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Actions of the endocrine disruptor methoxychlor and its estrogenic metabolite on in vitro embryonic rat seminiferous cord formation and perinatal testis growth

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Abstract

The current study examines the actions of methoxychlor and its estrogenic metabolite, 2, 2-bis-(p-hydroxyphenyl)-1, 1, 1-trichloroethane (HPTE), on seminiferous cord formation and growth of the developing rat testis. The developing testis in the embryonic and early postnatal period is likely more sensitive to hormonally active agents than at later stages of development. Embryonic day 13 (E13) testis organ cultures were treated with either 0.2, 2, or 20 μM methoxychlor or 1, 3, 6, 15, 30, or 60 μM HPTE to examine effects on cord formation. No concentration of methoxychlor completely inhibited cord formation. However, cord formation was abnormal with the presence of a reduced number of cords and appearance of "swollen" cords at the 2 and 20 μM concentrations of methoxychlor. The swollen cords were due to an increase in the number of cells in a cord cross section and reduction of interstitial cell numbers between cords. Treatment of embryonic day 13 (E13) testes with HPTE caused abnormal cord formation at the 3 μM and 6 μM concentrations, and completely inhibited cord formation at the 15, 30, and 60 μM concentrations. In addition to the estrogenic metabolite HTPE, methoxychlor can also be metabolized into anti-androgenic compounds. Therefore, to determine the spectrum of potential actions of methoxychlor on testis development, different concentrations of estradiol, testosterone, and an anti-androgen (flutamide) were utilized to determine their effects on E13 testis organ culture morphology. Estradiol (1 μM) and flutamide (0.1μM) both inhibited seminiferous cord formation in E13 testis organ cultures. Therefore, methoxychlor may be acting through the androgen and/or estrogen receptors to elicit its actions on seminiferous cord formation. Reverse transcription polymerase chain reaction (PCR) (RT-PCR) confirmed the presence of estrogen receptor alpha (ERα) mRNA from embryonic day 14 (E14) through postnatal day 5 (P5) while estrogen receptor beta (ERβ) mRNA did not appear until approximately E16 of testis development. Androgen receptor (AR) expression was present from E14 through P5 of testis development, but at apparently reduced levels at E14 and E16. Immunohistochemical analysis localized ERα to the cells of the seminiferous cords at E14 though P5 while ERβ was present in cells of the interstitium at E16 and P0. Androgen receptor was localized to germ and interstitial cells. The effects of methoxychlor, HPTE, estradiol, and testosterone on cell growth of perinatal testes was determined with a thymidine incorporation assay in postnatal day zero (P0) testis cell cultures. Methoxychlor (0.002, 0.02, and 0.2 μM) and HPTE (2 and 20 μM) stimulated thymidine incorporation in P0 testis cell cultures in a similar manner to estradiol (0.01, 0.1, and 1 μM). In addition, testosterone (0.1 μM) also stimulated thymidine incorporation in P0 testis cultures. Observations suggest that methoxychlor and its metabolite HPTE can alter normal embryonic testis development and growth. The actions of methoxychlor and HPTE are likely mediated in part through the steroid receptors confirmed to be present in the developing testis.

Keywords: Testis; Sertoli cell; Methoxychlor; HPTE; Mesenchymal-epithelial; Growth; Embryonic development; Gametogenesis

1. Introduction

Normal testis morphology and development is dependent upon critical somatic and germ cell differentiation and proliferation during the embryonic period. In the rat, crucial morphogenesis of the testis occurs at embryonic day 13.5 (E13.5; plug day considered to be E0) [1,2] when preperitubular cells migrate from the adjacent mesonephros to enclose Sertoli and germ cell aggregates forming seminiferous cords [1–4]. After seminiferous cord formation, so-

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matic and germ cells undergo the highest levels of cellular proliferation that occurs during testis development [5]. Embryonic somatic and germ cell proliferation ensures adequate numbers of germ and somatic cells are present for adult testicular spermatogenesis [6]. Therefore, many critical developmental events occur from E13 to postnatal day zero (P0) during gestation in the rat that allow for normal testis development.

Exposure to agents that interfere or alter morphogenic or proliferative events during embryonic testis development will impair the ability of the testis to develop, proliferate, or produce normal numbers of viable sperm [7–9]. Accumulating evidence has suggested that increasing amounts of synthetic endocrine active chemicals in the environment are compromising normal sex determination and the ability of testes to produce normal numbers of sperm [7–9]. The degree this is an issue with the human population remains to be elucidated; however, a number of wildlife species clearly are affected. Environmental agents can act as weak estrogens or anti-androgens and may contribute to the increase in abnormal sex determination [10], reduced sperm counts [11], and overall reductions in the ability of individuals to produce viable offspring in many species [10].

Methoxychlor is a chlorinated hydrocarbon pesticide currently used in the United States as a replacement for DDT [10] and has been categorized as weakly estrogenic. Methoxychlor can be metabolized by the liver into two demethylated compounds {(2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane, (HPTE) and 2,2-bis-(p-hydroxyphenyl)-1,1,1-dichloroethane, (HPDE)} and two 0-ring methylated compounds. Each of these metabolites have been demonstrated to act differentially at the estrogen and androgen receptors. The most active estrogenic metabolite is HPTE [12–14]. HPTE has been determined in several experiments to be weakly estrogenic [15–17]. HPTE stimulates the expression of estrogen receptors [18], and has been determined to cause decreased testosterone concentrations and decreased seminal and prostate weights when administered neonatally in rodents [19]. There are conflicting reports on whether methoxychlor elicits its response through the estrogen receptors [18,20]. Some of methoxychlor's actions may be through a metabolite that acts in an anti-androgenic manner [21]. Therefore, exposure to methoxychlor in the environment may impair or alter reproductive function in many domesticated and wildlife species through actions on both estrogen and androgen receptors.

