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Methoxychlor inhibits growth and induces atresia through the aryl hydrocarbon receptor pathway in mouse ovarian antral follicles

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Abstract

Methoxychlor (MXC) is an organochlorine pesticide used against pests that attack crops, vegetables, and livestock. MXC inhibits growth and induces atresia (death) of mouse ovarian antral follicles in vitro. Since several studies indicate that many chemicals act through the aryl hydrocarbon receptor (AHR) pathway, the current study tested the hypothesis that MXC binds to the AHR to inhibit growth and induce atresia of antral follicles. The data indicate that MXC binds to AHR. Further, a relatively high dose of MXC (100 µg/ml) inhibits growth and induces atresia in both wild-type (WT) and AHR null (AHRKO) follicles, whereas a lower dose of MXC (10 µg/ml) inhibits growth and induces atresia in WT, but not in AHRKO follicles. These data indicate that AHR deletion partially protects antral follicles from MXC induced slow growth and atresia. Collectively, these data show that MXC may act through the AHR pathway to inhibit follicle growth and induce atresia in antral follicles of the ovary.

Keywords

methoxychlor; antral follicles; ovary; mouse; aryl hydrocarbon receptor; atresia; growth

1 Introduction

Endocrine disrupting chemicals (EDCs) such as pesticides, plasticizers, cosmetics, solvents, paints and pollutants are present in the environment. EDCs exert their toxicity by affecting the synthesis, secretion, transport, binding, action, and elimination of variety of hormones present in the body [1]. The EDC methoxychlor (MXC) is an organochlorine pesticide that is widely used in many parts of the world, primarily to prevent or destroy pests that feed on crops and domestic animals [2]. MXC gained popularity in 1970s and replaced the potent

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Conflict of interest

The authors do not have any conflicts to disclose

chlorinated pesticide dichlorodiphenyltrichloroethane (DDT) after its usage was restricted in the US and other parts of the world. The use of MXC was banned in 2004 because of failure of registration with the Environmental Protection Agency (EPA) [3]. Globally, humans and domestic animals are exposed to MXC through extensive usage of this chemical and in the US, through imported agricultural products.

MXC has been shown to induce persistent vaginal estrus, decrease ovarian weights, and increase atresia (follicle death) of large follicles in adult female mice [4]. Exposure to MXC in utero increases atretic follicles in F1a litters and the residual effect of MXC induces premature vaginal opening in F1b litters. In addition, mothers exposed to MXC have an increased gestation period and increased number of dead fetuses compared to vehicle controls during their first pregnancy [5]. Neonatal exposure to MXC also induces ovarian atrophy, decreases relative ovarian weight, and decreases corpora lutea numbers [6]. Collectively, these previous studies indicate that MXC targets the ovary and affects fertility in laboratory rodents.

More recent studies indicate that MXC damages the ovary by inhibiting antral follicle growth and increasing atresia [7;8;9]. In vivo studies in mice indicate that MXC specifically targets antral follicles by increasing the number of atreticantral follicles and decreasing the percentage of healthy antral follicles in the ovary [7]. Several in vitro studies have also shown that MXC inhibits growth and increases atresia of mouse antral follicles [8;9].

While it is known that MXC inhibits growth and increases atresia of antral follicles, the mechanism by which it does so is unclear. Many studies indicate that other EDCs such as polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, and polyhalogenated biphenyls mediate their toxicity through the aryl hydrocarbon receptor (AHR) pathway [10;11]. However, limited information is available about whether MXC works via the AHR pathway. One study by Han et al. (2007) suggests that MXC may bind to the AHR, inhibiting the action of 2,3,4,8-tetrachlorodibenzo-*p*-dioxin (TCDD), in murine Hepa-1c1c7 cells [12]. Thus, the current study was designed to determine the involvement of the AHR pathway in mediating the effects of MXC treatment on antral follicles of the mouse ovary. Specifically, this study was designed to test the hypothesis that MXC works through the AHR to inhibit follicle growth and induce atresia in mouse antral follicles. We focused on the AHR pathway because of the work by Han et al. showing that MXC binds to the AHR in liver cells [12] and because the AHR plays many important physiological roles in the ovary [13]. Specifically, studies have shown that the AHR helps regulate the growth of pre-antral and antral follicles by promoting granulosa cell proliferation and that it plays a role in the regulation of steroidogenesis, ovulation, and corpora lutea formation in the ovary [13]. In this study, we used concentrations of MXC (1, 10, and 100 µg/ml) that are relevant to occupational exposure levels. The Food and Drug Administration (FDA) monitored the chemical contaminants in food products in the United States and calculated the average daily intake of MXC in adults was up to 4 ng/kg/day [2]. Normally, serum levels were found to be below the level of detection. However, a study involving an occupational exposure in farm workers showed that MXC concentrations in serum could reach as high as 5.16 µg/ml [2]. 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.

