To Mom and Dad
ACKNOWLEDGMENTS

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Hypospadias is a congenital malformation of the external genitalia in which the urethral tube fails to close and the prepuce (foreskin) and ventral penis do not completely form. Due to the dramatic increase in the incidence of hypospadias over the past 40 years, it has been proposed that endocrine-disrupting compounds (EDCs) may be the underlying cause. In this study, I administered the EDCs Bisphenol A, Methoxychlor and p,p’-DDE as well as the type II 5-α reductase inhibitor Finasteride during the androgen-dependent stage of development of external genitalia. In these studies, only finasteride at a dose of 200 mg/kg/day induced hypospadias and produced offspring with shortened anogenital distances. Treatment with p,p’-DDE showed a trend of shortened anogenital distances as the dose increased, although this trend was only marginally significant (p = 0.073). These studies suggest that type II 5-α reductase is important for normal genital development.

I also examined the effect of the fungicide Vinclozolin administered during development and the possible ameliorative effects of a-tocopherol (vitamin E) when given with Vinclozoin. Using a rating system devised to quantify the severity of genital
defects in this study, I observed a significant increase in the severity score of the
vinclozolin only treatment, but this significance disappeared with the supplementation of
vitamin E. This study suggests that vitamin E may diminish the effect of vinclozolin,
making it a candidate for a preventative treatment of hypospadias.
CHAPTER 1
INTRODUCTION AND AIMS

Development of the External Genitalia in Mice

Mammalian external genital development is a coordination of proximodistal outgrowth, patterning and tubulogenesis. The first phase of genital development is the outgrowth and patterning of the genital tubercle, which is the same in both males and females. This phase is considered to be under the control of genetic programming and independent of hormonal control, since the tubercle begins to develop at embryonic day (E) 10.5 (Perriton et al. 2002), and the testes do not produce testosterone until E12.5-13 (Gondos 1980). The second phase, however, is hormonally-controlled and involves the differentiation of the genital tubercle into a penis or clitoris by either continued or arrested outgrowth (Perriton et al. 2002). This proximal to distal outgrowth of the genitalia is patterned along three axes and coordinated with the formation of the urethral canal which will form the urethra.

In mice, the first phase of genitalia development begins at E10.5 with the formation of a pair of genital swellings of mesenchyme covered by surface epithelium that bud lateral to the cloacal membrane, posterior to the hindlimb buds (Perriton et al. 2002) (Fig. 1-1). These swellings will grow and fuse medially to form a single genital tubercle by E11.75. The urethral plate (the precursor to the male glandar urethra) will also form during this time along the dorsoventral axis of the tubercle when the cloacal endoderm extends into the tubercle, fusing in a distal to proximal direction. At E13.5, preputial swellings begin to form lateral to the genital tubercle, and will continue to grow both laterally and ventrally to form the prepuce. At E14.5, the proximal opening of the urethra can be seen, but begins to close at E15.5 as the preputial swellings fuse
ventrally and is no longer detected by E16.5. In the clitoris, the urethral plate epithelium will persist as an epithelial cord, and in the penis it will canalize to form a urethral tube. The genes and gene networks controlling development of the external genitalia are still in the early stages of being discovered, but several genes have been implicated as being important in outgrowth and patterning of the genital tubercle. Sonic Hedgehog (Shh) is essential for outgrowth of the genital tubercle, and mice lacking Shh or hedgehog pathway components have agenesis of the external genitalia (Haraguchi et al. 2001, Paulozzi et al. 1997). Hoxa13/Hoxd13 compound mutants fail to grow a genital tubercle, and heterozygosity for either causes patterning defects (Warot et al. 1997). Fgf signaling also regulates the proper development of the urethra, and Fgfr2-IIIb/- mice exhibit hypospadias (Petiot et al. 2005).

