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# Effect of Transient Embryonic In Vivo Exposure to the Endocrine Disruptor Methoxychlor on Embryonic and Postnatal Testis Development

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**ABSTRACT:** The current study was designed to examine the effects of a transient embryonic exposure to the pesticide methoxychlor, an endocrine disruptor, on *in vivo* rat testis development and function. Gestating female rats were transiently administered methoxychlor (MXC) from embryonic day 7 (E7; EO = plug date) through E15. Embryonic testes were collected at E16 and postnatal (PO = day of birth) testes at P4, P10, P17–20, and P60. Seminiferous cords formed in testes from MXC exposed males. However, at E16, there was a decrease in the area of cords and an increase in interstitial area in MXC exposed testes when compared with controls. At all postnatal ages collected, there did not appear to be differences in seminiferous cord/tubule area, interstitial area, or number of seminiferous cords/tubules between untreated controls and males exposed to MXC. Exposure to the endocrine disruptor also had no effect on the postnatal organ weights of a variety of different organs, nor were testosterone levels altered. Interestingly, there were reductions in the number of germ cells in testes from MXC-exposed males at P17–P20 when compared with untreated controls. Furthermore, there was a twofold increase in apoptotic cells in tubules from pubertal P17–P20–MXC exposed males when compared with untreated controls. Testes were collected from adult P60 males to determine if early embryonic and postnatal alterations in germ cell numbers or testis cellular composition had compromised spermatogenesis. In adult P60 MXC exposed testes there were no gross morphological changes in testis structure or cellular composition over that of controls. However, there was an increase in apoptotic cell number in elongating spermatids in MXC exposed testes. Four P60 males that were exposed to MXC during gestation and 4 control males were bred with unexposed females to determine their ability to produce offspring. All MXC exposed males were capable of impregnating females and had normal litter size and pup weights. Combined observations demonstrated that exposure to MXC during gestation at a critical stage of testis development (ie, sex determination) affects embryonic testis cellular composition, germ cell numbers, and germ cell survival. While alterations in these parameters does not affect the ability of males to produce offspring, there appears to be a reduced spermatogenic capacity associated with MXC treatment. Therefore, transient embryonic exposure to an endocrine disruptor (methoxychlor) during gestation can influence the germline and fertility in adult males.

Key words: Sertoli, mesenchymal-epithelial, growth, gametogenesis.
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Previous studies have demonstrated that endocrine disruptors can influence normal sex determination (Facemire et al, 1995; Crisp et al, 1998; Crain and Guillette, 1998), sperm viability (Linder et al, 1992), steroid production (Cumming and Laskey, 1993), and estrous cycles (Laws et al, 2000). Reports also suggest that there is a decline in sperm numbers (Carlsen et al, 1992; Swan et al, 1997), but the decline appears to be regional (Sharpe, 2001), suggesting a possible role of environmental factors in the decline. Potential environmental factors are endocrine disruptors, and these include common pesticides (eg, methoxychlor), fungicides (eg, vinclozolin), and plastics (eg, phthalates) that are used in our environment (Crisp et al, 1998; Cummings, 1997). These compounds can affect physiological functions by acting as weak estrogenic or antiandrogenic compounds (Kapoor et al, 1970; Bulger and Kupfer, 1985; Dehal and Kupfer, 1994; Kelce et al, 1997). Exposure to endocrine disruptors at critical stages of gonadal development (eg, early postnatal) can promote reproductive failure in adult rodents (Gray et al, 1989; Eroschenko et al, 1995; Eroschenko et al, 1997; Saunders et al, 1997; Swartz and Eroschenko, 1998; Atanassova et al, 1999).

Methoxychlor is a chlorinated hydrocarbon pesticide currently used in the United States as a replacement for DDT (Kapoor et al, 1970). Methoxychlor can be metabolized by the liver into two demethylated compounds (ie, mono-OH-M and bis-OH-M). The most active estrogenic metabolite is 2, 2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane, (HPTE) (Dehal and Kupfer, 1994; Cummings,

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1997; Kupfer et al, 1990). Other methoxychlor metabolites appear to have antiandrogenic activity (Kelce et al, 1997). HPTE is weakly estrogenic (Lamoureux and Feil, 1980; West et al, 1982; Bulger and Kupfer, 1985), and it stimulates the expression of estrogen receptor (ER) (Eroschenko et al, 1996). Recently it has been found that the estrogenic metabolite of methoxychlor HPTE has differential effects on ER-α and ER-β, being an ER-α agonist and ER-β antagonist (Gaido et al, 1999; Gaido et al, 2000). Other methoxychlor metabolites also have differential effects on ER-α and ER-β (Gaido et al, 2000). Therefore in examining the actions of methoxychlor or HPTE, the ER agonist and antagonist activities need to be considered as well as antiandrogenic activities. Consideration of these differential activities is critical in elucidating the mechanisms of action of endocrine disruptors such as methoxychlor. Previously, methoxychlor metabolites have been shown to act differentially on the ER of different species (Mathews et al, 2000). The effects of methoxychlor at an embryonic or early postnatal period can influence reproductive functions at later adult periods (Cooke and Eroschenko, 1990; Chapin et al, 1997). Neonatal exposure to methoxychlor can influence pregnancy (Swartz and Eroschenko, 1998), ovarian and hypothalamic function (Eroschenko et al, 1995), reproductive behavior (Palanza et al, 1999), and prostate development (Stoker et al, 1999). Therefore, transient embryonic exposure to an endocrine disruptor can reprogram or imprint effects that manifest in the adult on reproductive physiology. A study has shown that its effects on a gestating mother may influence subsequent pregnancies as well (Swartz and Corkern, 1992). The current study is designed to investigate the reproductive toxicology of MXC.

The potential environmental exposure concentration to MXC can be based on its recommended use as a pesticide (ACSCEQ, 1983; Cummings, 1997). For the control of mosquitoes, MXC has been used at a final surface water concentration of 1μM (ACSCEQ, 1983). In vivo exposures to 50 to 250 mg/kg/day have been reported (Gray et al, 1989; Facemire et al, 1995; Eroschenko et al, 1997; Swartz and Eroschenko, 1998; Atanassova et al, 1999). Estimating the body volume, the concentrations used in the current study, 50 to 150 mg/kg/day are similar to potential environmental exposures. Previous in vivo studies have used either intraperitoneal (IP) (Facemire et al, 1995; Eroschenko et al, 1997; Swartz and Eroschenko, 1998; Atanassova et al, 1999) or oral gavage (Gray et al, 1989). The IP route of administration controls the exposure dose more efficiently; it was therefore, used in the current study.