2 Materials and Methods

2.1 Reagents and Materials

2.1.1 Reagents—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 (1.33, 13.33, and

133.33 mg/ml) that permitted 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). For controls and MXC treatment groups, DMSO was used at 0.075%, which is able to solubilize MXC in aqueous media without exerting toxicity to follicles. These doses were selected for in vitro studies based on previously published studies showing that these concentrations of MXC induce toxicity in antral follicles and granulosa cell culture models [8;9]. 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 (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA).

2.1.2 Animals—C57BL/6 female wild-type (WT) and AHR homozygous null (AHRKO) mice were maintained and bred in the core animal facility located at College of Veterinary Medicine, University of Illinois and maintained on 12L: 12D cycles. Mice were housed in polystyrene cages and provided with corn cob bedding to limit as much as possible contamination from other EDCs. Mice were given *ad libitum* food and water, and temperature was maintained at 22±1 °C. Mice were fed with 2016 Teklad Global 16% Protein Rodent Diet (Harlan laboratories), which does not contain any phytoestrogens including alfalfa or soybean meal. Animals were euthanized at 30-35 days of age by carbon dioxide (CO₂) 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 (IACUC) approved all protocols involving animal care, euthanasia, and tissue collection.

2.1.3 Screening/genotyping AHRKO and WT mice—The offspring born to adult cycling mice were genotyped using polymerase chain reaction (PCR) - based assays. Briefly, ear punch tissues from pups were lysed in 25 µl of buffer (1M Tris pH 8.0, 5M NaCl, 0.5M EDTA, and 20% SDS) containing 2 µl of 20 mg/ml proteinase K (Qiagen Inc., Valencia, CA). Digestion was carried out at 55°C for 1h followed by enzyme inactivation at 100°C for 3 min. Molecular grade water (73 µl) then was added to the lysate and this mixture was subjected to PCR using primers (1) Neo F: 5'-TTGGGTGGAGAGGCTATTCG - 3' and (2) Neo R: 5'-CCATTTTCCACC ATGATATTCG - 3', which detect the insert in the AHR gene and primers (3) F: 5'-TCTTGGGCTCGATCT TGTGTCA - 3' and (4) R: 5'-TTGACTTAATTCCTTCAGCGG - 3', which detect the AHR gene.

The conditions for PCR were 94°C for 2 min of initial denaturation followed by 40 cycles at 94°C for 45 s, 55°C for 1 min, and 72°C for 3 min. PCR products then were subjected to agarose gel electrophoresis. The presence of a 672-bp fragment indicated that the mice were WT, the presence of a 580-bp band indicated that the mice were AHRKO, and presence of both 673- and 580-bp bands indicated that mice were heterozygotes. Only WT and AHRKO homozygous mice were used in these experiments.

2.2 Granulosa cell culture and xenobiotic response element (XRE) reporter assay

The XRE reporter assay was performed to determine the binding of MXC to AHR. To perform this assay, ovaries from 30-35 day old CD-1 mice were collected and cleaned in petri dishes containing collection media (α-MEM media, 10% FBS, 200 U/ml penicillin, 200 mg/ml streptomycin). The ovaries then were transferred to petri dishes containing supplemented media and were incubated for 20 - 30 min at 37° C. Supplemented media were prepared as previously described [14]. Granulosa cells (GC) were collected by

puncturing follicles using syringe needles. GCs were counted using a hemocytometer chamber, and approximately 20,000 GCs were added to each well of a 96 well microplate and incubated at 37°C for 3 days with medium changed daily.