Figure 1-1. Normal development of the external genitalia. Modified from Figure 1 in Perriton et al. 2002.
The second phase of genitalia development is dependent on androgen signaling by either testosterone or dihydrotestosterone (Perriton et al. 2002), which are produced by the Leydig cells of the testes. Testosterone and dihydrotestosterone production begins soon after Leydig cell differentiation, around E12.5-13 in mice (Gondos 1980). This is just prior to the onset of this second phase of genital development, which begins at E15.5 and results in the sex-specific development of the genital tubercle into the penis in males (extended outgrowth) and the clitoris in females (truncated outgrowth) (Seifert et al. 2008, Yamada et al. 2003). Androgen signaling also regulates the canalization of the urethral plate into the urethra in males (Yucel et al. 2003, Baskin et al. 2001), resulting in a final urethral opening at the distal end of the penis. Disruption of this tubulogenesis process is proposed to be the cause of hypospadias (Baskin et al. 2001). Androgens bind to androgen receptors, causing the receptors to be activated, form a homodimer, translocate into the nucleus, and bind to androgen response elements in DNA upstream from androgen-dependent genes. In this way, androgen receptors act as transcription factors, directly regulating the expression of these genes. Androgen receptors are abundant in the urethral epithelium of both the fetal human (Kim et al. 2002) and rodent (Murakami 1986) penises, and 5-α reductase, the enzyme that converts testosterone to 5α-dihydrotestosterone, is abundant in the stroma surrounding the urethra (Kim et al. 2002). In humans, mutations resulting in a nonfunctional androgen receptor or 5-α reductase type II enzyme can cause genital defects (Sultan et al. 2001).

**Endocrine-Disrupting Compounds and Hypospadias**

In the United States, the incidence of hypospadias approximately doubled between 1968 and 1993, without any obvious explanation, and this defect now affects
approximately 1 in 125 live male births (Paulozzi et al. 1997). Although children with this defect have been screened for mutation in candidate genes (Chen et al. 2007), there is still no known genetic basis for hypospadias. Thus, it is thought that the effect may instead be environmental, and this growing number of incidences may be due to exposure to endocrine-disrupting compounds (EDCs) in utero. EDCs are thought to exert their effect by disrupting the steroid hormone signaling pathways necessary for normal reproductive tract development. However, little is known about the effects of EDCs on the genetic networks involved in genitalia development. In my experiments, I sought to identify EDCs which could induce hypospadias in male offspring when administered to pregnant female CD-1 mice. For my initial screen of EDCs, I chose three compounds: bisphenol A, methoxychlor and p,p'-DDE.

Bisphenol A

Bisphenol A is an organic compound used to make polycarbonate plastic and epoxy resin and is known to have estrogenic and weak antiandrogenic activity. Its estrogenic activity was first identified more than 70 years ago (Dodds and Lawson 1936), yet it was still used to synthesize polycarbonate plastic for contact with food beginning in the 1950s. As part of a national health survey, the CDC reported that 93 percent of persons tested had detectable levels of BPA in their urine (Calafat et al. 2008). The US Environmental Protection Agency (EPA) and Food and Drug Administration (FDA) currently consider a dose of 50 µg/kg/day to be a safe level of human consumption, but studies by Vandenberg et al. (2007), led a panel of scientists at a National Institutes of Health conference on BPA to conclude that current levels of exposure already exceed this level (vom Saal et al. 2007). In laboratory studies using
rodents, low doses of BPA have been shown to cause a decrease in daily sperm production and an increase in prostate size (vom Saal et al. 1998) and a progression from hyperplasia of prostate basal (stem) cells in the primary prostatic ducts during development (Timms et al. 2005) to basal cell squamous metaplasia in adulthood (Ogura et al. 2007) and eventually to early stage prostate cancer in response to adult administration of testosterone and estradiol (Ho et al. 2006).

**Methoxychlor**

Methoxychlor is a synthetic organochlorine pesticide developed as a replacement to DDT during the 1970s. It was thought to be less toxic than DDT and to have a shorter half-life, but has still proven to be a formidable EDC. Methoxychlor has been shown to have both estrogenic and antiandrogenic properties and can inhibit spermatogenesis (Staub et al. 2002) and can induce apoptosis in the adult male rat testes (Vaithinathan et al. 2010). Methoxychlor has been shown to persist in the environment (National Research Council 1999).