The current study was conducted to determine if transient administration of methoxychlor prior to morphological testis development (seminiferous cord formation, embryonic day 13.5 of rat gestation; E0 = plug date) could have detrimental effects on testis morphology that result in impaired adult testis function. Previous studies have primarily examined effects of late embryonic and early postnatal exposure to MXC (Carlsen et al, 1992; Linder et al, 1992; Cummings and Laskey, 1993; Facemire et al, 1995; Swan et al, 1997; Crain and Guillette, 1998; Crisp et al, 1998; Laws et al, 2000). The hypothesis tested is that transient early embryonic exposure to the endocrine disruptor methoxychlor will alter testis morphogenesis and proliferative events during embryonic testis development and will subsequently influence adult male fertility.

## Materials and Methods

### In Vivo Procedures

Timed-pregnant Sprague-Dawley rats were obtained from Charles River (Wilmington Mass). Gestating mothers were exposed to methoxychlor (MXC) from E7 to E15 (E0 = plug date) during gestation. The normal gestation period of a rat is 21 to 23 days and was not affected by the MXC treatment used. The dose of methoxychlor (98% pure; Sigma, St Louis, Mo) was 50 or 150 mg/kg/day injected with sesame oil intraperitoneally. This dose represented the low end of in vivo doses previously found to affect reproductive tissues (Eroschenko et al, 1997; Swartz and Eroschenko, 1998; Atanassova et al, 1999). Gonads were collected from embryos in gestating mothers at E16 and from P4, P10, P17–P20, and P60 postnatal males. Testis and other organ weights, when appropriate, were determined on a microanalytical balance. All experiments were repeated a minimum of 3 times with 3 different mothers exposed and pups from 3 different litters for each data set. Approximately 5 to 7 males were collected for each time period for each treatment group (MXC treated or controls). P60 males from different litters from control and MXC exposed groups (per group) were used for breeding experiments to determine fertility. All protocols and procedures were approved by the University Animal Care and Use Committee at Washington State University, Pullman. The testosterone radioimmunoassays (testosterone double antibody RIA kit, Diagnostic Systems, Webster, Texas) were performed by the Center for Reproductive Biology Assay Core Laboratory.

### Embedding, Histology, and Immunohistochemistry

Tissues were fixed in Histochoice (Amresco, Solon, Ohio) and embedded in paraffin according to procedures previously described (Cupp et al, 1999). The tissue sections (3–5 μm) were deparaffinized, rehydrated, microwaved, and blocked in 10% goat serum for 30 minutes at room temperature. Immunohistochemistry was performed as described previously (Martinez and Swartz, 1991; Itoh et al, 1998). The germ cell nuclear antigen (GCNA1) antibody was a monoclonal antipeptide antibody generously provided by George Enders, University of Kansas, Kansas City. The GCNA1 antibody was diluted 1:50 in 10% goat serum. The antibody stains all germ cells in the mouse and mitotic germ cells in the rat. As a negative control, serial sections were put through the same procedure without any primary an-

tibody. The biotinylated goat antirabbit (Vector Laboratories, Burlington, Calif) was diluted 1:300. The secondary antibody was detected by using the Histo stain-SP kit (Zymed Laboratories, South San Francisco, Calif), and immunohistochemical images were digitized with a slide scanner. All sections utilized for negative controls had no positive staining. This demonstrated that the positive staining detected with the GCNA1 antibodies was not due to nonspecific staining or artifacts of tissue fixation and processing. Three different experiments were conducted for GCNA1 antibodies for each developmental time. In each experiment, 3 serial sections of 4 or 5 testes for each developmental age were analyzed. One serial section was used for the nonimmune control for each time period. There was uniform and repeatable staining at each developmental age for GCNA1 in all 3 experiments.

### *Testis Cellular Composition Analysis*

Sections from each treatment group at each developmental age were analyzed at a 200× magnification in the NIH image program for: area of seminiferous cords, area of interstitium, number of germ cells (through GCNA staining), and number of seminiferous cords. The total area represented for each testis section was approximately 308000 pixels. To determine the area of seminiferous cords or tubules per section, the cords/tubules were outlined and the area (in pixels) was calculated with the NIH image program. The area of interstitium was calculated by subtracting the area of the seminiferous cords/tubules from the total area within the section (308000 pixels). The number of seminiferous cords/tubules was determined by counting the number of cords or tubules within the designated testis section. This included any partial cords or tubules that were present. The total number of germ cells per section was determined by counting the GCNA positively stained cells within the section. For each parameter, 3 different experiments were done in replicate with approximately 8 to 12 sections analyzed per treatment group for each development age. Two independent measurements were taken for each section analyzed. These measurements were averaged for each parameter and statistically analyzed for differences between treatments. The data for each averaged area are depicted as number of pixels per designated testis area (ie, total pixels).

### *Apoptosis Analysis*

TUNEL analysis was conducted utilizing the Promega Apoptosis Detection System, fluorescein kit (Madison, Wisc). Briefly, histochoice-fixed and paraffin-embedded histological sections (ACSCEQ, 1983) were deparafinized and washed in phosphate-buffered saline (PBS) and then fixed in 4% methanol-free formaldehyde solution. The sections were then treated with a 20 ug/ml solution of Proteinase K for 8 minutes. Subsequently, they were washed in PBS, fixed in 4% methanol-free formaldehyde, and washed again with PBS. The sections were then placed in an equilibrating solution with a fluorescein-tagged TdT enzyme and nucleotide (fluorescein-12-dUTP) mix and incubated at 37°C for 1 hour in a humidified chamber. The reaction was terminated and washed with PBS to remove unincorporated fluorescein-12-dUTP. The slides were then coverslipped using a drop of Anti-Fade mounting solution (Molecular Probes, Eugene, Ore) and analyzed on fluorescent and confocal microscopes in the Center for Reproductive Biology, Histology Core Laboratory at Washington State University. Fluorescently labeled cells were counted per area of testis for each treatment group at each developmental age. Eight to 10 microscopic fields from each section were digitally imaged and the fluorescently labeled cells counted. Five to 7 sections were evaluated for each developmental age from 3 different experiments and averaged to obtain the data presented. Positive controls used DNAse I instead of Proteinase K to determine if enzyme was labeling properly, since DNAse I causes DNA strand breaks. Negative controls without enzyme were also conducted to determine the background of the fluorescent procedure.

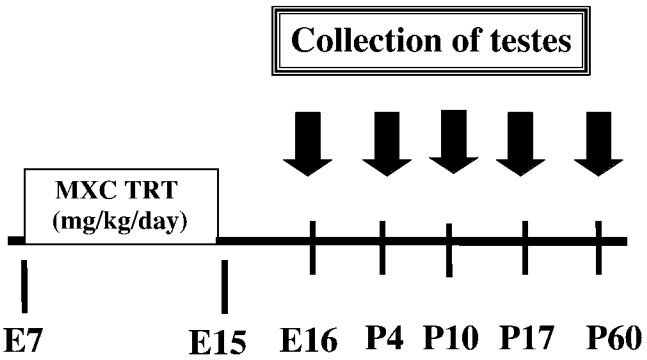


Figure 1. Experimental design of current study demonstrating transient treatment (TRT) of methoxychlor (MXC) and collection of testes at E16, P4, P10, P17, and P60.

