Early postnatal methoxychlor exposure inhibits folliculogenesis and stimulates anti-Mullerian hormone production in the rat ovary

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Abstract

Methoxychlor [1,1,1-trichloro–2,2-bis(4-methoxyphenyl) ethane; MXC] is a chlorinated hydrocarbon pesticide commonly used in the United States as a replacement for DDT [1,1,1-trichloro–2,2-bis(p-chlorophenyl)ethane]. While MXC is a weak estrogenic compound, its more active, major metabolite [2,2-bis(p-hydroxyphenyl)–1,1,1-trichloroethane; HPTE] shows estrogenic, anti–estrogenic, or anti–androgenic properties depending on the receptor subtype with which it interacts. Anti-Mullerian hormone (AMH) is a paracrine factor that suppresses initial follicle recruitment in the ovary. Studies have shown the effects of exposure to MXC on adult ovarian morphology and function. However, the effect of exposure to MXC at an early postnatal stage on pre-pubertal follicular development and ovarian AMH production has not been studied. Around postnatal day (P) 4, most of the primordial follicular assembly in rats is complete, and a large number of primordial follicles transition into the primary follicle stage, a process that is inhibited by estrogen. The objective of this study was to examine the effect of early postnatal (P3–P10) MXC exposure on ovarian morphology and size, follicle number, and AMH production in the pre-pubertal (P20) rat ovary and to investigate the effect of HPTE on AMH production in immature rat granulosa cells in $vitro$. Female rats were injected (s.c.) daily with vehicle (control) or 1, 10, 50, 100, or 500 mg MXC/kg per day (referred to here as 1MXC, 10MXC, and so forth.) between P3 and P10. On P20, uterine and ovarian weights were determined, ovarian histology was examined, and follicles were counted and classified into primordial, primary, secondary, pre-antral, or antral stages using the two largest serial sections at the center of the ovary. Ovarian AMH production

was examined using immunohistochemistry and western blot analysis. The effect of HPTE (0·5–25 µM) on AMH production in cultured immature rat granulosa cells was determined by western blot analysis. Ovarian weight was reduced by 50, 100, and 500MXC ($P < 0·01$). MXC treatment inhibited folliculogenesis. Both 100 and 500MXC had a reduced number of antral follicles ($P < 0·05$) with a concomitant increase in pre-antral follicles ($P < 0·05$). Follicle numbers were not significantly affected by 1, 10, or 50MXC. Total follicle number and the number of primordial, primary, or secondary stage follicles were not significantly different in all treatment groups. Immuno-histochemistry showed that MXC-treated ovaries had more AMH-positive follicles with stronger AMH immunostaining. Western blot analysis showed that AMH production was $1·6 \pm 0·2$, $1·85 \pm 0·6$, and $2·2 \pm 0·5$ times higher in the 50, 100, and 500MXC ovaries as compared with the control ovaries respectively ($P < 0·05$). Granulosa cells treated with 1 or 5 µM HPTE had significantly greater AMH production ($P < 0·05$). These results demonstrate that MXC inhibits early ovarian development and stimulates AMH production directly in the rat ovary. In addition, HPTE was shown to stimulate AMH production in rat granulosa cells. Endocrine disruptors are widespread in the environment, and MXC represents a model endocrine disruptor due to the multiple actions of its metabolites. This study confirms that the endocrine disruptor MXC inhibits follicular development and demonstrates for the first time that MXC and HPTE directly stimulate AMH production in the ovary. This novel finding suggests that elevated AMH may play a role in MXC's inhibitory effect in the ovary.

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Introduction

The primary functions of the mammalian ovary are steroidogenesis and ovulation, processes that are intricately connected to folliculogenesis. Primordial follicles are formed from oocyte nests after synchronous oocyte apoptosis occurs during the perinatal period in mice (Pepling & Spradling 2001). Each primordial follicle consists of an oocyte surrounded by squamous, non-growing pre-granulosa cells.

The formation is completed by around postnatal day (P) 3 or 4 in rats (Kezele & Skinner 2003). Most primordial follicles remain quiescent, but some grow and transition into the primary follicle stage. Each primary follicle consists of an oocyte surrounded by a single layer of proliferating cuboidal granulosa cells (Hirshfield 1991).

Early follicular development is primarily controlled by paracrine growth factors. Some growth factors, such as kit ligand and growth differentiation factor–9 stimulate follicular

development, and their dysfunction or absence results in deficiencies in this process (Huang *et al*. 1993, Bedell *et al*. 1995, Dong *et al*. 1996). Other growth factors, including anti-Mullerian hormone (AMH), inhibit early folliculogenesis. In general, AMH is not expressed in primordial follicles, but only expressed in the primary follicle through small antral follicle stages. In pre-ovulatory, large antral follicles, AMH expression disappears or is very limited. AMH-expressing follicles appear to inhibit initial recruitment of primordial follicles, since increased initial follicular recruitment occurs in AMH-deficient mice (Durlinger *et al*. 1999). In addition, cultured neonatal mice ovaries treated with AMH have fewer growing follicles as compared with untreated ovaries (Durlinger *et al*. 2002). Furthermore, AMH may also inhibit gonadotropin-stimulated cyclic follicle recruitment, since AMH inhibits follicle-stimulating hormone (FSH)-stimulated aromatase activity and luteinizing hormone (LH) receptor expression in granulosa cells (di Clemente *et al*. 1994), thereby attenuating the effects of FSH on follicular development in the ovary. Thus, AMH appears to be a physiological brake in the ovary.