**DDT/p,p’-DDE**

Dichlorodiphenylchloroethylene or p,p’-DDE is the main metabolite of the organochlorine pesticide DDT. Both p,p’-DDE and DDT are lipophilic and thought to persist in the environment and bioaccumulate in humans because of their long half lives (Longnecker 2005; Wolff et al. 2000). DDT was first identified as a potent pesticide in 1939 and was heavily used until it was banned in the United States in 1972. However, it is still used to control malaria-carrying mosquitoes in several Asian and African countries (Stockholm Convention on Persistent Organic Pollutants 2008). Shown to be a potent androgen receptor antagonist but a very weak estrogen receptor antagonist (Kelce et al. 1995), p,p’-DDE can reduce anogenital distance in male rats treated in
In utero as well as induce retained nipples and reduce the size of the ventral prostate in these rats (Kelce et al. 1995).

**Finasteride**

I also chose to also study the effect of the synthetic antiandrogen finasteride on male genital development. Finasteride acts by inhibiting 5-α reductase type II, the enzyme that converts testosterone to dihydrotestosterone (DHT). Finasteride is a drug that was originally approved by the FDA for the treatment of benign prostatic hyperplasia, but was later approved in 1997 to also treat male pattern baldness under the brand name Propecia. Treatment of rabbits in utero with only 10 mg/kg/day of finasteride during gestational days 19 to 28 resulted in reduced anogenital distances and hypospadias in male offspring (Kurzrock 2000).

**Vinclozolin**

Finally, I chose to look at the effects of the dicarboximide fungicide vinclozolin on development of the male genitalia. Vinclozolin is most commonly used to prevent fungus growth primarily in vineyards and strawberry crops but is used on a variety of fruits and vegetables. Gray et al. (1999) have shown that administration of vinclozolin during sexual differentiation in rats results in reduced anogenital distance in males as well as retained nipples, cleft phallus and hypospadias. Since this is an EDC known to cause hypospadias in rodents, I chose to use this compound to also study the effects of treating pregnant dams with vinclozolin while also supplementing with a dose of the antioxidant vitamin E (α-tocopherol).

**Possible Ameliorative Effects of Antioxidants on EDC Exposure**

Oxidative stress occurs when reactive oxidative species (ROS) such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and the hydroxyl radical (OH-) are
generated at a rate that exceeds an organism’s ability to detoxify these oxygen intermediates, causing damage to DNA, proteins and lipids (Bartsch and Nair 2000). ROS can interrupt lipid peroxidation (the biologically-essential oxidative degradation of lipids) by “stealing” electrons from the lipids of the cell membrane, which can cause extensive cell damage (Lundberg 1960).

The Leydig cells of the testes are located in the testicular interstitium, adjacent to the seminiferous tubules and produce testosterone in the presence of luteinizing hormone. They are especially susceptible to extracellular sources of ROS because they reside close to testicular interstitial macrophages, which can produce these species when stimulated (Hales et al. 1999). ROS can inhibit steroidogenesis by interfering with the transfer of cholesterol in MA-10 tumor Leydig cells (Stocco et al. 1993).

To counteract the deleterious effects of ROS, aerobic cells possess a number of antioxidant defense mechanisms. These are mainly antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase; antioxidant proteins such as thioredoxin and metallothionein; and small molecular antioxidants such as glutathione, vitamin E and vitamin C. The lipid-soluble small molecular antioxidant vitamin E can protect cell membranes from oxidative stress by interacting and neutralizing lipid radicals produced during lipid peroxidation (Dieber-Rotheneder et al. 1991).

The dicarboximide fungicides iprodion, procymidone and vinclozolin have all been shown to cause lipid peroxidation in studies of the fungus Botrytis cinerea (Choi et al. 1996, Lee et al. 1998). The effects of vinclozolin on this fungus were reversed by
growing the fungus in incubation medium containing α-tocopherol (vitamin E) (Choi et al. 1996).