### *Statistical Analysis*

Data were analyzed with the JMP 3.1 statistical analysis program (SAS Institute, Cary, NC). All values were derived from 3 different experiments done in replicate and are expressed as the mean ± SEM. Statistical analysis was performed using one-way ANOVA. Significant differences were determined using Dunnett's test for comparing with controls and the Tukey-Kramer significant difference tests for multiple comparisons. Statistical difference was confirmed at $P < .05$.

## *Results*

Gonads were collected at E16, P4, P10, P17–P20, and P60 from rats exposed to 50 mg/kg/day of methoxychlor from E7 to E15 of gestation (Figure 1). Previously, the range of methoxychlor in vivo doses used have been 50 to 250 mg/kg/day with intraperitoneal injection being the most common mode of administration (Facemire et al, 1995; Eroschenko et al, 1997; Swartz and Eroschenko, 1998; Atanassova et al, 1999). In the current study, the lowest previously shown effective in vivo dose was selected to provide the most relevant potential environmental exposure (ACSCEQ, 1983). Testes collected for analysis were fixed for histology and testis cellular composition examined. All experiments involved 3 different ex-

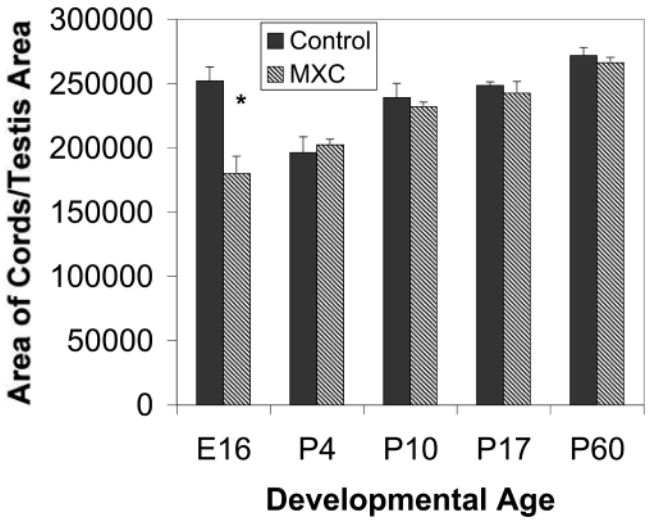


Figure 2. Effect of gestational exposure to methoxychlor (MXC) on area of seminiferous cords/tubules (pixels) per testis section area at each developmental age collected. Total testis area that was examined for each section was 308,000 pixels. Six testes were collected for each treatment at each developmental age and 6 to 12 sections were analyzed for area of seminiferous cords/tubules at each developmental age ($n > 9$). The mean ± SEM of 3 different experiments on replicate pups is presented with (*) indicating a statistical difference ($P < .05$) with control.

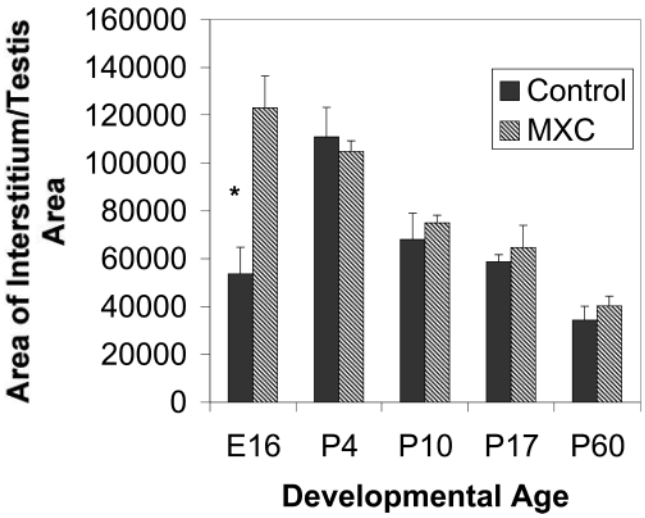


Figure 3. Effect of gestational exposure to methoxychlor (MXC) on area of interstitium (pixels) per testis section area at each developmental age collected. Total testis area that was examined for each section was 308,000 pixels. Six testes were collected for each treatment at each developmental age and 6 to 12 sections were analyzed for area of interstitium at each developmental age ($n > 9$). The mean ± SEM of 3 different experiments on replicate pups is presented with (*) indicating a statistical difference ($P < .05$) with control.

periments (ie, exposed gestating mothers) and multiple pups from different litters for analysis. The embryonic testes from E16 MXC exposed males had a decrease in the area of cords (Figure 2) and an increase in interstitial area (Figure 3) when compared with the control male testes. The number of cells within the tubules was found to decrease 20% in MXC treated testes compared with control testis (data not shown). There were no differences in the number of seminiferous cords presented in E16 control and MXC treated testes.

At P4, P10, P17, and P60 (Figures 2, 3, and 4) there were no differences in area of seminiferous cords/tubules, interstitial area, or in number of seminiferous cords between MXC and control testes. In addition, MXC did not appear to influence Sertoli cell or Leydig cell numbers (data not shown). Therefore, any changes in testis cellular composition that occurred at E16 did not influence the cellular composition of testis postnatally. However, there was a trend for a reduction (but not statistically significant) in the numbers of seminiferous tubules in P17 MXC exposed male testes when compared with controls (Figure 4).

Transient exposure to the low 50 mg/kg/day or higher 150 mg/kg/day methoxychlor had no effect on pubertal P20 (Figure 5) or adult P60 (Figure 6) testis weights. In addition, methoxychlor exposure did not influence the weights of a large number of different organs (Figures 5 and 6). Therefore, the transient embryonic exposure did not have any gross effects on general organ development.

Methoxychlor exposed males had reduced germ cell numbers at P17 of development as determined by positively stained cells for the germ cell nuclear antigen (GCNA) (Figure 7). No other developmental age demonstrated significant differences in number of germ cells per testis area. However, there were trends for reductions (but not statistically significant) in the number of germ cells present in MXC exposed males at P10 and P60 of

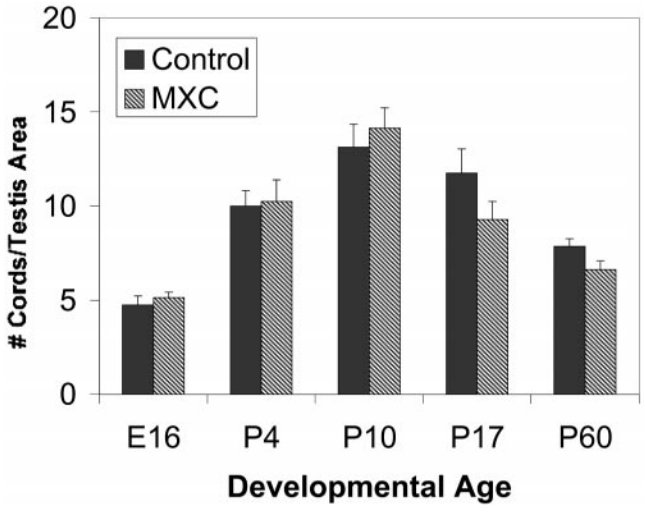


Figure 4. Effect of gestational exposure to methoxychlor (MXC) on number of seminiferous cords/tubules per testis section area at each developmental age collected. Six testes were collected for each treatment at each developmental age and 6 to 12 sections were analyzed for number of seminiferous cords/tubules at each developmental age ($n > 9$). The mean ± SEM of 3 different experiments on replicate pups is presented.

