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Methoxychlor reduces estradiol levels by altering steroidogenesis and metabolism in mouse antral follicles in vitro

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Abstract

The organochlorine pesticide methoxychlor (MXC) is a known endocrine disruptor that affects adult rodent females by causing reduced fertility, persistent estrus, and ovarian atrophy. Since MXC is also known to target antral follicles, the major producer of sex steroids in the ovary, the present study was designed to test the hypothesis that MXC decreases estradiol (E₂) levels by altering steroidogenic and metabolic enzymes in the antral follicles. To test this hypothesis, antral follicles were isolated from CD-1 mouse ovaries and cultured with either dimethylsulfoxide (DMSO) or MXC. Follicle growth was measured every 24 h for 96 h. In addition, sex steroid hormone levels were measured using enzyme-linked immunosorbent assays (ELISA) and mRNA expression levels of steroidogenic enzymes as well as the E₂ metabolic enzyme *Cyp11b1* were measured using qPCR. The results indicate that MXC decreased E₂, testosterone, androstenedione, and progesterone (P₄) levels compared to DMSO. In addition, MXC decreased expression of aromatase (*Cyp19a1*), 17 β -hydroxysteroid dehydrogenase 1 (*Hsd17b1*), 17 α -hydroxylase/17,20-lyase (*Cyp17a1*), 3 β hydroxysteroid dehydrogenase 1 (*Hsd3b1*), cholesterol side-chain cleavage (*Cyp11a1*), steroid acute regulatory protein (*Star*), and increased expression of *Cyp11b1* enzyme levels. Thus, these data suggest that MXC decreases steroidogenic enzyme levels, increases metabolic enzyme expression and this in turn leads to decreased sex steroid hormone levels.

Keywords

Methoxychlor; steroid hormones; steroidogenic enzymes; metabolic enzymes; antral follicles; ovary

INTRODUCTION

Many chemicals are known to be present in the environment. Many of them mimic endocrine hormone functions in the body, interfering with normal endocrine activity in

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CONFLICT OF INTEREST

The authors do not have any conflicts to disclose.

humans, wild-life, or laboratory animals, thus, acting as endocrine disrupting chemicals (EDCs) (Crisp *et al.*, 1998). The EDC methoxychlor (MXC) is a chlorinated organic pesticide, which is primarily used against various species of insects that attack field crops, trees, vegetables, fruits, gardens, stored grain, livestock and domestic pets (ATSDR, 2002). This chemical was first registered for use in 1948 and was widely used as a replacement for dichlorodiphenyltrichloroethane (DDT) until 2004 (Stuchal *et al.*, 2006). MXC is still being used in many countries on agricultural products that are imported to the United States (US), resulting in human exposure in the US. Further, MXC is persistent in soil and its residues are present even after 18 months of post-treatment with microbes that scavenge MXC (Golovleva *et al.*, 1984).

MXC targets the ovary and its exposure has adverse effects on reproductive function in adult female mice causing persistent estrus, reduced fertility, ovarian atrophy, and increased follicular atresia (Eroschenko *et al.*, 1997; Martinez and Swartz, 1991). Exposure of pregnant mice to MXC results in an increased percentage of atretic follicles in female pups compared to controls. Further, the next generation of female pups born to same mother display residual effects of MXC, showing accelerated vaginal opening (Swartz and Corkern, 1992). Exposure of adult mice to MXC increases lipid accumulation in the interstitial tissue and thecal cells of the ovary, mimicking estrogen effects (Martinez and Swartz, 1992). Further, MXC increases the number of pyknotic bodies in mouse granulosa cells, decreases the number of healthy antral follicles, increases number of atretic follicles, and increases ovarian surface epithelium height (Borgeest *et al.*, 2002; Swartz and Corkern, 1992). Under in vitro conditions, treatment of antral follicles with MXC decreases antral follicle growth and increases atresia (Gupta *et al.*, 2006; Miller *et al.*, 2005, 2006).

The effects of MXC on antral follicles are of concern because antral follicles are the structural and functional units of the ovary. Further, antral follicles are the only follicle types that have the ability to release eggs for fertilization and to produce sex steroid hormones such as 17 β -estradiol (E₂). The E₂ produced by antral follicles is essential for normal menstrual and estrous cyclicity, maintenance of the female reproductive tract, and maintenance of non-reproductive tissues such as bones, vascular tissues, and the brain (Britt and Findlay, 2002; Couse & Korach, 1998; Findlay *et al.*, 2001).

E₂ production by antral follicles is a complex process in which cholesterol is eventually converted to E₂. During steroidogenesis, the thecal cells in the follicle acquire cholesterol through endocytosis from blood and this cholesterol is stored in the cytoplasm as cholesterol esters. Cholesterol has to be transported across both mitochondrial membranes, and this process is aided by steroidogenic acute regulatory protein (STAR). The cholesterol side-chain cleavage enzyme (CYP11A1) is a rate limiting enzyme present in all the steroidogenic cells. This enzyme is attached to the inner mitochondrial membrane and converts cholesterol to pregnenolone. The next enzyme in the steroidogenesis pathway is 3 β -hydroxysteroid dehydrogenase (HSD3B1), which catalyzes the conversion of pregnenolone to progesterone (P₄). Progestins, pregnenolone and P₄ are converted irreversibly to androstenedione by 17 α -hydroxylase/17,20-lyase (CYP17A1). Androstenedione is then converted to the more potent androgen testosterone by 17 β -hydroxysteroid dehydrogenase (HSD17B1). This enzyme also catalyzes the conversion of estrone to a more potent estrogen, E₂. The androgens are then converted to estrogens by aromatase (CYP19A1) (Suter, 2004). E₂ then can be metabolized to catecholestrogens (Spink *et al.*, 1994).

Some studies have shown that MXC alters steroidogenesis in isolated granulosa cells and whole ovaries, thus acting as an endocrine disruptor (Chedrese and Feyles, 2001; Cummings, 1997). However, little is known about the effects of MXC on steroid production by antral follicles in mice. Therefore, the present study tested the hypothesis that MXC

decreases the sex steroid hormone levels (E_2 , testosterone, androstenedione, and P_4) produced by antral follicles in vitro. The present study also evaluated the mechanism by which MXC alters steroidogenesis by examining the effects of MXC on steroidogenic and metabolic enzymes.