The antioxidants vitamin C and E have been shown to have a significant protective role on free-radical-induced toxicity in testicular Sertoli and Leydig cell and epididymal sperm when given to PCB-exposed rats (Murugesan et al. 2005). Also, supplementation with vitamins C and/or E can have a beneficial effect on steroidogenic acute regulatory (StAR) protein and steroidogenic enzyme mRNA expression in the Leydig cells of adult male rats exposed to the PCB Aroclor 1254 by intraperitoneal injection (Murugesan et al. 2007). In the same study, serum testosterone levels were reduced for PCB-treated rats and near normal for rats treated with the PCB as well as vitamin C or E. Similar effects were seen by supplementing chromium treatments in rats with vitamin E, resulting in restored serum testosterone levels as well as sperm count and seminiferous tubule morphology (Chandra et al. 2010).

**Aims of Thesis**

The overall aim of this thesis was to further understand the role of endocrine-disrupting compounds in the etiology of the congenital malformation hypospadias. The incidence of hypospadias is alarmingly high at approximately 1 in 125 live males births (Paulozzi et al. 1997). Although this is one of the most common birth defects in the U.S., little is known of the etiology of hypospadias. Since it is widely suspected that endocrine disruptors are at least partly to blame, I sought to use the mouse model to begin to identify which compounds are capable of inducing hypospadias when administered during development.
Only in the last 10 years has a clear, staged study of normal external genital development in the mouse been available, as done by Perriton et al. in 2002, shown in part in Figure 1. Although this is fairly recent, our lab and others have made great progress in identifying genetic and hormonal processes necessary for external genital development in this model system. Work in this system also allows for the powerful genetic tools of transgenesis. Specifically to this work, mice can only be used not only for studies in knocking out essential genes, but also in labeling specific cell populations.

My first aim was to identify EDCs which could induce hypospadias, so that later these compounds could be studied in our model system more extensively after we established the lowest dose that could induce a phenotypic effect. I did this by administering a dose response series of each compound to pregnant female CD-1 mice during a critical stage of genital development. I then examined the male offspring both in whole mount and in histological section to determine if there was an effect on a morphological level.

I next wanted to test the hypothesis that the antioxidant α-tocopherol or vitamin E has an ameliorative effect on EDC exposure. I hypothesized that part of the effect of EDCs is inducing oxidative stress and that this could be at least partially prevented or amended by a supplement of an antioxidant such as vitamin E. I tested this hypothesis by co-administering vinclozolin at a fixed amount and α-tocopherol at increasing amounts to pregnant female CD-1 mice, according to the same protocol used in my EDCs screen. I then examined the offspring in whole mount and in histological section to see if there was a difference in morphology between treatment groups.
LIST OF SOLUTIONS AND REAGENTS

This chapter provides a list of the solutions and reagents used in these studies, as well as protocols for performing the experiments. The following list includes all reagents used in the experiments described, including the companies from which they were purchased. All catalog numbers for reagents are available upon request.

- $\alpha$-tocopherol (Sigma)
- $\alpha$-tocopherol-stripped corn oil (Sigma)
- Bisphenol A (Sigma)
- CD-1 mice (Hsd:ICR) (Harlan)
- Citrisolv (Fisher)
- Corn oil (Sigma)
- Dichlorodiphenyldichloroethylene (p,p'-DDE) (Sigma)
- Eosin Y (Fisher)
- Ethanol 200 proof (100%) ethanol (Fisher)
- Ethanol 190 proof (95%) ethanol (Fisher)
- Finasteride (Sigma)
- Harris Hemotoxylin (Fisher)
- Methoxychlor (Sigma)
- Paraplast tissue embedding media (Fisher)
- Permount mounting medium (Fisher)
- Phosphate buffered saline (PBS) tablets (Fisher)
  - Dissolve one tablet in 100 mL of water, and autoclave to sterilize.
- Tocopherol-stripped Corn Oil (MP Biomedicals)
- Vinclozolin (Sigma)
- Xylene (Fisher)

**Dissolving Bisphenol A in Corn Oil**

To make solutions for the 100 mg/kg dose, 40 mg of bisphenol A was dissolved in 50 µL of 200 proof ethanol by vortexing a few times at room temperature. Corn oil was then added to make 1 mL of solution, making the final concentration 40 mg/mL of BPA in a solution of 5% ethanol in corn oil. To make solutions for the 50 mg/kg dose, 20 mg of BPA was dissolved in the same manner to make a solution of 20 mg/mL of BPA in...
5% ethanol in corn oil. The 5 mg/kg and 0.5 mg/kg dose solutions were made by
diluting the 50 mg/kg dose solution by either 10 or 100 fold in corn oil.