Neonatal estrogen treatment inhibits folliculogenesis (Ikeda *et al*. 2001) and stimulates AMH expression in the ovary (Ikeda *et al*. 2002). A recent study showed that estradiol treatment inhibits the primordial-to-primary follicle transition in neonatal ovaries *in vitro*, suggesting a direct action of the steroid on the ovary (Kezele & Skinner 2003). Methoxychlor [1,1,1-trichloro–2,2-bis(4–methoxyphenyl) ethane; MXC] is an organochlorine pesticide used as a replacement for DDT [1,1,1-trichloro–2,2-bis(p-chlorophenyl)ethane]. MXC is estrogenic *in vivo* and has low affinity for the estrogen receptor (Hall *et al*. 1997). One of the primary metabolites of MXC is 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE). HPTE shows estrogenic, anti-estrogenic, or anti-androgenic activities depending on the receptor subtype (Maness *et al*. 1998, Gaido *et al*. 2000). MXC is considered to be a model endocrine disruptor (Cummings 1997) with direct effects on the antral follicle (Miller *et al*. 2005).

Studies have shown the effect of relatively long-term MXC exposure on adult ovarian morphology and female fertility. Exposure to MXC during the first 14 days of life leads to irregular estrous cycles and causes the formation of either follicular cysts or ovarian atrophy in adult mice depending on the dose (Eroschenko *et al*. 1995). Rats were exposed to 5–150 mg/kg per day MXC 1 week before and 6 weeks after birth, and reproductive parameters were examined (Chapin *et al*. 1997). Doses of 50 and 100 mg/kg per day MXC stimulated early vaginal opening, inhibited ovarian development, caused irregular reproductive cycles, and reduced fecundity in rats. Exposure to 32 mg/kg MXC for 20 days reduces antral follicle number by atresia in mice (Borgeest *et al*. 2002). However, the effect of brief neonatal (P3–P10) exposure to MXC on pre-pubertal (P20) ovarian folliculogenesis and AMH production in the ovary has not been studied.

The objective of this study was to examine the effect of the estrogenic endocrine disruptor MXC on the rat ovary when administered during the primordial-to-primary follicle transition (P3–P10), which Kezele & Skinner (2003) demonstrated to be inhibited by estradiol-17β. Tissues were collected for analysis at a pre-pubertal age (P20) before cyclic gonadotropin secretion starts in order to assess the effects of MXC on early folliculogenesis and AMH expression in the ovary. In the present study, as an exposure period P3–P10 was selected, since the formation of primordial follicles is mostly completed around P3 or P4 (Kezele & Skinner 2003). While most primordial follicles remain quiescent, a relatively large number of newly formed primordial follicles undergo the primordial-to-primary follicle transition right after follicle formation (McGee & Hsueh 2000). After the transition of primordial follicles into the primary follicle stage, they are destined for either ovulation or atresia (Hirshfield 1991). Both of these processes deplete the limited number of follicles, ending the reproductive lifespan of a female.

Materials and Methods

Animals

Neonatal female Sprague–Dawley rats were injected daily (s.c.) with 1, 10, 50, 100, or 500 mg/kg per day methoxychlor between P3 and P10 (P0 = day of birth). Control rats were injected with vehicle, 25 µl DMSO:sesame oil (1:2). The s.c. injection route was used for a more precise dosing.

To facilitate reading, the 1 mg/kg per day MXC group will be referred to as '1MXC', the 10 mg/kg per day MXC group will be referred to as '10MXC', and so forth (Chapin *et al*. 1997). The MXC dose was calculated according to the body weight (BW) on the first day of the injections. The average BWs were not different between the groups and ranged from 9·4 to 9·6 g on P3. The doses that were used in this study are comparable with the doses that have been used in previous studies (5–800 mg/kg per day; Chapin *et al*. 1997, Hall *et al*. 1997). Environmental levels of MXC range from 40 to 160 ppm (mg/l) in waters downstream of MXC-sprayed areas (cited in Miller *et al*. 2005), which is within the range of doses in the present study. Since the actual MXC intake by humans averages only 100 ng/day (Gunderson 1988), MXC is not a specific concern for human health. However, many endocrine disruptors show estrogenic, anti-estrogenic, or anti-androgenic properties. Since MXC's major metabolite, HPTE, has estrogenic, anti-estrogenic, and anti-androgenic actions, MXC was selected as a prototype endocrine disruptor in this study.

The animal care followed the Rutgers University Animal Care and Facilities Committee's guidelines. On P20, the rats were euthanized by CO_2 inhalation and the uteri and ovaries were collected. After organ weights were determined, one ovary was used for histology, follicle counting, and AMH immunohistochemistry (IHC) while the other was used for western blot analysis.

Ovarian histology

Ovaries were fixed in Bouin's fixative for 2 h at room temperature and stored in 70% ethanol at 4 °C until embedding in paraffin. Paraffin-embedded tissues were sectioned at 4–5 μM. One of the sections from the largest cross section at the center of the ovary was used for hematoxylin and eosin (H and E) staining and was imaged using light microscopy to determine size of the ovary and follicle numbers and stages in the ovary.

Follicle numbers and stages

Using two sections from the largest cross section of the ovary, the number of follicles in each developmental stage was counted (Parrott & Skinner 1999). The follicles was classified according to previously published studies (Oktay *et al.* 1995). Primordial: an oocyte surrounded by squamous pre-granulosa cells of which not more than one is an enlarged granulosa cell. Primary: a relatively larger oocyte surrounded by at least two or more cuboidal granulosa cells. At the early primary follicle stage, some of the granulosa cells may still be squamous. Secondary (transitional): an oocyte surrounded by two to three layers of cuboidal granulosa cells. Pre-antral: an oocyte surrounded by more than three layers of granulosa cells with no apparent antrum. Antral: an oocyte surrounded by multiple layers of granulosa cells with an antrum. Unknown follicles: granulosa cells surrounded by a theca layer without a clear oocyte present.