After 3 days of culture, when the cells reached 80-90 % confluence, GCs were transfected with negative control, positive control or xenobiotic response element (XRE) reporter plasmids (Cignal XRE reporter (luc) kit: SABiosciences) using Lipofectamine2000 (Invitrogen). Transfection was carried out according to manufacturer instructions for a transfection time of 18 h. After the transfection, medium was replaced with supplemented medium containing either DMSO or MXC (1, 10, and 100 µg/ml) or the positive control TCDD (10 nM) and incubated for 18 h. DMSO or MXC treatments were also added to negative controls to determine the specific effects and background reporter activity. The Dual-Glo Luciferase Reagent (Promega) was added directly to medium to measure both firefly and renillaluminescences. TCDD was used as a positive control for induction of XREs because several studies indicate it binds the AHR with high affinity [11;15]. No-treatment controls were used as a control for culture conditions. XRE reporter plasmids contain a mixture of inducible AHR-responsive firefly luciferase construct and constitutively expressing Renilla luciferase construct (40:1). Negative control contained a mixture of non-inducible firefly luciferase construct and constitutively expressing renilla luciferase construct (40:1). Positive controls contained a mixture of constitutively expressing green fluorescence protein, constitutively expressing firefly luciferase, and constitutively expressing renilla luciferase constructs (40:1:1). Renilla luciferase constructs acted as internal controls within each well.

2.3 Antral follicle culture

Antral follicles were isolated from ovaries of WT and AHRKO mice between 30-35 days old because this is the age at which mice are cycling young adults [14;16]. Antral follicles were isolated mechanically from the ovaries based on appearance and relative size (diameter 200-400 µm) [17] and interstitial tissue was removed using fine watch-maker forceps. About 2-3 mice were used per experiment and they yielded approximately 30-40 follicles per mouse. Once follicles were isolated, they were placed in each well in 96 well culture plates containing 150 µl of unsupplemented medium. A dose response regimen of MXC (1-100 µg/ml) and vehicle control (DMSO) was individually prepared in supplemented α-MEM. The unsupplemented medium was replaced with supplemented medium containing either DMSO or MXC (1, 10, and 100 µg/ml). Non-treated controls were used in each experiment as a control for culture conditions. The unsupplemented medium was removed slowly so that follicles could attach to the bottom of wells. Each treatment group in an experiment consisted of 10-15 follicles.

Follicles then were incubated for 168 hours (h) with medium changed at 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. Follicle diameter measurements were averaged among treatment groups and plotted to compare the effects of chemical treatments on growth over time. At the end of 168 h, media were collected and stored at -80°C for later use. In addition, some follicles were fixed in Dietrick's solution for histological evaluation of atresia and some follicles were collected, snap frozen, and stored at -80°C for quantitative real time PCR as described below. Follicle cultures were repeated three times to obtain enough power for statistical analysis.

2.4 Histological evaluation of atresia

The follicles collected in Dietrick's solution were processed through series of washes with ethanol: 70% for 10 min, 85% for 10 min, 95% for 7 min (2X), and 100% for 7 min (2X).

Then, they were embedded in plastic blocks using Technovit 7100 kits from HeraeusKulzer GmbH, Germany. The follicles were incubated in a pre-infiltration medium for one hour and then with infiltration medium overnight before they were embedded in plastic blocks using embedding medium. Pre-infiltration solution was prepared with one part 100% ethanol : one part base solution and infiltration solution was prepared with 1 gm of hardener-1 with 100 ml of base solution. Embedding medium was prepared by adding 1 ml of hardener-2 to 15 ml of infiltration solution.

The follicles embedded in plastic blocks were sectioned into 2 μ m thin sections using a microtome. Follicle sections were stained with Lee's methylene blue-basic fuchsin stain for 30 sec and washed with distilled water before they were cover slipped. Each follicle section was examined for level of atresia as evidenced by the presence of apoptotic bodies and reported at the highest level observed throughout the tissue. Specifically, follicles were rated on a scale of 1-4 for the presence of apoptotic bodies: 1= healthy, 2 = less than 10% apoptotic bodies (early atresia), 3 = 11-30% apoptotic bodies (mid atresia), 4 = greater than 30% apoptotic bodies (late atresia), as previously described by Miller et al. (2005) [8]. Ratings were averaged and plotted to compare the effect of chemical treatments on atresia levels.