**Dissolving Methoxychlor and p,p’-DDE in Corn Oil**

Solutions for both compounds at the 200 mg/kg dose were prepared by dissolving
80 mg of either methoxychlor or p,p’-DDE in 1 mL of corn oil. The solutions were
placed in a 65° incubator and periodically vortexted for approximately 2 hours to aid
dissolving. Solutions for lower doses were prepared in the same manner using 60
mg/mL solutions for the 150 mg/kg dose, 40 mg/mL solutions for the 100 mg/kg dose,
20 mg/mL solutions for the 50 mg/kg dose, and 10 mg/mL solutions for the 25 mg/kg
dose.

**Using Tocopherol-Stripped Corn Oil**

Since we suspected the tocopherols in the corn oil may be interfering with our
studies, we repeated the highest dose of BPA (100 mg/kg), methoxychlor (200 mg/kg)
and p,p’-DDE (200 mg/kg) using tocopherol-stripped corn oil. Tocopherol-stripped corn
oil which has been steamed to remove tocopherols. This type of corn oil is guaranteed
by the manufacturer to contain no more than 10 IU/kg (10 ppm) of tocopherols and less
than 0.5 mg/100g of α-tocopherol, specifically. For these repeated experiments, the
compounds were dissolved in the same manner as previously described, but
tocopherol-stripped corn oil was used in place of normal corn oil.

**Dissolving Finasteride in Tocopherol-Stripped Corn Oil**

Finasteride solutions were prepared by dissolving 80, 70, 60, 50 or 40 mg of
Finasteride in 50 μL of 200 proof ethanol for the 200, 175, 150, 125 and 100 mg/kg
doses, respectively. These were each diluted in stripped corn oil to a final volume of 1 mL.

**Dissolving Vinclozolin and α-Tocopherol in Stripped Corn Oil**

Vinclozolin was dissolved in concentrations of 60 mg/mL in stripped corn oil. Solutions were placed in a 65° incubator and periodically vortexed for approximately 3 hours to aid dissolving. Solutions of vinclozolin plus α-tocopherol were prepared by dissolving 60 mg of vinclozolin in an amount of corn oil equal to 1 mL minus the volume of α-tocopherol required for the dose, since the α-tocopherol is a viscous liquid. This solution was then heated and vortexed as previously described and then cooled to room temperature. The vitamin E was added and the solution was vortexed a few times at room temperature. The amount of α-tocopherol added to each solution was 210.5 µL for the vinclozolin plus 500 mg/kg α-tocopherol dose and 84.2 µL for the vinclozolin plus 200 mg/kg α-tocopherol dose. These calculations were made taking into account that the density of α-tocopherol is 0.95 g/mL, allowing a volumetric measurement of α-tocopherol to be added without weighing it.

**Dosing Pregnant CD-1 Dams by Oral Gavage**

CD-1 mice were time mated, and noon of the day a vaginal plug was observed was considered E0.5. Mice were dosed from E12.5 to E17.5 in all studies presented in this thesis. Each solution of BPA, methoxychlor, p,p’-DDE, finasteride, vinclozolin and vinclozolin plus α-tocopherol was prepared to be at a concentration that calls for a dose of 100 µL per 40 gram mouse. Therefore, each mouse was weighed every day and was gavaged with the volume of solution based on its weight, using the ratio of 100 µL per
40 grams. This same ratio of dosing was used for unstripped and stripped corn oil control groups to ensure the same volume of corn oil was administered to each group.

**Harvesting CD-1 Embryos and Pups**

Embryos were harvested at E18.5 for all BPA, methoxychlor, p,p’-DDE and finasteride experiments. Pregnant dams were euthanized by cervical dislocation, and embryos were dissected in PBS. Tail tissue was taken to genotype for the ZFY gene to identify males and females. Embryos were fixed in cold 4% PFA overnight.

