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16. Abstract (Limit: 200 words) The effects of exposure to methoxychlor (72435) (MXC) on reproductive functions in nonpregnant and pregnant mice were studied. Sexually mature virgin female CD-1-mice were exposed to 1.25, 2.5, or 5.0 milligrams (mg) MXC via oral gavage for 5 consecutive days each week for either 2 or 4 weeks. A dose dependency of MXC was noted in inducing persistent vaginal estrus. Ovaries of MXC and estradiol-17beta (E-17beta) exposed mice weighed significantly less than controls. There was also an increase in the number of atretic large follicles in the E-17beta group and in those mice treated with the two highest doses of MXC, indicating a potential reduction in the immediate fertility of the mice. Increased lipid accumulation was noted in interstitial cells and theca cells of both E-17beta treated and 5.0mg MXC treated mice. This chemical appears to closely mimic the effects on the female ovary induced by estrogen and raises concern whether such an exposure of an adult female will interfere with the normal hormonal environment and thus jeopardize future pregnancy. These alterations appear to be reversible once exposure stops. Pregnant mice were exposed to 2.5, 5.0, or 7.5mg of MXC by gavage from day six to 15 of gestation. Mice exposed to 7.5mg MXC were not able to carry their litters to term. Results revealed a significant increase in the length of gestation of mice exposed to both E-17 beta and 5.0mg MXC. Females from a subsequent unexposed litter displayed a significant advance in time of vaginal opening, a residual effect of MXC from a mother exposed during a previous pregnancy.

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FINAL PERFORMANCE REPORT

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Principal Investigator:	William J. Swartz, Ph.D.
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William J. Swartz, Ph.D.
Grant No. 0H00835

FINANCIAL STATUS REPORT

This is being submitted under separate cover by the Louisiana State University Medical Center Business Office.

EQUIPMENT INVENTORY

I have acquired one major piece of equipment totaling over $1000 under this grant. This item is a Tissue-Tek Microtome/Cryostat having a temperature control and an automatic defrost. It is used for sectioning frozen tissue for enzyme analysis.

It is manufactured by Miles Laboratories, Inc. Diagnostics Division, P.O. Box 70, Elkhart, IN, 46515. The serial number of the microtome is 9547 and the serial number for the cryostat is 6452. The manufacturer's model number is 4553.

It was purchased on March 7, 1989 at a cost of $8450 which was solely federal funds. This item is in excellent condition and will continue to be used to acquire data on my Continuation Grant.

FINAL INVENTION STATEMENT

No inventions were conceived under this grant.

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LIST OF ABBREVIATIONS

DES - diethylstilbestrol
E-17β - estradiol-17β
hCG - human chorionic gonadotropin
MXC - methoxychlor
PMSG - pregnant mare's serum gonadotropin
PVE - persistent vaginal estrus

LIST OF TABLES

SIGNIFICANT FINDINGS

A. Exposure of Non-Pregnant Adult Females to Methoxychlor

Exposure of adult female mice to MXC for five consecutive days per week for four weeks resulted in significant alterations in the reproductive system. Effects were realized in both physiological and biological parameters. Results revealed that MXC, at least in the higher doses employed in this study, affects the adult female reproductive system in some ways similar to those induced by estrogenic substances such as estradiol and DES and other estrogenic pesticides such as DDT and chlordecone when administered to prenatal or postnatal females. Four weeks of pesticide exposure was sufficient to significantly decrease ovarian weight in the mice treated with 2.5 or 5.0 mg MXC. Histologic observations revealed that this decrease in weight was due primarily to the small number of corpora lutea in ovaries of these mice, since there appears to be little, if any, ovulation occurring in these animals.

Additionally, there was a significant increase in the percentage of large follicles that were atretic in the ovaries of these mice. The need for concern about this centers around the fact that these large follicles constitute the pool from which oocytes are selected for ovulation. Therefore, there is a reduced number of oocytes available for ovulation which might affect the immediate fertility of the female. Even if some of the oocytes from the remaining healthy appearing follicles were induced to ovulate, it is unknown whether such oocytes would be viable and able to be fertilized.

Looking under the electron microscope at the different cell types within the ovary, one can observe MXC-induced effects. The most dramatic change occurred in the interstitial cells and in the theca cells. These cells contained large amounts of lipid in their cytoplasm. Similar lipid accumulations were observed in those mice treated with E17-β. It is possible that both MXC and exogenous estrogen alter the steroidogenic process with the resultant increase in lipid accumulation. It follows that any alteration of gonadal steroidogenesis could have serious consequences on normal reproductive activity of the female.

It does appear that these alterations in reproduction in the adult female may be reversible once exposure to MXC ceases. Mice exposed to MXC for four weeks and then maintained exposure-free for one month possessed ovaries of similar weight as the controls and filled with healthy corpora lutea, indicating a return to ovulation.

