Published in final edited form as:

Birth Defects Res B Dev Reprod Toxicol. 2015 December ; 104(6): 238–243. doi:10.1002/bdrb.21164.

Exposure Duration-Dependent Ovarian Recovery in Methoxychlor-Treated Mice

Lawrence V. Tannenbaum¹ and Jodi A. Flaws^{2,*}

¹Army Public Health Center, Aberdeen Proving Ground, Aberdeen, Maryland ²Department of Comparative Biosciences, University of Illinois at Urbana-Champaign, Illinois

Abstract

The pesticide methoxychlor (MXC) is known to target ovarian antral follicles in the mouse. In previous *in vivo* studies, MXC administration for 20 days increased atresia, but did not affect female fertility immediately after dosing. Thus, we hypothesized that perhaps not enough time had elapsed between the onset of MXC-induced atresia and actual follicle loss to result in reduced fertility. The current study was undertaken to determine whether MXC treatment for 20 days results in reduced antral follicle numbers and fertility at 30 and 60 days after dosing. To test this hypothesis, adult CD-1 female mice were dosed with vehicle control or MXC (64 mg/kg/day) for 20 days. At 30 and 60 days post-dosing, the mice were either subjected to fertility tests or their ovaries were collected and subjected to histological evaluation of follicle numbers and atresia. The results indicate that at 30 days after the completion of dosing, MXC significantly increased atresia and reduced primordial and total follicle numbers, but did not affect fertility compared to controls. At 60 days after completion of dosing, MXC did not significantly affect fertility, follicle numbers, or atresia compared to controls. Collectively, these data indicate that the ovary may be able to recover from MXC treatment for 20 days.

Keywords

methoxychlor; antral follicle; atresia; ovary; recovery

INTRODUCTION

For close to 30 years, the organochlorine pesticide methoxychlor (MXC) has been a model compound used in mammalian reproductive toxicology studies. MXC has been linked to a host of adverse reproductive effects in female mice, including premature estrus, ovarian atrophy, decreased ovulation, and increased atresia (death) of follicles (Gray et al., 1989;

*Correspondence to: Jodi A. Flaws, Department of Comparative Biosciences, University of Illinois at Urbana-Champaign, 2001 S. Lincoln Avenue, Urbana, IL 61802. jflaws@illinois.edu.

Use of a trademarked name does not imply endorsement by the U.S. Army, but is intended only to assist in the identification of a specific product.

The opinions or assertions contained herein are the views of the author and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

The authors do not have any conflicts of interest to declare.

Eroschenko and Cooke, 1990; Borgeest et al., 2002). With regard to the last of these, a feature of MXC exposure, demonstrated through both *in vitro* and *in vivo* studies with rodents, is its capacity for specifically targeting antral ovarian follicles (Martinez and Swartz, 1991; Swartz and Corkern, 1992; Borgeest et al., 2002; Gupta et al., 2006). In contrast to studies involving chemicals that target primordial follicles and cause permanent infertility (Hirshfield, 1997), testing with MXC may allow for the assessment of temporary infertility. Temporary infertility can occur when a chemical only targets antral follicles and not primordial and primary follicles. If the chemical can be removed from the body, the primordial and primary follicles can mature to replace the antral follicles targeted by the chemical, and fertility may be restored.

The practicality of MXC studies also extends to potentially gaining an improved understanding of premature reproductive senescence, mediated by the pesticide's ability to cause follicle atresia (Borgeest et al., 2002; Gupta et al 2006). Premature reproductive senescence constitutes a major concern because it may increase the risk of cardiovascular diseases, osteoporosis, and hypertension (Bagur and Mautzlen, 1992; Ashraf and Vongpatanasin, 2006; Gast et al., 2008).