2.5 Hormone measurements

The media samples from at least 10-15 individual wells distributed equally across 3-4 experiments were randomly selected and subjected to enzyme-linked immunosorbent assays (ELISA) as described previously [14]. Specifically, estradiol (E_2) levels were measured in the collected media using kits from DRG International (Mountainside, NJ). The sensitivity of the ELISA was 9.714 pg/ml for E_2 . The intra-assay coefficient of variation (CV) was 4.7% and the inter-assay CV was 7.8%. The cross reactivity with other hormones for each type of kit was negligible.

2.6 Statistical analysis

All data were analyzed using SPSS statistical software (SPSS Inc., Chicago, IL). For all comparisons, statistical significance was assigned at $p < 0.05$. Comparisons between DMSO and the different doses of MXC were conducted on data obtained from 3 to 4 separate experiments using two-way analysis of variance (ANOVA) or Kruskal-Wallis test followed by Tukey's post hoc test or Mann-Whitney test or a test for linear regression when applicable. Data are graphed using means $\pm$ standard error of the means (SE).

3 Results

3.1 Effect of MXC on XRE reporter activity

We found that MXC (10 and 100 μ g/ml) significantly induces XRE reporter activity. The level of induction of XRE reporter activity by 100 μ g/ml of MXC was similar to that induced by the positive control TCDD (Fig 1).

3.2 Effect of MXC on WT and AHRKO follicle growth

Given our finding that MXC binds to the AHR, we next determined if MXC works through the AHR to inhibit follicle growth. Previous studies have shown that MXC inhibits growth of mouse antral follicles by 96 h of culture [8;14]. In the present study, we evaluated the effects of MXC on growth of WT and AHRKO follicles through 168 h of culture. Evaluation of growth in WT follicles showed that MXC at 10 and 100 μ g/ml significantly inhibited follicle growth compared to vehicle control (DMSO) at 96-168 h. MXC at 1 μ g/ml did not inhibit follicle growth at any time point (Fig 2A). Interestingly, evaluation of growth in AHRKO follicles showed that only MXC 100 μ g/ml inhibited follicle growth compared

to DMSO (Fig 2B). MXC at 1 and 10 $\mu\text{g/ml}$ did not inhibit follicle growth and thus, AHRKO follicles grew similar to controls at these concentrations of MXC. Further, MXC inhibited follicle growth at an earlier time point in WT follicles compared to AHRKO follicles. Specifically, MXC 100 $\mu\text{g/ml}$ inhibited growth at 96 h in WT follicles (Fig 2A), but not until 120 h in AHRKO follicles (Fig 2B).

3.3 Effect of MXC on WT and AHRKO follicle atresia

Since MXC is also known to induce atresia of antral follicles in vivo and in vitro [7;8], we next compared the effect of MXC on follicular atresia in WT and AHRKO follicles to determine whether MXC induces atresia via the AHR pathway. In WT follicles, MXC at 10 and 100 $\mu\text{g/ml}$ significantly increased atresia compared to DMSO (Fig 3). However, MXC at 1 $\mu\text{g/ml}$ did not increase atresia compared to control. In AHRKO follicles, only MXC at 100 $\mu\text{g/ml}$ significantly increased atresia compared to DMSO. MXC at 1 $\mu\text{g/ml}$ did not increase atresia compared to control (Fig 3). When we compared atresia in treated and control follicles across WT and AHRKO genotypes, MXC at 10 $\mu\text{g/ml}$ significantly increased atresia in WT, but not AHRKO follicles (Fig 3).

3.4 Effect of MXC on estradiol levels in WT and AHRKO follicles

MXC is also known to inhibit E_2 levels [14]. Further, E_2 levels are thought to help regulate normal follicle growth and atresia [18;19]. Thus, we examined whether MXC decreases E_2 levels via the AHR. MXC significantly decreased estradiol levels in WT and AHRKO follicle types at the MXC 10 and 100 $\mu\text{g/ml}$ doses, but not the MXC 1 $\mu\text{g/ml}$ dose compared to DMSO (Fig 4).