Even with these alterations in the female reproductive system observed following four week exposure to MXC, there is no indication of any such changes visible externally. The female appears to be normal and healthy with no sign of tremors as is the case when such females are exposed to kepone for a similar duration.

Therefore, significant alterations in the reproductive system can occur in the absence of any external manifestation of toxicity. Thus, exposure levels of females in a setting where exposure to such an agent is a possibility need to be monitored, since there may be no external indication

of any reproductive alteration.

The similarities in the response of the female reproductive system to both MXC and E-17β is disturbing. Of concern, is the estrogenicity of MXC. The widely reported deleterious effects of another estrogenic agent, diethylstilbestrol (DES), should always make one wary when exposure to an estrogenic substance is a possibility. Exposure to DES has resulted in serious abnormalities within the female reproductive system.

B. Exposure of Pregnant Females to Methoxychlor

Pregnant mice exposed to either E-17β or to 5.0 mg MXC from Days 6-15 of gestation experienced a significant delay in the time of delivery. Although there is a delay in the time of parturition, there are no differences in the number and weight of the offspring. Additionally, no maternal toxicity was visible at any of the doses of MXC.

Animals exposed to either MXC, E-17β or sesame oil were cross-fostered at birth. In those female offspring born to a mother exposed during pregnancy to 5.0 mg MXC and subsequently lactating from a mother also exposed to 5.0 mg of MXC during pregnancy, most lacked corpora lutea in their ovaries. Obviously, these mice were not ovulating. Whether this signifies a trend toward a cessation of ovulation in all the members of the group is not known at this time.

There was a higher rate for atresia in large follicles found in female mice exposed to 5.0 mg MXC prenatally and/or lactating from a female who was similarly exposed during pregnancy. This effect on the population of large follicles was similar to that seen in this same population when adult females were exposed to MXC as mentioned above.

Additionally, this study has demonstrated a latent reproductive effect on offspring of mothers exposed to either 2.5 or 5.0 mg MXC during a previous pregnancy. Offspring who were not exposed to MXC during gestation but whose mothers had been exposed during a previous pregnancy displayed an acceleration of vaginal opening. Precocious vaginal opening has served to indicate some future reproductive impairment. This early onset of vaginal openings in the subsequent litter suggests that either some of the MXC used to expose the mother in the previous pregnancy still remains to exert a direct effect upon the next litter or it has altered the hormonal milieu of the mother and/or developing fetus which induces the early vaginal opening.

This fact is of paramount importance in that it demonstrates that a subsequent litter of a mother exposed only during a previous pregnancy to MXC can also be affected. Thus, there may be potentially deleterious reproductive effects on the offspring of a pregnancy during which no exposure occurred, if exposure had occurred prior to this pregnancy.

Another significant result emanating from this research is that exposure of pregnant females to doses above 5.0 mg MXC resulted in spontaneous abortions within 48 hours when administered beginning on Day 6 of pregnancy. Exactly how this cessation of pregnancy is induced and whether it is directed toward the developing embryo or whether the uterus is the

primary target site of MXC is not known. The fact that there appears to be extensive hemorrhaging in the lacunae of the developing placenta and that a bloody vaginal discharge is visible by the eighth day of gestation would seem to indicate that there might be some erosion of the uterine and/or developing embryonic vascular system.

ABSTRACT

The proposed study is designed to characterize the reproductive hazards that confront both non-pregnant and pregnant females engaged in occupations which subject them to potential exposure to MXC. Sexually mature (7-8 weeks) virgin female CD-1 mice were exposed to 1.25, 2.5 or 5.0 mg MXC (50% technical grade) via oral gavage for five consecutive days each week for either two or four weeks. Control groups received either 0.025 mg E-17β or the sesame oil vehicle for the same time period. Vaginal smears were taken daily and weights were recorded weekly. Twenty-four hours following the final exposure, animals were sacrificed. Ovaries and reproductive tracts were removed and weighed. One ovary from each animal was prepared for light microscopic evaluation and the other for electron microscopic evaluation. Results revealed a dose dependency of MXC in inducing PVE. Ovaries of MXC- and E-17β-exposed animals weighed significantly less than the sesame oil controls. In addition, there was an increase in the number of atretic large follicles in the E-17β group and in those mice treated with the two highest doses of MXC indicating a potential reduction in the immediate fertility of the animal. Ultrastructural observation revealed increased lipid accumulation in interstitial cells and theca cells of both E-17β-treated and 5.0 mg MXC-treated mice. This would suggest that these cells are unable to synthesize and secrete steroids. Thus, this commonly employed pesticide appears to closely mimic those effects on the female ovary induced by estrogen and raises concern whether such an exposure of an adult female who eventually desires pregnancy will interfere with the normal hormonal environment and thus jeopardize future pregnancy. It does, however, appear that these alterations are reversible once exposure ceases. The second portion of this study was designed to assess whether exposure to MXC during pregnancy would affect reproductive parameters not only in female offspring exposed prenatally but also in those of a subsequent litter. Mice exposed to 7.5mg MXC were unable to carry their litters to term. Results revealed a significant increase in the length of gestation of mice exposed to both E-17β and 5.0 mg MXC. Females from a subsequent unexposed litter displayed a significant advance in time of vaginal opening, an apparent residual effect of MXC from a mother exposed during a previous pregnancy.