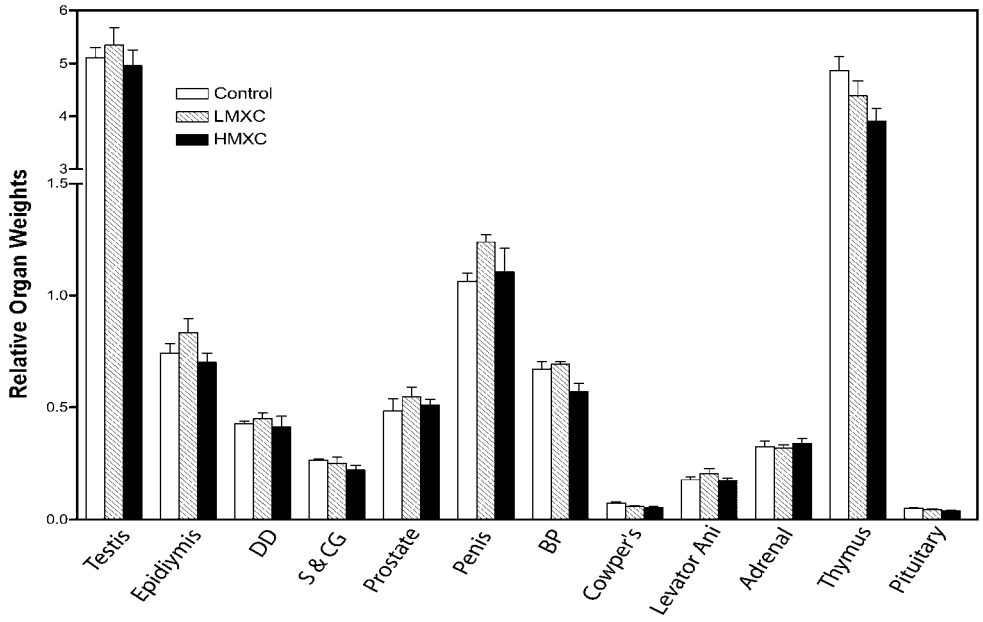


Figure 5. Relative organ weights (organ weight [mg]/ body weight [g]) from control (oil treated) (CTL), low 50 mg/kg/day methoxychlor-treated (LMXC) or high 150 mg/kg/day methoxychlor-treated (HMXC) animals. The mean ± SEM from a minimum of 3 animals at postnatal 20 days of age (P20) is presented. No statistical difference was observed between treatments. DD, ductus deferens; S & CG, seminal vesicle; coagulating gland; and BP, bulbus penis.

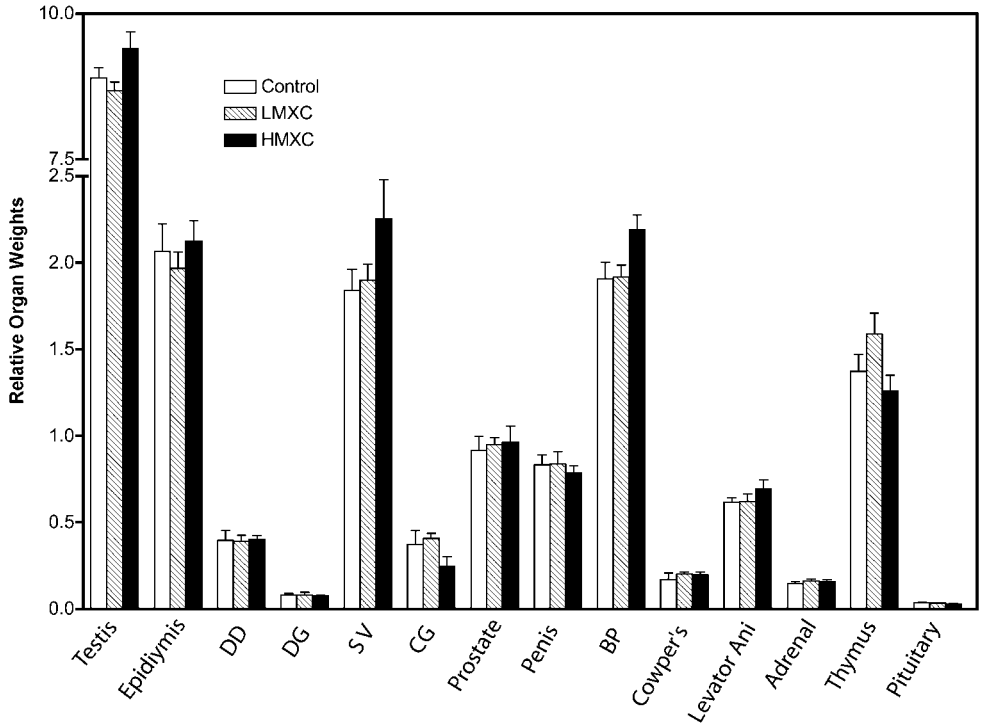


Figure 6. Relative organ weights (organ weight [mg]/ body weight [g]) from control (oil treated) (CTL), low 50 mg/kg/day methoxychlor-treated (LMXC) or high 150 mg/kg/day methoxychlor-treated (HMXC) animals. The mean ± SE from a minimum of 3 animals at postnatal 60 days of age (P60) is presented. No statistical difference was observed between treatments. DD, ductus deferens; DG, deferens gland; SV, seminal vesicle; CG, coagulating gland; and BP, bulbus penis.

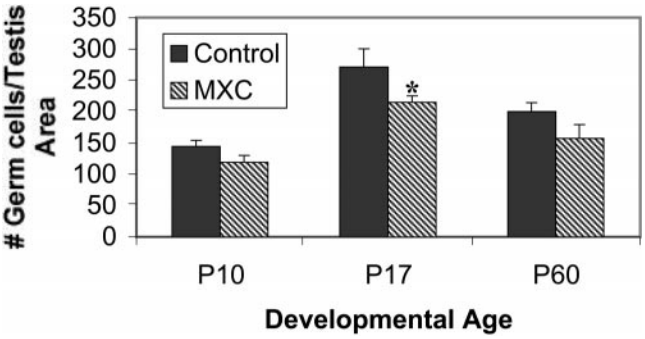


Figure 7. Effect of gestational exposure to methoxychlor (MXC) on number of mitotic germ cells per testis section area at P10, 17, and P60 of testis development. Germ cells were counted by positive staining for GCNA. Six testes were collected for each treatment at each developmental age and 6 to 12 sections were analyzed for number of GCNA positive germ cells at each developmental age ($n > 9$). The mean ± SEM of 3 different experiments on replicate pups is presented with (*) indicating a statistical difference ($P < .05$) with control.

development when compared with their counterpart controls.