Pups from the vinclozolin and vinclozolin plus α-tocopherol treatment groups were taken at postnatal day 0 (P0). Pups were euthanized by decapitation and dissected in PBS. Again, tail tissue was taken for genotyping, and embryos were fixed in cold 4% PFA overnight.

**Measuring and Analyzing Anogenital Distance**

Fixed embryos and pups were examined under a dissecting microscope fitted with an eyepiece reticle. The reticle was calibrated so that I was able to measure the distances in millimeters. These measurements were compared across the dose response by linear regressions.

**Whole Mount Analysis of Vinclozolin Treatment Groups**

Due to the wide range of phenotypes, a rating scale was developed to categorize the severity of hypospadias among specimens. A specimen with a completely distal placement of the urethral opening was given a rating of 0. One with an opening in the distal third of the penis was given a rating of 1. A specimen with a urethral opening reaching down into the middle third was given a rating of 2. One with an opening reaching into the most proximal third of the penis was given a rating of 3. Examples of each of these ratings are shown in Figure 2-1.
The specimens were removed from their labeled tubes and placed into randomly numbered tubes by postdoctoral researcher Dr. Krista McCoy of the Cohn lab, rated by myself and renumbered with random numbers, and then rated again by Dr. McCoy. These ratings were averaged together for each specimen and this average was used in all statistical analyses.

**Histological Analysis**

Embryos or pups were fixed over night in 4% PFA in PBS, then dissected and washed in PBS. They were then dehydrated in a graded ethanol series of 50%, 75%, 95%, and 100% twice, each wash lasting one hour. Embryos were then washed for one hour in a 1:1 mixture of 100% ethanol and either citrusolv or xylene, then two one-hour washes of citrusolv or xylene, then one hour in 1:1 citrisolve or xylene and paraffin wax. Finally, the tissue was incubated in two one-hour changes of paraffin wax followed by an overnight incubation in paraffin wax in a vacuum incubator. Samples were oriented with warmed forceps and allowed to set overnight.

Wax blocks were sectioned with a microtome into 8 µm sections, which were floated out on a waterbath, mounted onto glass slides and dried for at least 2 hours on a
slide warmer. Slides were put into racks and stained with hematoxylin and eosin as follows:

- Xylene or Citrisolv 1 – 10 minutes
- Xylene or Citrisolv 2 – 10 minutes
- 100% ethanol – 10 minutes
- 95% ethanol – 10 minutes
- 70% ethanol – 10 minutes
- Water – 5 minutes
- Hematoxylin – 1 minute
- Water – 5 minutes
- 70% ethanol – 1 minute
- Eosin – 30 seconds
- 95% ethanol – 1 minute
- 95% ethanol – 1 minute
- 100% ethanol – 1 minute
- Xylene or Citrisolv – 5 minutes

Sections were then coverslipped with Permount. An example of a transverse section of an E18.5 murine penis stained according to this method is shown in Figure 2-2.

Figure 2-2. Transverse section of normal penis of an E18.5 mouse embryo. G = glans, P = Prepuce, DV = dorsal vein, U = urethra, PG = preputial glands
CHAPTER 3

RESULTS

Effects of Endocrine-Disrupting Compounds on External Genital Development

Endocrine-disrupting compounds (EDCs) are suspected to be an underlying cause of the worldwide increased frequency of defects of the genitalia, including hypospadias. The first part of this project was designed to test the efficacy of EDCs and their ability to induce hypospadias in our model organism, the mouse. The following three EDCs were chosen to be screened using our dosing regime: Bisphenol A, a plasticizer with estrogenic and antiandrogenic activity; methoxychlor, a pesticide with estrogenic and antiandrogenic activity; and p,p’-DDE, a metabolite of the organochlorine pesticide DDT with estrogenic and antiandrogenic activity. The 5-α reductase type II inhibitor Finasteride was also screened. Pregnant CD-1 strain dams were orally gavaged from E12.5 to E17.5 with increasing concentrations of each compound, which had been dissolved in corn oil. Embryos were harvested at E18.5. After anogenital distances (AGDs) were measured, embryos were photographed in whole mount and then sectioned and stained with hematoxylin and eosin, in order to be analyzed for defects.