4 Discussion

The current studies were conducted to test the hypothesis that MXC inhibits follicle growth and induces atresia through the AHR pathway in mouse ovarian antral follicles. Our data indicate that MXC binds to AHR in ovarian cells and that it may work through the AHR to regulate the effects of 10 $\mu\text{g/ml}$ of MXC on follicle growth and atresia, but not the effects of MXC on estradiol levels.

In cells, upon ligand binding, the AHR is activated and translocates into the nucleus, where it forms a complex with aryl hydrocarbon nuclear translocator (ARNT). The liganded AHR-ARNT complex then acts as a transcriptional activator by binding to XREs present in 5'-flanking region of numerous genes including Cyp1a1 and Cyp1b1 [20]. Studies have shown that the 5'-flanking region containing AHR-responsive element (AHRE) is responsible for the induction of Cyp1a1 and Cyp1b1 [10]. In the current studies, we first assessed whether MXC binds to AHR by transfecting a XRE-reporter (luc) plasmid into granulosa cells. We showed that MXC induces the XRE-reporter plasmid, suggesting that it binds to the AHR in the ovary. Our results are consistent with previous findings that MXC binds to AHR in Hepa-1c1c7 cells [12]. Further, our results are consistent with an in vivo study in rats showing that MXC induces CYP1A1/1A2 activities in the liver [21]. They also are consistent with a previous study, which showed that MXC exposure increases *Cyp1b1* expression in mouse antral follicles in vitro [14]. Interestingly, our data indicate that MXC (100 $\mu\text{g/ml}$) induces the XRE-reporter plasmid to the same degree as TCDD (10nM). TCDD and other polycyclic aromatic hydrocarbons (PAHs) are known to bind to the AHR and induce the cytochrome P450 family of genes in different tissues, including the ovary [11;22;23]. Studies in granulosa cells also have shown that TCDD induces *Cyp1a1* and *Cyp1b1* by increasing expression of *Ahr* [24].

Previous studies have shown that MXC inhibits follicle growth and induces atresia in cultured antral follicles [9;17]. Given that MXC binds the AHR, we next tested whether MXC inhibits follicle growth and induces follicular atresia via the AHR pathway. We found that deletion of AHR can rescue follicles from MXC (10 µg/ml) induced inhibition of follicle growth and MXC (10 µg/ml) induced atresia. Interestingly, the rescue only occurred at the 10 µg/ml dose of MXC and not the 100 µg/ml dose of MXC. At the 10µg/ml dose, it is possible that MXC exclusively acts through the AHR pathway to inhibit follicle growth and atresia. Hence, genetic deletion of the AHR can rescue the MXC inhibition of follicle growth and atresia at that dose. However, at the 100 µg/ml dose, it is possible that in addition to AHR pathways, MXC works via other pathways such as non-genomic estrogen receptor pathways and androgen receptor pathways. This possibility is supported by previous studies, which suggest that MXC might work through non-genomic estrogen receptor (ER) or androgen receptor pathways.[17;25;26]. Specifically, Miller et al. (2006) showed that treatment with the antiestrogen ICI does not protect mouse antral follicles from MXC-induced inhibition of follicle growth or atresia [17]. However, treatment with E₂ protects mouse antral follicles from MXC-induced atresia, suggesting that MXC may act through non-genomic ER pathways rather than through classical ER pathways to induce atresia [17]. In addition, Waters et al. (2001) showed that the MXC metabolite HPTE increases ERβ levels similar to antiandrogen flutamide in the ovary suggesting the role of androgen receptor in response to MXC [26].

Our results are consistent with other studies showing that manipulation of the AHR rescues ovarian cells from other chemicals. For example, studies have shown that AHR disruption by α-naphthoflavone (ANF) reduces the ability of 9,10-dimethylbenz(a)anthracene-3,4-dihydrodiol (DMBADHD) to cause cell death and as a result, increases the number of fetal oocytes [27;28]. Another study also showed that AHR disruption by ANF reduces the effect of PAHs such as benzo(a)pyrene, 3-methylcholanthrene, and 7,12-dimethylbenz(a)anthracene (DMBA) on the ovary and as a result increases the numbers of primordial and primary follicular oocytes [29-31].