Previous studies have demonstrated the effects of methoxychlor on the female reproductive system that have resulted in reduced embryo implantation efficiency, increased abortion rate, and formation of smaller gonads with a higher incidence of germ cell apoptosis [22]. Exposure to methoxychlor either late embryonically or during the early postnatal period also has resulted in reproductive tract abnormalities [19] and altered reproductive behavior in males [23]. To date no experiments have been conducted to evaluate the effect of exposure of methoxychlor on early embryonic testis morphology (seminiferous cord formation) or growth. Therefore, the current study was conducted to determine if methoxychlor or its metabolite HPTE may alter morphologic sex determination (formation of seminiferous cords) and/or growth of the testis during the perinatal period in rats.

2. Materials and methods

2.1. Organ cultures

Timed pregnant Sprague-Dawley rats were obtained from Charles River (Wilmington MA). Plug day was considered to be E0. Embryonic day 13 (E13) gonads were dissected out with the mesonephros. The organs were cultured in drops of medium on Millicell CM filters (Millipore, Bedford MA) floating on the surface of 0.4 ml CMRL 1066 media (Gibco BRL, Gaithersburg MD) supplemented with penicillin-streptomycin, insulin (10 μg/mL) and transferrin (10 μg/mL). Methoxychlor at 0.2, 2, or 20 μM, HPTE at 1, 3, 6, 15, 30, or 60 μM, estradiol at 0.01. 0.1, or1 μM, testosterone at 0.1 μM, or flutamide at 0.1 μM were added to the medium every day. The medium was changed every day. E13 gonad plus mesonephros were typically kept for 3 days by which point cords were well developed in the controls [24]. Images of whole organs were obtained by an image analysis system [24].

Embryonic day 13 (E13) testis organ cultures were treated with different concentrations of methoxychlor or HPTE to examine effects on seminiferous cord formation. The initial concentration range selected was 0.1 to 10 μM, the approximate concentrations expected in the environment. Organ cultures were either treated daily for 3 days with greater than 99% pure methoxychlor or HPTE. Since the methoxychlor and HPTE were made up in DMSO, the controls were also treated with the same amount of DMSO. A minimum of three experiments with at least 6 testis pairs per experiment were performed (36 testis organ pairs per treatment). Since methoxychlor is normally metabolized in the body by the liver, its estrogenic metabolite HPTE was utilized in the testis organ cultures to examine effects on seminiferous cord formation. Testis organ cultures were treated with 1, 3, 6, 15, 30, or 60 μM HPTE.

2.2. Genomic DNA isolation and PCR for SRY

To determine the sex of E13 embryos, PCR for SRY was conducted on each embryo. Embryonic tails were collected to isolate genomic DNA by standard procedures. Briefly, the tissue was homogenized through a 25 gauge needle in digestion buffer (100 mM NaCl, 10 mM Tris pH 8, 25 mM EDTA, 0.5% SDS), and treated with proteinase K (0.15 mg/mL) for at least 4 h at 60°C. The samples were then extracted twice with an equal volume of phenol:chloroform: isoamyl alcohol (24:24:1), and once with chloroform:

isoamyl alcohol. The DNA was then precipitated by adding 1/10 volume 7.5 M ammonium acetate and 3 volumes cold ethanol and collected by centrifugation at 4°C for 30 min after an hour incubation at -20°C. Pellets were dried and resuspended in 10 μl distilled water. PCR was performed using 1 μl genomic DNA with primers to SRY. The sequences of the SRY primers are: 5′CGGGATCCATGTCAAGCGCCCCATGAATGCATTTATG3′ and 5′GCGGAATTCACTTTAGCCCTCCGATGAGGCTGATAT 3′. PCR was performed using an annealing temperature of 55°C for 30 cycles to yield a product of 240 bp [25].

2.3. Testicular cell culture and growth assay

The influence of methoxychlor and HPTE on testis growth was examined to confirm direct actions on testicular cells and to assess a parameter other than tissue morphogenesis. The amount of tissue required was not available from the E16 developmental stage; therefore, the P0 testis was used for the growth studies. The P0 testis cell cultures were synchronized in regards to the cell cycle (e.g. G1) and then cultured in the absence (control) or presence of testosterone, estradiol, methoxychlor, or HPTE. Affects on thymidine incorporation were assessed and data normalized per μg cell DNA and the data presented as relative growth with control cultures set at 1.0

To generate a testicular culture from P0 testis, the tunica was removed and the testis digested with 0.125% trypsin, 0.1% EDTA, and 0.02 mg/mL DNase in Hanks Balanced Salt Solution (HBSS), for 15 min at 37°C. After digestion, the trypsin was inactivated with 10% calf serum. The samples were triturated with a pipette tip and washed twice in 1 ml HBSS. The pellet was resuspended and either used in growth assays immediately or placed in 10 mm plates in F12 media supplemented with 10% bovine calf serum until confluent (approximately two days). For growth assays cells were plated at a 25% confluence in 24-well plates and allowed to settle overnight in DMEM media without thymidine. Media was replaced the next day and cells were treated for 24 h with different hormones or growth factors. Media were removed after the 24-h treatment period and media containing tritiated thymidine (10 μCi/mL) were placed on cells for 5 to 6 h. After 5 to 6 h, media were discarded and cells were either frozen or processed using the tritiated thymidine assay. Briefly, a solution of 0.5M NaH_2P0_4 (pH 7.3; 500 μl) was added to each well and cells were sonicated. Half of the sonicated cells were placed on DE-81 filters on a manifold and a vacuum was applied. After three washes with the NaH_2P0_4 buffer, the filters were dried, placed in counting vials with 5 mL scintillation fluid, and counted. The remaining sonicate was used for DNA assays to normalize number of cells per well [24,26].