MATERIALS AND METHODS

Chemicals

MXC (99% pure) was purchased from Chemservice (West Chester, PA). Stock solutions of MXC for in vitro experiments were prepared using dimethylsulfoxide (DMSO) (Sigma, St. Louis MO) as a solvent, and in various concentrations (2, 20, and 200 mg/ml) that permitted an equal volume of solvent to be added to individual culture wells for each treatment group. Thus, final concentrations of MXC in culture were 1, 10, and 100 $\mu\text{g/ml}$ (ppm). The doses used in these experiments were selected based on previously published studies showing that these concentrations of MXC induce toxicity in antral follicles, granulosa cell culture models, and in uterine leiomyoma cells (Chedrese and Feyles, 2001; Gupta *et al.*, 2006; Miller *et al.*, 2005). These concentrations are relevant to occupational exposure levels. The FDA monitored the chemical contaminants in food products in the United States and calculated the average daily intake of methoxychlor in adults was up to 4 ng/kg/day (ATSDR, 2002). Normally, serum levels were found to be below the level of detection. However, a study involving an occupational exposure in farm workers has shown that MXC concentration in serum can reach up to 5.16 $\mu\text{g/mL}$ (ATSDR, 2002). Thus, the occupational exposure dose is much higher than normal human exposure and lies between the doses used in the present experiments: MXC 1 $\mu\text{g/ml}$ and MXC 10 $\mu\text{g/ml}$. For controls and MXC treatment groups, DMSO was used at 0.05%, which is able to solubilize MXC in aqueous media. DMSO, ITS (insulin, transferrin, selenium), penicillin, and streptomycin were obtained from Sigma-Aldrich (St. Louis, MO). Alpha-minimal essential media (α -MEM) was obtained from Invitrogen (Carlsbad, CA). Human recombinant follicle stimulating hormone (rFSH) was obtained from Dr. A.F. Parlow from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA). Fetal bovine serum (5% FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA).

Animals

Adult female cycling CD-1 mice were purchased from Charles River Laboratories (Charles River, CA) and housed in the core animal facility located at College of Veterinary Medicine, University of Illinois and maintained on 12L:12D cycles. Mice were housed in the animal facility for at least two days to relieve transportation stress, given *ad libitum* food and water, and temperature was maintained at 22 ± 1 °C. Animals were euthanized at 35–39 days of age by carbon dioxide (CO_2) inhalation followed by cervical dislocation. The ovaries were removed and antral follicles were isolated as explained below. The University of Illinois Institutional Animal Care and Use Committee approved all protocols involving animal care, euthanasia, and tissue collection.

Antral follicle culture

Ovaries were removed and antral follicles were isolated from ovaries of mice between 35–39 days old because this time point was used in previous studies, and this is the age at which mice are cycling young adults (Borgeest *et al.*, 2004; Miller *et al.*, 2005). Antral follicles were isolated mechanically from the ovaries based on relative size and interstitial tissue was removed using fine watch maker forceps. About 3–4 mice were used per experiment and they yielded approximately 20–30 follicles per mouse. Once follicles were isolated, they were placed individually in wells of a 96-well culture plate with 150 μl of unsupplemented α -MEM prior to treatment. Each treatment group in an experiment consisted of 10–15

follicles. Supplemented α -MEM was prepared with: 1% ITS (10 ng/ml insulin, 5.5 ng/ml transferrin, 5.5 ng/ml selenium), 100 U/ml penicillin, 100 mg/ml streptomycin, 5 IU/ml rFSH and 5% FBS. A dose response regimen of MXC (1–100 μ g/ml) and DMSO controls were individually prepared in supplemented α -MEM. For treatment, unsupplemented α -MEM was removed from each well and replaced with 150 μ l of supplemented α -MEM containing MXC or DMSO. Follicles were then incubated for 24, 48, and 96 hours (h) at 37°C in 95% air and 5% CO₂. Non-treated controls (supplemented media alone) were used in each experiment as a control for culture conditions. At the end of 24, 48, and 96 h follicle cultures, media were collected, and stored at –80°C for later use. In addition, follicles were collected, snap frozen, and stored at –80°C for later use.

Analysis of follicle growth

Antral follicles were cultured as described above for 96 h. Follicle growth was examined in 24 h intervals by measuring follicle diameters across perpendicular axes with an inverted microscope equipped with a calibrated ocular micrometer. Antral follicles were considered as those follicles having diameters of 200 μ m or greater (Miller *et al.*, 2006), which correlates with the histological appearance of antral follicles. At least three separate culture experiments were performed for each chemical treatment. In the present study, we first averaged the sizes of follicles in each treatment group within each experiment and then data were averaged and analyzed across different experiments (n=3 separate experiments).

Hormone measurements

The media samples from at least 9–12 individual wells distributed equally across 3–4 experiments were randomly selected and subjected to enzyme-linked immunosorbent assays (ELISA) as described previously (Craig *et al.*, 2010). E₂, testosterone, androstenedione, and P₄ levels were measured in the media using kits from DRG International (Mountainside, NJ). These sex steroid hormones were selected since they are the major intermediates in the biosynthesis of E₂ (Jones and DeCherney, 2005). The sensitivities for each kit were 9.714 pg/ml for E₂, 0.083 ng/ml for testosterone, 0.019 ng/ml for androstenedione, and 0.045 ng/ml for P₄. The intra-assay coefficients of variation (CVs) were 4.7% for E₂, 7.1% for testosterone, 10.2% for androstenedione, and 11.4% for P₄. The inter-assay CVs were 7.8% for E₂, 3.6% for testosterone, 6.5% for androstenedione, and 10.4% for P₄. The cross reactivity with other hormones for each type of kit was negligible.

Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated from frozen follicles using the RNeasy Micro Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol. RNA was treated with DNase to remove any possible genomic DNA contamination. The concentration of RNA in each sample was measured at 260 nm using the Nanodrop ND1000 UV-Vis spectrophotometer (Nanodrop Technologies, Wilmington, DE). The cDNA was synthesized by reverse transcribing the mRNA (200 ng) using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Then cDNA was diluted to 1:4 with nuclease free water.

qPCR was conducted using CFX96 Real-time System C1000 Thermal Cycler (Bio-Rad). All samples were measured in triplicate, and each reaction contained 2 μ L of diluted cDNA, 0.66 μ L (330nM) of gene-specific primers (Integrated DNA Technologies, Inc, Coralville, IA, Table 1), 2.34 μ L of nuclease-free water, and 5 μ L of SsoFast EvaGreen Supermix (Bio-Rad) for a final volume of 10 μ L. The qPCR program consisted of an enzyme activation step (95 °C for 1 min), an amplification and quantification program [40 cycles of 95 °C for 10 sec, annealing/extension for 10 sec (Table 1), single fluorescence reading], a 72 °C for 5 min

step, a melt curve (65 – 95°C heating 0.5 °C per second with continuous fluorescence readings) and a final step at 72 °C for 5 min.

Primer sequences are shown in the Table 1. The specificity of each primer was tested using BLASTN2.218+ and by the presence of single peak in melt curve analysis. In addition, each product was run on 2% agarose gel to confirm the product size. A standard curve was generated from six serial dilutions of one of the samples (1:2, 1:4, 1:8, 1:16, 1:32, and 1:64); thus, allowing analysis of the amount of cDNA in the exponential phase. β -Actin was used as a reference gene. Final values were expressed as genomic equivalents and were calculated as the ratio of each gene to β -actin. The reported data were obtained from the mean expression values of 3–4 separate culture experiments.

Statistical analysis

All data were analyzed using SPSS statistical software (SPSS Inc., Chicago, IL). For all comparisons, statistical significance was assigned at $p \leq 0.05$. Comparisons between DMSO and the different doses of MXC were conducted on data obtained from 3 to 4 experiments using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test or a test for linear regression when applicable.

RESULTS

Effect of MXC on steroid hormone levels

Previous studies have shown that MXC inhibits growth of mouse antral follicles after 96 h of in vitro culture (Gupta *et al.*, 2006; Miller *et al.*, 2005). In the present study, we confirmed these previous findings and showed that MXC inhibits follicle growth at 48, 72, and 96 h (Fig. 1). We further investigated whether MXC alters E_2 levels. At 24h, MXC did not alter E_2 levels. However, at 48 (MXC 1, 10 and 100 μ g/ml) and 96 h (MXC 10 and 100 μ g/ml), MXC exposure significantly decreased E_2 levels compared to DMSO (vehicle control) (Fig. 2).

In addition to E_2 levels, we also investigated whether MXC affects biosynthetic hormone precursors of E_2 such as testosterone, androstenedione, and P_4 . MXC did not affect testosterone levels at 24 and 48 h, but it decreased the levels at 96 h (MXC 10 and 100 μ g/ml) compared to controls (Fig. 3). MXC did not affect androstenedione levels at 24 h, but it decreased the levels at 48 (MXC 1, 10, and 100 μ g/ml) and 96 h (MXC 1, 10, and 100 μ g/ml) compared to controls (Fig. 4). MXC did not alter P_4 levels significantly at any time point, however, the test for linearity using regression analysis showed that P_4 levels trended downwards at 96 h compared to DMSO controls ($p=0.065$) (Fig. 5).

Effect of MXC on expression of enzymes that synthesize ovarian steroids

Since MXC decreased levels of steroid hormone precursors and E_2 levels, we next examined whether the decreased steroidogenesis could be due to effects of MXC on mRNA expression of steroidogenic enzymes. We focused on expression levels because previous studies have shown that the activities of enzymes are directly related to their transcriptional regulation (Jones and DeCherney, 2005; Miller, 1989).

MXC significantly affected expression of *Cyp19a1* levels, the enzyme that converts testosterone to E_2 (Fig. 6). At 24 h (MXC 1 μ g/ml), MXC significantly increased expression of *Cyp19a1* enzyme levels compared to DMSO (vehicle control). However, at 48 (MXC 100 μ g/ml) and 96 h (MXC 10 and 100 μ g/ml), MXC significantly decreased expression of *Cyp19a1* compared to DMSO. In the MXC 1 μ g/ml treatment group, the enzyme levels were significantly decreased at 48 and 96 h compared to 24 h (Fig. 6).

MXC significantly decreased levels of *Hsd17b1*, the enzyme that converts androstenedione to testosterone (Fig. 7). In the MXC 100 µg/ml treatment groups, *Hsd17b1* expression levels were significantly decreased at 24, 48, and 96 h compared to DMSO groups. In the MXC 1 µg/ml group, the *Hsd17b1* enzyme levels were significantly decreased at 96 h compared to 24 h (Fig. 7).

MXC also significantly decreased *Cyp17a1* levels, the enzyme that converts P₄ to androstenedione (Fig. 8). These enzyme expression levels were significantly decreased at 48 h (MXC 10 and 100 µg/ml) and showed a significantly decreased trend at 96 h compared to DMSO. The levels in the MXC 10 µg/ml group were significantly decreased at the 48 and 96 h time points compared to 24 h (Fig. 8).

MXC significantly decreased *Hsd3b1* levels, the enzyme that converts pregnenolone to P₄ and dehydroepiandrosterone to androstenedione (Fig. 9). *Hsd3b1* expression levels were significantly decreased as early as 24 h (MXC 100 µg/ml) and continued to be decreased at 48 (MXC 100 µg/ml) and 96 h (MXC 10 and 100 µg/ml) compared to DMSO groups. In the MXC 10 µg/ml treatment group, *Hsd3b1* expression levels were significantly different across different time points. The levels were significantly decreased at 48 and 96 h compared to 24 h. In the MXC 100 µg/ml treatment group, *Hsd3b1* expression levels were significantly decreased at 48 h compared to 24 h (Fig. 9).

MXC significantly decreased *Cyp11a1* expression levels, the mitochondrial enzyme that converts cholesterol to pregnenolone (Fig. 10). *Cyp11a1* expression levels were significantly decreased at 48 (MXC 100 µg/ml) and 96 h (MXC 10 and 100 µg/ml) compared to DMSO groups. There were no differences between expression levels in each treatment group when compared across different points (Fig. 10).