One other research area to potentially gain from controlled MXC exposure studies with rodents pertains to the field of ecological risk assessment (ERA). The goal of ERA is to anticipate population impacts on animals that inhabit chemically contaminated sites such as hazardous waste sites, where mammals constitute the dominant group studied in terrestrial settings. With reproduction being the toxicological endpoint of greatest concern in ERA (Tannenbaum, 2013), it would be helpful to know the extent to which follicle numbers in the small rodents of contaminated sites, serving as surrogates for larger mammals (e.g., fox), need to be reduced for reproductive compromise. Such knowledge would allow for the development of a field-based reproductive health assessment scheme for female mammals that parallels an existing patented scheme for male assessment, Rodent Sperm Analysis (RSA; U.S. Army Public Health Command, 2009; Tannenbaum et al., 2007). The RSA method is enabled through knowledge of the degree to which sperm counts need to be reduced to result in fewer litters, smaller-sized litters, and other correlates of reproductive impairment.

Previous mouse MXC exposure studies have, in part, endeavored to identify a dosing regimen that consistently induces a certain antral (and/or pre-antral) follicle count reduction, with the intent of learning of the threshold for reducing the production of offspring. To this end, our most recent efforts involved mating MXC-exposed adult cycling female mice with untreated proven breeder males to evaluate fertility (Paulose et al., 2012). This previous study showed that although MXC treatment for 20 and 30 days significantly increased atresia of antral follicles, it did not significantly affect fertility immediately after dosing (Paulose et al, 2012). Because we examined atresia and fertility immediately after dosing with MXC, we hypothesized that perhaps not enough time had elapsed between the onset of atresia and actual follicle loss to result in reduced fertility (Paulose et al, 2012). Thus, the current study was undertaken to determine whether MXC treatment for 20 days results in reduced antral follicle numbers and fertility at 30 and 60 days after completion of dosing.

MATERIALS AND METHODS

Chemicals

MXC (99%), as a white crystalline powder, was purchased from Sigma-Aldrich (St. Louis, MO). MXC was dissolved in sesame oil (SES) as the vehicle to achieve 64 mg/kg/day dosages when administered in volumes of 2.5 mL/kg body weight. SES, as a clear yellow liquid, was purchased from Sigma-Aldrich (St. Louis, MO).

Animals

Four week-old CD-1 mice (*Mus musculus*) were obtained from Charles River Laboratories, Inc. (CRL) and allowed to acclimate for seven days. Females (64 in total) were housed four per cage until mating, at which time they were singly housed with proven male breeders. Proven breeder males (16 in total), also obtained from CRL, were housed 1 per cage until mating. Animals were uniquely identified by ear punch. Cages were made of polycarbonate plastic with micro-isolator tops, measuring 17 cm wide by 28 cm long by 13 cm high. Absorbent heat-treated hardwood bedding (Northeastern Products Corp., Warrensburg, NY) was changed twice per week, and once per week when animals were single-housed. Temperature in the animal room ranged from 20.4 - 25.2°C during the course of the study. The animal room was set to a 12/12 hour light/dark cycle, and all animals received food and reverse osmosis treated tap water *ad libitum* throughout the study, with bottle changes once per week. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Illinois and were in compliance with the Animal Welfare Act Regulations, 9 CFR 1-4, and animals were handled and treated in accordance with applicable policy (ILAR, 2011).

Experimental Design

In the first set of experiments, female mice were dosed either with vehicle control (SES) (n=16) or MXC at 64 mg/kg/day (n=16) for 20 consecutive days via intraperitoneal (ip) injection. Dose volume was based on individual animal daily body weight. This dosing regimen was selected because previous studies indicate that it significantly increases atresia of antral follicles in mice (Borgeest et al., 2002). At 30 days following the last dosing day, half of the MXC animals (n=8) and half of the control animals (n=8) were euthanized by carbon dioxide inhalation, followed by exsanguination. Ovaries of all euthanized animals were excised and placed into Dietrich's fixative for subsequent histological evaluation as described below. Further, serum was collected for measurement of hormone levels as described below. The other half of the mice were subjected to breeding studies as described below.