2.4. RNA Isolation and RT-PCR

Total RNA was obtained using Tri Reagent (Sigma, St. Louis MO). Briefly tissue or cells were lysed in Tri Reagent (1 mL per 50 to 100 mg tissue or 1 mL/100 mm culture plate). After adding 0.2 ml chloroform/mL Tri Reagent, the mixture was centrifuged at 12,000 *g* for 15 min at 4°C, the colorless upper aqueous phase was transferred to a fresh tube, and 0.5 ml isopropanol/mL Tri Reagent was added to pellet the RNA. Reverse transcription was performed using MMLV-reverse transcriptase under standard conditions [27]. The ERα primers that were used for RT-PCR were: 5′ TAT GGG GTC TGG TCC TGT GA 3′ and 5′ GGG CGG GGC TAT TCT TCT TA 3′. PCR was performed using an annealing temperature of 58°C for 30 cycles to yield a product of 334 bp [28]. The ERβ was amplified with: 5′ CCA TCT ACA TCC CTT CCT CCT ACG 3′ and 5′ TCC CTC TTT GCG TTT GGA CTA 3′ with a PCR reaction using an annealing temperature of 58°C for 30 cycles to yield a product of 348 bp [29]. The androgen receptor (AR) primers that were used for RT-PCR were: 5′ CTC TCT CAA GAG TTT GGA TGG CT 3′ and 5′ CAC TTG CAC AGA GAT GAT CTC TGC 3′ with a PCR reaction using an annealing temperature of 60°C for 30 cycles to yield a product of 342 bp

2.5. Embedding, histology and immunohistochemistry

Tissues were fixed in Histochoice (Amresco, Solon OH) and embedded in paraffin according to standard procedures [24]. The tissue sections (3 to 5 μm) were deparaffinized, rehydrated, microwaved, and blocked in 10% goat serum for 30 min at room temperature. Immunohistochemistry was performed as described previously [30,31]. The ERα antibody was an anti-ERα peptide antibody (MC-20) (Santa Cruz Biotechnology, Santa Cruz, CA). Its epitope maps at the C-terminus of the mouse ERα and is specific to ERα. With this antibody, a blocking peptide was used (MC-20P) to determine antibody specificity. The ERβ antibody was an anti- ERβ peptide antibody (PA1–310) (Affinity Bioreagents, Golden Colorado). PA1–310 was produced by immunizing rabbits with a synthetic peptide corresponding to the C-terminal amino acid residues 467 through 485 of the rat ERβ. The AR antibody was an anti-AR peptide antibody (Affinity BioReagents, Golden CO). Antiserum was produced by the immunization of rabbits with a 62kDa fusion protein from the N-terminal domain of the human AR expressed by bacteria. The ERα, ERβ, and AR antibodies were diluted 1:100 in 10% goat or rabbit serum. As a negative control, serial sections were put through the same procedure without any primary antibody. Additional negative control sections were incubated with 50× to 100× excess of synthetic immunizing peptide and the anti-ERα, anti-ERβ, or anti-AR. The biotinylated goat anti-rabbit and rabbit anti-goat secondary antibody (Vector Laboratories, Burlington, CA) were diluted 1:300. The secondary anti-

