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Long term reproductive organ and gonadotropin changes in female B6C3F1 mice following administration of the ovotoxin 4-vinylcyclohexene

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The University of Arizona, 1992
LONG TERM REPRODUCTIVE ORGAN AND GONADOTROPIN CHANGES
IN FEMALE B6C3F1 MICE FOLLOWING
ADMINISTRATION OF THE OVOTOXIN 4-VINYLCYCLOHEXENE

by
Deborah Parker Douds

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For the Degree of
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In the Graduate College
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1992
STATEMENT BY AUTHOR

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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF ILLUSTRATIONS</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>7</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>8</td>
</tr>
</tbody>
</table>

CHAPTER

1. INTRODUCTION

4-Vinylcyclohexene

Toxicology of VCH
Disposition and Metabolism of VCH

Reproductive Physiology

Hormonal Changes
Estrous Cycle of the Mouse

Reproductive Toxicology

Oocyte Destruction
Specific Ovarian Toxicants
Ovarian Tumorigenesis

Statement of Problem

2. MATERIALS AND METHODS

Animals
Chemicals
Methods

General
Vaginal Smear Determination of the Stage of
Estrous Cycle
Radioimmunoassay for FSH
Data Analysis
TABLE OF CONTENTS (CONTINUED)

3. RESULTS .................................................. 35
   Follicle Stimulating Hormone................................ 35
   Follicle Number.......................................... 36
   Ovarian Weight........................................... 36
   Body Weight.............................................. 42
   Estrous Cycles Per 30 Days............................... 44
   Uterine Weight........................................... 44

4. DISCUSSION .............................................. 47
   Oocyte Loss.............................................. 47
   Ovarian Weight.......................................... 48
   Body Weight............................................. 49
   Follicle Stimulating Hormone............................. 50
   The Number of Estrous Cycles Per 30 Days............... 52
   Uterine Weight.......................................... 53

APPENDIX A: Estrogenic activity of sesame seed and corn oil in female, B6C3F1 mice ................. 56

APPENDIX B: Follicle stimulating hormone (FSH) radioimmunoassay procedure ......................... 61

REFERENCES .............................................. 65
LIST OF ILLUSTRATIONS

Figure

1. Structure and physical properties of VCH.................9
2. The normal plasma levels of FSH and estrogen in the estrous cycle of the female mouse....................16
3. Diagram of the control of the estrous cycle............17
4. Vaginal cytology of the rodent estrous cycle...........19
5. Labeled and unlabeled FSH compete for binding to the anti-FSH antibody.................................33
6. Effect of VCH treatment on plasma FSH levels (ng FSH/ml plasma as % of control) after ip administration daily for 30 days......................35
7. Effect of 4-vinylcyclohexene (VCH) treatment on small and growing follicle numbers in mice after ip administration for 30 days..................37
8. Ovaries from mice treated with either VCH (800 mg/kg) or sesame seed oil (2.5 ml/kg) ip for 30 days and killed immediately after day 30 when the vaginal smear indicated they were in diestrus................38
9. Mouse ovary after treatment with sesame seed oil (2.5 ml/kg) or VCH (800 mg/kg) daily ip for 30 days and killed after 360 days.........................39
10. Effect of VCH treatment on the weight of both ovaries of mice after ip administration for 30 days..........40
11. Mouse ovary after treatment with sesame seed oil or VCH and killed after 360 days.........................41
12. Body weight measurements of control and VCH treated mice during the 30 days of treatment and at the time the mice were killed..........................43
13. Effect of VCH treatment on the number of estrous cycles per 30 days after ip administration..............45
14. Effect of VCH treatment on wet and dry uterine weight (expressed as a percent of body weight) after ip administration for 30 days...............46
LIST OF TABLES

Table

I. Decrease in ovarian weight (as a percent body weight) in the VCH treated animals..........................42
4-Vinylcyclohexene (VCH) is released during rubber manufacture as an environmental contaminant. It causes destruction of ovarian follicles and ovarian cancer in mice. To determine its long term effects on the female reproductive system, female B6C3F1 mice were administered sesame seed oil or VCH (800 mg/kg) ip daily for 30 days. Mice were killed when in diestrus at 30, 60, 120, 240, or 360 days after VCH administration. Ovaries and uteri were weighed and ovaries prepared for histology. Primary and secondary follicles were counted and FSH determined by RIA. The number of estrous cycles differed from controls in the 30 day group. Uterine weight differed in the 30, 60, and 360 day groups. Primary follicles, secondary follicles and ovarian weight decreased in the VCH groups. FSH increased in the 240 and 360 day VCH groups. These studies indicate that VCH causes a dramatic and permanent reduction in ovarian follicles with a reduction in ovarian weight the most obvious indicator of follicle destruction.
**INTRODUCTION**

4-VINYLCYCLOHEXENE

4-vinylcyclohexene (VCH) is a six-member carbon ring with a vinyl side chain in the 4 position. At room temperature it is a colorless liquid with a very pungent odor. VCH is used as an intermediate in the production of vinylcyclohexene diepoxide (VCD), which is used in the manufacturing of polyesters, coatings, and plastics. It is also used in the manufacture of flame retardants and in the production of insecticidal compounds with ethylene chlorohydrin. During the manufacture of rubber, VCH is produced as an environmental contaminant by a dimerization reaction of 1,3-butadiene (IARC, 1985)(Figure 1.).

![Structure of 1,3-Butadiene and 4-Vinylcyclohexene](image)

**Figure 1. Structure and physical properties of VCH.**

- **Molecular weight:** 108.18 g/mole
- **Boiling point:** 128.9°C
- **Melting point:** -109°C
- **Density:** 0.8299 g/ml
Therefore, during the manufacture of rubber products and in the process of tire retreading and tire curing, workers in these plants are exposed to VCH by inhalation (IARC, 1985; Rappaport and Fraser, 1976 and 1977). It is also present in gases discharged from rubber stock during vulcanization and in the stock effluent of tire curing (Rappaport and Fraser, 1976 and 1977).

**TOXICOLOGY OF VCH.** The acute toxicity of VCH was examined in a study by Collins and Manus (1987). Male and female Fischer 344 rats and B6C3F1 mice were dosed orally for 14 days with VCH at 300, 600, 1250, 2500, and 5000 mg VCH/kg body weight or 13 weeks with 50, 100, 200, 400, and 800 mg VCH/kg body weight for the rats and 75, 150, 300, 600, and 1200 mg VCH/kg body weight for the mice (n=10 per group). All of the rats receiving 1250 mg/kg or greater, 3 of male mice receiving 1250 mg/kg, and all mice receiving 2500 mg/kg or greater died during the course of the study. After 14 days of dosing there were no compound-related gross or histological changes noted in either the F344 rats or B6C3F1 mice of either sexes. After 13 weeks hyaline droplet degeneration of the proximal convoluted tubule was seen in the dosed male rats but not the female rats or mice of either sex. The hyaline droplet degeneration was the most severe in the
group receiving 800 mg/kg. Of greatest interest to these studies, was a reduction in the number of primary follicles and mature Graafian follicles in the ovaries of the mice. This was not seen in the ovaries of the rats.

