interesting questions. Many chemicals in our society contain vinyl and/or cyclohexene substituents. Understanding how such substituents affect ovarian toxicity is therefore quite important. The results presented here suggest that the vinyl group of VCH appears to be more critical with respect to
ovotoxicity. Metabolic and ovotoxicity studies with other structurally related compounds are necessary to establish how structures influence toxicity. If the presence of specific structural groups can increase or decrease the ovotoxicity of chemicals then it is possible that this knowledge could better predict ovotoxicants in humans.
Capillary gas-liquid chromatography conditions for 4-phenylcyclohexene and 1-phenylcyclohexene

Objective: 1) Determine capillary gas-liquid chromatography (GLC) conditions for 4-phenylcyclohexene (4PC) and 1-phenylcyclohexene (1PC) (internal standard).

2) Determine if 4PC remains linear over the concentrations 0.001 - 0.1 mg/ml when using GLC analyses.

Equipment and Chemicals:

5890A Hewlett Packard gas chromatograph with a flame ionization detector (FID)
3392A Hewlett Packard Integrator
RSL 300 Altech Capillary Column 25m, 0.32mm
Fisher Scientific HPLC grade Hexane H302-4
Aldrich Chemical Co. 1-Phenylcyclohexene P2,230-3
  -Purity 95%
  -F.W. 158.24
  -bp 251-253°
  -d 0.994
Wiley Organics 4-Phenylcyclohexene 7808.00
  -Purity 98%
  -F.W. 158.24
  -bp 235°
  -d 0.994

Design:

4PC was diluted in a 10 ml volumetric flask with hexane for a final concentration 10 mg/ml and subsequently serially diluted to 1 mg/ml. The 1PC was diluted in a similar fashion. An injection volume of 1 ul of 4PC or 1PC (1 mg/ml) was used to determine chromatographic conditions. This was accomplished by varying the column temperature, and or the flow rate until both compounds could be separated and eluted from the column within five minutes.

In addition, a standard curve of 4PC was made to determine if the concentrations 0.001 mg/ml to 0.1 mg/ml were linear.
Results:

The retention times were determined to be 4.00 minutes for 4PC and 5.00 minutes for IPC under the following column conditions.

Column Conditions:
Flow Rate of N₂ carrier gas 1.25 ml/min
Split Vent 20 ml/min
FID Flow Rate for H₂, N₂, and Air 30, 30, and 240 ml/min
Injector, Oven, and Detector Temp. 200°C, 150°C, and 300°C

A standard curve of 4PC was analyzed and determined to be linear over the concentrations 0.001-0.1 mg/ml (Figure 1a).

Figure 1a. Standard curve of 4PC using gas-liquid chromatography. Each point represents the mean (x ± S.D) of three separate 1 ul injections. The correlation coefficient for these values is 0.9959.
Appendix B

Purity of 4-phenylcyclohexene

Objective: Determine the purity of 4PC by GLC analysis.

Equipment and Chemicals:
Refer to Appendix A

Design:

A sample of 4PC (0.1 mg/ml) which was obtained from Wiley Organics and a sample from Dr. Mark Van Ert from the University of Arizona, Department of Toxicology of known purity (99.5%) (Walsh, 1986) were analyzed by the conditions described in Appendix A.

An average of three 1 ul injections of each sample was analyzed individually to determine the purity by performing the sample calculations shown below. The two samples were also combine to see if they co-chromatographed.

Calculation for Purity:

\[
\text{Purity} = \left( \frac{\text{4PC Area Response}}{\text{Total Area} - \text{Solvent Front Area}} \right) \times 100\%
\]

4PC sample from University of Arizona:
\[
\frac{926980}{42911000 - 41963000} \times 100\% = 97.7\%
\]

4PC from Wiley Organics:
\[
\frac{1250200}{42866000 - 41585000} \times 100\% = 97.59\%
\]

Results:

Both the 4PC received from Wiley Organics and Dr. Van Ert demonstrated a purity of approximately 98%. Both samples co-chromatographed as well, demonstrating similarity between the sample compounds.
Appendix C

Degradation products of 4-phenylcyclohexene after being prepared in sesame seed oil and stored at 4°C

Objective: 1) Determine if 4PC breaks down after being stored at 4°C.

2) Determine if 4PC breaks down over time in sesame seed oil dosing preparations.