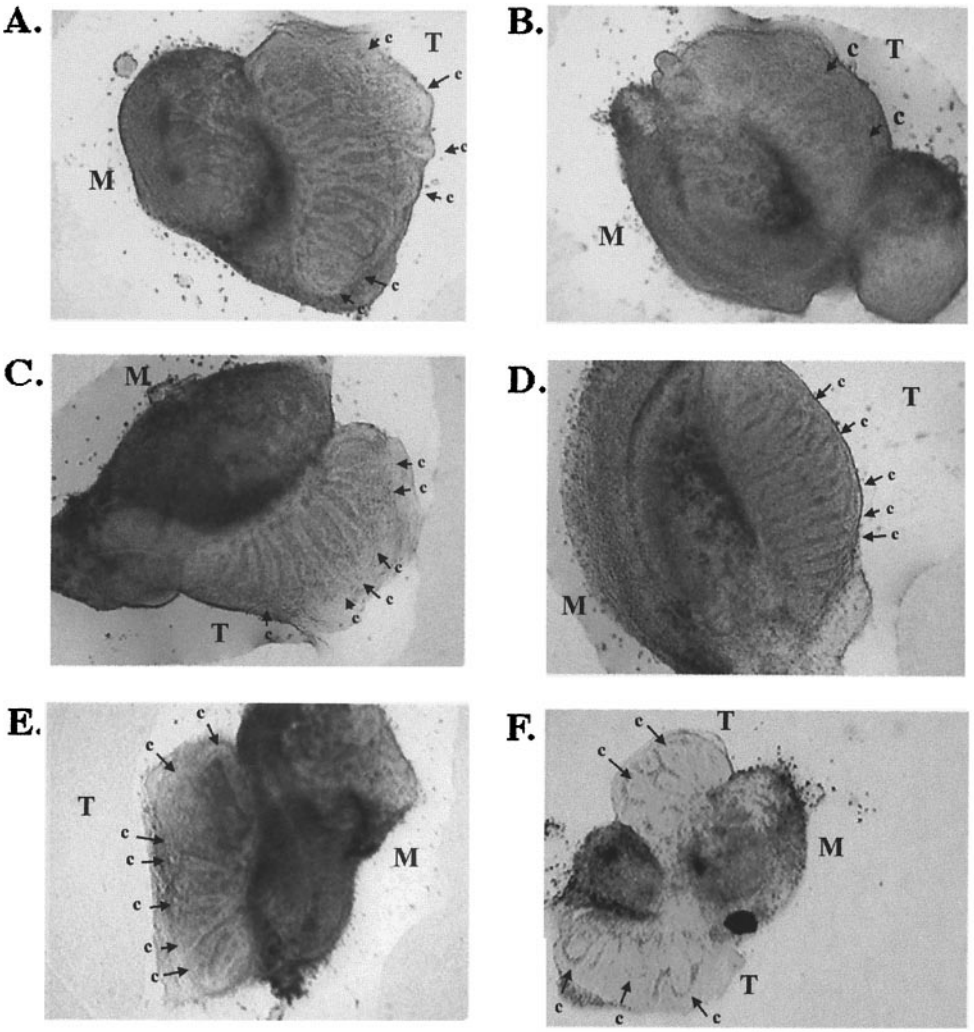

Fig. 1. Effects of methoxychlor on seminiferous cord formation. A) Paired control for 0.2 μM methoxychlor concentration; B) 0.2 μM methoxychlor; C) Paired control for 2 μM methoxychlor concentration; D) 2 μM methoxychlor; E) Paired control for 20 μM methoxychlor concentration; F) 20 μM methoxychlor. Gonads were cultured with intact mesonephros for three days. Photographs are representative of at least three different experiments with 6 or 7 testis organ pairs per experiment. M = mesonephros, T = testis. Cords are marked with arrows in selected micrographs.

body was detected by using the histo stain-SP kit (Zymed Laboratories, South San Francisco, CA) and immunohistochemical images were digitized with a slide scanner. All sections utilized for negative controls (without specific primary antibody or with excess synthetic blocking peptide) had no positive staining. This finding demonstrated that the positive staining detected with the ERα, ERβ, and AR antibodies were not due to nonspecific staining or due to artifacts of tissue fixation and processing. Three different experiments were conducted for ERα, ERβ, and AR antibodies. In each experiment, three serial sections of four or five testes for each developmental age were analyzed (E14, E16, P0; only P0 data are shown). One serial section was utilized for the non-immune control for each time period

2.6. Statistical Analysis

Data were analyzed with the JMP 3.1 statistical analysis program (SAS Institute, Cary, NC). All values are expressed as the mean ± SEM. Statistical analysis was performed using one-way ANOVA. Significant differences were determined using Dunnett's test for comparison to controls and using the Tukey-Kramer honesty significant difference tests for multiple comparisons. Statistical difference was confirmed at $P < 0.05$

3. Results

3.1. Effects of methoxychlor and HPTE on seminiferous cord formation in E13 testis organ cultures

No concentration of methoxychlor completely inhibited seminiferous cord formation (Fig 1). However, the 2 and 20 μM concentrations caused abnormal seminiferous cord formation with swollen and altered cord morphology (Fig 1). The swollen cords were due to an increased number of cells