In a study on the long term effects of VCH, female B6C3F1 mice and F344 rats were dosed orally with VCH at 0, 200, or 400 mg/kg, 5 days per week for 103 weeks (Collins, Montali, and Manus, 1987). Both the 200 and 400 mg/kg dose groups of mice had an increase in the occurrence of ovarian neoplasms including mixed benign tumors and granulosa cell tumors and/or carcinomas. There was no increase in the incidence of tumors in rats.

**DISPOSITION AND METABOLISM OF VCH.** After exposure to VCH, mice have a loss of ovarian follicles and an increase in ovarian neoplasia which is not seen in rats. The possibility of a species difference in the disposition of VCH between rats and mice was investigated by Smith, et al. (1990a). It was determined that after a single oral dose of $^{14}$C-labeled VCH at 400 mg/kg, 50-60% of the dose was excreted in the urine of both B6C3F1 mice and F344 rats and 30-40% was excreted in the expired air. Eight hours after administration of VCH at 400 mg/kg, adipose tissue contained the highest concentration of the parent compound in both the rat and the mouse. The time of the
peak concentration in the adipose tissue was different between species, however. In mice the highest concentration of VCH occurred 1-2 hours after treatment and in the rat it occurred 8 hours after treatment. The appearance of a monoepoxide metabolite of VCH, VCH 1,2-epoxide, was measured in the blood 0.5 to 6 hours after intraperitoneal (ip) treatment with 800 mg/kg VCH. Large differences in the amount of the monoepoxide measured in the blood of the rats and mice were seen. The monoepoxide appeared at the highest concentration (41 nmol/ml) 2 hours after ip treatment in the mouse and remained below the level of detection in the rat at all times. Neither 14C-VCH nor the parent compound was selectively distributed to the ovaries in either rats or mice. In an in vitro study using hepatic microsomes, the rate of epoxidation of VCH to the 1,2-epoxide was 4 to 6 times greater in the mouse than in the rat.

Intraperitoneal treatment with VCH daily for 30 days produced a dose dependent decrease in primary follicles in the ovaries of B6C3F1 mice, but not in F344 rats (Smith et al., 1990b). However, treatment with VCH 1,2-epoxide and vinylcyclohexene diepoxide (VCD) produced a dose dependent decrease in primary follicles in both species with VCD causing a greatest amount of destruction than either the
monoepoxide or VCH. Therefore, a critical step in VCH induced ovotoxicity in mice appears to be conversion of the parent compound to the epoxide metabolite.

REPRODUCTIVE PHYSIOLOGY

The ovary is the primary reproductive organ in the female. It is formed in the medial section of the embryo from thickenings in epithelium of the urogenital ridges. Early in embryonic development, primordial germ cells are formed in the yolk sac region of the extraembryonic coelom. These germ cells migrate to and settle in the genital ridge where they give rise to a definitive population of oocytes which undergo the first meiotic division and then remain in a prolonged resting (dictyate) stage of prophase until approximately 12 hours before ovulation. This process is termed oogenesis. In mammals oogenesis is completed before birth. The maximum number of oocytes are present prenatally with only 30\% remaining at the time of birth. By the start of puberty only 3 to 4 percent of the original number of oocytes remain (Briggs, 1986). Each oocyte is incorporated into a follicle which consists of an oocyte, a single layer of granulosa cells and a basement membrane that separates it from the adjacent interstitial tissue. For toxicological evaluations, the follicles are classified based on the
size of the oocyte, the size of the follicle (defined by the number of granulosa cells surrounding the oocyte), and the morphology of the follicle (Pedersen and Peters, 1968).

Small follicles (primordial or primary follicles) consist of a small oocyte with a single, and sometimes incomplete, layer of granulosa cells surrounding it. Secondary or growing follicles contain a growing oocyte and several layers of granulosa cells. Graafian or preovulatory follicles are surrounded by many layers of granulosa cells and the follicles contain an antrum or fluid filled cavity. Cells of primary follicles contain receptors for estradiol (E2) and follicle stimulating hormone (FSH) (Erickson, 1978). The theca interna, which is a layer of modified stroma with hypertrophied stromal cells surrounding the basement membrane and the granulosa cells, first appears at the primary follicle stage. During differentiation of the secondary follicle the thecal cells develop LH receptors. FSH receptors are only found on the granulosa cells. The growth and differentiation of the follicles is dependent on FSH. In absence of sufficient FSH the follicles undergo atresia and die. The growing and preovulatory follicles have the ability to produce the steroid hormones: androgens (such
as androstenedione), progestins (progesterone and pregnenolone), and estrogens (such as estradiol). It is believed that synthesis of the steroids occurs in two different cell types. LH stimulates the thecal cells to produce androgens which diffuse into the granulosa cells where FSH stimulates aromatase enzymes to convert the androgens to estrogen. 17-β Estradiol is the major estrogen that is present.

**HORMONAL CHANGES.** FSH and LH are synthesized and stored in the anterior pituitary gland. Release of these two hormones is stimulated by pulsatile release of gonadotropin releasing hormone (GnRH) from the hypothalamus. In the normal cycling female, estrogen which is produced by the growing follicle and progesterone (produced by both the growing follicle and the corpus luteum) exert a negative feedback on the production of FSH by inhibiting both the GnRH release from the hypothalamus and the release of FSH itself from the pituitary (Fig. 2). The release of FSH causes follicular growth and the associated secretion of estradiol from the growing follicle which once again exerts negative feedback on FSH release at the hypothalamus and pituitary gland. Estradiol released on the morning of proestrus from mature Graafian follicles is the stimulus for LH release. At
this time, estrogen exerts positive feedback on the hypothalamus causing release of GnRH (See Fig. 3). GnRH stimulates release of LH and FSH from the pituitary. The absolute concentration and timing of progesterone in relation to the amplitude and duration of the preovulatory estradiol surge is important for the LH and FSH peak.

Figure 2. The normal plasma levels of FSH and estrogen in the estrous cycle of the female mouse. Revised from Campbell et al. (1977).
Figure 3. Diagram of the control of the estrous cycle. FSH and LH released from the pituitary promote growth of the follicles within the ovary. These follicles produce estrogen and progesterone which exert both negative and positive feedback on the hypothalamus and pituitary. (Fink, 1988.)

The peaking of LH and FSH initiates ovulation of a mature Graafian follicle. After ovulation the remaining granulosa cells in the ruptured follicle undergo luteinization (differentiation to luteal cells) and form the corpus luteum (CL). The CL's main function is to produce progesterone and estrogen. In the mouse however, the CL is essentially nonfunctional and progesterone is not elevated during the latter part of the cycle. Therefore, estrogen and progesterone remain low and FSH and LH increase. The cycle then repeats itself.
ESTROUS CYCLE OF THE MOUSE. The estrous cycle encompasses growth of the follicle, ovulation, production of the corpus luteum, and return to conditions favorable for another ovulation. It lasts approximately 4 to 6 days in the mouse and can be broken down into 5 different stages: proestrus, estrus, metestrus 1, metestrus 2, and diestrus. Proestrus is a period of augmented growth of the ovarian follicle, while estrus is the period of sexual excitement or "heat". Metestrus 1 and 2 are a period of return to the diestrus conditions and diestrus is a period of quiescence. Proestrus usually lasts less than one day and estrus 1-2 days. Metestrus 1 and 2 usually last one day each. The length of diestrus is highly variable lasting from 1 to 3 days. In 1922 Allen devised a system to determine the stage of the estrous cycle based on changes in the epithelial cells lining the vagina. The quantity and type of cells present on the vaginal epithelium respond to the plasma estrogen levels and correspond to the stage of the animal's estrous cycle. During proestrus there are predominantly oval, light staining epithelial cells with round nuclei (Figure 4). Estrogen induces cornification of the stratified, squamous epithelium of the uterus and vagina, therefore, in estrus there are flattened, angular, nonnucleated cornified cells.
Figure 4. 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
present. Metestrus 1 has many nonnucleated cornified cells that are clumped in masses. In metestrus 2, nonnucleated cornified cells remain with the addition of large numbers of leukocytes, predominantly neutrophils. Only a very few cells are present during diestrus; predominantly rounded, nucleated cells with a few leukocytes.