MXC significantly decreased *Star* expression levels, the enzyme that is involved in the transport of cholesterol from the cytoplasm into mitochondria during steroidogenesis in the theca cells (Fig. 11). When treatment groups were compared within each time point, *Star* expression levels were significantly increased at 48 h (MXC 100 µg/ml) and significantly decreased at 96 h with MXC 10 µg/ml ($p=0.01$) and trended toward decreased expression with MXC 100 µg/ml ($p=0.065$) compared to DMSO. When the expression levels of each treatment group were compared across the different time points, *Star* expression levels in DMSO and MXC 1 µg/ml treatment groups were increased at 96 h compared to 24 h (Fig. 11).

Effect of MXC on expression of an enzyme that metabolize E₂

MXC significantly altered expression of *Cyp1b1* levels, the enzyme that converts E₂ to catecholestrogen. Specifically, MXC (100 µg/ml) significantly reduced expression of *Cyp1b1* levels by 24 h and did not affect the levels at 48 h, but significantly induced expression levels by 96 h compared to DMSO controls (Fig. 12). In the MXC 10 µg/ml treatment group, expression levels were increased at 48 h compared to 24 h and in the MXC 100 µg/ml treatment group, expression levels were increased at 96 h compared to 24 h. The ability of MXC to increase expression of *Cyp1b1* at 96 h was similar to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD: 10nM), a known inducer of *Cyp1b1* expression (Dasmahapatra *et al.*, 2001).

DISCUSSION

The present studies were designed to test the hypothesis that MXC decreases E₂ levels produced by antral follicles by altering steroidogenic and metabolic enzymes. This study confirms results from previous studies that MXC inhibits follicle growth in cultured mouse

antral follicles (Gupta *et al.*, 2006; Miller *et al.*, 2005). This study also expands previous studies by examining the effects of MXC on the steroidogenesis pathway. In the current study, MXC decreased follicle growth, hormone levels, and most enzyme mRNA levels as early as 48 h. However, MXC decreased *Hsd17b1* and *Hsd3b1* expression levels as early as 24 h. Thus, this study shows that some of the earliest induced changes by MXC may be in some steroidogenic enzymes. These early changes in *Hsd17b1* and *Hsd3b1* may lead to the decrease in hormone levels observed in MXC-treated follicles. Further, since adequate levels of sex steroids are required for follicle growth (Drummond *et al.*, 2006), the changes in hormones may contribute to MXC-induced inhibition of follicle growth.

Our data showed that MXC decreases E₂ levels in cultured mouse antral follicles. These results are consistent with previous findings performed in rat ovarian culture studies (Cummings and Laskey, 1993) and studies on bovine granulosa cells showing that MXC (1 µg/ml) decreases E₂ levels (Tiemann *et al.*, 1996). However, the present study demonstrates that MXC disrupts E₂ produced by intact mouse antral follicles and expands previous studies by showing that MXC disrupts steroid precursors made in both thecal and granulosa cells of the follicle. The data obtained using intact antral follicles are significant since antral follicles are the functional units of the ovary that produce sex steroid hormones, which are involved in various important physiological functions in the body, including ovulation.

The present studies also showed that MXC inhibits testosterone production and causes a decreased trend in P₄ levels in cultured mouse antral follicles. These data are consistent with previous studies in rat ovarian cultures showing that MXC decreases testosterone, but not P₄ levels in the media (Cummings and Laskey, 1993) and that MXC decreases P₄ levels in vivo (Chapin *et al.*, 1997). Our data on P₄ levels are also similar to data from studies on porcine granulosa cells showing that MXC decreases P₄ levels (Chedrese and Feyles, 2001). In addition, another study in cultured bovine granulosa cells showed that MXC decreases P₄ synthesis (Tiemann *et al.*, 1996). Other studies have also shown that MXC decreases testosterone levels in male rat testes in vivo (Muroño *et al.*, 2006). Thus, our data in conjunction with others from rat, porcine, and bovine studies suggest that MXC may have similar effects on steroidogenesis across various tissues and species. The present study also expands previous studies by measuring another steroid hormone - androstenedione, which also is significantly decreased in response to MXC. Thus, suggesting that in addition to E₂, testosterone, and P₄, MXC has significant negative effects on androstenedione levels.

Some of the effects of MXC on steroid levels are similar to those produced by metabolites of MXC. Previous studies in liver cells have shown that MXC is metabolized to metabolites such as 2-(p-hydroxyphenyl)-2-(p-methoxyphenyl)-1,1,1-trichloroethane (mono-OH MXC) and 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) (Kapoor *et al.*, 1970) by cytochrome P450 (Cyp) enzymes *Cyp1a2* and *Cyp2c29* (Stresser and Kupfer, 1998). These Cyp enzymes are expressed in the ovary and can be altered by MXC treatment (Paulose *et al.*, 2011; Symonds *et al.*, 2006). Thus, ovary is equipped with Cyp enzymes to metabolize MXC to mono-OH MXC and HPTE. Mono-OH MXC has been shown to decrease E₂, androgen, and P₄ levels by altering the expression of steroidogenic enzymes in the antral follicles from mouse ovaries (Craig *et al.*, 2010) and HPTE been shown to inhibit P₄ and E₂ production in rat theca-interstitial and granulosa cultures by inhibiting steroidogenic enzymes (Akgul *et al.*, 2008; Zachow and Uzumcu, 2006). This raises the possibility that the effects of MXC on steroid levels observed in this study are due to MXC metabolites and not just MXC alone. It is possible that ovarian follicles have the capacity to convert MXC to mono-OH MXC and/or HPTE and that these metabolites inhibit steroid hormone levels. Alternatively, it is possible that the ovarian follicles do not convert MXC to its metabolites and that MXC itself can inhibit steroid levels. Future studies should distinguish between these possibilities.