Interestingly, in the present study, we observed that AHR deletion did not rescue E₂ levels in follicles. These data are consistent with previous studies, which show that MXC decreases E₂ levels by decreasing the expression of steroidogenic enzymes *Cyp19a1*, *Hsd17b1*, *Cyp17a1*, *Hsd3b1*, and *Cyp11a1* in antral follicles of the ovary [14]. Our results are also consistent with previous studies which show that MXC affects testicular steroidogenesis in rats by decreasing Δ⁵ Hsd17b and Δ⁵ Hsd3b1 activities [32]. Another study in rats has also shown that MXC alters steroidogenesis by decreasing the Cyp11a1 immunoreactivity in large ovarian antral follicles [33]. It is known from studies that MXC may bind directly to ERs and AR [34], which are thought to bind to response elements on most of these steroidogenic enzymes. One study has shown that AHR response elements are present in the Cyp19a1 promoter and thus, are controlled by at least partially by AHR [35]. Thus, it is possible that in the absence of AHR, MXC can bind to other partners, which further bind to response elements present on most of steroidogenic enzymes and regulate their expression, finally altering steroid levels.

5 Conclusions

In conclusion, these results provide mechanistic information that MXC (10 µg/ml) exerts toxicity through the AHR pathway in antral follicles. The present data showed that MXC binds to AHR in granulosa cells of the ovary. In addition, current data confirmed previous studies that MXC inhibits follicle growth and atresia in WT follicles. Further, the current study expanded previous work by demonstrating that MXC-induced inhibition of follicle growth and induction of atresia were rescued in AHRKO follicles, but not in WT follicles.

Collectively, these data show that MXC partially acts through the AHR pathway to inhibit follicle growth and induce atresia in antral follicles of the ovary.

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HIGHLIGHTS

Methoxychlor (MXC; 10 and 100 $\mu\text{g/ml}$) binds to aryl hydrocarbon receptor (AHR) in mouse ovarian cells.

MXC (10 $\mu\text{g/ml}$) inhibits growth and induces atresia through the AHR pathway in mouse ovarian antral follicles.

The effects of MXC on sex steroid hormone levels may not be mediated by the AHR pathway.

Fig 1. Effect of in vitro MXC exposure on XRE reporter activity

Granulosa cells (GCs) were collected from ovaries of CD-1 mice, transfected with negative or positive or XRE-reporter plasmids, and then treated with vehicle (DMSO), MXC (1-100 $\mu\text{g/ml}$), or TCDD (10 nM). Dual-glo luciferase reagent was added and luminescence was measured using a luminometer. Data represent means $\pm$ SE from 3 separate experiments (* indicates significant difference from vehicle controls; $n=4-6$ ovaries per experiment; $p < 0.05$).

Fig 2. Effect of in vitro MXC exposure on WT and AHRKO follicle growth

Antral follicles isolated from ovaries of WT (panel A) and AHRKO (panel B) mice were exposed to vehicle (DMSO) or MXC (1-100 µg/ml) for 168 h. Growth of follicles was monitored during culture, recorded in µm and reported as percentage change. Data represent means ± SE from 3 separate experiments (* indicates significant difference from vehicle controls; **a** indicates significant difference from MXC 100 µg/ml; n= 12-16 follicles per treatment; p 0.05).

Fig 3. Effect of in vitro MXC exposure on atresia of WT and AHRKO antral follicles
Antral follicles isolated from ovaries of WT or AHRKO mice were exposed to vehicle (DMSO) or MXC (1-100 µg/ml) for 168 h. At the end of culture, follicles were subjected to histological analysis of atresia. Data represent means $\pm$ SE from 3 separate experiments (* indicates significant difference between WT and AHRKO genotypes; Bars with different letters are significantly different from each other withingenotype; n= 4 - 6 follicles per treatment; p 0.05).

Fig 4. Effect of in vitro MXC exposure on estradiol (E_2) levels

Antral follicles isolated from ovaries of wild-type (WT) or AHR null (AHRKO) mice were exposed to vehicle (DMSO) or MXC (1-100 $\mu\text{g/ml}$) for 168 h. The media was subjected to measurements of E_2 levels by enzyme-linked immunosorbent assay (ELISA). Data represent means $\pm$ SE from 3 separate experiments (Bars with different letters are significantly different from each other; $n = 10 - 12$ follicles per treatment; $p < 0.05$).