**REPRODUCTIVE TOXICOLOGY**

The reproductive cycle is vulnerable at many points to the influence of both direct and indirect acting foreign substances. The direct compounds act either by structural similarity to a hormone or other vital substance or by nonspecific chemical reactivity. With indirect compounds the xenobiotic is either metabolized to a direct acting substance or it may cause some enzyme modification, such as inhibition or induction. This change in the enzyme behavior could result in altered synthesis of a reproductive hormone or other substance that is vital to the reproductive system. Although the vast majority of metabolism of xenobiotic compounds occurs in the liver (Sipes and Gandolfi, 1986), it has been shown that the ovary also has the ability to metabolize xenobiotic compounds (Mattison and Thorgeirsson, 1978). Both hepatic and ovarian metabolism of a xenobiotic can
potentially result in disruption of some event necessary for reproduction.

**OOCYTE DESTRUCTION.** The age that the ovary is depleted of oocytes is controlled by many factors. These include the number of oocytes formed during oogenesis, the rate of loss due to ovulation, failure of folliculogenesis to occur, the rate of atresia, and toxicity. One type of reproductive toxicity is manifested when xenobiotics block oogenesis or destroy oocytes contained within follicles.

Compounds that destroy follicles at different stages of development will have different impacts on the reproductive cycle. Destruction of growing and preovulatory follicles will cause an immediate decrease in fertility during the period of exposure. The symptoms will mimic postmenopausal symptoms and will continue until the primary follicles mature to growing and preovulatory follicles that are capable of producing the steroid hormones necessary for reproduction. Xenobiotics which destroy primary follicles are initially silent reproductive toxicants. They are only identifiable at first by a decrease in primary oocytes (by direct oocyte quantification) then by a premature onset of menopause.

Many compounds that destroy oocytes do so by binding to or causing damage to DNA. Pedersen and Mangia (1978)
demonstrated that growing and resting mouse oocytes are capable of excision repair mechanisms to the damaged DNA. In their studies, they exposed both growing and resting oocytes to ultraviolet light (254 nm). The oocytes were then incubated in medium containing $^3$H-labeled thymidine. After autoradiographic exposure they were able to detect unscheduled DNA synthesis, thus showing that mouse oocytes appear to have repair enzymes for excision and replacement of DNA bases damaged by ultraviolet light.

Sensitivity to oocyte destruction by chemicals varies with species. Mattison (1979) demonstrated that dimethylbenzantracene (DMBA), benz(a)pyrene (BP), and 3-methylcholanthrene (MC) had a greater ability to destroy oocytes in C57B1/6N (B6) and DBA/2N (D2) mice than in Sprague Dawley rats. The mice and rats were dosed with BP, MC, or DMBA and were killed either 6 or 14 days after treatment. Six days after treatment with DMBA the percentages of primordial oocytes left were 5.6 and 0.6% of control in the D2 and B6 mice respectively. In the rats, 50% of the primordial oocytes remained 14 days after treatment with DMBA. Treatment with MC destroyed greater than 99% of the oocytes in the B6 mice, 97% in the D2 mice, but only 33% in the rats. BP had a similar effect. It destroyed 97% of the oocytes in the B6 mice, 61% in the
D2 mice, and 30% in the rats. One reason for these differences may be due to species and strain differences in polycyclic aromatic hydrocarbon (PAH) inducible ovarian microsomal P-450 dependent monooxygenase.

Aryl hydrocarbon hydroxylase (AHH) is a cytochrome P450 enzyme that metabolizes PAHs to reactive intermediates. Mattison and Thorgeirsson (1978 and 1979) showed that AHH is present in the ovaries of mice and rats. The amount of ovarian AHH activity was measured and it was similar in the B6 and D2 mice. However, after induction with MC an increase in AHH activity was seen only in the B6 mice, which may explain the increased toxicity in the B6 mice. In the rat there was a 2-3 fold increase in ovarian AHH activity after MC treatment. If the inducibility of the AHH enzymes is responsible for the increased toxicity then it would be expected that the rats would demonstrate a higher degree of toxicity to MC than the D2 mice. In reality the rats displayed a lesser degree of toxicity and this could mean that the rat has a greater ability to detoxify the MC or repair damage from the MC.

Sensitivity to toxicants also varies with follicular size. Small, immature oocytes in primordial follicles are more sensitive to ionizing radiation than the mature
oocytes in large, growing follicles (Mattison and Schulman, 1980; Dobson and Felton, 1983). In addition, age of the animal also plays a role in the sensitivity of the oocyte to foreign compounds. Turbyfill and Chang (1969) irradiated hamsters at ages of 1, 7, 14, 21, or 30 days after birth. The hamsters were then killed on day 33 and the ovaries examined for the number of follicles. Follicles of all stages appeared to be most sensitive on days 7 and 14, as ovaries from the hamsters irradiated on these days had the lowest oocyte counts.

**SPECIFIC OVARIAN TOXICANTS.** Many different chemicals are known to destroy oocytes. In one study, of the 77 chemicals tested, 21 caused oocyte loss. These 21 chemicals all had known mutagenic/carcinogenic properties (Dobson and Felton, 1983).

Some of the chemicals that destroy oocytes are environmental contaminants. Polycyclic aromatic hydrocarbons (PAH), which are present in automobile exhaust, smokestack emission, and cigarette smoke, have been shown to destroy oocytes in weanling and sexually mature rats and mice (Mattison, 1981). PAHs are metabolized to chemically reactive intermediates that bind covalently to cellular macromolecules. The end result is destruction of oocytes and premature ovarian failure. It
is not known if PAHs destroy human oocytes, but smoking does produce a dose-dependent decrease in the age of onset of menopause (Mattison, 1981).

Intraovarian injection of 3-methylcholanthrene (MC) or 7,12-dimethylbenz(a)anthracene (DMBA) destroys oocytes in C57BL/6N and DBA/2N mice (Shiromizu and Mattison, 1985). However, treatment with alpha-napthoflavone (ANF), a competitive inhibitor of the microsomal monooxygenases which metabolize PAHs, inhibits the oocyte destruction of MC and DMBA. These results indicate that DMBA and MC are indirect acting toxins and require metabolic activation. It also suggests that the ovary has enzymes that are needed to biotransform DMBA and MC to ovotoxic metabolites.