Equipment and Chemicals:

Refer to appendix A.
Sesame seed oil
Ethanol

Design:

To determine if 4PC does or does not degrade over time after being stored at 4°C, a stock solution of 10 mg/ml which was prepared five months earlier was compared to a freshly prepared solution. These solutions were analyzed by GLC as per appendix B.

To determine if 4PC does not break down in dosing preparations, a 4PC solution of the equivalent concentration to a 5 mmole/kg dosing solution was prepared in sesame seed oil and stored at 4°C for 1 week. After one week, a second fresh solution was prepared. Both of these solutions as well as an equal amount of sesame seed oil were extracted with ethanol three times. Ethanol was the solvent of choice since previous experiments had shown that ethanol was not miscible with the sesame seed oil. The extractions were pooled for each solution and analyzed by GLC. The 4PC was compared quantitatively for each solution.

Results:

This data suggests that 4PC does not degrade after storage at 4°C. In addition, 4PC does not break down or volatilize when prepared as a dosing solution in sesame seed oil (Table Ia).
Table Ia. Percent of 4PC recovered after storage at 4°C or after prepared in sesame seed oil

<table>
<thead>
<tr>
<th>Solution</th>
<th>Preparation Date</th>
<th>Analysis Date</th>
<th>Purity by GLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mg/ml 4PC in hexane</td>
<td>9-25-89</td>
<td>2-16-90</td>
<td>98.0%</td>
</tr>
<tr>
<td>10 mg/ml 4PC in hexane</td>
<td>2-12-90</td>
<td>2-16-90</td>
<td>97.8%</td>
</tr>
<tr>
<td>5 mmole/kg 4PC in sesame seed oil</td>
<td>2-11-90</td>
<td>2-19-90</td>
<td>98.1%</td>
</tr>
<tr>
<td>5 mmole/kg 4PC in sesame seed oil</td>
<td>2-19-90</td>
<td>2-19-90</td>
<td>97.8%</td>
</tr>
</tbody>
</table>
Appendix D

Extraction efficiency of 4-phenylcyclohexene from blood

Objectives: Determine the extraction efficiency of 4PC from blood and establish an extraction volume by GLC analysis

Equipment and Chemicals:

Refer to Appendix A.
Rat blood
Methanol (HPLC grade) Fisher Scientific

Design:

<table>
<thead>
<tr>
<th>Extracted samples</th>
<th>Blank samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>4PC</td>
</tr>
<tr>
<td>0.5ml</td>
<td>0.01ml</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

For the extracted blood samples, blood (0.5 ml) was placed in 2 ml glass vials and 0.01 ml of 4PC (2.5 mg/ml made up in MEOH) was added. Varying amounts of hexane (as shown in the table above) were added to the samples, they were then capped, vortexed and shaken for 5 minutes. The phases were separated by centrifugation (1500 x g for 10 minutes). The organic phases were transferred to glass, crimp top vials and analyzed. The blank samples were prepared by spiking hexane with the 4PC in a similar fashion (as shown in the above table).

The samples were analyzed by the GLC method described in appendix A. Each extracted sample was done in duplicate and compared to the blank samples as shown in the calculation.

Calculation:

\[
\text{Peak Area Sample Extracted from Blood} \times 100 = \% \text{ Extracted}
\]

\[
\text{Peak Area Spiked Hexane}
\]
Results:

The results indicated that 4PC can best be recovered by using an extraction volume of 0.5 ml when extracting 0.5 ml of blood (Table IIa).

Table IIa. Percent of 4PC recovered after extraction with hexanea

<table>
<thead>
<tr>
<th>Volume of hexane extract (ml)</th>
<th>% Extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>91</td>
</tr>
<tr>
<td>0.50</td>
<td>100</td>
</tr>
<tr>
<td>1.0</td>
<td>81</td>
</tr>
<tr>
<td>2.0</td>
<td>83</td>
</tr>
</tbody>
</table>

a Each sample represents duplicate extractions of 25 ug of 4PC.
Appendix E

Amount of 4-phenylcyclohexene found in the blood and expired air of female, B6C3F1 mice after ip administration

Objective: 1) Determine blood concentrations of 4PC over time after administration in B6C3F1 female mice.
2) Determine amount of 4PC expired.