BODY OF REPORT

Background

The significance of reproductive hazards in the workplace has been underscored by the fact that the National Institute for Occupational Safety and Health (NIOSH) ranks it as the sixth of the ten leading work-related diseases and injuries (1). This rating is based on the numbers of workers exposed to known toxic agents or agents suspected of being toxic to human reproductive functions. Thus, it is apparent that research designed to delineate specific causes of reproductive dysfunction is necessary, if the toxicity induced by these agents on the reproductive system is to be reduced.

The manufacture of pesticides is a major industry in this country. Exposure to pesticides is a fact of life for many workers both skilled and unskilled. Research chemists formulating and synthesizing such toxic agents are vulnerable. Individuals involved in the mixing, packaging and transport of pesticides also face potential risks as do exterminators, farmers and the migrant worker population.

Much research has been directed toward observing the effects of pesticides on the reproductive system. However, the majority of this work has been directed toward the male because it has been the male who has found himself in the position of potential exposure in the work place. However, with the advent of women entering the workforce in increased numbers (2) there is a widespread unawareness of chemical exposure risk to the female worker and her employer because of an insufficient data base.

Most experimental studies implicating pesticides as reproductive toxins have been concerned with the organochlorine class of pesticides. Some of the members of this group of pesticides, including DDT and chlordecone (Kepone), have been shown to exhibit estrogenic properties which allow them to compete with endogenous estrogen for receptor sites in target tissues. Toxicants that possess steroidal activity have been reported to affect the fertility of the exposed individual. DDT caused decreased reproductive capacities in mice (3), and dieldrin produced a decrease in reproduction indices in rats as measured by dam survival, conception rates, pup survival and weaned litter sizes (4). Chlordecone (Kepone), a chlorinated hydrocarbon known to be extremely persistent in the environment, caused decreases in fertility in Japanese quail (5) and rodents (6) and cellular alterations in the liver, gonads and adrenals (5, 7). Eroschenko (8) reported an accelerated ciliation and secretory activity in oviductal and uterine cells exposed to chlordecone which were similar to the effects elicited by estrogens.

Chlordecone's estrogenic activity has accounted for its serving as an important chemical model in assessing the effects of environmental chemicals with estrogenic action on reproductive toxicology. Unfortunately, or fortunately, as the case might be, this compound is no longer manufactured and presently unavailable in sufficient quantities to continue in-depth studies. It is imperative that such studies be continued to explore toxic cellular effects on the reproductive system elicited by estrogenic pesticides, since there are not only pesticides with estrogenic activity still being manufactured and widely used, but also there are other diverse classes of

compounds with reported estrogenic activity, such as polychlorinated biphenyls (PCBs) and organosiloxane fluids, the latter used in cosmetic formulations (9).

Methoxychlor (MX) has been selected as the pesticide with which to continue our work. MXC is a structural analog of DDT and is currently used world-wide for the control of a wide spectrum of insects (10). It is an excellent compound with which to continue these studies, since it has been shown to exhibit effects on the reproductive system similar to those induced by chlordecone. These include increase in uterine weight and vaginal cornification in mice and rats (10,11) and endometrial hyperplasia in swine (12). However, little attention has been paid to the effects of this estrogenic agent on the ovary itself.

The proposed study intends to develop an in-depth picture of the toxic effects of MXC on the female reproductive system, especially the ovary, by focusing on three distinct physiological targets for reproductive toxins. These are the ovaries of the non-pregnant animal, the pregnant mouse and the prenatal female mouse.

Too little attention has been directed toward evaluating toxic exposure of the non-pregnant individual. The adult female working in industry where exposure to such an agent is a possibility shows a relative lack of concern about toxic reproductive effects of the said chemical, since she is not pregnant and, therefore, believes special precautions are unnecessary. Information is needed to determine whether exposure of the non-pregnant female to MXC might prove detrimental to the future fecundity and fertility of the individual and to assess morphological and biochemical changes occurring within the ovary of such an individual.

Most of the reports detailing toxic effects on the reproductive system have been based on prenatal exposure of this system. Data from these studies, however, have dealt mainly with tabulations of numbers of live offspring, sex ratios and teratological defects. The reproductive fates of these offspring need to be evaluated to ascertain whether they can lead a normal reproductive life.