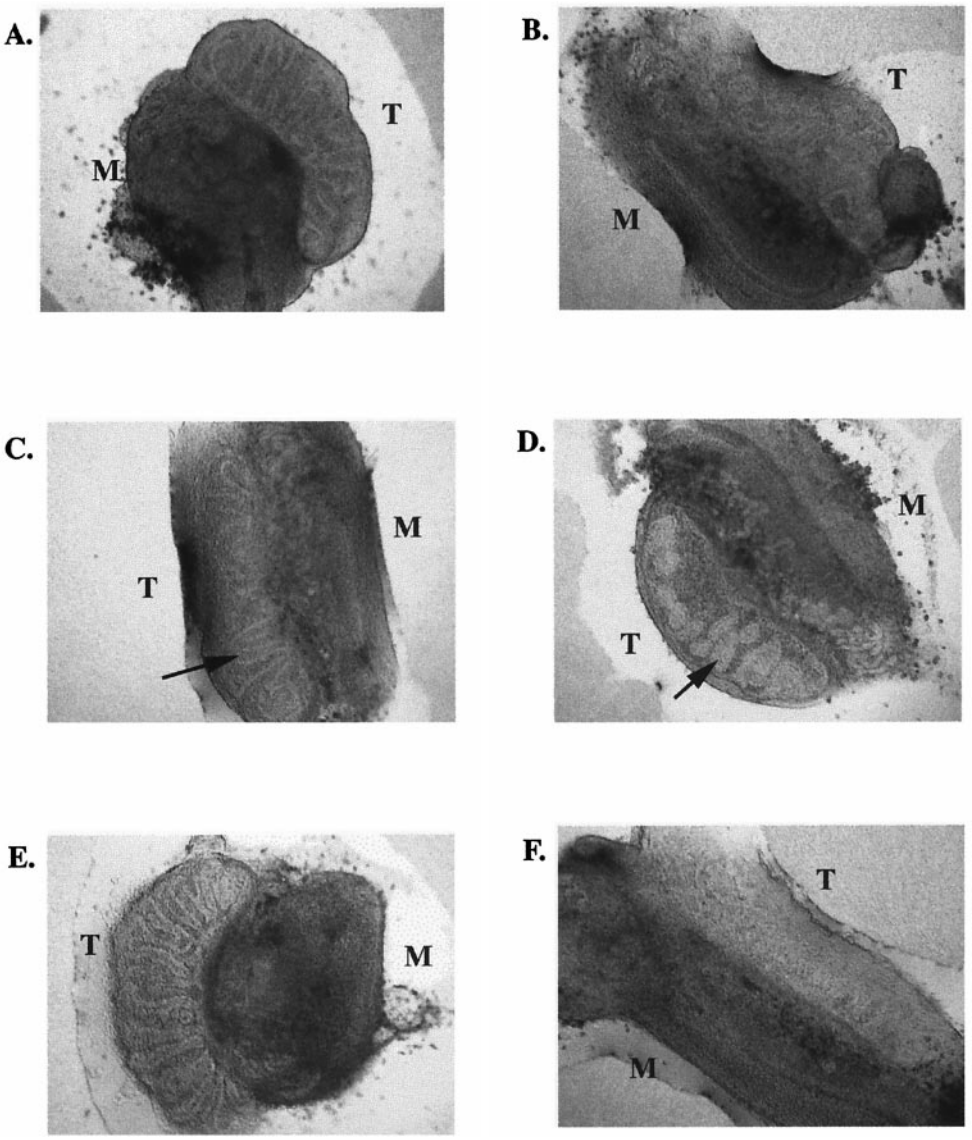

Fig. 2. Effects of HPTE on seminiferous cord formation. A) Paired control for 3 μM HPTE concentration; B) 3 μM HPTE; C) Paired control for 6 μM HPTE concentration; D) 6 μM HPTE; E) Paired control for 15 μM HPTE concentration; F) 15 μM HPTE. Gonads were cultured with intact mesonephros for 3 days. Photographs are representative of at least three different experiments with 6 or 7 testis organ pairs per experiment. M = mesonephros, T = testis. Cords are marked with arrows in selected micrographs.

in cord cross sections and a decreased number of interstitial cells between cords. Seminiferous cord formation was abnormal (swollen cords) with the 3 and 6 μM concentrations of HPTE (Fig 2b, d). These results with 3 and 6 μM HPTE were similar to the effects of 2 and 20 μM methoxychlor on cord formation (Fig 1). In some testis organ culture pairs, the 6 μM concentration of HPTE completely inhibited seminiferous cord formation. However, there was a more consistent inhibition of cord formation in the organ cultures treated with the 15 μM concentration of HPTE (Fig 2). Higher concentrations of HPTE (30 and 60μM; data not shown) were potentially toxic to the organ culture system and the testes did not develop normally. In organ cultures treated with 30 or 60 μM of HPTE, the gonad was indistinguishable from the mesonephros and large numbers of cells sloughed (i.e. became displaced) from the organ culture.

The actions of methoxychlor may be elicited through the estrogen or androgen receptor since methoxychlor can be metabolized into both estrogenic and anti-androgenic metabolites. Therefore, different concentrations of estradiol (0.01, 0.1, and 1.0μM), testosterone (0.1μM) and the anti-androgen flutamide (0.1μM) were used in E13 testis organ cultures to examine effects on seminiferous cord formation. Treatment with estradiol 17-β (1 μM) and flutamide (0.1 μM; Fig 3) both inhibited seminiferous cord formation in E13 testis organ cultures. These results were similar to those seen with concentrations of HPTE that inhibited seminiferous cord formation. However, there was no effect of testosterone (0.1 μM; data not shown) on seminiferous cord

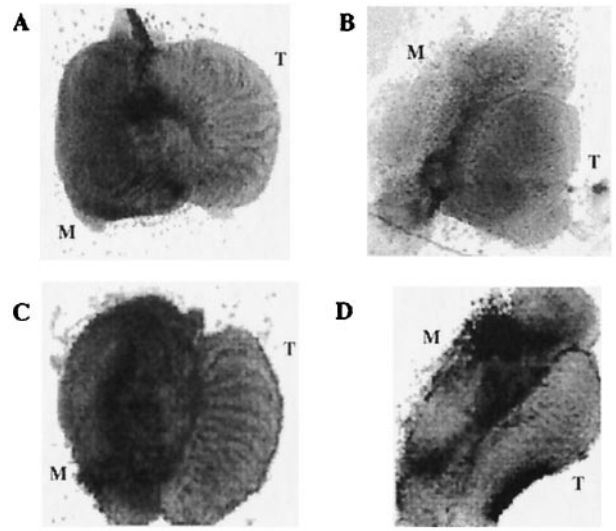

Fig. 3. Effects of estrogen and flutamide on seminiferous cord formation. A) Paired control to B; B) estradiol at 1 μM; C) Paired control to D; and D) flutamide at 0.1 μM. Gonads were cultured with intact mesonephros for 3 days. Photographs are representative of at least three different experiments with 6 or 7 testis organ pairs per experiment. M = mesonephros, T = testis

formation. Therefore, HPTE could be eliciting its effects on cord formation through steroid receptor agonist or antagonist mediated mechanisms (e.g. estrogen receptor agonist or androgen receptor antagonist).

3.2. Expression and localization of steroid receptors in the developing testis

The expression of mRNA for ERα was present in E14 through P5 of testis development (Fig 4A), as demonstrated by RT-PCR. In contrast, expression of mRNA for ERβ was not present until E16 of testis development (Fig 4B). Therefore, only ERα appeared present during the developmental period when the testis organ cultures were initially treated. Since the testis organ cultures were treated for 3 days, by the end of the treatment period, ERβ should have been expressed. ERα protein was localized to the nucleus of cells within the seminiferous cords and in the interstitium of E16 through P0 testis (Fig 5). At P5 and P0, ERα was localized to specific germ cells within the seminiferous cords (Fig 5). In contrast, ERβ was localized to the cytoplasm of cells within the interstitium and cords at E16 (data not shown). By P0 of testis development, ERβ was mainly localized to the Sertoli and interstitial cells (Fig 5). More thorough analysis is not shown since immunohistochemical data have already been reported using these antibodies in testis [32–34]