Equipment and Chemicals:
Refer to Appendix A.
Mouse glass metabolism cage
MSA air pump
Standard size SKC organic vapor charcoal tubes

Design:
Female B6C3F1 mice were given a single intraperitoneal dose of 4PC (6 mmole/kg) in sesame seed oil (2.5 ml/kg). The animals were killed by CO₂ asphyxiation at 0.5, 1, 2, 4, and 8 hours after 4PC administration. Blood was drawn into heparinized plastic syringes by cardiac puncture. Blood (0.5 ml) was dispensed into a screw-top glass vial and extracted with 0.5 ml hexane containing 1-phenylcyclohexene as the internal standard (12.5 ug/ml). Vials were shaken for 5 minutes and the phases separated by centrifugation (1500 X g for 10 minutes). The hexane phase was removed and sealed in a 300 ul crimp-top vial. The hexane extract was analyzed for 4PC by capillary gas-liquid chromatography as described in Appendix A.

Expired air samples were analyzed for 4PC by placing an animal in a glass metabolism cage. The cage was attached to a MSA air pump and a vacuum was drawn through a standard charcoal tube at 1.00 ml/minute. The charcoal tubes were changed at 0.5, 1, 2, 4, and 8 hours after administration of 4PC. The charcoal samples were desorbed in hexane (1 ml) for one hour with an efficiency of 90%. The hexane was removed and analyzed for 4PC by capillary gas-liquid chromatography as described in Appendix A.
Example Calculations:

Amount of 4PC found in the hexane extract of the mouse blood when compared to a blood extracted standard curve, ie. 30 minute time point.

\[
11.18 \text{ ug/ml} = 0.00001118 \text{ g/ml x mole/158.24g x 10}^6 \text{ umole/mole} \\
= 0.0706 \text{ umole/ml x 1.26 ml (total blood volume in mouse)} \\
= 0.089 \text{ umoles of 4PC}
\]

Total amount of 4PC dosed to the mouse.

Volume of 4PC x Dose
\[
0.0525 \text{ ml x 0.379 g/ml} = 0.0198 \text{ g/158.24 g/mole} \\
\times 10^6 \text{ umole/mole} \\
= 126 \text{ umoles of 4PC administered}
\]

% of total 4PC dose found at the 30 minute time point.

Amount found in blood / Total Dose
\[
0.089 \text{ umoles} / 126 \text{ umoles} = 0.000706 \times 100\% \\
= 0.071 \% \text{ of total dose}
\]

Results:

The amount of 4PC (nmole of 4PC/ml of blood) found in the mice over time is represented by Figure 2a. No more than 0.225% of the 4PC dose (2 hour time point) was found in the blood at any of the time points examined. % Dose was calculated as demonstrated by the example calculations above. The concentration of 4PC peaked between 2 and 4 hours, and decreased at 8 hours (Figure 2a). These data suggest that 4PC is slowly absorbed into the blood within the first 2-4 hours after administered. It is very likely that the 4PC remains sequestered in the peritoneum in the dosing vehicle.

Negligible amounts of 4PC were collected from the expired air samples as well. After 8 hours less than 0.03% of the total dose was desorbed from the charcoal samples. This may indicate that expiration is not a major route of 4PC elimination in mice.
Figure 2a. Blood concentrations of 4PC (parent compound) in female mice over time after treatment with 4PC (6 mmole/kg, ip). Each point represents the mean of 2 animals.
Appendix F

Estrogenic activity of sesame seed oil in female, B6C3F1 mice

Objective: Determine if sesame seed oil has estrogenic activity in female B6C3F1 mice.

Equipment and Chemicals:
Sartorius 1501 balance
Sesame seed oil, Sigma Chemical Co.
Saline

Rational and Design:

Corn oil, a common vehicle in toxicology experimentation, has been shown to cause estrogenic activity by increases in uterine weight and by producing vaginal cornification in ovariectomized rats and mice (Evans et al., 1941; Sharaf and Negm, 1973). Therefore, sesame seed oil, a common vehicle used in reproductive physiology experimentation, was chosen as the vehicle in this thesis. However, since a reference specifically stating that sesame seed oil does or does not have estrogenic activity could not be found, we designed an assay to determine this fact.