The third group of females to be evaluated in this investigation is probably the most overlooked group of all. Although many studies have centered around chemical exposure of pregnant females, end points of the experiments have been the observation of the offspring, as stated above. Little attention was paid to the mother, unless she failed to survive the pregnancy. Our observations with studies involving chlordecone exposure, another estrogenic pesticide, revealed that the mother must not be ignored once she delivers her young. Preliminary results revealed that offspring resulting from a mating following the delivery of young exposed prenatally, also exhibited reproductive alterations. The mean day of vaginal openings of these females was significantly lower than that of controls. The extent and ramifications of this is unknown; however, what is known is that the maternal organism can still exert a significant reproductive effect on offspring following an exposure during a previous pregnancy. Therefore, it is important to closely follow the maternal organism not only during the pregnancy during which exposure occurs, but also during the subsequent pregnancy with a follow-up on the young of this latter pregnancy.

Although the ovaries are the primary organs of reproduction in the female, they are not the only components of the female reproductive system. Other structures such as the uterus, vagina, and oviducts are responsive to hormones and also are governed by the activities of the ovaries. Longer term future objectives of this study include examination of the effects of pesticide exposure on these extra-ovarian components which are also extremely important in the overall reproductive process. Furthermore, it is hoped such endeavors will stimulate other researchers to pursue aspects of ovarian toxicity of other chemical agents. It is hoped that techniques such as employed in this proposed study may be able to be utilized to evaluate how other chemical agents, classified as hazards in the workplace, exert their deleterious effects on the female reproductive system.

SPECIFIC AIMS

The overall objective of the proposed research is to critically evaluate the effects of pesticide exposure on specific components and functions of the female reproductive system in order to initiate the implementation of adequate safeguards and warnings for women who are subject to exposure to such agents in the workplace.

An understanding of reproductive toxins and how they exert their toxic effects and to what specific cell or groups of cells these effects are directed is imperative, since reproduction is essential for the continued survival of any species. So that the inherent occupational hazards of exposure to certain pesticides during development and maturation of the female reproductive system can be assessed, the specific aims are:

1) to provide a comprehensive evaluation of the reproductive toxic effects of methoxychlor on the female reproductive system by examining its toxic effects on three physiologically distinct states of the female reproductive system:
   a) the non-pregnant female
   b) the pregnant female
   c) the prenatal female

2) to characterize methoxychlor-induced changes in these organisms by evaluation of the following parameters:
   a) histological assessment of morphological changes in the different cellular compartments of the ovary
   b) tabulation of number of different follicle populations in order to detect differential follicular sensitivity to the pesticide
   c) histochemical evaluation of certain enzymes that are involved in catalyzing specific metabolic activities necessary for proper functioning of the cell, tissue or organ as a whole

3) to be able to provide additional information to both female worker and employer concerning the risks of pesticides on the reproductive integrity of the female so that solid judgements about their roles in positions where exposure to such agents is a possibility can be made.

PROCEDURES

The experimental animal used in this study was the CD-1 female mouse obtained from Charles River Laboratories. All mice were fed a standard laboratory chow and water ad libitum. They were maintained on a 14:10 light:dark cycle.

1. Assessment of methoxychlor-induced effects on the adult non-pregnant female reproductive system.

Virgin female mice (7-10 weeks old) were exposed to methoxychlor for two or four weeks. Weekly exposures consisted of administering the pesticide for five consecutive days followed by two days of non-exposure. This timetable was selected to mimic a normal 5-day work week which would be an optimal daily exposure regimen for a female in the workplace. The dosages of methoxychlor used were 1.25, 2.5 or 5.0 mg. These dosages obtained from the literature have been shown to increase uterine weight (estrogenic effect) in ovariectomized mice (11). Each was dissolved in sesame oil and administered in a 0.2 ml volume via oral gavage. Two other groups of mice were employed. The first received similar daily exposures of 25 μg of estradiol-17β (E-17β) dissolved in sesame oil and another group only the sesame oil vehicle. A group receiving E-17β was included in order to determine whether effects seen in methoxychlor-treated animals were due to the estrogenicity of this pesticide or rather to its own inherent toxicity.

The mice were weighed weekly and vaginal smears taken daily. Weights were recorded to insure that the toxic effects observed on the reproductive system were not simply a general toxic response of the whole organism. Vaginal smears were performed daily to evaluate the effects of methoxychlor on the estrous cycle and the initiation of persistent vaginal estrus.

Twenty-four hours following the final exposure of methoxychlor at either two or four weeks, some of the mice were sacrificed by cervical dislocation in preparation for histological, histochemical and gas chromatographic evaluations. Other mice from each of the groups were maintained for evaluation of these parameters at six months following cessation of exposure.

a. Histological evaluations

Ovaries were removed at these different time periods, trimmed of all fat and weighed. Preparation for histological evaluations consisted of fixing the ovaries in Bouin's fixative for 72 hours. Following dehydration, the tissues were embedded in paraffin and the ovaries serially sectioned at 8μ. Sections were stained with hematoxylin and eosin.