Other chemicals which have been shown to destroy oocytes are drugs such as alkylating agents used in the treatment of neoplastic and some nonneoplastic diseases. One such antineoplastic drug is cyclophosphamide which destroys oocytes in an age and dose-dependent manner. Young females are more sensitive to the compound, as are small or resting oocytes (Mattison, 1981). Mattison et al. (1981) studied the effects of cyclophosphamide, azathioprine (AZA), and 6-mercaptopurine (6-MP) on oocyte and follicle number in C57BL/6N mice. A single dose of
cyclophosphamide at 100 mg/kg destroyed 63% of the small oocytes with no effect on the large or medium follicles. While 6-MP produced sterility in female offspring exposed in-utero, neither 6-MP nor AZA, a structural analog, produced any oocyte loss when given ip at 100 mg/kg for nine days to 28 day old mice. It was concluded that 6-MP exerted its toxicity during prenatal exposure by an alteration in oogenesis.

OVARIAN TUMORIGENESIS. Ovarian tumor formation often occurs after destruction of the oocytes. Twenty-one day old mice were treated with DMBA either by direct ovary painting, oral gavage or intraperitoneal (ip) injection. At 3, 6, 9, and 12 months of age the mice were killed and the ovaries were examined. Upon histological examination of the ovaries it was found that only approximately 6% of the small oocytes remained and that there was degeneration of the follicles. Follicles appeared as empty-rings (i.e. rings of granulosa cells without oocytes), and pseudo-follicles, which were described as solid balls of granulosa cells surrounded by a layer of flattened thecal cells. There was also an increase in luteinization of the stroma and a persistence and merging of corpora lutea. At 6 to 12 months there was a high incidence of ovarian tumors, such as luteomas and granulosa cell tumors.
(Krarup, 1967). These preneoplastic changes did not begin until most of the small oocytes had been destroyed. Krarup (1970) also showed that the faster the oocytes are destroyed by the toxicant 9,10-dimethyl-1,2-benzanthracene (DMBA) the earlier the tumors appeared. Tumors developed faster after ip injection of DMBA than oral administration, which correlated with the more rapid destruction of oocytes in the ip treated mice. Krarup hypothesized that the DMBA was the tumor initiator and some pituitary influence was the promoter.

Marchant (1961) studied the effect of hypophysectomy on the development of ovarian tumors in mice treated with DMBA. In this study hypophysectomy did not affect the ability of DMBA to destroy ovarian follicles and cause premature ovarian failure. It did, however, prevent the development of tumors. Marchant divided ovarian tumorigenesis by DMBA into two phases. The first phase is the preneoplastic phase where the ovary atrophies resulting from an increased rate of atresia of oocytes and follicles in the ovary. The second phase is the appearance and growth of tumors of the granulosa cell type. This phase requires stimulation from the pituitary.

Plasma gonadotropin levels were measured during early stages of ovarian tumorigenesis in mice of the W\textsuperscript{v}/W\textsuperscript{v}
genotype (Murphy and Beamer, 1973). These mice have less than 1% of the normal complement of oocytes at birth and develop bilateral ovarian tubular adenomas in 95% of the mice by 5 months of age. Thirty-eight percent of the mice surviving to 16 months of age had granulosa cell tumors and luteomas. Mice with the W<sup>x</sup>/W<sup>v</sup> genotype and the wildtype +/+ mice were killed at ages 30, 60, 90, 120, and 210 days and plasma LH and FSH were measured. At all ages FSH and LH levels were elevated in the W<sup>x</sup>/W<sup>v</sup> mice.

In a study by Jarrell, et al. (1988) direct radiation of the ovaries of 30 day old Sprague-Dawley rats caused a dose related loss of ovarian follicles and a dose related increase in serum FSH. There was a linear relationship between serum FSH and the number of preantral, but not healthy antral or primordial follicles. This showed that the pituitary is stimulated to produce more FSH upon oocyte loss.

**STATEMENT OF PROBLEM**

Although industrial workers are exposed to VCH during the manufacture of rubber products, the effect of VCH on the ovaries of humans has not been determined. Currently, the only indication of oocyte loss in humans is premature ovarian failure and early menopause. The goal of this research was to examine the long term effects of
VCH on the female reproductive system of mice and to examine any possible changes that could be used as a marker for the detection of chemically induced oocyte loss. To do this the correlation between oocyte loss and plasma FSH levels was examined. Since VCH causes a reduction in the number of primary follicles, with an eventual loss of preantral follicles, it would be expected that the estrogen production from the ovary would be decreased. Therefore, it was hypothesized that plasma FSH levels would increase and FSH could be used as a marker of chemically induced oocyte loss. Ovarian weight, uterine weight, and estrous cyclicity were also examined, since they are influenced by the circulating hormone levels.
MATERIALS AND METHODS

ANIMALS

Twenty-one day old female, B6C3F1 mice were obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN). They were housed 5 per cage in sawdust bedding and had free access to food (Teklad®, Harlan Sprague Dawley, Inc. Madison, WI) and water. The animals were maintained on a 12 hour light/dark cycle and acclimated to this environment for 7 days before use.

CHEMICALS

4-vinylcyclohexene (VCH) containing t-butylcatechol as an antioxidant was purchased from Aldrich Chemical Co. (Milwaukee, WI). Sesame seed oil, used as a control, was purchased from Sigma Chemical Co. (St. Louis, MO). Rat FSH-reference preparation 2, FSH for iodination, and antirat-FSH antiserum used in the FSH radioimmunoassay were obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). Antibody against the antirat-FSH antiserum used for the separation of the bound and free fractions was obtained from the laboratory of Dr. Mark Wise, Animal Science Department, University of Arizona.
METHODS

GENERAL. Twenty-eight day old female mice were given either VCH (800 mg/kg ip) dissolved in sesame seed oil or sesame seed oil alone as a control (2.5 ml/kg ip) daily for 30 days. Although corn oil is usually used as a vehicle control in toxicologic studies, it has been shown to have estrogenic effects (Sharaf and Negm, 1973 and Booth et al., 1960). Studies in reproductive physiology usually use sesame seed oil as a vehicle, since no estrogenic effects have been associated with its use. In a study that we performed in ovariectomized mice we compared corn oil and sesame seed oil for their ability to cause estrogenic effects (See appendix A). Neither corn oil nor sesame seed oil caused estrogenic effects in the study we performed. We chose to use sesame seed oil as the vehicle instead of corn oil. A dose of 800 mg/kg of VCH was selected based on studies done by Smith et al. (1990b) that showed this dose would cause maximum oocyte destruction. The animals were weighed daily for the first 30 days and their the stage of estrous was determined by examining their vaginal cytology by vaginal smear. Control mice and treated mice (n=10 per group) were killed by CO₂ inhalation at approximately 30, 60, 120, 240, or 360 days after the first day of treatment. The actual
days were determined by the stage of their estrous cycle. They were killed when their vaginal cytology indicated they were in diestrus. Blood was collected for FSH determination by cardiac puncture. The blood was centrifuged, the plasma collected and stored at -70°C until assayed for FSH. The ovaries were removed, weighed together, fixed in Bouin's solution for 24 hours, and then transferred to 70% ethanol prior to routine processing and embedding in paraffin. Sections were cut at 6-8 μm and stained with hematoxylin and eosin. The oocytes were identified by the method of Pedersen and Peters (1968) and counted in every 20th section. The oocyte counts from individual sections were summed to calculate the total oocyte count for each ovary. The uterus was removed and wet and dry weights were recorded.