In addition to differences in germ cell numbers at P17, there was also an increase in germ cell apoptosis in P17 MXC exposed testes when compared with controls (Figures 8 and 9). At P17–20, an increase in germ cell apoptotis at low MXC exposure is shown in Figure 8B and D. The high MXC exposure has a similar effect on germ cell apoptosis (Figure 9). Testes collected at earlier developmental timepoints did not have differences in apoptotic cells between MXC exposed and control testes (data not shown). Although testis composition was not different in P17, there were alterations in germ cell number and germ cell viability in MXC treated testes when compared with controls (Figures 7, 8, and 9). In MXC exposed P60 testes there appeared to be an increase in germ cell apoptosis in elongating spermatids associated with stage X–XI of the spermatogenic cycle (Figure 8E and F). Apoptotic cell numbers in tubules other than stage X–XI of the treated testis were higher than control but the difference was statistically not significant ($P > .05$). However, in the MXC-treated group, the number of X–XI stage tubules with 5 or more apoptotic cells was dramatically higher. While approximately ⅓ of the stage X–XI tubules in control group had 5 or more apoptotic germ cells, approximately ⅔ of the X–XI stage tubules in the treated group had 5 or more apoptotic germ cells. The number of tubules with apoptotic germ cells was doubled by the MXC treatment. Low-level exposure (50 mg/kg/day) MXC treatment did not have consistent effects on apoptotic germ cell number at P60 (data not shown).

Testosterone levels in serum collected from P20 and P60 MXC treated animals and control animals were tested to determine potential endocrine effects of the MXC exposure. Serum levels of testosterone (ng/ml) in MXC treated animals compared with control demonstrated no significant change in testosterone levels (Figure 10). Therefore, the transient embryonic exposure to MXC does not appear to influence the endocrine status of the adult male.

Postnatal day 60 MXC exposed males (n = 4) and control males (n = 4) were allowed to mate with control 60-day-old females. All females became pregnant and produced normal litter sizes and pup weights with no difference between control and MXC treated animals (data not shown). Therefore, the MXC exposed males were determined to be fertile even though some sperm cells may have been compromised. Preliminary analysis of caudal epididymal sperm showed no change in sperm number, a small (15%) decrease in the percentage of motile sperm and forward movement, and a small increase in sperm with abnormal morphology (data not shown). However, the majority of sperm had normal morphology and motility; this remains to be more thoroughly investigated.

## Discussion

Neonatal and in utero exposure to methoxychlor has been demonstrated to cause alterations in uterine morphology (Swartz and Eroschenko, 1998), reduction in age at vaginal opening (Palanza et al, 1999), and reduction in the efficiency of superovulation in the female (Eroschenko et al, 1997). Previous experiments have demonstrated the ability of methoxychlor and its metabolite HPTE to affect or inhibit seminiferous cord formation in vitro with rat testis organ cultures (Cupp and Skinner, 2001). No previous experiments have addressed the effects of transient in utero exposure to methoxychlor at this critical timepoint in testis development (ie, cord formation) on subsequent testis cellular composition and male fertility. The current study was designed to determine the effects of a transient in vivo embryonic exposure to methoxychlor, an estrogenic endocrine disruptor, on testis cellular composition, growth, and germ cell development.

The effects of exposure to methoxychlor in utero resulted in an imbalance of seminiferous cords to interstitium at embryonic day 16 (E16) of testis development. Exposure to methoxychlor was at a time when both seminiferous cord formation and somatic and germ cell proliferation were occurring. Different testicular cell types are required to migrate during this period of testis morphogenesis (Magre and Jost, 1991; Karl and Capel, 1998; Levine et al, 2000). Some interstitial cells (eg, preperitubular and endothelial) are derived from the migration of mesonephric cells into the testis to promote formation of the seminiferous cords, while some Sertoli cells migrate from the coelomic epithelium (Karl and Capel, 1998). Previous in vitro studies (Cupp and Skinner, 2001)