In the second set of experiments, mice were dosed either with vehicle control (SES) (n=16) or MXC at 64 mg/kg/day (n=16) for 20 consecutive days via ip injection. Dose volume was based on individual animal daily body weight. At 60 days following the last dosing day, half of the MXC animals (n=8) and half of the control animals (n=8) were euthanized by carbon dioxide inhalation, followed by exsanguination. Ovaries of all euthanized animals were excised and placed into Dietrich's fixative for subsequent histological evaluation as

described below. The other half of the mice were subjected to breeding studies as described below.

Histological Evaluation of Follicle Numbers and Atresia

The ovaries were completely serially sectioned at 8 μ m intervals, mounted on glass slides, stained with Weigert's hematoxylin-picric acid methyl blue, and mounted with Permaslip (Alban Scientific Inc, St. Louis MO). In every tenth section, the numbers of primordial, primary, and preantral follicles were counted as previously described (Borgeest et al., 2002). Only follicles containing a visible nucleus were counted to avoid double-counting, and all counting was done without knowledge of treatment group. Follicles were counted as primordial if they contained an oocyte surrounded by flattened granulosa cells, or a mixture of less than seven flattened and cuboidal granulosa cells. Follicles were counted as primary if they contained an oocyte surrounded by a single layer of cuboidal granulosa cells. Preantral follicles were counted as those containing an oocyte surrounded by two or more complete layers of granulosa cells. Antral follicles were counted as those containing an oocyte surrounded by more than two layers of granulosa cells and an antral space.

To count atretic versus healthy follicles, each section used to obtain follicle counts was subjected to morphological evaluation. Follicles were classified as atretic if 10% of the granulosa cells were apoptotic, the granulosa cell layer was disorganized, the oocyte was degenerating or its nucleus was fragmented (Borgeest et al., 2002).

Breeding Studies

All females to be mated were placed with males for up to seven days. The date of the vaginal plug was recorded and considered gestational day (GD) 1. Beginning with GD 1, female mice were weighed every 3 to 5 days to monitor pregnancy. Females were allowed to deliver litters naturally (postnatal day 0), and the following day (postnatal day 1), pups were counted and individually weighed.

Hormone Analysis

Blood was drawn from the mice on random days of the estrous cycle immediately following euthanasia, and serum was subjected to enzyme-linked immunosorbent assays (ELISAs) or radioimmunoassays (RIAs) at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. ELISAs were used to measure estradiol levels and RIAs were used to measure follicle-stimulating hormone (FSH) levels.

Statistical Analysis

Data analysis was conducted using SPSS statistical software (SPSS, Inc., Chicago, IL). Data were expressed as means and standard error of the means (SEM). Comparisons between normally distributed experimental groups were made using t-tests. Statistical significance was assigned at $p = 0.05$.

RESULTS

Effects of MXC on Follicles Numbers and Atresia at 30 and 60 Days Post-dosing

At 30 days post-dosing, MXC treatment (64 mg/kg/day) significantly decreased the number of primordial follicles and the number of total follicles compared to vehicle controls (Figure 1A). Overall, the MXC-treated mice had 31% fewer primordial follicles and 17.5% fewer total follicles than did their vehicle-treated counterparts at 30 days after completion of dosing. Further, MXC treatment significantly increased the number of atretic follicles compared to controls (Figure 2A).

In contrast, 60 days post-dosing MXC treatment (64 mg/kg/day) did not significantly affect the numbers of primordial, primary, pre-antral, or antral follicles compared to controls (Figure 1B). Further, it did not significantly affect the number of atretic follicles compared to controls (Figure 2B).

MXC and Breeding Outcomes at 30 and 60 Days Post-dosing

MXC-treatment did not significantly affect litter size at 30 or 60 days post-dosing relative to controls (Table 1). Similarly, MXC treatment did not affect the mean pup weights at 30 or 60 days post-dosing compared to controls (Table 1).