Next, to test the possibility that α-tocopherol could decrease the effect of these compounds, I repeated the experiments in tocopherol-stripped corn oil. The highest dose from the normal corn oil series for each compound was dissolved in tocopherol-stripped corn oil and administered to pregnant dams in the same manner as before. Embryos were harvested and analyzed in the same manner as well.

Lastly, the possible ameliorative effect of a supplementation of α-tocopherol (vitamin E) when administered with an EDC was tested. Pregnant dams were treated with vinclozolin, a dicarboximide fungicide with antiandrogenic activity, at a dose that
would cause hypospadias in 100% of male offspring. Other dams were treated with the same dose of vinclozolin and supplemented with increasing doses of vitamin E. All dams were dosed from E12.5 to E17.5 and pups were collected at P0 for analysis as previously described.

**Effect of Bisphenol A - Dose Series in Normal (Unstripped) Corn Oil**

Analysis of male embryos from dams treated with either 0.5, 5, 50 or 100 mg/kg/day of Bisphenol A (BPA) showed no obvious defects when analyzed in whole mount (Fig. 3-1). The prepuce of the tubercle appears to be intact on the ventral side, and the urethral opening appears to be in its normal place. This was also confirmed by analyzing sectioned stained with hematoxylin and eosin (Fig. 3-2). Table 3-1 provides a summary of the number of embryos harvested as well as the anogenital distances. A linear regression plotting these AGD averages is shown in Figure 3-3. There is no significant difference in the AGD with regard to the increase in the dose of BPA ($p = 0.149$).

![Figure 3-1. BPA dose series. Whole mount pictures of representative E18.5 male embryos from each litter of the dose series. All specimens appear normal.](image)
Figure 3-2. BPA dose series. Sections of high dose specimens from series. Oriented so that dorsal is to the top and ventral to the bottom of each. All specimens appear normal.

Table 3-1. Results of bisphenol A dose response series

<table>
<thead>
<tr>
<th>Dose</th>
<th># of Litters</th>
<th>Average AGD (mm)</th>
<th>Genital Defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg/kg/day</td>
<td>2</td>
<td>1.23</td>
<td>None</td>
</tr>
<tr>
<td>0.5 mg/kg/day</td>
<td>1</td>
<td>1.33</td>
<td>None</td>
</tr>
<tr>
<td>5 mg/kg/day</td>
<td>1</td>
<td>1.21</td>
<td>None</td>
</tr>
<tr>
<td>50 mg/kg/day</td>
<td>1</td>
<td>1.17</td>
<td>None</td>
</tr>
<tr>
<td>100 mg/kg/day</td>
<td>1</td>
<td>1.10</td>
<td>None</td>
</tr>
</tbody>
</table>

Figure 3-3. Linear regression of average AGDs in bisphenol A treatment. No significant change in the average anogenital distances is seen with regard to the increasing BPA dose ($p = 0.149$).
Effect of Bisphenol A – Dose Series in Tocopherol-Stripped Corn Oil

The highest dose tested in normal corn oil (100 mg/kg/day) was next tested in tocopherol-stripped corn oil according to the same dosing regime. Again, the male tubercles appeared normal in both whole mount (Fig. 3-1) and section (Fig. 3-2).

Effect of Methoxychlor – Dose Series in Normal (Unstripped) Corn Oil

Analysis of male embryos from dams treated with 25, 50, 100, 150 or 200 mg/kg/day of Methoxychlor showed no defects in whole mount (Fig. 3-4). The prepuce of the tubercle appears to be intact on the ventral side, and the urethral opening appears to be in its normal place. This was also confirmed by analyzing sections stained with hematoxylin and eosin (Fig. 3-5). Table 3-2 shows a summary of the number of embryos harvested and the anogenital distances. A linear regression plotting these AGD averages is shown in Figure 3-6. There is no significant difference in the AGD with regard to the increase in the dose of methoxychlor (p = 0.937).

Figure 3-4. Methoxychlor dose series. Whole mount pictures of representative E18.5 male embryos from each litter of the dose series. All specimens appear normal.
Figure 3-5. Methoxychlor dose series. Sections of high dose specimens from series. Oriented so that dorsal is to the top and ventral to the bottom of each. All specimens appear normal.