Since we observed alterations in hormone levels in response to MXC, we investigated the mechanism by which MXC alters hormone levels by measuring steroidogenic and metabolic enzymes and found that MXC does indeed decrease steroidogenic enzymes and increase the metabolic enzyme, *Cyp11b1*. Some of the changes in metabolic enzymes may explain our data on follicle growth and steroid levels. At 48 h, MXC 1 µg/ml inhibits E₂ levels, but these levels return to normal at 96 h. MXC also increases expression of *Cyp19a1* at 24 h. Thus, we speculate that MXC increases *Cyp19a1* protein and activity and that this turn leads to an increase in conversion of testosterone to E₂, which restores E₂ levels. Because E₂ is important for follicle growth, the normal E₂ levels could then restore follicle growth at the 1 µg/ml of MXC.

While there are limited other data on the effects of MXC on steroidogenic enzymes, a few studies have shown that metabolites of MXC may affect steroidogenic enzymes in other tissues and culture systems. Studies in rat granulosa cells showed that the MXC metabolite, HPTE (10 µM; 48 h), blocks FSH-stimulated mRNA expression levels of *Cyp19a1* and that these levels remain suppressed in the presence of dibutyryl-cAMP (db-cAMP) (Zachow and Uzumcu, 2006). Another metabolite of MXC, mono-OH MXC, also inhibits *Cyp19a1* mRNA expression levels in mouse antral follicles (Craig *et al.*, 2010). Studies in human and rat testes indicate that MXC and HPTE decrease HSD17B1 activity (Hu *et al.*, 2010; Vaithinathan *et al.*, 2008) and studies in mouse antral follicles show that mono-OH MXC decreases expression of *Cyp17a1* levels (Craig *et al.*, 2010). The previous studies have shown that MXC and its metabolite HPTE inhibit HSD3B1 activity in human and rat testes (Hu *et al.*, 2010; Vaithinathan *et al.*, 2008). Studies on rat ovaries showed that in-vivo exposure to MXC (100 mg/kg) significantly reduces luteinizing hormone receptor expression in large antral follicles and significantly reduces CYP11A1 immunoreactivity in the ovaries (Armenti *et al.*, 2008). In addition, another study in rat Leydig cells showed that HPTE decreases testosterone production by inhibiting the mRNA levels of *Cyp11a1* (Akingbemi *et al.*, 2000). Another study in rat testis showed that MXC reduces STAR protein levels (Vaithinathan *et al.*, 2008). In the present study, MXC up-regulated the *Star* mRNA expression levels transiently at 48 h and inhibited *Star* expression by 96 h. A similar increase in *Star* levels was previously reported in granulosa cells at 48 h in response to HPTE in the presence of db-cAMP (Zachow and Uzumcu, 2006). These data suggest that MXC may affect STAR expression differently in the testes and ovary. In the ovary, it is possible that *Star* overexpression is an attempt to increase the transfer of cholesterol to the mitochondria to increase substrate for *Cyp11a1* and compensate for inhibition of hormonal levels present downstream in the steroidogenic pathway.

In addition to E₂ biosynthetic enzymes, E₂ metabolic enzymes such as *Cyp1a1* and *Cyp1b1* are important in maintenance of E₂ levels. TCDD is the classical halogenated aromatic hydrocarbon that has been shown to induce *Cyp1a1* and *Cyp1b1* enzymes in different tissues, including granulosa cells of the ovary (Bofinger *et al.*, 2001; Dasmahapatra *et al.*, 2001; Whitlock, 1993). *Cyp1a1* and *Cyp1b1* are involved in C-2 and C-4 hydroxylations of E₂ respectively, leading to formation of catecholestrogens (Spink *et al.*, 1992, 1994). In the present study, levels of *Cyp1b1* were measured as they are shown to be expressed constitutively at higher levels compared to *Cyp1a1* in the ovary (Dasmahapatra *et al.*, 2001). *Cyp1b1* levels were reduced at 24 h (MXC 100 µg/ml) and reached normal levels by 48 h, but significantly increased at 96 h to levels similar to those produced by TCDD (10nM). At 24 h, MXC did not alter the expression of steroidogenic enzymes, but it decreased expression of metabolic enzymes, resulting in no change in steroidogenesis and leading to no change in E₂ levels. At 48 h, MXC inhibited steroidogenic enzymes, but not metabolic enzymes, resulting in decreased steroidogenesis with no changes in E₂ metabolism, which finally lead to decreased E₂ levels. At 96 h, MXC decreased steroidogenic enzymes and increased metabolic enzymes, resulting in decreased synthesis and increased clearance of E₂.

thus leading to further decrease in E₂ levels. Thus, these data show that both steroidogenesis and metabolism of E₂ were affected by MXC over time.

Collectively, our results show that MXC decreases steroidogenic enzymes and increases metabolic enzymes (*Cyp11b1*) in mouse antral follicles. The reduction in steroidogenic enzymes likely leads to decreased synthesis of androgens and E₂. Further, increase in the metabolic enzyme *Cyp11b1* likely increases metabolism of E₂ in follicles. Together, with decreased availability of hormone precursors and increased expression of metabolic enzyme *Cyp11b1*, MXC causes significant decrease in E₂ levels and these low levels of steroids may contribute to MXC-induced inhibition of follicle growth. The reasons for all these MXC-induced changes are unknown, but could include one or more common factors upstream of enzyme expression. MXC as well as its metabolites have been shown to bind both nuclear and membrane estrogen receptors. Thus, it is possible that MXC binds estrogen receptors and that this leads to changes in gene expression, which eventually leads to changes in steroidogenesis and follicle growth. Future studies will determine whether this is the case.