Twenty-eight day old female B6C3F1 mice were ovariectomized and given daily intraperitoneal (ip) treatments of sesame seed oil (2.5 ml/kg) or saline for 21 days. These mice were examined for the stage of estrous cycle by vaginal smear. Intact mice of the same age served as naive controls. On day 22 the mice were killed by CO₂ inhalation, their uteri were removed and weighed on a sensitive balance.

Results:

The ovariectomized mice treated with either the sesame seed oil or saline remained in a state of diestrus throughout the assay. The uterine weights of both sesame seed oil and saline treatment groups when compared to the naive controls demonstrated a dramatic decrease in weight (Figure 3a).

The combine evidence, lack of normal estrus cycle or cornified epithelium in the saline or sesame seed oil treatment groups and a decrease in uterine weight in these
groups suggest that sesame seed oil does not produce an estrogenic activity in female B6C3F1 mice.

Figure 3a. Effect of saline or sesame seed oil on uterine weights of ovariectomized female mice treated daily for 21 days. The control uterine weights are from untreated, intact mice. Each group represents the mean ± S.D. of 5 animals. The saline and sesame seed oil groups were significantly different from the control \(^a\), and subsequently did not differ from each other \(^b\) as determined by ANOVA and a Newman-Keuls range test (p < 0.05).
Appendix G

Balb C studies

Objective: 1) Determine the approximate lethal dose of 1-phenylcyclohexene (IPC), vinylcyclohexane (VCHA), and cyclohexene (CHE) in female Balb C mice after a single intraperitoneal injection.

2) Continue dosing the survivors of the respective compounds for two weeks to establish lethality of repeated doses.

3) Obtain primary follicle counts on the mice which survive the respective treatments for two weeks.

Chemicals:

IPC Aldrich Chemical Co. P2, 230-3
CHE Aldrich Chemical Co. 12, 543-1
VCHA Aldrich Chemical Co. 11, 140-6

Design:

Refer to the "Range finding studies" in the materials and methods section of this thesis.

Species: Female Balb C Mice, 20-25g
Number: 2 animals per dose
Vehicle: Sesame seed oil
Dosing Volume: 5.0 ml/kg
Doses: g/Kg
IPC 4.0, 3.5, 3.0, 2.0 and 1.0
VCHA 2.4, 1.6, 1.2, 0.8 and 0.4
CHE 2.5, 2.0, 1.5, 1.0 and 0.5

Structures:

IPC MW=158.24
CHE MW=82.15
VCHA MW=110.2
Results:

Table IVa. Lethality of a single treatment of IPC, CHE, or VCHA in female Balb C mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (g/Kg)</th>
<th>Survivala</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPC</td>
<td>4.0</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>1/2</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2/2</td>
</tr>
<tr>
<td>CHE</td>
<td>2.5</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>2/2</td>
</tr>
<tr>
<td>VCHA</td>
<td>2.4</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>2/2</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>2/2</td>
</tr>
</tbody>
</table>

a Survival 24 hours post-dosing.

Table Va. Survival of female Balb C mice after treatment with IPC, CHE, or VCHA for 14 consecutive days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dosea</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPC</td>
<td>2.0</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2/2</td>
</tr>
<tr>
<td>CHE</td>
<td>1.0</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>2/2</td>
</tr>
<tr>
<td>VCHAb</td>
<td>4.0</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>3.2</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>2.4</td>
<td>1/2</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>2/2</td>
</tr>
</tbody>
</table>

a g/kg/day;ip  
b Additional concentrations were added to find a lethal dose for VCHA
Figure 4a. Effect of IPC, CHE, or VCHA administration on primary follicle counts in female Balb C mice after 14 days of consecutive treatment. Ovaries were removed, fixed, and counted by the method described in the materials and methods of this thesis, "Fourteen day study in female Balb C mice". Each box represents the counts of 1-2 animals.

Based on the morality observed in this study (Table IVa and Va) IPC could be administered to Balb C mice repeatedly at a dose of 1.0 g/kg, CHE at a dose of 0.5 g/kg, and VCHA at a dose of 1.6 g/kg.

These data also suggest that all three compounds may cause depletion of primary follicles in Balb C mice. The naive controls had an average count of 256 (n=2), the mice dosed with IPC had counts of 138 (n=2), those dosed with CHE and VCHA had counts of 151 (n=1), and 133 (n=1) respectively (Figure 4a). However, it is stressed that the number of animals per group is much too small to draw conclusions.
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