The message for androgen receptor (AR) was present in the testis from E14, E16, E18, P0, and P5 (Fig 4C). Although not quantitative, AR levels of expression were apparently low in the E14 and E16 testis compared to more advanced stages of testis development. This finding will

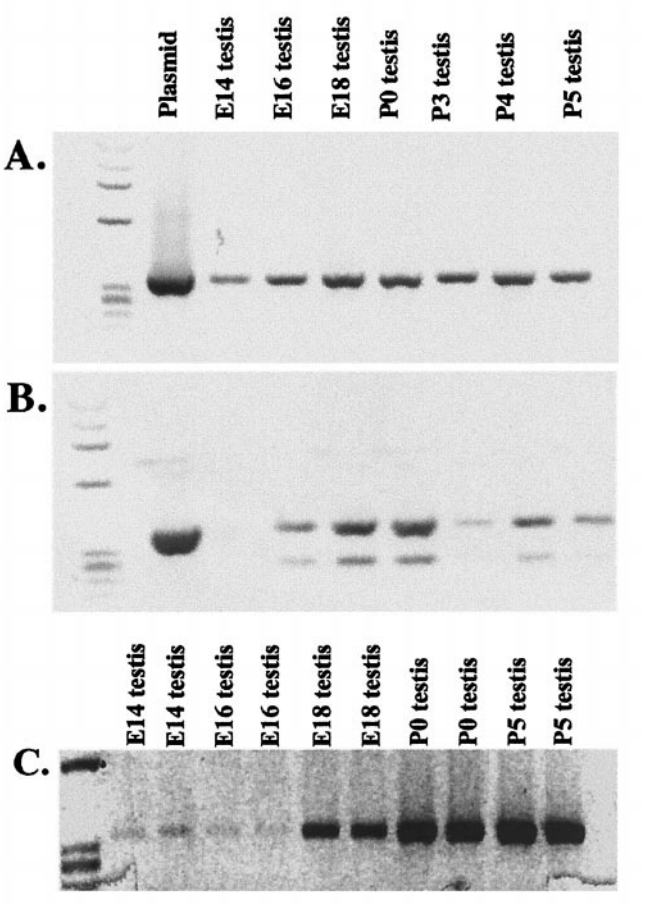

Fig. 4. Reverse transcription PCR RT-PCR for A) ERα, B) ERβ, and C) AR. Samples of embryo day (E)14, E16, E18, Postnatal day (P)0, P3, P4, and P5 testis were obtained for RNA isolation and PCR analysis. The PCR products for ERα 334bp, ERβ 348bp, and AR 342bp were isolated and confirmed with sequence analysis. Positive control plasmid is shown for ERα and ERβ, while duplicate samples are shown for AR from different testis. Marker bands are shown in the far left lane. Photographs are representative of three different experiments.

need to be confirmed with more quantitative procedures. Therefore, AR was present during the organ culture and may have been a potential receptor for the transduction of methoxychlor activity. Expression of AR protein through immunohistochemistry was present at E14, E16, P0, P3, P5 (data not shown), and P0 (Fig 5). AR was present in most cell types in the embryonic testis, followed by localization to germ cell and interstitial cells at P0 (Fig. 5). The staining appeared cytoplasmic. Negative controls with no primary antibody (Fig. 5) or excess receptor peptide antigen demonstrated no staining (data not shown).

3.3. Effects on perinatal P0 testis growth

Testosterone stimulated growth of P0 testis cells approximately 2-fold above control (Fig 6). Estradiol also stimulated P0 testis thymidine incorporation with the optimal concentration being less than 0.1 μM. Methoxychlor at a

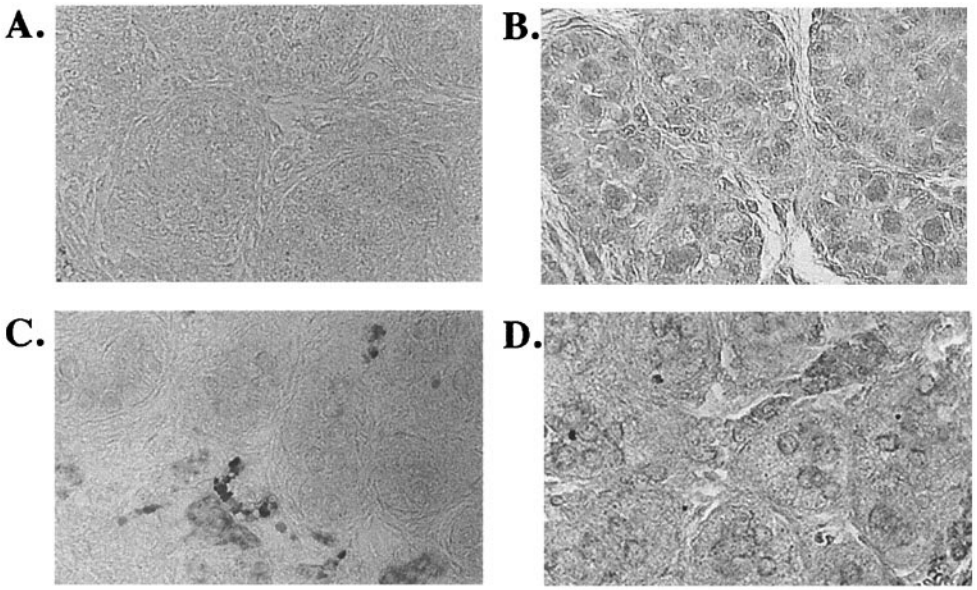

Fig. 5. Immunohistochemistry for steroid receptors in Postnatal day 0 testis: A) nonimmune control; B) ERα; C) ERβ; and D) AR. Dark stain indicates positive immuno-staining and B was lightly counterstained with hemotoxylin to indicate nuclear staining. Data are representative of at least three different experiments conducted with these antibodies.