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REFERENCE LIST

- Akgul Y, Derk RC, Meighan T, Rao KMK, Murono EP. The methoxychlor metabolite, HPTE, directly inhibits the catalytic activity of cholesterol side-chain cleavage (P450_{scc}) in cultured rat ovarian cells. *Reprod. Toxicol.* 2008; 25:67–75. [PubMed: 18065196]
- Akingbemi BT, Ge RS, Klinefelter GR, Gunsalus GL, Hardy MP. A metabolite of methoxychlor, 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane, reduces testosterone biosynthesis in rat Leydig cells through suppression of steady-state messenger ribonucleic acid levels of the cholesterol side-chain cleavage enzyme. *Biol. Reprod.* 2000; 62:571–578. [PubMed: 10684797]
- Armenti AE, Zama AM, Passantino L, Uzumcu M. Developmental methoxychlor exposure affects multiple reproductive parameters and ovarian folliculogenesis and gene expression in adult rats. *Toxicol. Appl. Pharmacol.* 2008; 233:286–296. [PubMed: 18848953]
- ATSDR. Toxicological profile for methoxychlor. Atlanta, GA: Agency for Toxic Substances and Disease Registry; 2002.
- Bofinger DP, Feng L, Chi LH, Love J, Stephen FD, Sutter TR, Osteen KG, Costich TG, Batt RE, Koury ST, Olson JR. Effect of TCDD exposure on CYP1A1 and CYP1B1 expression in explant cultures of human endometrium. *Toxicol. Sci.* 2001; 62:299–314. [PubMed: 11452143]
- Borgeest C, Miller KP, Gupta R, Greenfeld C, Hruska KS, Hoyer P, 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]
- 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]
- Britt KL, Findlay JK. Estrogen actions in the ovary revisited. *J Endocrinol.* 2002; 175:269–276. [PubMed: 12429025]
- Chapin RE, Harris MW, Davis BJ, Ward SM, Wilson RE, Mauney MA, Lockhart AC, Smialowicz RJ, Moser VC, Burka LT, Collins BJ, Haskins EA, Allen JD, Judd L, Purdie WA, Harris HL, Lee CA,

- Corniffe GM. The effects of perinatal/juvenile methoxychlor exposure on adult rat nervous, immune, and reproductive system function. *Toxicol. Sci.* 1997; 40:138–157.
- Chedrese PJ, Feyles F. The diverse mechanism of action of dichlorodiphenyldichloroethylene (DDE) and methoxychlor in ovarian cells in vitro. *Reprod. Toxicol.* 2001; 15:693–698. [PubMed: 11738522]
- Couse JF, Korach KS. Exploring the role of sex steroids through studies of receptor deficient mice. *J. Mol. Med.* 1998; 76:497–511. [PubMed: 9660168]
- Craig ZR, Leslie TC, Hatfield KP, Gupta RK, Flaws JA. Mono-hydroxy methoxychlor alters levels of key sex steroids and steroidogenic enzymes in cultured mouse antral follicles. *Toxicol. Appl. Pharmacol.* 2010; 249:107–113. [PubMed: 20840852]
- Crisp TM, Clegg ED, Cooper RL, Wood WP, Andersen DG, Baetcke KP, Hoffmann JL, Morrow MS, Rodier DJ, Schaeffer JE, Touart LW, Zeeman MG, Patel YM. Environmental endocrine disruption: An effects assessment and analysis. *Environ. Health Perspect.* 1998; 106:11–56. [PubMed: 9539004]
- Cummings AM. Methoxychlor as a model for environmental estrogens. *Crit. Rev. Toxicol.* 1997; 27:367–379. [PubMed: 9263644]
- Cummings AM, Laskey J. Effect of methoxychlor on ovarian steroidogenesis: Role in early pregnancy loss. *Reprod. Toxicol.* 1993; 7:17–23. [PubMed: 8448411]
- Dasmahapatra AK, Wimpee BAB, Trewin AL, Hutz RJ. 2,3,7,8-Tetrachlorodibenzo-p-dioxin increases steady-state estrogen receptor- β mRNA levels after CYP1A1 and CYP1B1 induction in rat granulosa cells in vitro. *Mol. Cell. Endocrinol.* 2001; 182:39–48. [PubMed: 11500237]
- Drummond AE. The role of steroids in follicular growth. *Reprod. Biol. Endocrinol.* 2006; 4
- Eroschenko VP, Swartz WJ, Ford LC. Decreased superovulation in adult mice following neonatal exposures to technical methoxychlor. *Reprod. Toxicol.* 1997; 11:807–814. [PubMed: 9407591]
- Findlay JK, Britt K, Kerr JB, O'Donnell L, Jones ME, Drummond AE, Simpson ER. The road to ovulation: The role of oestrogens. *Reprod. Fertil. Dev.* 2001; 13:543–547. [PubMed: 11999304]
- Golovleva LA, Polyakova AB, Pertsova RN, Finkelshtein ZI. The fate of methoxychlor in soils and transformation by soil microorganisms. *Journal of Environmental Science and Health, Part B: Pesticides, Food Contaminants, and Agricultural Wastes.* 1984; 19:523–538.
- 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]
- Hu GX, Zhao B, Chu Y, Li XH, Akingbemi BT, Zheng ZQ, Ge RS. Effects of methoxychlor and 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane on 3β -hydroxysteroid dehydrogenase and 17β -hydroxysteroid dehydrogenase-3 activities in human and rat testes. *Int. J. Androl.* 2010
- Jones, E.; DeCherney, A. The female reproductive system. In: Boron, W.; Boulpaep, E., editors. *Medical physiology: a cellular and molecular approach.* Philadelphia, PA: Elsevier Saunders; 2005. p. 1141–1189.
- Kapoor IP, Metcalf RL, Nystrom RF, Sangha GK. Comparative metabolism of methoxychlor, methiochlor, and DDT in mouse, insects, and in a model ecosystem. *J. Agric. Food Chem.* 1970; 18:1145–1152. [PubMed: 5483054]
- Martinez EM, Swartz WJ. Effects of methoxychlor on the reproductive system of the adult female mouse: 2. Ultrastructural observations. *Reprod. Toxicol.* 1992; 6:93–98. [PubMed: 1562805]
- 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]
- Miller KP, Gupta RK, Flaws JA. Methoxychlor metabolites may cause ovarian toxicity through estrogen-regulated pathways. *Toxicol. Sci.* 2006; 93:180–188. [PubMed: 16760418]
- Miller KP, Gupta RK, Greenfeld CR, Babus JK, Flaws JA. Methoxychlor directly affects ovarian antral follicle growth and atresia through Bcl-2 and Bax-mediated pathways. *Toxicol. Sci.* 2005; 88:213–221. [PubMed: 16081520]
- Miller WL. Regulation of mRNAs for human steroidogenic enzymes. *Endocr. Res.* 1989; 15:1–16. [PubMed: 2667966]