Each of the ovaries was be examined for gross aberrations such as the presence of ovarian cysts, hemorrhagic follicles and the absence of corpora lutea. Populations of the different follicle classifications were tabulated for the two and four week exposure periods. Follicles were

classified according to the characteristics of Pederson and Peters (13) modified by Chen et al. (14). The classification is as follows:

1. small follicles - smallest oocyte to an oocyte surrounded by no more than a single layer of follicle cells
2. medium follicles - structures containing growing oocytes surrounded by more than one layer of follicle cells with no antrum
3. large follicles - follicles possessing an antrum

Every tenth section of each ovary was be examined and the follicle counts made from these sections. This is a widely accepted method of assessing follicle populations and insures no duplication in the counting of follicles. This determines whether selectivity of this toxin exists toward a specific follicle population. Statistical analysis of the follicle populations was performed using the Student t test.

b. Histochemical evaluation

Several histochemical methods were employed to characterize pesticide-induced changes in the metabolic activities within the different cellular compartments of the ovaries. Ovaries from methoxychlor-treated mice and control mice from each of the exposure periods were fixed in 10% neutral buffered formalin and embedded in glycol methacrylate (GMA) and sectioned on a JB-4A microtome.

The histochemical studies were to demonstrate the methoxychlor-induced alterations in the presence and localization of specific enzymes whose activities are known to be present within the ovary. Changes in the qualitative appearance and localization of these enzymes serves as a basis for understanding the target site(s) and mechanism(s) of actions of such toxic agents. Alkaline and acid phosphatase, β-glucuronidase and adenosine triphosphatase were be examined in both treated and control mice following all three periods of exposure. Although the enzymes selected for this study comprise only a small fraction of the enzymes present in the cells of the ovary, they were selected since they play critical roles in catalyzing metabolic activities occurring within cells and the presence of these enzymes indicate important cellular activities and reactions available to the cells.

The histochemical procedures employed for the different enzymes are as follows:

1. Adenosine triphosphatase (ATPase) - lead method of Wachstein et al. (15)
2. Alkaline phosphatase (ALP) - azo dye method of Gomori (16)
3. Acid phosphatase (AP) - azo dye method of Burstone (17)
4. β-glucuronidase - method of Hayashi et al. (18)

All of the studies involving histochemical localization of the above enzymes involved ascertaining the qualitative behavior of these enzymes following the different periods of pesticide exposures. The enzyme reactions were classified according to the density of the reaction

product. The intensity of the reaction product in these cells was visually graded (-) to (+++), with (-) designating no demonstrable reaction and (+++) a maximal reaction.

c. Gas chromatographic analysis of methoxychlor incorporation

The livers were removed at the same time as the ovaries. They were weighed and their weights recorded as per cent of body weight. They were frozen in preparation of gas chromatographic analysis for the purpose of assessing the amount of uptake of this chemical by the liver to verify that methoxychlor is being taken up by the organism. Mr. Roy Schutzmann, a chemist, at the United States Environmental Protection Agency Environmental Chemistry Laboratory in NSTL, MS has performed these assays. He has performed similar assays for me localizing both DDT and chlordecone.

d. Ovulatory response to exogenous gonadotropins following four week exposure to methoxychlor

On the second day of the fourth week of exposure exogenous gonadotropin treatment was initiated in some of the animals in each of the treatment groups. This regimen consisted of an intraperitoneal (IP) injection of 10 I.U. of pregnant mare's serum gonadotropin (PMSG) followed 48 hours later by an IP injection of 10 I.U. of human chorionic gonadotropin (hCG). This sequential administration of PMSG and hCG stimulates a superovulatory response in mice within 12-15 hours following exposure to hCG (19). Animals were sacrificed 15-20 hours following the hCG exposure at which time their oviducts were removed and placed in a dish containing physiological saline. The ampullae of the oviducts were punctured with a 25 gauge needle and the ovulated oocytes released into a dish. Ovulated oocytes were counted and the average numbers of oocytes from the different treatment groups compared. Statistical differences among groups were ascertained by employing the student t test.

2. Assessment of methoxychlor-induced effects on the reproductive system of the prenatally exposed female.

This segment of the study was specifically designed to determine whether prenatal exposure of the developing mammalian female reproductive system resulted in reproductive toxicity and to determine whether such toxicity is permanent, naturally reversible with time, or is able to be circumvented with the use of exogenous hormonal supplementations.

Timed-pregnant CD-1 mice were obtained form Charles River Laboratories. The day of the appearance of the vaginal plug was considered Day 0 of pregnancy. Pregnant females were exposed daily via oral gavage to the different dosages of methoxychlor from Day 6-15 of pregnancy. These dosages were 0.5, 1.0, 5.0 and 10 mg methoxychlor. Another group was exposed to 25 μg of E17-β. A vehicle control group receiving only the sesame oil vehicle was also employed.