MXC and Hormone Levels at 30 and 60 Days Post-dosing

MXC treatment did not significantly affect estradiol levels 30 or 60 days after the completion of dosing compared to controls (Figure 3). Similarly, MXC treatment did not significantly affect FSH levels 30 or 60 days after the completion of dosing relative to controls (Figure 4).

DISCUSSION

The present study indicates that at 30 days after the completion of dosing, MXC treatment led to a significant reduction in the numbers of primordial and total follicles and a significant increase in the number of atretic follicles compared to controls. In contrast, at 60 days after the completion of dosing, MXC treatment did not cause any significant changes in follicle numbers or atresia compared to controls. Further, our previous study indicates that MXC treatment reduces the number of antral follicles and increases atresia immediately after dosing (Paulose et al., 2012). Collectively, these data indicate that MXC treatment reduces follicle numbers and increases atresia, but not in a permanent manner. In fact, it appears that the ovary may be able to recover from the MXC-induced increase in atresia and loss of antral follicles over time. This is supported by our data showing that at 60 days after the completion of dosing, the numbers of follicles are similar for MXC-treated and control groups. Additionally, these data are supported by the decline in primordial follicles observed in response to MXC treatment at 30 days after the completion of dosing. It is possible that the primordial follicles were recruited to grow at a faster than normal rate from the primordial to antral stage to recover from the MXC-induced decrease in antral follicles. It is unlikely that MXC treatment is reducing primordial follicles by killing them because

previous studies indicate that MXC selectively targets antral follicles (Borgeest et al., 2002; Borgeest et al., 2004).

The potential recovery from the MXC-induced loss of antral follicles over time by increasing recruitment of primordial follicles to the antral stage may have its drawbacks. When accelerated folliculogenesis is called upon to ensure that antral follicle numbers are normal, the potential exists for the primordial cell pool to become severely depleted over time. In theory, since mammals are born with a finite number of primordial follicles (Hirshfield, 1997; Da Silva-Butkus et al., 2009), acceleration of growth from this pool can lead to premature reproductive senescence. In the case of wild rodents exposed to environmental chemicals, it is doubtful that a nearly obliterated primordial follicle pool condition would arise because the lifespan is only about one year (Burt and Grossenheider, 1980; Millar and Innes, 1983), which may not be long enough for the animal to enter reproductive senescence. To determine whether reduction in follicle numbers at 30 days after the completion of dosing and the recovery at 60 days were due to changes in hormone levels, we examined both estradiol and FSH levels at 30 and 60 days post-dosing. Our data indicate that at both of these time points, MXC did not significantly affect estradiol or FSH levels compared to controls. These data suggest that these hormones are not involved in either the follicle loss or increased atresia in response to MXC observed at 30 days, or the recovery observed in response to MXC at 60 days post-dosing. However, it is important to note that the serum samples used for hormone measurements were collected at random days of the estrous cycle, leading to variability in hormone levels. This variability may have made it difficult to observe significant differences in hormone levels between control and MXC-treated groups.

Our data also indicate that MXC treatment followed by a 30 or 60 day post-dosing period is not sufficient to cause reductions in fertility. The MXC treated mice gave birth to normal sized litters when mated at 30 and 60 days after the completion of dosing. Collectively, these data indicate that the degree of loss of follicles at 30 days is not enough to compromise fertility in female mice.

Although more study is needed, it may be that artificially inducing a condition whereby MXC causes a sustained reduction in numbers of antral follicles, a situation that could lead to reduced fertility, is not achievable. This is supported by studies showing that higher doses of MXC than those used in this study do not impact female fertility (Paulose et al., 2012) and our current data indicating that the ovary may be able to recover from MXC-induced increases in atresia. Further corroboration is supplied by the ERA field in both general and specific senses. First, documented cases of population impacts at contaminated terrestrial sites for a host of species (mammals prominently among them) exposed to a broad range of environmental chemicals (reproductive toxins prominently among them) are lacking (Tannenbaum, 2003, 2005, 2013). Presumably over time, mammals in the wild develop a resiliency to chemicals that initially stand to interfere with gamete production and other elements of their reproductive biology. Second, the absence of critically reduced sperm counts in all RSA applications to date, involving approximately 10 different rodent species, also suggest that sustained reproductive impacts do not occur (Tannenbaum et al., 2007). With reproductive toxicity risk assessment guidance, understanding both (rodent) sexes to

respond similarly to equivalent toxic exposures (U.S. EPA, 1996), long-term/sustained antral follicle reductions, like absent sperm count reductions, would not be anticipated.