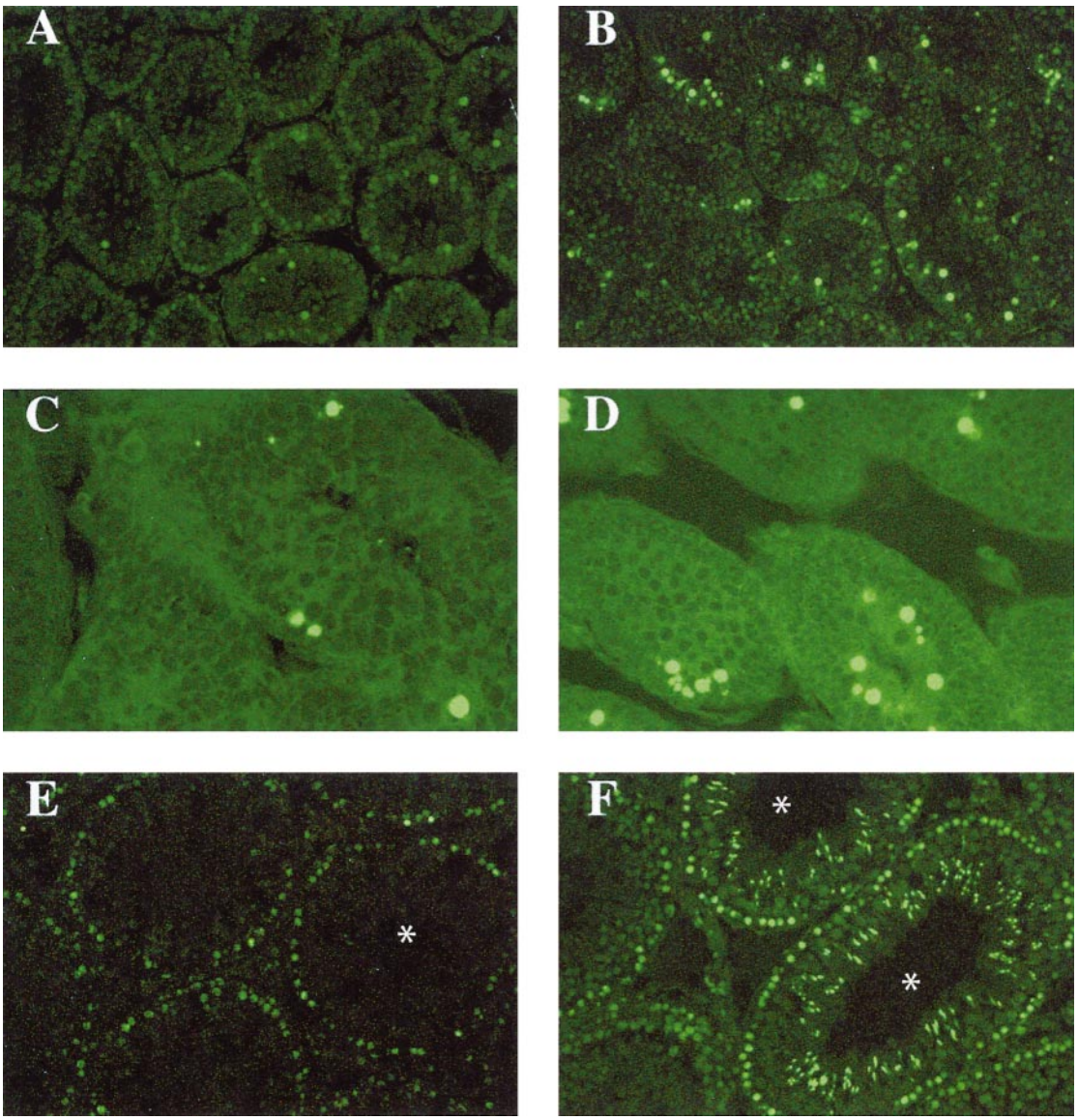


Figure 8. Histological sections demonstrating effect of gestational exposure to methoxychlor on DNA fragmentation through the use of a TUNEL assay on P17–20 (**B, D**) or P60 (**F**) testes. Panels are control sections for P17–20 (**A, C**) and P60 testes (**E**). The apoptotic cells were in stage X–XI tubules which were marked with (*) (E, F). Data are representative of 5 to 7 different testes from 3 separate experiments analyzed to determine the number of apoptotic cells per treatment at each age. Magnification 200× (A, B, E, and F) and 400× (C, D).

have shown that both MXC and its metabolite HPTE interfere with or inhibit cord formation. More recent studies (Uzumcu et al, 2002; Cupp et al, 2003) have shown that one of the reasons for abnormal cord formation is the inhibition of mesonephros cell migration.

Sertoli, interstitial, and peritubular cells are rapidly proliferating from E14 to P0 of testis development (Akingbemi et al, 2000; Levine et al, 2000). Germ cells rapidly proliferate until approximately E18, when mitosis arrests until it resumes after birth (Orth, 1982). Since the area of seminiferous cords was reduced, observations suggest that either the germ cell numbers, Sertoli cell numbers, or size may have been affected by methoxychlor exposure. Since there was no apparent change in germ cell numbers at E16, reduction in Sertoli cell number or size appears to at least contribute to the reduced seminiferous cord area. Alternatively, the number or size of the Leydig cells may be affected by the methoxychlor exposure, which subsequently can alter the normal ratio between cord and interstitium. This is possible since some of the MXC metabolites (eg, HPTE) are antiandrogenic (Maness et al, 1998) which may have led to hypertrophy or hyperplasia of the Leydig cell. A similar mechanism was suggested for phthalates. When prepubertal rats were exposed to shorter (14 days) or longer (28 days) exposure to phthalates, the shorter exposure reduced testosterone

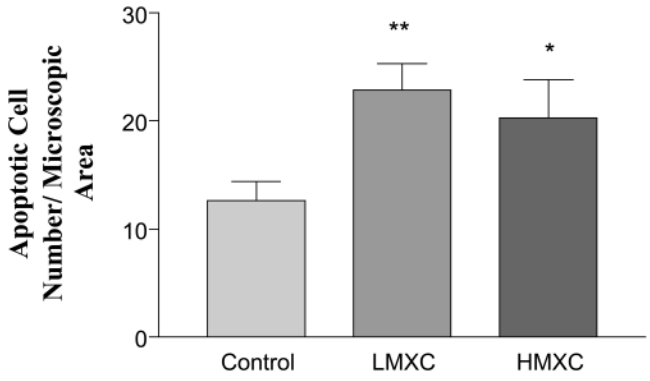


Figure 9. Effect of gestational exposure to 50 mg/kg/day (LMXC) or 150 mg/kg/day (HMXC) methoxychlor on apoptotic cells in P17–20 testes. The apoptotic germ cell number/micrograph area is presented. Five to 7 testes were collected and 10 sections from each testis were analyzed for number of apoptotic cells per treatment. The mean ± SEM of 3 different experiments is presented. (**) or (*) indicates statistical significant at $P < .01$ or $P = .06$ levels, respectively.

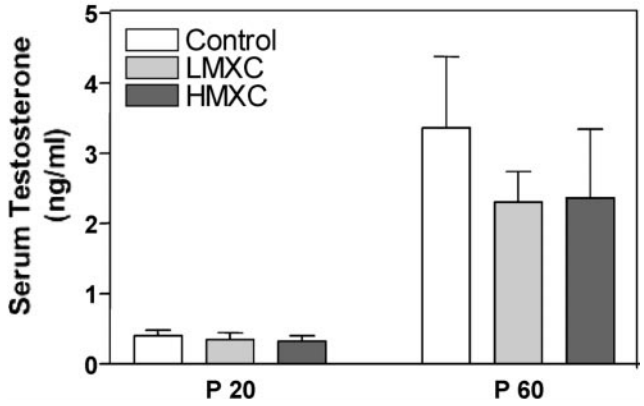


Figure 10. Serum testosterone levels (ng/ml) from control (oil treated) (control), low 50 mg/kg/day methoxychlor (LMXC) or high 150 mg/kg/day methoxychlor (HMXC)-treated animals. The mean ± SEM from a minimum of 6 different animals is presented. No statistical difference in treatments was found for postnatal P20 and P60 rats.

production but longer exposure increased testosterone and LH levels, potentially leading to Leydig cell hypertrophy (Akingbemi et al, 2001). Thus, the exposure to methoxychlor may have resulted in inefficient migration, differentiation, or proliferation of Sertoli cells or hypertrophy of Leydig cells that would result in a relative reduction of seminiferous cord area. This reduction was transient since no further alterations in seminiferous cord/tubule area or interstitial cell area was observed in subsequent testis collections. Furthermore, the size of the adult testes in the methoxychlor exposed males appears to be similar to controls. Therefore, any alteration in testis cellular composition observed at E16 did not result in differential size or abnormal structures within the testes collected at the postnatal ages. Similar transient effects (eg, reduction in serum testosterone) in rats that were in utero exposed to phthalates were observed (Akingbemi et al, 2001).

