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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 (E_2) 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 E_2 metabolic enzyme *Cyp1b1* were measured using qPCR. The results indicate that MXC decreased E_2, testosterone, androstenedione, and progesterone (P_4) levels compared to DMSO. In addition, MXC decreased expression of aromatase (*Cyp19a1*), 17β-hydroxysteroid dehydrogenase 1 (*Hsd17b1*), 17α-hydroxylase/17,20-lyase (*Cyp17a1*), 3β hydroxysteroid dehydrogenase 1 (*Hsd3b1*), cholesterol side-chain cleavage (*Cyp11a1*), steroid acute regulatory protein (*Star*), and increased expression of *Cyp1b1* 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.

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 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 the same mother displays 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 17β-estradiol (E_2). The E_2 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

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as bones, vascular tissues, and the brain (Britt and Findlay, 2002; Couse & Korach, 1998; Findlay et al., 2001).

E_2 production by antral follicles is a complex process in which cholesterol is eventually converted to E_2. 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 3β-hydroxysteroid dehydrogenase (HSD3B1), which catalyzes the conversion of pregnenolone to progesterone (P_4). Pregnenolone and P_4 are eventually converted irreversibly to androstenedione by 17α-hydroxylase/17,20-lyase (CYP17A1). Androstenedione is then converted to the more potent androgen testosterone by 17β-hydroxysteroid dehydrogenase (HSD17B1). This enzyme also catalyzes the conversion of estrone to a more potent estrogen, E_2. The androgens are then converted to estrogens by aromatase (CYP19A1) (Suter, 2004). E_2 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 μg/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 that 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 μg/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 μg/ml and MXC 10 μg/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, and 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 2 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 and 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, and 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 μg/ml) and DMSO controls was individually prepared in supplemented α-MEM. For treatment, unsupplemented α-MEM was removed from each well and replaced with 150 μl 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% CO_2. 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 μm 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). E_2, testosterone, androstenedione, and P_4 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 E_2 (Jones and DeCherney, 2005). The sensitivities for each kit were 9.714 pg/ml for E_2, 0.083 ng/ml for testosterone, 0.019 ng/ml for androstenedione, and 0.045 ng/ml for P_4. The intra-assay coefficients of variation (CVs) were 4.7% for E_2, 7.1% for testosterone, 10.2% for androstenedione, and 11.4% for P_4. The inter-assay CVs were 7.8% for E_2, 3.6% for testosterone, 6.5% for androstenedione, and 10.4% for P_4. The cross reactivity with other hormones for each type of kit was negligible.