CONCLUSION

This study has shown that MXC-induced follicle atresia does not lead to a reduction in follicle counts at 30 and 60 days after the completion of dosing. Our results also reveal that the mouse ovary may be able to recover from MXC-induced toxicity over time. Future studies should examine the potential mechanisms by which the ovary recovers from MXC-induced toxicity.

ACKNOWLEDGEMENT

The authors thank Liying Gao and Emily Brehm for the assistance with the ovarian histological evaluations and they thank Sherri Hutchens and Jeffrey Leach for the manuscript review. The University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core is supported by the Eunice Kennedy Shriver NICHD/NIH (NCTRI) Grant P50-HD28934.

Grant support: The authors thank the Army Environmental Command (Mr. Jim Daniel) for funding this study. This work was also supported by the National Institute of Environmental Health Sciences grant R01ES019178.

LITERATURE CITED

- Ashraf MS, Vongpatanasin W. Estrogen and hypertension. *Curr Hypertens Rep.* 2006; 8:368–376. [PubMed: 16965722]
- Bagur AC, Mautzlen CA. Risk for developing osteoporosis in untreated premature menopause. *Calcif Tiss Int.* 1992; 51:4–7.
- Borgeest C, Symonds D, Mayer LP, Hoyer PB, Flaws JA. Methoxychlor may cause ovarian follicular atresia and proliferation of the ovarian epithelium in the mouse. *Toxicol Sci.* 2002; 68:473–478. [PubMed: 12151644]
- Borgeest C, Miller KP, Gupta R, Greenfield C, Hruska KS, Hoyer PB, Flaws JA. Methoxychlor-induced atresia in the mouse involves Bcl-2 family members, but not gonadotropins or estradiol. *Biol Reprod.* 2004; 70:1828–1835. [PubMed: 14973267]
- Burt, WH.; Grossenheider, RP. *A Field Guide to Mammals.* Houghton Mifflin; Boston: 1980.
- Da Silva-Buttkus P, Marcelli G, Franks S, Stark J, Hardy K. Inferring biological mechanisms from spatial analysis: Prediction of a local inhibitor in the ovary. *PNAS.* 2009; 106:456–461. [PubMed: 19122142]
- Eroschenko VP, Cooke PS. Morphological and biochemical alterations in reproductive tracts of neonatal female mice treated with the pesticide methoxychlor. *Biol Reprod.* 1990; 42:573–583. [PubMed: 2340337]
- Gast GC, Grobbee DE, Pop VJ, Keyzer JJ, Wijnands-van Gent CJ, Samsioe GN, Nilsson PM, van der Schouw YT. Menopausal complaints are associated with cardiovascular risk factors. *Hypertension.* 2008; 51:1492–1498. [PubMed: 18391100]
- Gray LE Jr, Ostby J, Ferrell J, Rehnberg G, Linder R, Cooper R, Goldman J, Slott V, Laskey J. A dose-response analysis of methoxychlor-induced alterations of reproductive development and function in the rat. *Fundam Appl Toxicol.* 1989; 12:92–108. [PubMed: 2925022]
- Gupta RK, Miller KP, Babus JK, Flaws JA. Methoxychlor inhibits growth and induces atresia of antral follicles through an oxidative stress pathway. *Toxicol Sci.* 2006; 93:382–389. [PubMed: 16807286]
- Hirshfield AN. Overview of ovarian follicular development: considerations of the toxicologist. *Environ Mol Mutagen.* 1997; 29:10–15. [PubMed: 9020302]
- Institute of Laboratory Animal Resources. *Guide for the Care and Use of Laboratory Animals.* National Academy Press; Washington, DC: 2011.