A major effect of methoxychlor exposure was the reduction in number of mitotic germ cells detected at P17 through positive germ cell nuclear antigen (GCNA) staining. This reduction in germ cell number was also supported by an increase in germ cell DNA fragmentation in P17–20 in males exposed to methoxychlor during gestation. Therefore, the transient exposure to methoxychlor appears to result in abnormal germ cell survival or development during puberty. The abnormal germ cell development or accelerated germ cell apoptotis may be caused by inappropriate cell differentiation after fetal exposure to methoxychlor. This may result in an insensitivity of cells, such as the Sertoli cell, to respond adequately to FSH or Leydig cells to respond to LH. Several studies have demonstrated that exposure to high doses of estrogen or estrogenic endocrine disruptors reduces serum testosterone levels (Akingbemi et al, 2000; McKinnell et al, 2001) and alters or inhibits fetal Leydig cell differentiation (Abney, 1999); it can also abolish androgen receptor populations (Abney, 1999) within the testis. In a similar manner, methoxychlor has been demonstrated in the female to cause an insensitivity to gonadotropins that would normally cause superovulation (Eroschenko et al, 1997). The metabolite of methoxychlor-HPTE has been demonstrated to reduce Leydig cell function in cell culture (Akingbemi et al, 2000). Therefore, methoxychlor may act in an estrogenic manner to inhibit testosterone function and induce abnormal Leydig cell differentiation or insensitivity to gonadotropin stimulation.

There also appeared to be an effect of methoxychlor exposure on adult animal germ cells that are at stage X–XI of the spermatogenic cycle. In P60, high-dose, methoxychlor exposed testes, the majority of the stage X–XI seminiferous tubules had apoptotic, elongated spermatids. This stage of the seminiferous tubule is especially sensitive to testosterone withdrawal and to antiandrogens such as flutamide that inhibit the development of spermatids. A 5-alpha reductase inhibitor also reduced spermatid development, but not to the extent of flutamide (O'Donnell et al, 1999). Analysis of serum testosterone demonstrated that the transient embryonic methoxychlor exposure did not influence the endocrine status of the pubertal or adult males. The lack of methoxychlor effect on any of the reproductive organ weights examined correlated with the normal testosterone levels. Embryonic testis development does not require endocrine control; however, the effects of methoxychlor treatment on embryonic and early postnatal serum testosterone remains to be elucidated. It is speculated that a transient embryonic exposure to methoxychlor may alter the abilities of Leydig and Sertoli cells to respond to gonadotropins, which promotes a reduction of viable germ cells within the testis. The reduction in germ cells does not appear to inhibit reproductive function because the P60 littermate males

were capable of producing offspring with normal litter size and pup weights.

In summary, the current study demonstrates that a transient in vivo embryonic exposure to the endocrine disruptor methoxychlor influences testis development and function. Males exposed to methoxychlor during E7 to E15 of gestation appear to have inappropriate differentiation of cells during the initial stages of embryonic testis differentiation and growth. The altered testis differentiation may subsequently lead to accelerated germ cell apoptosis or altered germ cell development in the adult male. While the reduction in germ cell numbers and increased germ cell apoptosis may not result in infertility, there is the possibility that offspring of matings between individuals subjected to methoxychlor exposure may have reproductive problems. Future studies will investigate the potential transgenerational effects of estrogenic endocrine disruptors (eg, methoxychlor) on male reproduction. Although the current study selected a relatively low-dose exposure of methoxychlor to provide relevance to environmental exposure levels (Atanassova et al, 1999; ACSCEQ, 1983), the possibility that a higher dose exposure would cause more profound effects on testis development or male fertility also will be investigated in future studies.

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## References

Abney TO. The potential roles of estrogens in regulating Leydig cell development and function: a review. *Steroids.* 1999; 64:610–617.

ACSCEQ (Association Committee on Scientific Criteria for Environmental Quality). Impact Assessments in Lotic Environments—Methoxychlor. Publication *NRCC* 1983; 20645, National Research Council of Canada, Ottawa.

Akingbemi BT, Youker RT, Sottas CM, Ge R, Katz E, Klinefelter GR, Zirkin BR, Hardy MP. Modulation at rat Leydig cell steroidogenic function by di(2-ethylhexyl)phthalate. *Biol Reprod.* 2001; 65:1252–1259.

Akingemi BT, Ge RS, Klinefelter GR, Gunsalus GL, Hardy MP. A metabolite of methoxychlor, 2,2-bis(p-hydroxyphenyl)-1,1,1-trichlorethane, reduces testeosterone biosynthesis in rat Leydig cells through suppression of steady-state messenger ribonucleic acid levels of the cholesterol side-chain cleavage enzyme. *Biol Reprod.* 2000; 65:571–578.

Atanassova N, McKinnel C, Walker M, et al. Permanent effects of neonatal estrogen exposure in rats on reproductive hormone levels. Sertoli cell number and the efficiency of spermatogenesis in adulthood. *Endocrinology.* 1999; 140:5364–5373.

Bulger WH, Kupfer D. Estrogenic activity of pesticides and other xenobiotics on the uterus and male reproductive tract. In: Thomas JA, Korach KS, McLachlan JA, eds. *Endocrine Toxicology.* New York: Raven Press; 1985:1–33.

Carlsen, E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during past 50 years. *Br Med J.* 1992; 305: 609–613.

Chapin RE, Harris HW, Davis BJ et al. The effects of perinatal/juvenile methoxychlor exposure on adult rat nervous, immune, and reproductive system function. *Fundam Appl Toxicol.* 1997; 40:138–157.

Cooke PS, Eroschenko VP. Inhibitory effects of technical grade methoxychlor on development of neonatal male mice reproductive organs. *Biol Reprod.* 1990; 42:585–596.

Crain DA, Guillette LJ Jr. Reptiles as models of contaminant-induced endocrine disruption. *Anim Reprod Sci.* 1998; 58:77–86.

Crisp TM, Clegg ED, Cooper RL, et al. Environmental endocrine disruption: an effects assessment and analysis. *Environ Health Perspect.* 1998; 106(suppl 1):11–56.

Cummings AM, Laskey J. Effect of methoxychlor on ovarian steroidogenesis: role in early pregnancy loss. *Reprod Toxicol.* 1993; 7:17–23.

Cummings AM. Methoxychlor as a model for environmental estrogens. *Crit Rev Toxicol.* 1997;27:367–379.

Cupp AS, Uzumcu M, Skinner MK. Chemotactic role of nerotropin 3 in the embryonic testis that facilitates male sex determination. *Biol Reprod.* 2003; Jan 8;68:2033–2037.

Cupp AS, Skinner MK. Actions of the endocrine disruptor methoxychlor and its estrogenic metabolite on embryonic seminiferous cord formation and perinatal testis growth *Reprod Toxicol.* 2001; 15:317–326.

Cupp AS, Kim G, Skinner MK. Expression and action of transforming growth factor beta (TGFb1, TGFb2, and TGFb3) during embryonic testis development. *Biol Reprod.* 1999; 60:1304–1313.