AMH immunohistochemistry

One of the sections was used for IHC as previously described (Uzumcu *et al.* 2000). The sections were deparaffinized, rehydrated, and microwaved in 0·01 M citrate buffer (pH 6·0) for antigen retrieval. The sections were then blocked with 1·5% normal serum for 10 min at room temperature. A polyclonal goat anti-AMH antibody (sc-6886, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the primary antibody. The sections were incubated with the primary antibody (1:200) overnight at room temperature. The negative control sections, instead of the primary antibody, were incubated with normal goat IgG or with primary antibody plus excess (10×) blocking peptide. Immunoreactivities were visualized using the ABC Staining System (Santa Cruz Biotechnology) following the manufacturer's instructions. Sections were counterstained with Mayer's hematoxylin.

Granulosa cell culture

The granulosa cells were prepared as described previously with some modification (Uzumcu & Lin 1994, Zachow & Uzumcu 2006). Briefly, to increase cell harvest, immature female rats (21–23 days old) were injected (s.c.) with 250 μg diethylstilbestrol (DES) in 100 μl sesame oil daily for 5 days.

Eight hours after the last DES injection, the rats were killed. Ovaries were removed from the animal, isolated from the associated fat, oviduct, and bursa ovary, and then placed in ice-cold Dulbecco's modified Eagle's medium: Ham's nutrient mixture F-12 (DMEM/F-12). Granulosa cells were isolated using a non-enzymatic needle puncture method with a sterile bundle of beading needles. Following two washes, the cells were plated at a density of approximately 4×10^5 viable cells/ml in a culture containing 10 ml DMEM/F-12 medium supplemented with 5% FBS, 100 U/ml penicillin, and 100 g/ml streptomycin sulfate for 24 h. The cells were treated with HPTE (0·5, 1, 5, 10, or 25 μM) in 5 ml serum-free DMEM/F-12 for 24 h. The cells were harvested for AMH western blot analysis at the end of treatment. The experiment was repeated with granulosa cells harvested from immature rats that were injected (s.c.) with 15 IU equine chorionic gonadotropin (eCG).

Western blot analysis

Western blot analysis was performed as previously described (Uzumcu *et al.* 2002). The ovaries were lysed with 1× cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA). The protein concentration in the supernatants was estimated using the DC Protein Assay Kit II (Bio-Rad). Approximately, 60 μg protein in the sample-loading buffer were boiled for 5 min and electrophoresed on a 10–12% SDS gel. The protein was subsequently transferred onto a nitrocellulose membrane and probed with specific antibodies for AMH and actin (AMH, sc-6886; actin, sc-1616; Santa Cruz Biotechnology). Following incubation with a secondary antibody, the specific antigen–antibody complex was visualized using ECL Advance Western Blotting Detection Kit (Amersham Biosciences) and viewed by autoradiography. The relative intensity was determined using actin as the loading control.

Statistical analysis

Organ weight, follicle numbers, and western blot data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). The values were expressed as the mean ± S.E.M. Statistical analysis was performed and the difference between the means of treatments and the respective controls was determined using one-way ANOVA followed by Dunnett's multiple comparison test. For the ovarian weight, histology, IHC, follicle number, and whole-ovary AMH western blot analysis, experiments were repeated two to five times, using at least two to three animals for each treatment in each experimental repeat. Therefore, 5–11 animals were used for each treatment group. The granulosa cell culture AMH western blot analysis experiment was repeated thrice using one replicate (i.e. one culture flask) for each treatment in one experiment. For each experiment, the cells were obtained from seven to eight animals and combined. A statistically significant difference was confirmed at $P < 0·05$.

Results

Ovarian weights

Daily MXC treatment between P3 and P10 decreased ovarian weight and increased uterine weight by P20 (Fig. 1). The decrease in ovarian weight was significant for 50, 100, and 500MXC ($P<0.01$). Only 100 and 500MXC caused a significant increase in uterine weight ($P<0.01$). None of the MXC doses caused systemic toxicological effects as indicated by general appearance, and no significant change in body weight occurred. On P20, the average BWs for all the groups ranged between 51·0 and 52·5 g ($P>0.05$). In a separate experiment, the body weight of rats that were treated daily with MXC (1–500 mg/kg per day) did not significantly change during the treatments (data not shown).