- Martinez EM, Swartz WJ. Effects of methoxychlor on the reproductive system of the adult female mouse. 1. Gross and histologic observations. *Reprod Toxicol*. 1991; 5:139–147. [PubMed: 1807545]
- Millar JS, Innes DG. Demographic and life cycle characteristics of montane deer mice. *Can J Zool*. 1983; 61:1981–1985.
- Paulose T, Tannenbaum LV, Borgeest C, Flaws J. Methoxychlor-induced ovarian follicle toxicity in mice: Dose and exposure duration-dependent effects. *Birth Defects Res*. 2012; 95:219–224.
- Swartz WJ, Corkern M. Effects of methoxychlor treatment of pregnant mice on female offspring of the treated and subsequent pregnancies. *Reprod Toxicol*. 1992; 6:431–437. [PubMed: 1463923]
- Tannenbaum LV. Can ecological receptors really be at risk? *Hum Ecol Risk Assess*. 2003; 9:5–13.
- Tannenbaum LV. A critical assessment of the ecological risk assessment process: A review of misapplied concepts. *Integrated Environ Assess Mngmt*. 2005; 1:66–72.
- Tannenbaum LV, Thran BH, Williams KJ. Demonstrating ecological receptor health at contaminated sites with wild rodent sperm parameters. *Arch Environ Contam Toxicol*. 2007; 53:459–465. [PubMed: 17612787]
- Tannenbaum, LV. Wiley Blackwell; Sussex: 2013. *Alternative Ecological Risk Assessment: An Innovative Approach to Understanding Ecological Assessments for Contaminated Sites*.
- U.S. Army Public Health Command. Rodent Sperm Analysis. 2009. U.S. Patent 7,627,434

Figure 1. Effect of MXC on follicle numbers at 30 and 60 days post-dosing

Female CD-1 mice were dosed with vehicle or MXC (64 mg/kg/day) for 20 days. At 30 and 60 days, post-dosing, ovaries were collected and subjected to histological evaluation of follicle numbers. Panel A = 30 days post-dosing and panel B = 60 days post-dosing. The asterisks indicate significant difference from control (n = 8 per treatment group; $p < 0.05$).

Figure 2. Effect of MXC on atresia at 30 and 60 days post-dosing

Female CD-1 mice were dosed with vehicle or MXC (64 mg/kg/day) for 20 days. At 30 and 60 days, post-dosing, ovaries were collected and subjected to histological evaluation of atresia. Panel A = 30 days post-dosing and panel B = 60 days post-dosing.

Figure 3. Effect of MXC on estradiol levels at 30 and 60 days post-dosing

Female CD-1 mice were dosed with vehicle or MXC (64 mg/kg/day) for 20 days. At 30 and 60 days, post-dosing, sera were collected and subjected to measurements of estradiol levels. Panel A = 30 days post-dosing and panel B = 60 days post-dosing.

Figure 4. Effect of MXC on FSH levels at 30 and 60 days post-dosing

Female CD-1 mice were dosed with vehicle or MXC (64 mg/kg/day) for 20 days. At 30 and 60 days, post-dosing, sera were collected and subjected to measurements of FSH levels.

Panel A = 30 days post-dosing and panel B = 60 days post-dosing.

Table 1

Litter size and pup weight analysis for MXC-treated and SES-treated mice

Treatment	Litter size (live pups; mean $\pm$ SD)	Mean pup weight (g) (mean $\pm$ SD)
SES (vehicle)	12.1 $\pm$ 1.5	1.8 $\pm$ 0.2
MXC / 30 days post-dosing	10.4 $\pm$ 1.8	1.8 $\pm$ 0.2
SES (vehicle)	11.1 $\pm$ 1.6	1.8 $\pm$ 0.2
MXC / 60 days post-dosing	11.3 $\pm$ 1.4	1.8 $\pm$ 0.2