Dehal SS, Kupfer D. Metabolism of the proestrogenic pesticide methoxychlor by hepatic P450 monooxygenases in rats and humans. Dual pathways involving novel ortho ring-hydroxylation by CYP2B. *Drug Metabolism and Disposition.* 1994; 22:937–946.

Eroschenko VP, Abuel-Atta AA, Grober MS. Neonatal exposures to technical methoxychlor alters ovaries in adult mice. *Reprod Toxicol.* 1995; 9:379–387.

Eroschenko VP, Rourke AW, Sims WF. Estradiol or methoxychlor stimulates estrogen receptor (ER) expression in uteri. *Reprod Toxicol.* 1996; 9:265–271.

Eroschenko VP, Swartz WJ, Ford LC. Decreased superovulation in adult mice following neonatal exposures to technical methoxychlor. *Reprod Toxicol.* 1997; 11:807–814.

Facemire CF, Gross TS, Guillette LJ Jr. Reproductive impairment in the Florida panther: nature or nurture? *Environ Health Perspect* 1995; 103(suppl 4):79–86.

Gaido KW, Leonard LS, Maness SC, Hall JM, McDonnell DP, Saville B, and Safe S. Differential interaction of the methoxychlor metabolite. 2,2-bis-(p-hydorxyphenyl)-1,1,1-trichlorethane with estrogen receptors alpha and beta. *Endocrinology.* 1999; 140:5746–5753.

Gaido KW, Maness SC, McDonnell DP, Dehal SS, Kupfer D, Safe S. Interaction of methoxychlor and related compounds with estrogen receptor α and β, and androgen receptor: structure-activity studies. *Mol Pharmacol.* 2000; 58:852–858.

Gray LE Jr, Ostby J, Ferrell J, et al. A dose-response analysis of methoxychlor-induced alterations of reproductive development and function in the rat. *Fundam Apl. Toxicol.* 1989; 12:92–108.

Itoh N, Patel U, Cupp AS, Skinner MK. Developmental and hormonal regulation of transforming growth factor-β1 (TGFβ1), -2 and -3 gene expression in isolated prostatic epithelial and stromal cells: epidermal growth factor and TGFβ interactions. *Endocrinology.* 1998; 139: 1378–1388.

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.

Karl J, Capel B. 1998. Sertoli cells of the mouse testis originate from the coelomic epithelium. *Dev Biol.* 1998; 203:323–333.

Kelce WR, Lambright CR, Gray LE, Roberts KP. Vinclozolin and *p,p′*-DDE alter androgen-dependent gene expression: in vivo confirmation of an androgen receptor-mediated mechanism. *Toxicol Appl Pharmacol.* 1997; 142:192–200.

Kupfer D, Bulger WH, Theoharides AD. Metabolism of methoxychlor by hepatic P-450 monooxygenases in rat and human. 1. Characterization of a novel catechol metabolite. *Chem Res Toxicol.* 1990; 3:8–16.

Lamoureux CH, Feil VJ. Gas chromatographic and mass spectrometric characterization of impurities in technical methoxychlor. *J Assoc Offic Anal Chem.* 1980; 63:1007–1037.

Laws SC, Carey SA, Ferrell JM, Bodman GJ, Cooper RL. Estrogenic activity of octylphenol, nonylphenol, biosphenol A and methoxychlor in rats. *Toxicol Sci.* 2000; 54:154–167.

Levine E, Cupp AS, Miyashiro L, Skinner MK. Role of transforming growth factor-alpha and the epidermal growth factor receptor in embryonic rat testis development. *Biol Reprod.* 2000; 62:477–490.

Linder RE, Strader LF, Slott VL, Suarez JD. Endpoints of spermatotoxicity in the rat after short duration exposures to fourteen reproductive toxicants. *Reprod Toxicol.* 1992; 6:491–505.

Magre S, Jost, A. Sertoli cells and testicular differentiation in the rat fetus. *J Electron Microsc Tech.* 1991; 19:172–188.

Maness SC, McDonnell DP, Gaido KW. Inhibition of androgen receptor-dependent transcriptional activity by DDT isomers and methoxychlor in HepG2 human hepatoma cells. *Toxicol Appl Pharmacol.* 1998; 151: 135–142.

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.

Mathews J, Celius R, Halgren R, Zacharewski T. Differential estrogen receptor binding of etrogenic substances: a species comparison. *J Steroid Biochem Mol Biol.* 2000; 74:223–234.

McKinnell C, Atanassova N, Williams K, Fisher JS, Walker M, Turner KJ, Saunders TK, Sharpe RM. Suppresion of androgen action and the induction of gross abnormalities of the reproductive tract in male rats treated neonatally with diethylstilbestrol. *J Androl.* 2001; 22:323–338.

O'Donnell L, Pratis K, Stanton PG, Robertson DM, McLachlan RI. Testosterone-dependent restoration of spermatogenesis in adult rats is impaired by a 5alpha-reductase inhibitor. *J Androl.* 1999; 20:109–117.

Orth JM. Proliferation of Sertoli cells in fetal and postnatal rats: a quantitative autoradiographic study. *Anat Rec.* 1982; 203:485–92.

Palanza P, Morellini F, Parmigiani S, vom Saal FS. Prenatal exposure to endocrine disrupting chemicals: effects on behavior development. *Neurosci Biobehav Rev.* 1999; 23:1011–1027.

Saunders PT, Majdic G, Parte P, Millar MR, Fisher JS, Turner KH, Sharpe RM. Fetal and perinatal influence of xenoestrogens in testis gene expression. *Adv Exp Med Biol.* 1997; 424:99–110.

Sharpe RM. Hormones and testis development and the possible adverse effects of environmental chemicals. *Toxicol Lett.* 2001; 120:221–232.

Stoker TE, Robinette CL, Cooper RL. Perinatal exposure to estrogenic compounds and the subsequent effects on the prostate of the adult rat: evaluation of inflammation in the ventral and lateral lobes. *Reprod Toxicol.* 1999; 13:463–472.

Swan SH, Elkin EP, Fenster L. Have sperm densities declined? A reanalysis of global trend data. *Environ Health Perspect.* 1997; 105: 1228–1232.

Swartz WJ, Eroschenko VP. Neonatal exposure to technical methoxychlor alters pregnancy outcome in female mice. *Reprod Toxicol.* 1998; 12: 565–573.

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.

Uzumcu M, Westfall SD, Dirks KA, Skinner MK. Embryonic testis cord formation and mesonephric cell migration requires the phosphotidylinositol 3-kinase signaling pathway. *Biol Reprod.* 2002; 67:1927–1935.

West PR, Chaudhary SK, Branton GR, Mitchel R. High performance liquid chromatographic analysis of impurities and degradation products of methoxychlor. *J Assoc Offic Anal Chem.* 1982; 65:1457–1470.