Ovarian histology

Daily MXC treatment between P3 and P10 caused a reduction in the size of the ovary and inhibited folliculogenesis (Fig. 2). The 50 and 500MXC treatments reduced ovarian size by approximately 35 and 80% respectively (Fig. 2C and E), as compared with the controls (Fig. 2A). The size reduction by 100MXC was approximately 75% (not shown). While control ovaries had many small, medium, and large antral follicles (Fig. 2B), there was a dose-dependent reduction in the number of antral follicles in the ovaries of 50MXC (Fig. 2D), 100MXC (not shown), and 500MXC (Fig. 2F). The size, histology, and follicle composition of 1 and 10MXC were similar to those of the controls (not shown). The 50MXC dose eliminated most of the large antral follicles, but some small and medium-sized antral follicles remained (Fig. 2D). Ovaries of 500MXC had few or no antral follicles (Fig. 2F). The 100MXC effect was similar to that of 500MXC (data not shown).
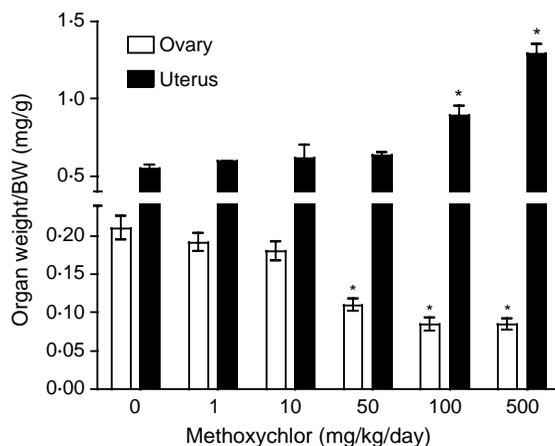
Figure 1 Effect of early postnatal methoxychlor (MXC) exposure on P20 rat ovarian and uterine weights. The procedures were carried out as described in Materials and Methods. Daily MXC injections (s.c.) between P3 and P10 reduced ovarian weight and increased uterine weight relative to body weight (BW) by P20 ($n=5$–11). *$P<0.01$.

Follicle numbers and stages

In order to determine more precisely any effects of different doses of MXC on follicle numbers and stages, follicles in all stages of development were counted in two serial sections from the largest cross-sectional area at the center of the ovary (Fig. 3). On average, a section in control ovaries had 99·28 ± 8·0 total follicles that were distributed among primordial follicles, 9·0 ± 2·0; primary follicles, 22·9 ± 4·2; transitional (secondary) follicles, 4·1 ± 1·9; pre-antral follicles, 7·7 ± 3·3; and antral follicles, 12·3 ± 1·5. The total number of follicles was unaffected by any of the treatments. Both 100 and 500MXC reduced the antral follicle number significantly with a concomitant increase in the pre-antral follicle stage ($P<0.05$). The number of follicles in primordial, primary, and transitional stages did not differ among all treatment groups ($P>0.05$), nor did the number of unknown follicles differ between treatment groups ($P>0.05$).

AMH immunohistochemistry

Immunohistochemistry showed that AMH was produced by granulosa cells of primary, secondary, pre-antral, and small antral follicles (Fig. 4). In medium and large antral follicles, AMH immunostaining was reduced. This limited AMH production was primarily observed in cumulus granulosa cells (Fig. 4A; arrowheads). MXC-treated ovaries had many more AMH-positive follicles with stronger AMH immunostaining (Fig. 4C, E and G) as compared with the control ovaries (Fig. 4A). In 1 and 10MXC ovaries, the number of AMH-positive follicles and the intensity of the staining was similar to the control (not shown). In ovaries of 50MXC, the number of AMH-positive follicles increased and most follicles showed a more widespread and stronger staining (Fig. 4C and D). The majority of growing follicles, with the exception of some antral follicles (Fig. 4C; arrowhead), had strong, widespread staining in the granulosa cells. Antral follicles stained only in cumulus granulosa cells. In 500MXC ovaries, nearly all follicles other than primordial follicles showed strong AMH immunostaining (Fig. 4G and H). In 100MXC ovaries, the number of AMH-positive follicles and staining intensity was intermediary to the 50 and 500MXC (Fig. 4E and F). Higher magnification micrographs revealed stronger immunoreactivity in granulosa cells in 50, 100, and 500MXC ovaries (Fig. 4D, F, and H insets) as compared with granulosa cells in control ovaries (Fig. 4B inset). In control ovaries, granulosa cells had relatively limited AMH immunoreactivity in follicles beyond the primary follicle stage as compared with MXC-treated ovaries. Negative-control ovaries showed no specific immunoreactivity (data not shown).