VAGINAL SMEAR DETERMINATION OF THE STAGE OF ESTROUS CYCLE. Each mouse was picked up by the base of her tail, a fire-polished tip of a Pasteur pipet was inserted into the vagina and one drop of tap water was expelled. The water containing vaginal epithelial cells and leukocytes was then collected by an inoculating loop and placed on 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).

**RADIOIMMUNOASSAY FOR FSH.** Radioimmunoassay (RIA) is based on the binding of an antigen to a fixed amount of antibody in the presence of different amounts of radiolabeled antigen. $^{125}$I-labeled FSH and unlabeled FSH, either a standard or sample, compete for binding to an anti-FSH antibody (Figure 5). The distribution of the antigen, in this case FSH, between the bound and free

\[
125I-\text{FSH} + \text{Ab} \rightarrow 125I-\text{FSH-Ab} \\
+ \\
\text{FSH (unlabeled)} \\
\downarrow \\
\text{FSH-Ab}
\]

**Figure 5.** Labeled and unlabeled FSH compete for binding to the anti-FSH antibody.
phases is directly related to the total amount of antigen present and provides a means of quantifying the amount of antigen present. The percent of $^{125}$I-FSH bound to the antibody is compared to a standard curve to obtain the correct concentration of the sample. FSH was iodinated by a revised Chloramine-T method of Hunter and Greenwood (1962) in the laboratory of Dr. Mark Wise, Animal Science Department, University of Arizona. 10 ul of $^{125}$I (approximately 1 uCi) and 5 ug of FSH were used in the iodination procedure. The iodinated FSH was diluted with phosphate buffered saline to approximately 10,000 cpm per 100 ul. A sample size of 100 ul of plasma was used and each sample was done in duplicate when there was an adequate amount of plasma available.

DATA ANALYSIS

All data were analyzed using Number Cruncher Statistical System 5.0 (1988), (Kaysville, Utah). A student's t-test was used to determine significant differences between 2 group means. One-way analysis of variance (ANOVA) was used to make multiple comparisons. When significant differences were detected within the ANOVA, individual groups were compared using a Newman-Kuel's range test. The level of significance for all tests was $p < 0.05$. 
RESULTS

FOLLICLE STIMULATING HORMONE

Follicle stimulating hormone (FSH) was measured in the plasma of the mice. The mice were killed during diestrus when the plasma FSH levels should be at the lowest point in the estrous cycle. Plasma FSH levels remained equivalent to controls in the VCH treated animals until day 240 when the plasma FSH levels in the VCH treated mice were greater than 2 fold that of the controls (Figure 6).

![Graph showing effect of VCH treatment on plasma FSH levels](image)

* p < 0.05

**Figure 6. Effect of VCH treatment on plasma FSH levels (ng FSH/ml plasma as % of control) after ip administration daily for 30 days.** Each bar represents the mean (x ± S.E.) of 6-10 animals. The asterisks indicate the groups which are significantly different from controls (p<0.05).
FOLLICLE NUMBER

As was expected, when the ovaries were inspected by light microscopy the number of primary follicles was greatly reduced in the VCH treated animals. As compared to controls, ninety percent of the primary follicles and 78% of the growing follicles were destroyed in the group of mice killed at 30 days (See figures 7 and 8). Besides a decrease in the number of follicles in the VCH treated animals, there was the appearance of empty rings, which were described by Krarup (1967) as rings of granulosa cells with no corresponding oocyte in the middle. The number of primary and growing follicles continued to decline in the groups that were killed at the later time points (Figure 9). At the 240 day time point the ovaries of the VCH treated mice contained the appearance of small nests of hyperplastic ovarian cells. The hyperplasia was much more evident at the 360 day time point. With the great reduction in follicle number the size of the ovaries was also significantly different as can be seen in Figure 10. These two pictures are from either control or VCH treated mice in the 360 day time point and are taken at the same magnification. This size difference is also reflected in the change in ovarian weight.
OVARIAN WEIGHT

The weight of both ovaries (as a percent of body weight) was significantly decreased at the 30 day time point in the VCH treated animals (See figure 11). At the 30 day time point the mean ovarian weight (as a percent body weight) in the VCH treated group was 0.042; the corresponding control mean ovary weight was 0.057. The ovary weight remained significantly lower in the VCH treated mice than their respective controls throughout the study period (See Table I).

Table I. Decrease in ovarian weight (as a percent body weight) in the VCH treated animals.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>TREATMENT</th>
<th>OVARIAN WEIGHT (% body weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 Day</td>
<td>Control</td>
<td>.057</td>
</tr>
<tr>
<td></td>
<td>VCH</td>
<td>.042</td>
</tr>
<tr>
<td>60 Day</td>
<td>Control</td>
<td>.073</td>
</tr>
<tr>
<td></td>
<td>VCH</td>
<td>.046</td>
</tr>
<tr>
<td>120 Day</td>
<td>Control</td>
<td>.064</td>
</tr>
<tr>
<td></td>
<td>VCH</td>
<td>.044</td>
</tr>
<tr>
<td>240 Day</td>
<td>Control</td>
<td>.054</td>
</tr>
<tr>
<td></td>
<td>VCH</td>
<td>.023</td>
</tr>
<tr>
<td>360 Day</td>
<td>Control</td>
<td>.042</td>
</tr>
<tr>
<td></td>
<td>VCH</td>
<td>.018</td>
</tr>
</tbody>
</table>
Figure 7. Effect of 4-vinylcyclohexene (VCH) treatment on small and growing follicle numbers in mice after ip administration for 30 days. Each box represents the mean (x ± S.E.) of ten animals. The asterisk indicates the groups which are significantly different from the controls (p<0.05).
Ovaries from mice treated with either VCH (800 mg/kg) or sesame seed oil (2.5 ml/kg) ip for 30 days and killed immediately after day 30 when the vaginal smear indicated they were in diestrus. (440X) The control ovary (a) had 278 primary follicles and 54 growing follicles while the ovary from the VCH treated animal (b) had 26 small follicles and 23 growing follicles. The large arrows indicate growing follicles, small arrows indicate small follicles and E indicates empty rings.
Figure 9. Mouse ovary after treatment with sesame seed oil (2.5 ml/kg) or VCH (800 mg/kg) daily ip for 30 days and killed after 360 days. (220X) The control ovary had 7 small follicles and 19 growing follicles. The VCH treated ovary did not have any small or growing follicles. The large arrow indicates growing follicles and CL indicates a corpus luteum. The hyperplastic cells are denoted with an H.
Figure 10. Mouse ovary after treatment with sesame seed oil or VCH. Mice were treated for 30 days by ip administration and killed at day 360. Magnification is the same for both (46X) to show the size difference. The control ovary had 2 small follicles and 30 growing follicles. The VCH treated ovary did not have any small or growing follicles.
Figure 11. Effect of VCH treatment on the weight of both ovaries of mice after ip administration for 30 days. The data is presented as the weight of both ovaries as a percent of body weight. Each box represents the mean (x ± S.E.) of 10 mice. The asterisk indicates the groups which are significantly different from controls (p<0.05).