0.2-μM concentration caused a 5-fold increase in testis cell thymidine incorporation (Fig 6). Methoxychlor concentrations as low as 0.002 μM stimulated thymidine incorporation similar to 1 μM estradiol. HPTE also stimulated thymidine incorporation to the same degree as testosterone and estradiol at 2 μM and 20 μM concentrations. Therefore, both methoxychlor and HPTE can influence P0 testis cell DNA synthesis in a similar manner to androgens and estrogens

4. Discussion

The primary objective of the current study was to determine if exposure to methoxychlor and its metabolite HPTE during critical stages of embryonic development affected normal testis morphology and growth. Previous experiments with estrogen and methoxychlor have evaluated the effects of neonatal exposure on adult testis function [19,23, 35]. Neonatal exposure to estrogens resulted in detrimental effects on normal Sertoli cell function, germ cell numbers, and Leydig cell sensitivity to gonadotropins [35] In utero exposure to estrogenic chemicals has also demonstrated diminished production of steroidogenic enzymes such as 3-β hydroxy steroid dehydrogenase (3βHSD) [36]. However, effects of hormonally active agents on critical stages of embryonic testis development have not been thoroughly examined. In particular, effects on seminiferous cord formation during embryonic testis development have not been evaluated. The process of seminiferous cord formation requires aggregation of Sertoli and primordial germ cells and migration of pre-peritubular cells from the adjoining mesonephros to enclose aggregates forming cord-like structures [3,4]. The process of seminiferous cord formation is thought to be regulated by the secretion of pre-peritubular migration factors from the Sertoli cells [3,4,37]. Therefore, normal differentiation of Sertoli cells is necessary to initiate the development of seminiferous cords.

In the current experiments methoxychlor at three concentrations, 0.2, 2, and 20 μM, did not totally inhibit the formation of seminiferous cords. However, the structures of the cords were abnormal and appeared to be swollen. This swollen appearance was also seen when 3 μM and 6 μM of HPTE was used in testis organ cultures. The swollen cord appearance in testis organs was due to an increased number of cells in cord cross sections and reductions in interstitial cell numbers. Leydig cells may originate from cells that migrate from the mesonephros [38] or from cells that arise from the coelomic epithelium [39]. In either instance, inappropriate differentiation of cells or migration may have caused reductions in the interstitial area between the cords, which influenced the swollen cord appearance. HPTE has been shown to affect leydig cell function and potentially differentiation. Previous research with primary leydig cell cultures observed reduced testosterone production in response to LH stimulation from cell cultures treated with HPTE. Furthermore, prepubertal leydig cells treated with HPTE were more sensitive to treatment and had a reduced ability to produce testosterone compared to adult HPTE-treated leydig cell cultures [40]. Neonatal exposure to estrogens also affected Sertoli cell numbers, steroidogenic enzyme production and the ability of germ cells to develop within the testis [35,36]. Therefore, HPTE treatment can cause reductions in cells between the seminiferous cords and alterations in the growth and proliferation pattern of cells within the cords.

HPTE is weakly estrogenic and can effectively compete with DES for binding to the estrogen receptor at concentrations of 15 μM and higher [41,42]. Since estrogen at 1 μM can inhibit seminiferous cord formation, it was hypothesized that higher concentrations of HPTE might completely inhibit seminiferous cord formation. Total inhibition of cord formation occurred at a 15 μM HPTE concentration. The higher concentrations used in the current study (30 and 60 μM HPTE) also completely prevented cord formation, but these concentrations may be toxic to the organ cultures since the outer layers of cells sloughed off the organs.

A consideration in the current study is whether the concentrations of methoxychlor and HPTE used are relevant to environmental exposure levels. In Canada, the concentration of methoxychlor used in surface water to control black flies and mosquitoes is 1 μM final concentration [43]. It is not known if methoxychlor or HPTE can bioaccumulate to higher levels after an exposure of 1 μM. Since the concentrations of methoxychlor and HPTE found to affect cord formation and testis growth were in the micromolar range, it appears that the concentrations used are relevant to potential environmental exposure levels. Interestingly, the concentrations required to influence testis cell growth were in the nanomolar range (i.e. 0.002 μM).