Effect of MXC on ovarian AMH production *in vivo*

To quantify ovarian AMH production, western blot analysis was performed. The results of western blot analysis
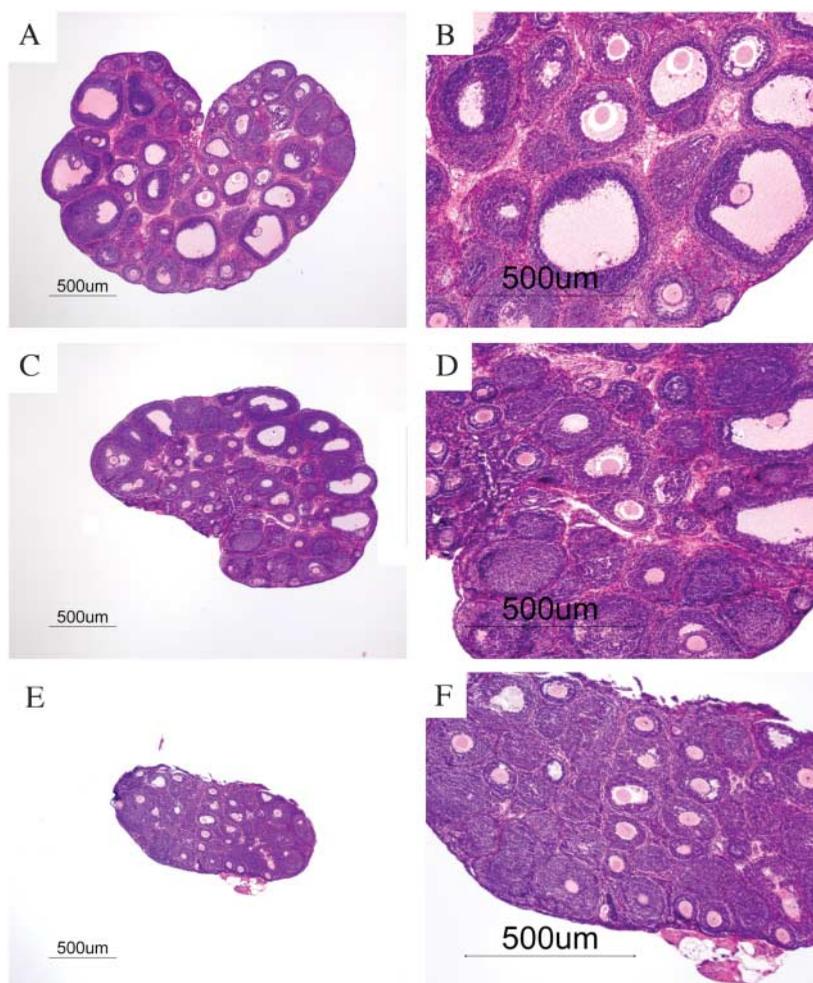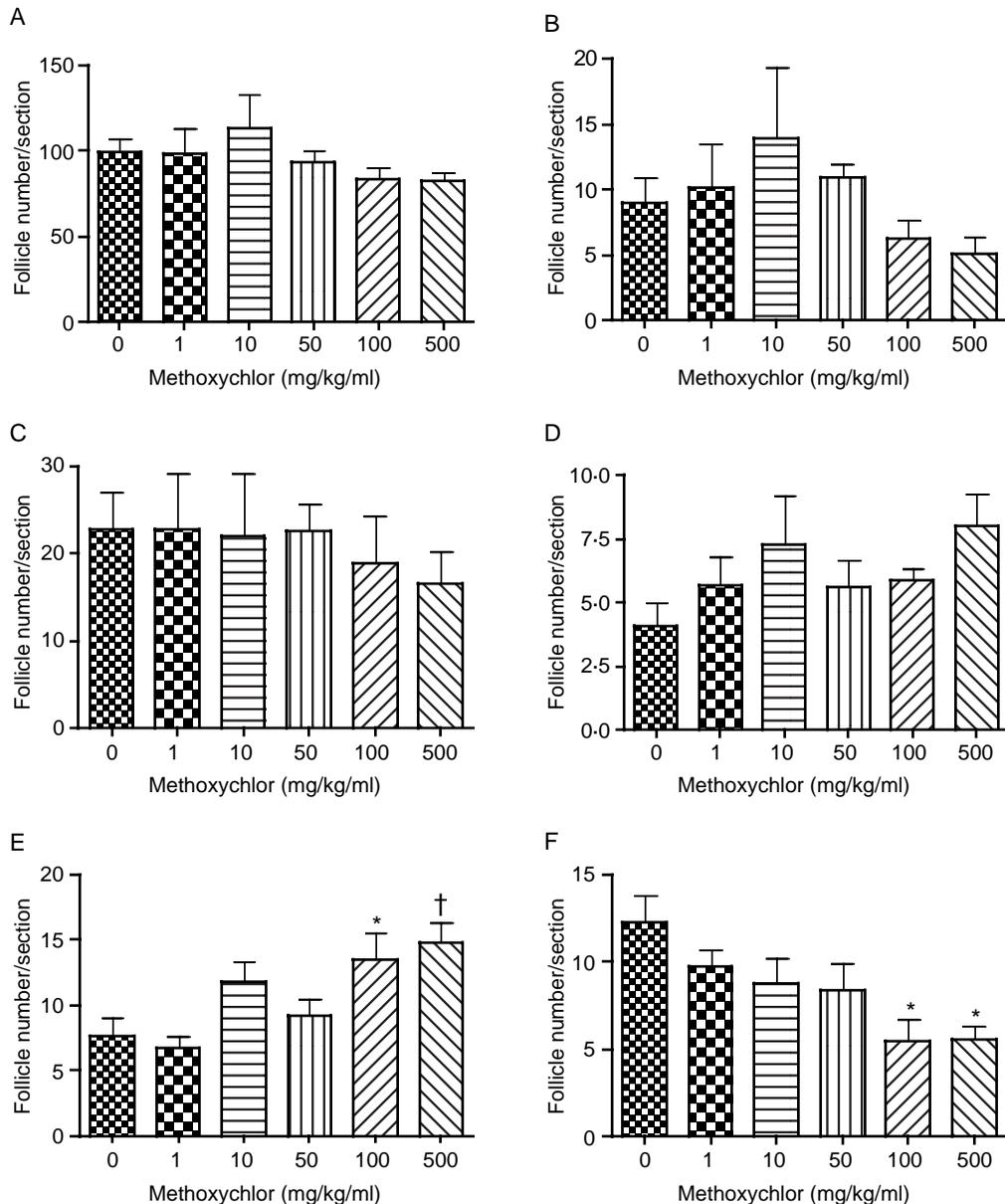
Figure 2 Effect of early postnatal methoxychlor (MXC) exposure on size of and folliculogenesis in the rat ovary. Histology was performed as described in Materials and Methods. (A and B), controls; (C and D), 50 mg/kg per day MXC; (E and F), 500 mg/kg per day MXC. Daily MXC injections (s.c.) between P3 and P10 reduced ovarian size in a dose-dependent manner by P20 (C and E) compared with controls (A). In addition, while control ovaries had many small, medium, and large antral follicles (B), MXC treatment caused dose-dependent reduction in the number of medium and large antral follicles in the ovary (D and F). Ovaries treated with 500 mg/kg per day MXC had no medium or large antral follicles (F). The figures are representative of approximately ten ovaries in each treatment group.

supported our observations in IHC and showed that ovaries of 50, 100, and 500MXC–treated females contained 1.6 ± 0.2, 1.85 ± 0.6, and 2.2 ± 0.5 times more AMH protein than the control ovaries respectively (Fig. 5A and B), the differences being statistically significant ($P < 0.05$). The AMH protein production in ovaries of 1 and 10MXC was not significantly different from control ovaries ($P > 0.05$). The molecular weight of the AMH band was approximately 65 kDa, which is similar to a previously reported result for western blot analysis of bovine AMH separated under reducing conditions (Wilson *et al*. 1993).