BODY WEIGHT

Body weight measurements were taken over the 30 days of treatment and at the time the animals were killed. The body weight was compared for each group between the control and the VCH treated animals. Since the animals were randomly assigned to the different groups there was no difference in body weight at the beginning of the study and weight gain was similar for the two groups at 5 and 10 days. However, at 15, 20, and 30 days body weight of the control animals was statistically greater than the VCH treated animals. Body weight measurements taken at the
time the animals were killed were not statistically different between the two groups (Figure 12).

Figure 12. Body weight measurements of control and VCH treated mice during the 30 days of treatment and at the time the mice were killed. Each point in the graph of body weight over the first 30 days (a) represents the mean (x ± S.E.) of 50 mice. Each point in the second graph (b) of the body weights at the time the mice were killed represents the mean (x ± S.E.) of 10 mice. The asterisks indicate the groups which significantly different from control (p<0.05).
**ESTROUS CYCLES PER 30 DAYS**

During the 30 days prior to being killed the number of estrous cycles was recorded for the 30, 60, and 120 day groups. Due to an outbreak of mouse hepatitis virus in the animal facilities the 240 and 360 day mice were in quarantine. Although our mice never tested positive for the virus, it was not possible to perform vaginal smears on these mice for the 30 days prior to being killed. However, when we performed vaginal smears on the mice to determine when they were in diestrus prior to killing them, the 240 day mice appeared to still be cycling at that time. While the 360 day VCH treated mice did not appear to be cycling at 360 days, their respective controls were still cycling. The vaginal cytology indicated the 360 day VCH treated mice were in Metestruis 2 for the entire two weeks prior to being killed. The mice used were prepubertal at the time the study started and did not start cycling regularly for approximately 2 weeks into the study. The number of estrous cycles was significantly decreased in the 30 day VCH treated group as compared to sesame seed oil controls, but was equivalent to controls in the 60 and 120 day groups (Figure 13).

**UTERINE WEIGHT**

The uterus was removed at the point just above the
cervix and wet and dry uterine weight were measured. The uterus was weighed wet, dried for 24 hours at room temperature, and then dry weight was obtained. Both the wet and dry uterine weight of the VCH treated mice were statistically lower than controls in the 30 and 60 day groups, but the weights were equivalent in the 120 and 240 day groups (Figure 14).

Figure 13. Effect of VCH treatment on the number of estrous cycles per 30 days after ip administration. Each bar represents the mean (x ± S.E.) of 10 mice. The asterisks indicate the groups which are significantly different from controls (p<0.05).
Figure 14. Effect of VCH treatment on wet and dry uterine weight (expressed as a percent of body weight) after ip administration for 30 days. Each bar represents the mean (x ± S.E.) of 10 mice. The asterisks indicate the groups which are significantly different from the control (p<0.05).
DISCUSSION

Various chemicals can cause oocyte destruction (Dobson and Felton, 1983). It has been shown in previous studies that VCH destroys primary follicles in the ovaries of B6C3F1 mice (NTP, 1986; Collins and Manus, 1987; and Smith et al., 1990a,b) and also causes ovarian tumors (NTP, 1986; Collins, Montali, and Manus, 1987). In the previous studies the only parameters examined were oocyte loss and tumor formation. Other factors related to the integrity of the reproductive system were not measured. The purpose of this study was to determine the long term reproductive tissue and gonadotropin changes that occur after VCH induced depletion of primary follicles but before the occurrence of ovarian tumors. The parameters examined in this study were plasma FSH levels, follicle number, ovarian weight, body weight, uterine weight, and the number of estrous cycles per 30 days. Within this study, there were 5 different groups of mice, each dosed with VCH at 800 mg/kg for 30 days and killed at five different time points: 30, 60, 120, 240, and 360 days after the start of the study.

OOCYTE LOSS

Collins and Manus (1987) reported a reduction in the number of primary and mature Graafian follicles in the
ovaries of mice, but not rats, treated with VCH at 1200 mg/kg by oral gavage for 13 weeks. In a study by Smith et al. (1990b) the dose of VCH which reduced the number of primary follicles by 50% of the controls was calculated to be 2.7 mmol/kg (292 mg/kg). At a dose of 7.4 mmol/kg (800 mg/kg) a significant amount of primary follicles was lost after 15 days of treatment. In this study more than 90% of the oocytes were destroyed after 30 days of daily dosing with VCH. These findings correspond well with those of Smith et al. (1990a,b).

Treatment with VCH by oral gavage at 400 mg/kg for 2 years caused an increase in the incidence of uncommon ovarian neoplasms in mice, namely mixed benign tumors and granulosa cell tumors, but not in rats (Collins, Montali, and Manus, 1987). At the 240 day time point small nests of ovarian hyperplastic cells were found in the ovaries of the mice treated with VCH and ovarian cell hyperplasia was seen in the 360 day mice treated with VCH. Since ovarian tumors are found after exposure to VCH, the ovarian cell hyperplasia could be a beginning of the formation of ovarian tumors.

**OVARIAN WEIGHT**

In the study being reported we saw a decrease in the weight of both ovaries (reported as a percent of body weight...
weight) in the all of the VCH treated groups. This decrease in weight was not surprising since at this time nearly all of the ovarian follicles were destroyed and the ovaries had decreased in size. The small amount of granulosa cell hyperplasia seen in the 240 day VCH treated mice did not cause a significant increase in the ovarian weight in this group.

**BODY WEIGHT**

Body weight can be used as an indicator of stress and for this reason we measured the body weight of the animals daily over the 30 days of dosing and at the time each mouse was killed. The VCH treated animals had a statistically significant lower body weight than controls at days 15, 20, and 30 of the dosing period (n=50 per group). These results suggest that the dosing may have caused some stress. There was no difference between the body weight of the VCH treated and control mice at the later time points after dosing (n=10 per group). Since during the 30 days of dosing all groups of mice that were given VCH were treated exactly alike and all controls were treated alike, the different time groups were combined and the number of mice for each group was 50. While the difference in weight could be due to the stress of dosing with VCH, it is also possible that the greater number of
mice per group increased the statistical power for the t test. Therefore, the body weight data for the first 30 days of dosing was calculated to be significant at 15, 20, and 30 days by a student's t test with an n=50 when it would not be significant if the group number was only 10.

**FOLLICLE STIMULATING HORMONE**

FSH is produced by the pituitary gland and causes follicular growth in the ovary. GnRH which is produced in the hypothalamus causes synthesis and release of FSH from the pituitary gland. Both release of GnRH from the hypothalamus and FSH from the pituitary are controlled by negative feedback from steroid hormones produced in the ovary. Estrogen and progesterone, as well as inhibin, regulate release of FSH directly, by inhibiting its release from the pituitary and indirectly by inhibiting release of GnRH from the hypothalamus.

If the negative feedback system of estrogen and progesterone on FSH production is interrupted, the plasma FSH levels will increase. For example, mean serum FSH concentrations increase 8-12 fold in women after ovariectomy (Yen and Tsai, 1971). Jarrell et al. (1980) showed that after destroying the follicles of the ovary with irradiation, FSH increases in a dose dependent manner and this increase in FSH correlates with the number of
preantral, or growing, follicles. With these facts in mind, it was hypothesized that plasma FSH levels should increase with the decreasing number of follicles after treatment with VCH. In our model we did not see an increase in FSH until 240 days after treatment began.