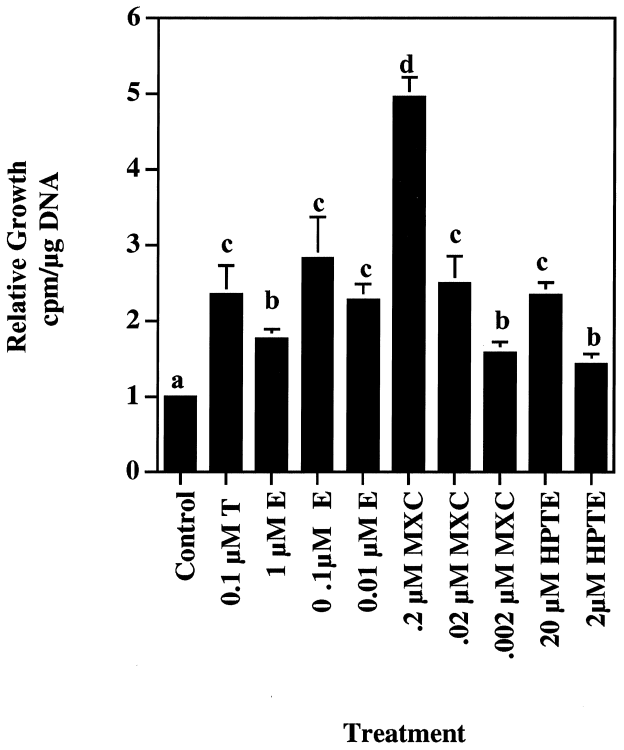

Fig. 6. Effect of estradiol (E), testosterone (T), methoxychlor (MXC), and HPTE on Postnatal day 0 testicular cell culture thymidine incorporation. The final concentration is listed. Data are the mean ± SEM for thymidine cpm/μg cell DNA and are presented as relative growth compared to untreated control cells set at 1.0. Data are representative of 4 to 6 experiments conducted in triplicate for each treatment. Different subscript letters represent significance difference at $P < 0.05$.

Endocrine disruptors are hypothesized to elicit their actions through steroid receptors. The endocrine disruptor in the current study, methoxychlor, has both weakly estrogenic and anti-androgenic metabolites that may result in different responses dependent upon the receptors that they bind [44–46]. Recent literature has determined that the different metabolites of methoxchlor can elicit contrasting actions through the steroid receptors. For example, HPTE can act as an agonist on ERα but as an antagonist on ERβ and AR [46]. Therefore, it was important to determine when the steroid receptors are present and then determine the potential mechanisms of action of these compounds on testis development.

Previous experiments have demonstrated the presence of mRNA for both ERα and ERβ in the fetal and postnatal testis from E16 until adulthood [32,47]. However, the current study demonstrates the presence of message for ERα and AR at E14 while ERβ was not present until E16. The information is crucial, since the testis organ culture treatments start at E13 prior to seminiferous cord formation. This finding suggests that any effects that these endocrine disruptors have on steroid receptors would be through AR and ERα, but not ERβ.

The localization of ERα in the current study was found to be widespread in the testis at E14 with localization to Sertoli, germ, and Leydig cells. At P0, ERα protein was in the nucleus of germ cells with some staining in the interstitium. This pattern of localization is similar to that of other reports [32] utilizing the same ERα antibody. However, the localization of ERβ is distinct from other reports in the developing testis. In the current study, ERβ was localized to cells in the cords and interstitium at E16 with localization to the germ and interstitial cells at P0. This finding is in contrast to experiments utilizing a different ERβ antibody in the fetal testis [34]. In these experiments, there was no localization of ERβ to interstitial cells at E16 and later in development (P4). The ERβ was localized to Sertoli cells and different stage spermatogonia. These differences in localization may be predominantly due to different antibodies utilized. Both the ERα and ERβ antibodies used in the current study were made to the conserved C-terminus of each estrogen receptor and in many other reports have been specific to ERα and ERβ [32,48]. Localization at P0 and E14 has not been previously reported. Therefore, the localization presented in this study demonstrates more dynamic changes in ERα and ERβ than were previously reported.

The cellular localization of AR was similar to other reports with AR being present in germ, interstitial [49] and Sertoli cells at E14 and E16. Subsequently, localization was observed to germ and interstitial cells at P0 (Fig 6). The pre-peritubular cells that migrate from the mesonephros into the gonad have receptors for androgen receptor [50]. The process of seminiferous cord formation is dependent on these cells migrating and forming around the Sertoli and germ cell aggregates [3]. HPTE has been demonstrated to be anti-androgenic in some of its actions [44]. Thus, the effects

of an anti-androgen (flutamide) was also examined in the present experiment. Flutamide had similar effects as estrogen and higher concentrations of HPTE (15, 30, and 60 μM) on inhibition of seminiferous cord formation. Therefore, HPTE may be inhibiting seminiferous cord formation through both estrogenic and anti-androgenic mechanisms in the developing testis.

The effects of both methoxychlor and HPTE on growth of the perinatal testis (P0) were examined with thymidine incorporation assays in P0 testis cell cultures. All concentrations of methoxychlor and HPTE caused an increase in thymidine incorporation in P0 testicular cell cultures. A 0.2 μM concentration of methoxychlor caused a dramatic 5-fold increase in thymidine incorporation, which exceeded the actions of estradiol or testosterone. The low 0.002-μM concentration of methoxychlor increased thymidine incorporation similar to the actions of estradiol and testosterone. HPTE also increased thymidine incorporation similar to the steroids. Results demonstrate that methoxychlor and its metabolite HPTE can influence perinatal cell growth potential. Although this tissue included a mixed cell population, the data confirm that methoxychlor and HPTE can alter testis cell proliferation and complement the tissue morphogenesis data provided. The mechanism of how these agents act on the testis will likely involve additional factors to the steroid receptors alone.

Combined observations demonstrate that methoxychlor and its estrogenic metabolite HPTE are antagonistic to normal embryonic testicular development. Exposure to methoxychlor or HPTE results in abnormal seminiferous cord formation and changes in cell growth characteristics. Therefore, environmental exposure to methoxychlor could result in abnormalities in embryonic seminiferous cord formation and perinatal cell proliferation, which may reduce reproductive efficiency (spermatogenesis) in the adult male. Examination of steroid receptor localization and actions of estrogen and androgen suggest the effects of methoxychlor and HPTE are likely mediated in part through the estrogen receptor, however, potential anti-androgenic actions need to be further investigated. Methoxychlor is an estrogenic compound [11–19] and other estrogenic chemicals are anticipated to have similar actions on embryonic testis development. Further analysis of the mechanistic actions of these agents on embryonic and early postnatal testis development will elucidate how estrogens and anti-androgens may influence male fertility and reproduction.

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