Effect of HPTE on AMH production in rat granulosa cells in vitro

To assess whether increased ovarian AMH production *in vivo* was due to a direct action of MXC or its metabolites in the ovary, we examined the effect of HPTE on granulosa cells as HPTE is more active (e.g. has a higher affinity for estrogen receptor (ER)) than MXC. While $0.5 \mu M$ HPTE stimulated AMH production, this was not significant ($P > 0.05$). However, 1 and $5 \mu M$ HPTE significantly stimulated AMH production ($P < 0.05$; Fig. 6). Higher doses of HPTE (10 and $25 \mu M$) stimulated the AMH production but this was not
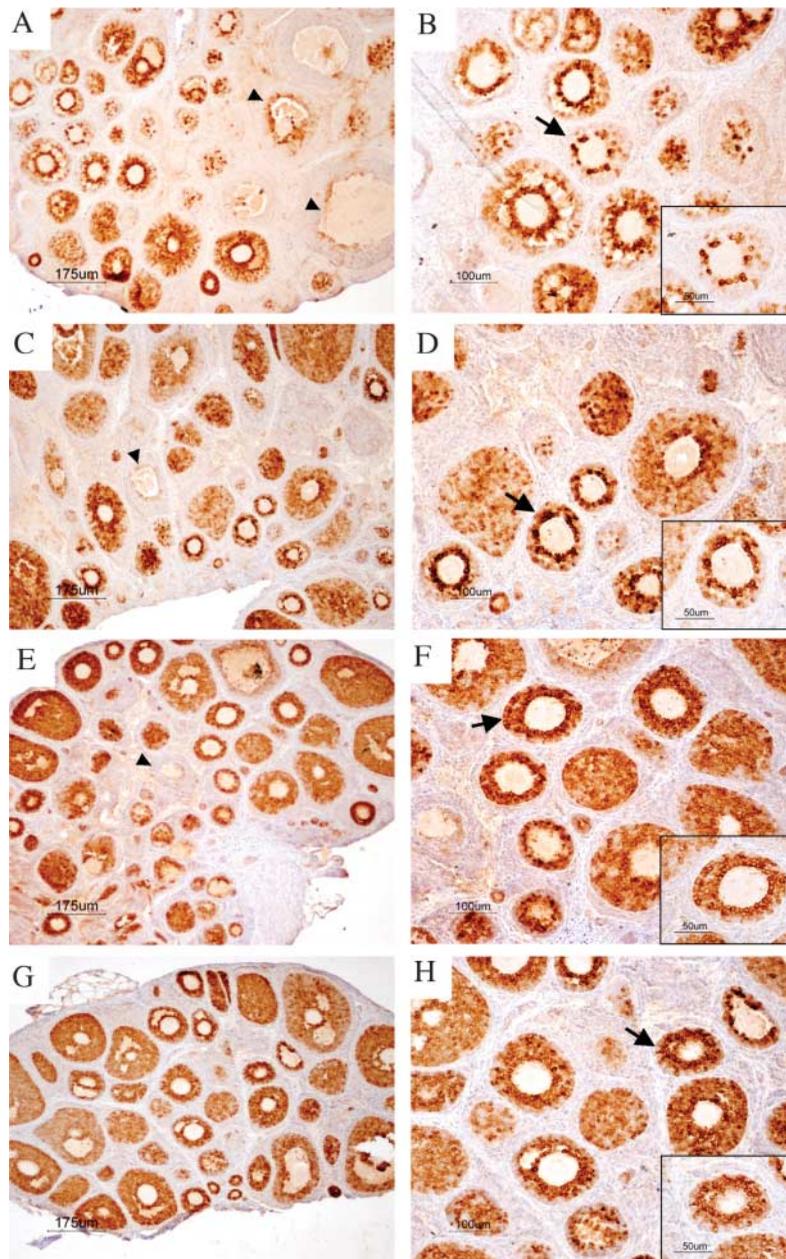
Figure 3 Effect of early postnatal MXC exposure on follicle number and stage in the P20 rat ovary. The rats were injected (s.c.) daily with MXC (1–500 mg/kg per day) between P3 and P10; (A) total follicle numbers were determined; and follicles were assigned into (B) primordial, (C) primary, (D) secondary, (E) pre-antral, or (F) antral stages as described in Materials and Methods. Exposure to 100 and 500 mg/kg per day MXC decreased antral follicles with a concomitant increase in pre-antral follicle numbers. Total follicle number and the number of primordial, primary, and secondary follicles were not different between the treatment groups. The experiments were repeated two to five times with at least two to three animals in each experimental repeat. The data are representative of 5–11 ovaries in each treatment group. *$P<0.05$, †$P<0.01$.

significant ($P>0.05$), and 25 μM HPTE appeared to be toxic to the cells since some of the cells detached (not shown). A similar result was obtained when the experiment was done using granulosa cells harvested from immature rats, primed with 15 IU eCG (s.c.) for 24 h (data not shown).