There are several reasons that would explain this delay in the increase in serum FSH. First, although greater than 90% of the primary follicles were gone, there were still growing follicles present. These are capable of producing estrogen and exerting the negative feedback. Perhaps all of these growing follicles have to mature to antral follicles and ovulate or become atretic before estrogen production is stopped. At day 240 there was an average of 0.8 growing follicles remaining in the VCH treated mice, compared to 50 in the controls. Second, Gosden et al. (1983) showed that aged mice with less than 100 total follicles are still capable of ovulating and cycling. Since these animals are still capable of ovulating they should still have normal levels of circulating estrogen and FSH. He also suggested that there may be some other factor that determines when the older mouse will stop cycling besides total number of follicles. Third, there may be a factor that slows down the amount of atresia that occurs when the ovary is
depleted of follicles. Gosden also showed that older mice with approximately 90% less follicles than younger mice still shed approximately the same number of ova and were capable of having a normal litter size. He attributed this to a reduction in the rate of atresia.

**THE NUMBER OF ESTROUS CYCLES PER 30 DAYS**

The vaginal epithelium is responsive to the different levels of the cycling hormones. Estrogen causes cornification of the stratified, squamous epithelium and during estrus there are flattened angular, nonnucleated, cornified cells present. Since the vaginal epithelium is responsive to the cycling hormone levels, it was hypothesized that with the VCH induced loss of oocytes the estrogen concentration would be decreased and, therefore, the estrous cycles would be irregular.

During the course of our study, the number of estrous cycles was measured by vaginal smear for the 30 days prior to termination for the 30, 60, and 120 day groups. For the first 30 days of the study the number of estrous cycles in the VCH treated group was significantly lower than the controls. At 60 and 120 days, however, the number of estrous cycles per 30 days in the VCH treated groups was equivalent to the controls. From these data and the FSH data it appears that the decrease in estrous
cycles for the first 30 days was not due to any hormonal changes that were occurring, but to a direct effect of the VCH. The VCH was dosed ip and was transiently very irritating to the mice which may have been enough stress to the mice that the estrous cycle was affected. Another possibility is that VCH could have caused a direct chemical effect on the cells of the uterine and vaginal walls, decreasing the amount of estrogen induced cornification.

In a study done by Parola (1991) treatment with VCH or 4-phenylcyclohexene (4PC, a structural analog of VCH) at 6 mmol/kg ip for 30 days caused a significant decrease in the number of estrous cycles as compared to controls over the 30 day time period of the study. While 4PC did cause a significant reduction in the number of estrous cycles, it did not cause a reduction in the number of ovarian follicles. This supports our theory that the VCH induced decrease in the number of estrous cycles per 30 days was not a result of a change in circulating hormones.

**UTERINE WEIGHT**

Estrogen causes an increase in uterine weight through proliferation of the endometrium and myometrium. With a decrease in ovarian follicles and estrogen production it was hypothesized that uterine weight would be decreased in
the VCH treated animals. Both wet and dry uterine weight were decreased in the 30 and 60 day groups in the VCH treated groups, but were equivalent to controls in all remaining groups except the 360 day group where the VCH treated mice had a lower uterine weight (both wet and dry). A direct effect of VCH on the uterine wall could explain the loss in uterine weight for the 30 and 60 day groups. If this was the case, then the vaginal epithelium recovered from the VCH treatment faster than the uterus did. If VCH had caused irritation, with the accompanying inflammation and edema, it would be expected that the VCH treated animals would have an increase in uterine weight. This is the opposite of what was seen. At this time the reason for the decrease in uterine weight in the 30 and 60 day groups remains unexplained. In the 360 day VCH treated group the animals were no longer cycling and the FSH levels were greatly elevated. Estrogen causes an increase in uterine weight and when there is no estrogen present the uterine weight will be reduced (See appendix A).

In conclusion, the first and most obvious result of the VCH treatment was a reduction in ovarian weight. This reduction in ovarian weight correlated with the decreased number of ovarian follicles that occurred after treatment.
with VCH. The decrease in the number of estrous cycles per 30 days that occurred for the first 30 days may be a direct effect of the VCH and was not a result of any hormonal changes. The change in uterine weight that occurred also appeared to be a chemical effect and not an hormonal effect, except at 360 days when the reduced weight in the VCH treated group was a result of the reduced estrogen concentration. FSH did increase in the VCH treated animals, but not until the 240 day time point when almost all of the growing follicles had been depleted.

Many people, including industrial workers, are exposed to VCH as well as other reproductive toxicants in the work place. Currently, total ovarian failure with premature menopause is the only sign that indicates the ovarian follicles have been destroyed. It is important to find a biomarker that can be used to determine when ovarian follicles are being destroyed. Further work to elucidate a method to diagnose damage to the ovary in the early stages, while it is occurring before all of the follicles are destroyed needs to be found to prevent this type of injury from happening.
Title:  Estrogenic activity of sesame seed and corn oil in female, B6C3F1 mice.

Objective:  To determine if there is a difference between the estrogenic activity of sesame seed oil and corn oil.

Equipment and Chemicals:
- Sesame Seed Oil, Sigma Chemical Co.
- Mazola Corn Oil
- 0.9% Saline
- Estradiol monobenzoate, Sigma Chemical Co.

Introduction:
In toxicological studies, corn oil is a frequently used vehicle for lipid soluble chemicals. Because of the potential estrogenic effects of corn oil in female mice (Booth et al., 1960; Sharaf and Negm, 1973) sesame seed oil is the more frequently used vehicle in reproductive physiological studies. Since there are no published reports comparing the possible estrogenic effects of sesame seed oil and corn oil in short or long term studies we compared the effects of sesame seed oil and corn oil in ovariectomized mice. The parameters examined in this study were plasma FSH, uterine weight, and vaginal
cytology. Estrogen is produced by the ovaries and exerts a negative feedback on the production of FSH at both the hypothalamus and pituitary. In ovariectomized mice plasma estrogen concentrations are low and therefore, FSH levels are high. Therefore, any estrogenic activity of the oil will cause a decrease in plasma FSH. In mice, uterine weight is increased by the administration of estrogen or compounds with estrogenic activity (Evans, et al., 1941). The uterus of each mouse was weighed at the end of the study as an indicator of estrogenic activity. Estrogen causes cornification of the vaginal epithelium and, therefore, vaginal smears were used as another indicator of estrogenic activity.

Materials:

Twenty-eight day old, B6C3F1 mice were ovariectomized and treated daily for 30 days by ip injection with either estradiol monobenzoate (5 ug/kg) as a positive control, saline as a negative control; sesame seed oil, or corn oil (n = 5 per group). Vaginal smears were examined daily to determine the amount of estrogen-induced cornification. On day 31 the mice were killed by CO₂ inhalation and exsanguination by cardiac puncture. Plasma was collected for FSH determination by radioimmunoassay and their uteri were weighed.
Results:

Mice treated with estradiol had maximum cornification of the vaginal epithelium. Cornification was not seen in the vaginal smears of the other groups (See Figure 1a). The vaginal cytology was scored by the amount and kind of cells that were present in the vaginal smear (See Table 1a). An increase in uterine weight was present only in the estrogen treated groups (Figure 2a). FSH levels were not different in any of the groups (Table 2a).

Figure 1a. Vaginal Smear Cytology. Mean score per group.
Table 1a. Vaginal smear scoring used to determine stage of estrous cycle.