The mothers were allowed to deliver and the following parameters were recorded at birth:

a) number of offspring
b) weight of offspring
c) sex of offspring
d) externally visible abnormalities

Female offspring from the different groups were identified as to the treatment of their mother during pregnancy. This was followed by random cross-fostering of the offspring within 48 hours after birth. In order to maintain proper identification of the neonates when they were cross-fostered, India ink was subcutaneously injected in different extremities of the newborn. This means of tagging has been very successful in our laboratory. The cross-fostering procedure consisted of placing a female offspring exposed prenatally to a particular agent with a mother exposed to a different or the same agent during her pregnancy. Thus, mice exposed to one agent during pregnancy were allowed to lactate from a mother exposed to a different or the same agent during her pregnancy. This method allows us to determine the relative importance of prenatal and/or postnatal exposure of methoxychlor on observed toxic reproductive effects. The different groups formed from this cross-fostering procedure were as follows:

1. 0.5 mg methoxychlor/0.5 mg methoxychlor
2. 0.5 mg methoxychlor/sesame oil
3. 1.0 mg methoxychlor/1.0 mg methoxychlor
4. 1.0 mg methoxychlor/sesame oil
5. 5.0 mg methoxychlor/5.0 mg methoxychlor
6. 5.0 mg methoxychlor/sesame oil
7. 10.0 mg methoxychlor/10.0 mg methoxychlor
8. 10.0 mg methoxychlor/sesame oil
9. sesame oil/0.5 mg methoxychlor
10. sesame oil/1.0 mg methoxychlor
11. sesame oil/5.0 mg methoxychlor
12. sesame oil/10.0 mg methoxychlor
13. sesame oil/E-17β
14. E-17β/sesame oil
15. sesame oil/sesame oil

The first chemical listed signifies the prenatal exposure agent and the second indicates the exposure of the mother, from which the offspring is lactating. For example, an offspring who was exposed to 0.5 mg MXC prenatally and is placed, after birth, with a mother who had been exposed to sesame oil during her pregnancy is indicated as 0.5mg methoxychlor/sesame oil.

The day of vaginal opening was recorded for each of the mice. Some of the female offspring from these 15 different groups were sacrificed at two months of age. Ovaries were removed at each of these time intervals and prepared for the histological and histochemical procedures as outlined above. These data were then compared to E-17β-treated mice and sesame oil control mice.

3. Latent effects of methoxychlor on the female exposed during pregnancy as evidenced by reproductive assessments following a second pregnancy.

Pregnant females, who were exposed in Experiment 2 of this project, are the same ones to be employed here. In Experiment 2 the offspring were followed and monitored. In this segment of the project the exposed mothers were the subjects. Following weaning of their first litter, the mothers were examined daily to verify whether they return to a normal estrous cycle. If this

occurred, the females were mated again with normal males and their mating ability assessed by observing the presence of a vaginal plug. If pregnancy and parturition occurred, the offspring were counted, weighed, and categorized according to sex. Female offspring from this second pregnancy were monitored to determine whether there was an acceleration in the time of vaginal opening as seen in our laboratory with another estrogenic pesticide, chlordecone.

RESULTS and DISCUSSION

The present project was designed to address several aspects of exposure of the pesticide methoxychlor on the female ovary.

There are no changes in the specific goals of the entire project. However, there is an addition to the methods employed in this study as originally described. In September of 1988, one of the graduate students in the Anatomy Department at LSU elected to work toward her Master of Science degree in my laboratory on my present research funded by NIOSH. This student, Emilia Martinez, is from Costa Rica and was on a Fulbright Scholarship. She returned to Costa Rica two years ago upon completing her degree requirements. Ms. Martinez developed an expertise in electron microscopy and she employed her talents to examine the effects of two- and four-week MXC exposure on alterations in organelles of the different cellular compartments of the mammalian ovary. This provided more depth to the first segment of the present study, which was not originally proposed. She was successful in preparing two manuscripts with me which have been published. Copies of these are included with this report.

One additional experiment was also performed. This study was originally designed to use a formulation of MXC containing approximately 50% of the pesticide. Significant results on the ovary were obtained. It was then suggested by Dr. Joe Lary of NIOSH that I try a higher concentration of MXC to determine whether effects observed with 50% MXC were caused by MXC itself or the additional inert ingredients. I have duplicated the two- and four-week exposures using material containing 90% MXC. Although all of the ovaries have not been tabulated, there appears to be similar high rates of atresia in 4-week animals exposed to 90% MXC as was previously seen in those exposed to 50% MXC. After consultation with Dr. Lary via the telephone, it was decided to continue using the 50% MXC material as originally proposed, since this is the primary form in which it is manufactured and sold and is the form to which individuals would most likely be exposed.