- Murono EP, Derk RC, Akgul Y. In vivo exposure of young adult male rats to methoxychlor reduces serum testosterone levels and ex vivo Leydig cell testosterone formation and cholesterol side-chain cleavage activity. *Reprod. Toxicol.* 2006; 21:148–153. [PubMed: 16226009]
- Paulose T, Hernández-Ochoa I, Basavarajappa MS, Peretz J, Flaws JA. Increased sensitivity of estrogen receptor alpha overexpressing antral follicles to methoxychlor and its metabolites. *Toxicol. Sci.* 2011; 120:447–459. [PubMed: 21252393]
- Spink DC, Eugster HP, Lincoln DW II, Schuetz JD, Schuetz EG, Johnson JA, Kaminsky LS, Gierthy JF. 17 β -Estradiol hydroxylation catalyzed by human cytochrome P450 1A1: A comparison of the activities induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin in MCF-7 cells with those from heterologous expression of the cDNA. *Arch. Biochem. Biophys.* 1992; 293:342–348. [PubMed: 1536570]
- Spink DC, Hayes CL, Young NR, Christou M, Sutter TR, Jefcoate CR, Gierthy JF. The effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on estrogen metabolism in MCF-7 breast cancer cells: Evidence for induction of a novel 17 β -estradiol 4-hydroxylase. *J. Steroid Biochem. Mol. Biol.* 1994; 51:251–258. [PubMed: 7826886]
- Stresser DM, Kupfer D. Human Cytochrome P450-catalyzed conversion of the proestrogenic pesticide methoxychlor into an estrogen. *Drug Metab. Dispos.* 1998; 26:868–874. [PubMed: 9733665]
- Stuchal LD, Kleinow KM, Stegeman JJ, James MO. Demethylation of the pesticide methoxychlor in liver and intestine from untreated, methoxychlor-treated, and 3-methylcholanthrene-treated channel catfish (*ictalurus punctatus*): evidence for roles of cyp1 and cyp3a family isozymes. *Drug Metab. Dispos.* 2006; 34:932–938. [PubMed: 16510540]
- Suter, D. Ovarian physiology. In: Hoyer, P., editor. *Ovarian Toxicology*. CRC Press; 2004. p. 1-16.
- 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]
- Symonds DA, Miller KP, Tomic D, Flaws JA. Effect of methoxychlor and estradiol on cytochrome P450 enzymes in the mouse ovarian surface epithelium. *Toxicol. Sci.* 2006; 89:510–514. [PubMed: 16280380]
- Tiemann U, Pöland R, Schneider F. Influence of organochlorine pesticides on physiological potency of cultured granulosa cells from bovine preovulatory follicles. *Theriogenology.* 1996; 46:253–265. [PubMed: 16727895]
- Vaithinathan S, Saradha B, Mathur P. Transient inhibitory effect of methoxychlor on testicular steroidogenesis in rat: an in vivo study. *Arch. Toxicol.* 2008; 82:833–839. [PubMed: 18401575]
- Whitlock J. Mechanistic aspects of dioxin action. *Chem. Res. Toxicol.* 1993; 6:754–763. [PubMed: 8117913]
- Zachow R, Uzumcu M. The methoxychlor metabolite, 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane, inhibits steroidogenesis in rat ovarian granulosa cells in vitro. *Reprod. Toxicol.* 2006; 22:659–665. [PubMed: 16737795]

Fig. 1. Effect of in-vitro MXC exposure on antral follicular growth

Antral follicles isolated mechanically from CD-1 mice were exposed to DMSO or MXC (1–100 µg/ml) for 96 h. Growth of follicles was monitored during culture, recorded in µm and reported as percentage change. Graphs represent means $\pm$ SE from 3 separate experiments [* indicates significant difference from controls ($n=12-16$ follicles per treatment; $p\leq 0.05$)].

Fig. 2. Effect of in-vitro MXC exposure on estradiol (E₂) levels

Antral follicles isolated mechanically from CD-1 mice were exposed to DMSO or MXC (1–100 µg/ml) and cultured for 24, 48, and 96 h. The media was subjected to measurements of E₂ levels by enzyme-linked immunosorbent assay (ELISA). (A) E₂ levels at 24 h (B) E₂ levels at 48 h (C) E₂ levels at 96 h. Graph represents means ± SEM from 3 separate experiments [* indicates significant difference from controls ($n=9-12$ follicles per treatment; $p \leq 0.05$)].

Fig. 3. Effect of in-vitro MXC exposure on testosterone levels

Antral follicles isolated mechanically from CD-1 mice were exposed to DMSO or MXC (1–100 µg/ml) and cultured for 24, 48, and 96 h. The media was subjected to measurements of testosterone levels by ELISA. (A) testosterone levels at 24 h (B) testosterone levels at 48 h (C) testosterone levels at 96 h. Graph represents means $\pm$ SEM from 3 separate experiments [* indicates significant difference from controls ($n=9-12$ follicles per treatment; $p \leq 0.05$)].

Fig. 4. Effect of in-vitro MXC exposure on androstenedione levels

Antral follicles isolated mechanically from CD-1 mice were exposed to DMSO or MXC (1–100 µg/ml) and cultured for 24, 48, and 96 h. The media was subjected to measurements of androstenedione levels by ELISA. (A) androstenedione levels at 24 h (B) androstenedione levels at 48 h (C) androstenedione levels at 96 h. Graph represents means $\pm$ SEM from 3 separate experiments [* indicates significant difference from controls (n=9–12 follicles per treatment; $p \leq 0.05$)].

Fig. 5. Effect of in-vitro MXC exposure on progesterone (P_4) levels

Antral follicles isolated mechanically from CD-1 mice were exposed to DMSO or MXC (1–100 $\mu\text{g/ml}$) and cultured for 24, 48, and 96 h. The media was subjected to measurements of P_4 levels by ELISA. (A) P_4 levels at 24 h (B) P_4 levels at 48 h (C) P_4 levels at 96 h. Graph represents means $\pm$ SEM from 3 separate experiments [$\dagger p=0.065$ using linear regression ($n=9\text{--}12$ follicles per treatment)].