<table>
<thead>
<tr>
<th>Score</th>
<th>Cytology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>Very few cells of any type</td>
</tr>
<tr>
<td>1.5</td>
<td>Many neutrophils, very small number of nucleated epithelial cells, cornified cells, and cell debris.</td>
</tr>
<tr>
<td>2.0</td>
<td>3/4 nucleated epithelial cells and 1/4 cornified epithelial cells.</td>
</tr>
<tr>
<td>3.0</td>
<td>1/2 nucleated epithelial cells and 1/2 cornified epithelial cells.</td>
</tr>
<tr>
<td>4.0</td>
<td>1/4 nucleated epithelial cells and 3/4 cornified epithelial cells.</td>
</tr>
<tr>
<td>5.0</td>
<td>Almost entirely cornified epithelial cells in very large number, often clumping.</td>
</tr>
</tbody>
</table>

Table 2a. Follicle Stimulating Hormone (FSH) Plasma Concentrations.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean ± S.D. (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>4</td>
<td>47.7 ± 6.6</td>
</tr>
<tr>
<td>Estradiol</td>
<td>5</td>
<td>50.3 ± 7.3</td>
</tr>
<tr>
<td>Sesame Seed Oil</td>
<td>5</td>
<td>46.7 ± 11.1</td>
</tr>
<tr>
<td>Corn Oil</td>
<td>5</td>
<td>48.0 ± 4.8</td>
</tr>
</tbody>
</table>
Discussion:

Neither sesame seed oil nor corn oil caused an increased in uterine weight or cornification of the vaginal epithelium, but increases in vaginal cornification and uterine weight were seen in the estradiol treated groups. FSH did not decrease in any of the treated groups, but this is believed to be a result of the time that has elapsed since the last injection of estradiol. Since the animals were killed 24 hours after the last injection, the estradiol has been cleared from the system, and any effect it would have had on lowering FSH levels has been terminated. In conclusion, in our model neither corn oil nor sesame seed oil had estrogenic effects following 30 days of treatment.
Appendix B

Title: Follicle Stimulating Hormone (FSH) Radioimmunoassay Procedure.

Objective: A detailed description of the RIA procedure for measuring plasma FSH levels.

Materials:

Buffer (PBS gel):
- Dibasic Sodium phosphate 0.05 M (7.098 gm/L)
- Gelatin 0.5 gm/L
- EDTA 0.05 M (18.61 gm/L)
- pH 7.5

FSH Standard:

FSH standard is obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) and is the vial marked rFSH-RP-2. It is stored in the freezer in room 4122 in 50 ul aliquots. Two vials will be needed to make one standard curve. The concentrations that will be needed for the standard curve are 15 ng/.1 ml, 10 ng/.1 ml, 7.5 ng/.1 ml, 5 ng/.1 ml, 2.5 ng/.1 ml, 1.0 ng/.1 ml, 0.5 ng/.1 ml, and 0.1 ng/.1 ml.

To make the first concentration of 15 ng/.1 ml add 2.45 ml to the first 50 ul vial of the rFSH-RP-2. This will give a concentration of 20 ng/.1 ml. Take 1.50 ml of
the 20 ng/.1 ml solution and dilute up to 2 ml with PBS gel. This will give a 15 ng/.1 ml concentration.

To make a concentration of 10 ng/.1 ml add 4.95 ml of PBS gel to the second 50 ul vial of the RP-2. Use this vial to make the rest of the standard curve diluting to the proper concentration.

- 7.5 ng/.1 ml: 1.5 ml of 10 ng/.1 ml, dilute to 2 ml with PBS gel.
- 5.0 ng/.1 ml: 1.0 ml of 10 ng/.1 ml, dilute to 2 ml with PBS gel.
- 2.5 ng/.1 ml: 0.5 ml of 10 ng/.1 ml, dilute to 2 ml with PBS gel.
- 1.0 ng/.1 ml: 200 ul of 10 ng/.1 ml, dilute to 2 ml with PBS gel.
- 0.5 ng/.1 ml: 200 ul of 5 ng/.1 ml, dilute to 2 ml with PBS gel.
- 0.1 ng/.1 ml: 200 ul of 1.0 ng/.1 ml, dilute to 2 ml with PBS gel.

\[ {_{125}I-FSH} \]

Obtained from Dr. Mark Wise, Animal Sciences Department. There should be approximately 10,000-8,000 cpm per 100 ul.

rFSH antibody:

Use at a dilution of 1:31,250. 10 ul of stock into 25 ml of PBS gel containing NRS (normal rabbit serum) at 1:400. The antibody is obtained from the NIDDK and is in the freezer in room 4122. It is at a dilution of 1:12.5 in the vial. Use 100 ul per tube in the assay.
Second Antibody:

The second antibody is used to separate the bound fraction from the unbound fraction. It recognizes the anti-rFSH antibody and binds it making it heavier so it will separate out upon centrifugation. It is obtained from Dr. Mark Wise, Animal Sciences Department, University of Arizona and used at a dilution of 1:20.

Assay Procedure:

Number and set up the tubes according to Table 3a preparing duplicates for each standard and sample.

1. Shake or vortex the tubes and incubate at room temperature for 24 hours under the hood.

2. After 24 hours add 250 ul of the second antibody to all tubes except the TC tubes and vortex or shake.

3. Incubate at room temperature for 24 hours under the hood.

4. Centrifuge in Sorvall at 5,000 rpm for 15 minutes.

5. Count TC tubes directly. Pour off supernate of all others and drain on a paper towel without turning the tube right side up. Let the tubes drain upside down for 10 minutes than take a Q-tip and wipe off any excess moisture from the rim. Count the pellet on gamma counter for 1 minute.

6. There should be 20-30% total binding in the assay. This is determined by the following formula:

\[
\frac{(0-NSB)}{(TC-NSB)} \times 100
\]
The concentrations of the first and second antibodies may have to be changed to get the required amount of binding. Perform a binding check before the samples are run using different dilutions of the antibody. The binding check is performed by preparing Total Count (TC) tubes, nonspecific binding tubes (NSB), and tubes with 0 ng/.1ml (See Table 3a). Use different dilutions of the first antibody: 1:15,125, 1:31,250, and 1:62,500. More or less concentrated antibody may be used to obtain 20-30% total binding.

**Table 3a. FSH Radioimmunoassay set up.**

<table>
<thead>
<tr>
<th>Contents (ng/.1ml)</th>
<th>PBS gel (ul)</th>
<th>Std/sample (ul)</th>
<th>1st Ab (ul)</th>
<th>$^{125}$I-FSH (ul)</th>
</tr>
</thead>
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<tr>
<td>TC</td>
<td>300</td>
<td>--</td>
<td>--</td>
<td>100</td>
</tr>
<tr>
<td>NSB</td>
<td>300</td>
<td>--</td>
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<td>100</td>
</tr>
<tr>
<td>0</td>
<td>200</td>
<td>--</td>
<td>100</td>
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</table>

TC—Total count  NSB—nonspecific binding
References


National Toxicology Program (1986) Toxicology and Carcinogenesis studies of 4-vinylcyclohexene in F344/N rats and B6C3F1 mice. NTP technical report no. 303. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Public Information, National Toxicology Program, P. O. Box 12333, Research Triangle Park, N.C.