During the past three years significant inroads have been made toward accomplishing the specific aims of the present project. The research efforts of this project are directed toward evaluating the toxic effects of two or four week exposure to MXC on the ovary of the adult non-pregnant female and the effect of exposing the pregnant female, evaluating both the ovaries of her offspring and those of the mother herself.

Exposure of Adult Non-Pregnant Females

There was no growth retardation in mice exposed to MXC for either two or four weeks. There was a significant increase in the mean percentage of weight gain in both the group exposed to 5.0 mg MXC and that treated with estradiol for two weeks, but this leveled off at the end of four weeks. Following four weeks of exposure, there were no significant differences in the mean percentage of body weight change among any of the groups (20).

There was a dose-related response to MXC with respect to the onset of persistent vaginal estrus (PVE). Animals exposed to 2.5 and 5.0 mg MXC exhibited PVE in less than six days. Interestingly enough, animals exposed to estradiol took a mean of a little more than seven days to acquire PVE. The group receiving 1.25 mg MXC took slightly longer than the estradiol group to reach PVE (20).

Ovarian weights did not differ significantly among the groups following two weeks exposure to MXC. However, four weeks of pesticide exposure was enough to significantly decrease ovarian weight in the two higher MXC-treated groups.

Data acquired from tabulating the condition of large follicles (greater than 300μ) following two weeks of exposure to MXC revealed no significant changes in the percentage of large follicles that were atretic. Following four weeks of exposure to MXC, there was a significant increase in the percentage of large follicles that were atretic in mice treated with 2.5 and 5.0 mg methoxychlor when compared to that of controls. Similarly, the estradiol-17β group also exhibited a significant increase in the percentage of atresia in large follicles (20).

There were no significant increases in the liver weights expressed as percent of body weight following exposure to MXC for two weeks. However, after four weeks, those mice exposed to the two higher doses of methoxychlor (2.5 and 5.0 mg) had livers significantly smaller than those of controls (Table 1). This was the exact opposite of results seen in our laboratory when chlordecone, another organochlorine pesticide, was examined. In those animals the livers increased four times in weight. Gas chromatographic analyses of the livers of all mice exposed to the different doses of MXC revealed that there was no incorporation of this pesticide in the livers except in those exposed to 5.0 MXC for four weeks (Table 2). In this group the level was only 0.1 ppm.

Electron microscopical studies of ovaries from animals exposed to 5.0 mg MXC for four weeks revealed significant morphological changes when compared to ovaries of sesame oil controls. The most dramatic change occurred in the interstitial compartment with cells containing large amounts of lipid in their cytoplasm. Similarly, there was an increase in the accumulation of lipid and rough endoplasmic reticulum within theca cells surrounding both large atretic and large healthy follicles in ovaries from animals treated with either estradiol or methoxychlor when compared to controls (21).

The MXC and estradiol-treated groups not only showed striking similarities in their ultrastructural characteristics but also in the decreased weight of their ovaries and increased atresia seen in their large follicles. Thus, the similarity in effects induced by MXC and estradiol would support the reported estrogenicity of MXC. It has been theorized that the high accumulation of lipid in such tissues as corpora lutea indicates a minimal level of steroid release. It is possible that both exogenous estrogen and methoxychlor alter the steroidogenic process with a resultant increase in lipid accumulation. These cells apparently retain their ability to

synthesize lipid, but lose their capacity to convert the lipids into steroids. It is apparent that any alteration of gonadal steroidogenesis could have serious consequences on normal reproductive activity.

Even after four weeks of exposure to MXC, animals when exposed to a superovulatory regimen of gonadotropin ovulated a number of oocytes similar to those ovulated in the controls. This response is unlike that observed following exposure to chlordecone, another estrogenic pesticide examined previously in this laboratory in which there was a significant reduction in the ovulatory response to exogenous gonadotropins. It appears that MXC exhibits a toxicity dissimilar to that of chlordecone. Whether these ovulated eggs are viable could not be ascertained from this study.

In those experiments in which animals were exposed to MXC for four weeks and then maintained exposure-free for 6 months, it was interesting to observe that the reduced ovarian weight observed immediately following four weeks of exposure to MXC was no longer present at 6 months following cessation of exposure. The ovaries of mice from each group contained healthy corpora lutea of several generations. It does appear that all of these animals were ovulating during the period of non-exposure.

This portion of the study indicates that in the adult mouse, the ovaries are susceptible to impairment by exposure to the pesticide MXC. The increase in atresia seen in the large follicles exposed for four weeks to the higher doses of MXC may result in an immediate infertility, but it appears that once exposure ceases, normal ovulatory processes return, at least, by four weeks. What is not known is whether the oocytes present are normal, able to be fertilized and able to develop into normal individuals.