Discussion

The present study shows that neonatal MXC treatment inhibits follicular development and reduces antral follicle numbers in the ovary. In addition, MXC treatment increases the level of AMH protein production in the ovary. This study
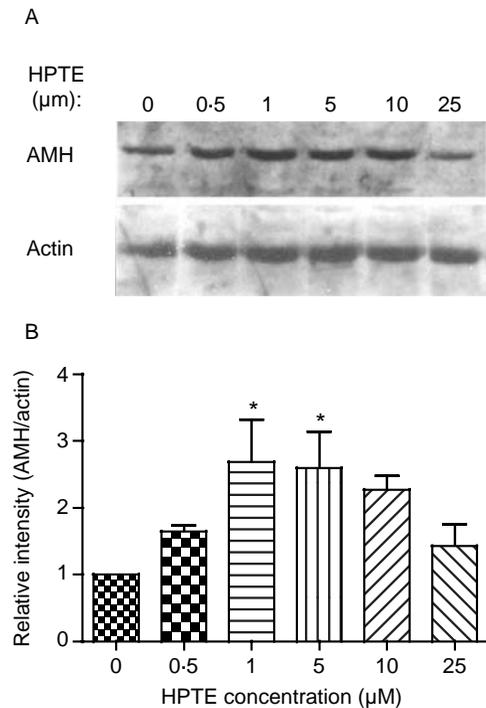
Figure 4 Effect of early postnatal methoxychlor (MXC) exposure on AMH immunolocalization in the P20 rat ovary. Neonatal female rats were injected (s.c.) daily with 0–500 mg/kg per day MXC between P3 and P10, and ovaries were collected on P20. AMH immunohistochemistry was performed in the ovaries as described in Materials and Methods. (A and B), Controls; (C and D), 50 mg/kg per day MXC; (E and F), 100 mg/kg per day MXC; (G and H), 500 mg/kg per day MXC. AMH protein immunoreactivity (brown color) was observed in granulosa cells of growing pre-antral and early antral follicles. In some antral follicles, staining was limited to cumulus granulosa cells or the staining was absent (arrowheads). There were more AMH-positive follicles in MXC-treated ovaries (C, E, and G) as compared with control ovaries (A). AMH immunostaining intensity in the follicles (i.e. granulosa cells) was stronger in MXC-treated ovaries (D, F, and H) as compared with control ovaries (B). A representative follicle (arrows) is shown at a larger magnification (B, D, F, and H; and insets). Negative control sections that were incubated with normal goat IgG or AMH antibody and excess blocking peptide instead of anti-AMH antibody showed no specific staining (not shown). The figures represent approximately ten ovaries for each treatment group.
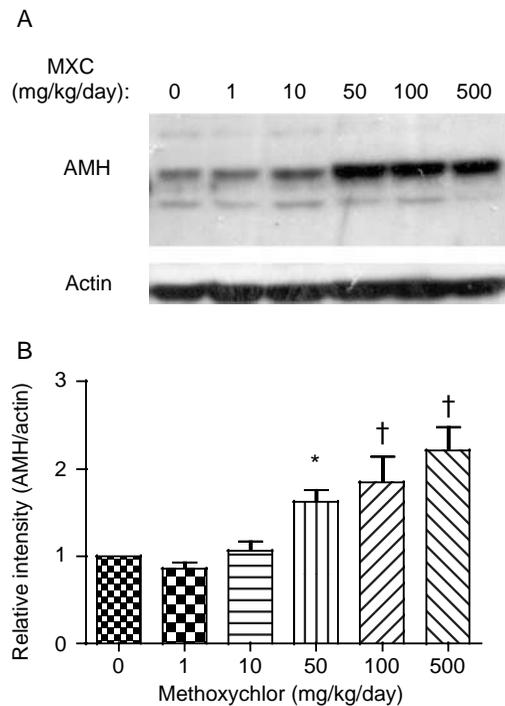
Figure 5 Effect of early postnatal methoxychlor (MXC) exposure on AMH production in the P20 rat ovary. Neonatal female rats were injected daily between P3 and P10, and ovaries were collected on P20. Western blot analysis was conducted in the ovaries as described in Materials and Methods. (A) Representative western blots of AMH protein. Actin was used as a loading control. (B) Mean and s.e.m. of percent relative intensity of AMH bands in western blot analysis of three independent experiments from ovaries of MXC-treated rats in comparison with the controls. The control band intensities were adjusted to 1 ($n=3$). *$P<0\cdot05$, †$P<0\cdot01$.

Figure 6 Effect of HPTE on AMH production in rat granulosa cells *in vitro*. DES-primed immature rat granulosa cells were prepared and treated with HPTE (0–25 μM HPTE), and western blot analysis was conducted using these granulosa cells as described in Materials and Methods. (A) Representative western blots of AMH protein. Actin was used as a loading control. (B) Mean and s.e.m. of percent relative intensity of AMH bands in western blot analysis of three independent experiments with HPTE-treated granulosa cells in comparison with the control cells. The control band intensities were adjusted to 1 ($n=3$). *$P<0\cdot05$.

shows for the first time that the production of AMH by granulosa cells is increased following HPTE treatment. Overall, these data suggest a local inhibitory effect of MXC directly on the ovary.