Fig. 6. Aromatase (Cyp19a1) levels in response to MXC

Antral follicles isolated mechanically from CD-1 mice were exposed to DMSO or MXC (1–100 $\mu\text{g/ml}$) and cultured for 24, 48, and 96 h. *Cyp19a1* mRNA expression was measured in antral follicles using qPCR. Graph represents means $\pm$ SEM from 3 separate experiments [n=12–14 follicles per treatment; * $p \leq 0.05$ compared to 24 h; Bars with different letters are significantly different from each other within each time point].

Fig. 7. 17 β -hydroxysteroid dehydrogenase (Hsd17b1) levels in response to MXC
Antral follicles isolated mechanically from CD-1 mice were exposed to DMSO or MXC (1–100 $\mu\text{g/ml}$) and cultured for 24, 48, and 96 h. *Hsd17b1* mRNA expression was measured in antral follicles using qPCR. Graph represents means $\pm$ SEM from 3 separate experiments [n=12–14 follicles per treatment; * $p \leq 0.05$ compared to 24 h; Bars with different letters are significantly different from each other within each time point].

Fig. 8. 17 α -hydroxylase/17,20-lyase (Cyp17a1) levels in response to MXC

Antral follicles isolated mechanically from CD-1 mice were exposed to DMSO or MXC (1–100 μ g/ml) and cultured for 24, 48, and 96 h. *Cyp17a1* mRNA expression was measured in antral follicles using qPCR. Graph represents means $\pm$ SEM from 3 separate experiments [n=12–14 follicles per treatment; * $p \leq 0.05$ compared to 24 h; Bars with different letters are significantly different from each other within each time point; † $p \leq 0.05$ using linear regression].

Fig. 9. 3 β -hydroxysteroid dehydrogenase (Hsd3b1) levels in response to MXC

Antral follicles isolated mechanically from CD-1 mice were exposed to DMSO or MXC (1–100 μ g/ml) and cultured for 24, 48, and 96 h. *Hsd3b1* mRNA expression was measured in antral follicles using qPCR. Graph represents means $\pm$ SEM from 3 separate experiments [n=12–14 follicles per treatment; * $p \leq 0.05$ compared to 24 h; Bars with different letters are significantly different from each other within each time point].

Fig. 10. Cholesterol side chain cleavage enzyme (Cyp11a1) levels in response to MXC
Antral follicles isolated mechanically from CD-1 mice were exposed to DMSO or MXC (1–100 µg/ml) and cultured for 24, 48, and 96 h. *Cyp11a1* mRNA expression was measured in antral follicles using qPCR. Graph represents means $\pm$ SEM from 3 separate experiments [n=12–14 follicles per treatment; Bars with different letters are significantly different from each other within each time point].

Fig. 11. Steroidogenic acute regulatory protein (Star) in response to MXC

Antral follicles isolated mechanically from CD-1 mice were exposed to DMSO or MXC (1–100 $\mu\text{g/ml}$) and cultured for 24, 48, and 96 h. *Star* mRNA expression was measured in antral follicles using qPCR. Graph represents means $\pm$ SEM from 3 separate experiments [n=12–14 follicles per treatment; * $p \leq 0.05$ compared to 24 h; Bars with different letters are significantly different from each other within each time point].

Fig. 12. Cytochrome P450 1b1 (Cyp1b1) levels in response to MXC

Antral follicles isolated mechanically from CD-1 mice were exposed to DMSO or MXC (1–100 $\mu\text{g/ml}$) and cultured for 24, 48, and 96 h. *Cyp1b1* mRNA expression was measured in antral follicles using qPCR. Graph represents means $\pm$ SEM from 3 separate experiments [n=12–14 follicles per treatment; * $p \leq 0.05$ compared to 24 h; Bars with different letters are significantly different from each other within each time point].

Table 1

Real-time qPCR primer accession numbers, abbreviations, and sequences.

Accession no.	Gene name	Abbreviation	Annealing/ extension temperature (°C)	Forward	Reverse
NM_007393.3	<i>actin, beta</i>	<i>Actb</i>	57.3	5'-GGGACACAGTGTGGGTGA C- 3'	5'-CTGGCACCAACACCTTCTAC-3'
NM_009994.1	<i>cytochrome P450, family 1, subfamily b, polypeptide 1</i>	<i>Cyp1b1</i>	60	5'-GCGACGATTCTCCGGGC TG- 3'	5'-TGCACGCGGGCCTGAACAT C- 3'
NM_019779.3	<i>cytochrome P450, family 11, subfamily a, polypeptide 1</i>	<i>Cyp11a1</i>	60	5'-AGATCCCTTCCCTTGGTG ACAATG- 3'	5'-CGCATGAGAAAGAGTATCGA CGCATC- 3'
NM_007809.3	<i>cytochrome P450, family 17, subfamily a, polypeptide 1</i>	<i>Cyp17a1</i>	56.2	5'-CCAGGACCCCAAGTGTGTT CT- 3'	5'-CCTGATACGAAGCACCTTCT CG- 3'
NM_007810.3	<i>cytochrome P450, family 19, subfamily a, polypeptide 1</i>	<i>Cyp19a1</i>	55.3	5'-CATGGTCCCGGAAACTGT GA- 3'	5'-GTAGTAGTTGCAGGCACCTT C- 3'
NM_008293.3	<i>hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1</i>	<i>Hsd3b1</i>	59.4	5'-CAGGAGAAAGAACTGCAG GAGGTC- 3'	5'-GCACACTTGCTTGAACACA GGC- 3'
NM_010475.1	<i>hydroxysteroid 17-beta) dehydrogenase 1</i>	<i>Hsd17b1</i>	58	5'-ACTGTGCCAGCAAGTTTG CG- 3'	5'-AAGCGGTTCGTGGAGAAAGT AG- 3'
NM_011485.4	<i>Steroidogenic acute regulatory protein</i>	<i>Star</i>	57.5	5'-CAGGGAGAGGTGGCTAT GCA- 3'	5'-CCGTGTCCTTTTCCAATCCTC TG- 3'