Exposure Of Pregnant Females

Examination of the F_1 Offspring

This segment of the proposal was designed to evaluate the toxic effects of MXC resulting from exposure of the pregnant female. Pregnant mice were exposed to 7.5, 5.0 or 2.5 mg of 50% MXC, 25 μg estradiol-17β or the sesame oil vehicle via oral gavage from Days 6-15 of pregnancy. Pregnant animals were allowed to deliver and the offspring were assessed according to their number, weight and sex. With the exception of the 7.5 mg methoxychlor-exposed group, there were no significant differences in any of these criteria between the different groups. It should be noted that the mice in the group exposed to 7.5 mg MXC were unable to carry their litters to term. Within two days of the beginning of exposure to this dosage, vaginal bleeding ensued and the embryos died.

The gonads of some of the surviving female offspring in the different treatment groups were removed and prepared for enzyme analysis. This data is currently being evaluated.

In the groups receiving 2.5 and 5.0 mg MXC, those exposed to E-17β and those exposed to the vehicle control, their remaining newborn offspring (F_{1a}) animals were cross-fostered following delivery. That is, some of those who were exposed to MXC prenatally were placed with mothers who were exposed to only sesame oil during pregnancy and vice versa. This was done in order to determine whether prenatal exposure or postnatal exposure to MXC via lactation was most or equally important in eliciting alterations in future reproductive activity of these female offspring.

The cross-fostered F_{1a} offspring were sacrificed after eight weeks of development. Ovaries were removed, weighed and prepared for histological evaluation. There were no significant differences in ovarian weight among the different cross-fostered offspring. Preliminary data does reveal, however, that there is a larger percentage of atretic follicles in the ovaries of mice exposed prenatally and lactating from a previously MXC-exposed mother when compared to animals exposed to sesame oil pre- and postnatally. Thus, there is an indication that prenatal exposure and exposure to MXC through lactation induces an increase in large follicle atresia which might affect the fertility of these offspring (22).

Examination of Mothers Exposed During Gestation

One of the most overlooked entities in experiments which involve injecting a toxic substance into a pregnant mother is the mother herself. Attention is usually directed toward the offspring. One segment of the present study was directed toward doing a follow-up study on the exposed mother to determine whether there is any inherent residual effect of the MXC administered during her first pregnancy that might interfere with or affect a later pregnancy. Thus, following weaning of their first group of offspring (F_{1a} generation), previously exposed mothers were allowed to mate again without any further exposure to MXC, estradiol or sesame oil. All previously exposed MXC females became pregnant again and delivered another litter (F_{1b}). These offspring were of similar number and weight when compared to sesame oil and estrogen controls. There was a significant difference, however, in the time of vaginal opening of these groups. Mice of the F_{1b} litters whose mothers had been previously exposed to sesame oil presented vaginae which opened in a mean of 25.1 days, whereas those female offspring from mothers exposed to MXC during their first pregnancy had vaginae which opened significantly earlier at 23.3 days (22). Thus, there appears to be a residual effect of MXC on young from a mother exposed during a previous pregnancy. This illustrates the need for concern not only for offspring prenatally exposed to a chemical agent but also for the subsequent offspring of a mother who may have been exposed during a previous pregnancy. More work needs to be directed toward this end. This group of F_{1b} mice were not examined beyond the time of vaginal opening. Results obtained from the experiments dealing with exposure of pregnant mice to MXC are reported in a manuscript which has been submitted to the journal Reproductive Toxicology. A copy of this manuscript accompanies this report.

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TABLE I

LIVER WEIGHTS IN MICE TREATED WITH METHOXYCHOR AND ESTRADIOL - 17β

Treatment	Dose (mg)	No. of mice	mg	% body weight
2 Weeks				
SO	--	10	1.3 ± 0.1	5.0 ± 0.2
E-17β	0.025	16	1.3 ± 0.1	5.0 ± 0.1
MXC	1.25	9	1.1 ± 0.1	4.7 ± 0.3
MXC	2.5	12	1.2 ± 0.0	5.0 ± 0.1
MXC	5.0	13	1.1 ± 0.0*	4.5 ± 0.2
4 Weeks				
SO	--	8	1.2 ± 0.0	4.8 ± 0.1
E-17β	0.025	12	1.2 ± 0.1	4.8 ± 0.1
MXC	1.25	9	1.1 ± 0.0	4.6 ± 0.2
MXC	2.5	9	1.1 ± 0.1	4.5 ± 0.1*
MXC	5.0	9	1.2 ± 0.0	4.5 ± 0.1*

*Statistically significant ($P < 0.05$)

TABLE 2

**Accumulation of Methoxychlor in Mouse Livers (ppm)
After Exposure for Two and Four Weeks**

	Duration of Treatment (weeks)	
Treatment	2	4
Sesame oil	ND[1]	ND
E-17β	ND	ND
1.25 mg MXC	ND	ND
2.5 mg MXC	ND	ND
5.0 mg MXC	ND	0.1

[1]Non-detectable at the minimum detectable level.

Present and Future Publications

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