MXC treatment causes a reduction in the weight and size of the ovary. In addition, while the ovaries of each MXC treatment had approximately the same total number of follicles, MXC-treated ovaries had a dose-dependent reduction in the number of antral follicles with a concomitant increase in pre-antral stage follicles. This is similar to previous observations that neonatal estrogen treatment causes a reduction in the number of medium and large antral follicles (Ikeda *et al*. 2001, 2002). A recent *in vitro* study has shown that estrogen treatment reduces the number of growing pre-antral follicles, suggesting that estrogen inhibits the transition from primordial to primary follicle stage (Kezele & Skinner 2003). In the present study, MXC-treated ovaries had fewer antral follicles without an apparent reduction in total follicle number. This suggests that the effect of MXC on follicular development can be at any stage, such as the primordial-to-primary follicle transition, growth of the primary follicle into a more advanced pre-antral follicle, or recruitment of

pre-antral follicles into the antral follicle stage. Based on the follicle numbers, it seems that the inhibition of MXC may be on the latter: the recruitment of pre-antral follicles into the antral follicle stage. Further investigation, such as follicle counts in multiple sections throughout the entire ovary, is needed to confirm this finding.

Due to its estrogenic actions, MXC may provide a negative feedback on the hypothalamus and pituitary and reduce gonadotropin secretion, which can inhibit ovarian folliculo-genesis in treated rats. The pituitary hormones are present in pre-pubertal rats. The level of FSH is low at birth and reaches its peak value between P12 and P15, after which it returns to the initial low level and stays low until P45 (Ojeda & Ramirez 1972). Estradiol normally cannot induce a negative feedback on the hypothalamus and pituitary in pre-pubertal rats because of serum α-fetoprotein, which binds to serum estradiol and does not allow it to move into tissues, such as the hypothalamus and pituitary (Greenstein 1992). Whether MXC can transfer into these tissues is not known. However, if it does, MXC can potentially act on these tissues because ERα and ERβ are expressed in the hypothalamus and pituitary of neonatal female rats (Nishihara *et al*. 2000, Ikeda *et al*. 2003). It has been

speculated that neonatal MXC exposure leading to cycle irregularities and ovarian pathology in mice is probably due to altered hypothalamic–pituitary function (Eroschenko *et al.* 1995). However, whether MXC affects the hypothalamus and pituitary is controversial. Injection of MXC (8–64 mg/kg per day) in adult mice for 20 days did not change the serum FSH levels or levels of the FSH receptor in the ovary (Borgeest *et al.* 2004). In addition, feeding MXC (24–1200 ppm) to pregnant rats between embryonic day 15 (E15) and P10 does not affect the size of the sexually dimorphic nucleus of the pre-optic area of the hypothalamus in the female offspring (Masutomi *et al.* 2003). On the other hand, perinatal (E15–P10) exposure to MXC has been reported to inhibit serum LH levels in adult female rats (Suzuki *et al.* 2004). Similarly, treatment with 50 or 100 mg/kg per day MXC for 1 week before and 6 weeks after birth suppressed FSH levels during estrus (Chapin *et al.* 1997). In addition, preliminary results from our laboratory suggest that MXC treatment between P3 and P10, suppressed serum FSH levels on P8–P16 (Clayton & M Uzumcu, unpublished observations). Studies are underway to confirm the preliminary results and to further investigate the exact role of suppressed gonadotropins in the observed inhibition of folliculogenesis in the present study.

In addition to its potential action through the hypothalamic-pituitary-ovarian (HPO) axis, MXC and/or its metabolites probably act directly on the ovary. It is possible that MXC stimulates ovarian AMH production directly, which in turn inhibits folliculogenesis. In the present study, the increased intensity of the AMH immunostaining in individual granulosa cells or follicles in MXC-treated ovaries suggests that MXC acts on the ovary directly. This suggestion was clearly supported by our results which show that HPTE, one of the major metabolites of MXC, significantly stimulated AMH production in DES-primed rat granulosa cells *in vitro*. This novel finding is somewhat expected, since MXC has been shown to directly increase antral follicle atresia through several mechanisms, including the Bcl-2 and Bax-mediated pathway (Miller *et al.* 2005), oxidative stress-mediated pathway (Gupta *et al.* 2006), and estrogen signaling-mediated pathway (Miller *et al.* 2006, Tomic *et al.* 2006) in a follicle culture system. In addition, estrogen and estrogenic compounds, such as MXC and its metabolites, are known to regulate ovarian AMH gene expression. The AMH promoter has a 13 base pair sequence that differs only by one nucleotide from the estrogen responsive element (ERE) of the vitellogenin gene found 1722 base pairs upstream of the human gene. Co-transfection of ER and ERE inverted repeat as 35 base pair oligonucleotide linked to a reported gene confers estrogen responsiveness on a heterologous reporter (Guerrier *et al.* 1990). Elevated AMH can inhibit folliculogenesis, since AMH inhibits the primordial-to–primary follicle transition (Durlinger *et al.* 2002) and FSH-induced follicular development (di Clemente *et al.* 1994) in the ovary. Data presented from the present study as well as studies by others suggest that the inhibition of folliculogenesis that was observed in this study can be due to direct action on the ovary – probably through increased ovarian AMH production.

In conclusion, this study shows that neonatal exposure to the endocrine disruptor MXC inhibits ovarian follicular development and reduces antral follicle numbers in rats. In addition, MXC, possibly through its major metabolite HPTE, stimulates AMH production in the ovary. Thus, the inhibitory effect of MXC on the ovary may, at least in part, be directly on the ovary and mediated by the local paracrine factor AMH. This study highlights the potential adverse effects of endocrine disruptors on ovarian pathology and female fertility, which can be mediated by local paracrine factors such as AMH.

Acknowledgements

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