Table 3-2. Results of methoxychlor dose response series

<table>
<thead>
<tr>
<th>Dose</th>
<th># of Litters</th>
<th>Average AGD (mm)</th>
<th>Genital Defects</th>
</tr>
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<tbody>
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<td>0 mg/kg/day</td>
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<td>None</td>
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<td>25 mg/kg/day</td>
<td>1</td>
<td>1.28</td>
<td>None</td>
</tr>
<tr>
<td>50 mg/kg/day</td>
<td>1</td>
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<td>None</td>
</tr>
<tr>
<td>100 mg/kg/day</td>
<td>1</td>
<td>1.23</td>
<td>None</td>
</tr>
<tr>
<td>150 mg/kg/day</td>
<td>1</td>
<td>1.30</td>
<td>None</td>
</tr>
<tr>
<td>200 mg/kg/day</td>
<td>1</td>
<td>1.27</td>
<td>None</td>
</tr>
</tbody>
</table>

Figure 3-6. Linear regression of average AGDs in methoxychlor treatment. No significant change in the average anogenital distances is seen with regard to the increasing methoxychlor dose (p = 0.937).
Effect of Methoxychlor – Dose Series in Tocopherol-Stripped Corn Oil

The highest dose tested in normal corn oil (200 mg/kg/day) was next tested in tocopherol-stripped corn oil according to the same dosing regime. Again, the male tubercles appeared normal in both whole mount and section (Figs. 3-4 and 3-5).

Effect of p,p’-DDE – Dose Series in Normal (Unstripped) Corn Oil

Analysis of male embryos from dams treated with either 25, 50, 100, 150 or 200 mg/kg/day of p,p’-DDE showed no defects in whole mount (Fig. 3-7). This normal morphology was confirmed by analyzing sections stained with hematoxylin and eosin (Fig. 3-8). Embryos from some treatment groups (50 mg/kg and 150 mg/kg) harvested at E18.5 appeared slightly younger than control embryos taken at the same stage, but the tubercles still had the normal morphology of an E17.5 tubercle. Table 3-3 shows a summary of the number of litters harvested and the anogenital distances. A linear regression of these average anogenital distances is shown in Figure 3-9. The AGDs show a marginally significant trend (p = 0.073) of shortening as the dose of p,p’-DDE increases.

Figure 3-7. p,p’-DDE dose series. Whole mount pictures of representative E18.5 male embryos from each litter of the dose series.
Figure 3-8. p,p’-DDE dose series. Sections of high dose specimens from series in both unstripped and stripped corn oil and a stripped corn oil control specimen. Sections are oriented so that dorsal is at the top and ventral at the bottom of each. All specimens appear normal.

Table 3-3. Results of p,p’-DDE dose response series

<table>
<thead>
<tr>
<th>Dose</th>
<th># of Litters</th>
<th>Average AGD (mm)</th>
<th>Genital Defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg/kg/day</td>
<td>2</td>
<td>1.28</td>
<td>None</td>
</tr>
<tr>
<td>25 mg/kg/day</td>
<td>1</td>
<td>1.20</td>
<td>None</td>
</tr>
<tr>
<td>50 mg/kg/day</td>
<td>1</td>
<td>1.20</td>
<td>None</td>
</tr>
<tr>
<td>100 mg/kg/day</td>
<td>1</td>
<td>1.30</td>
<td>None</td>
</tr>
<tr>
<td>150 mg/kg/day</td>
<td>1</td>
<td>1.10</td>
<td>None</td>
</tr>
<tr>
<td>200 mg/kg/day</td>
<td>1</td>
<td>1.00</td>
<td>None</td>
</tr>
</tbody>
</table>

Figure 3-9. Linear regression of average AGDs in p,p’-DDE treatment. There is a marginally significant change in the average anogenital distances with regard to the increasing methoxychlor dose (p = 0.073).
Effect of p,p'-DDE – Dose Series in Tocopherol-Stripped Corn Oil

The highest dose tested in normal corn oil (200 mg/kg/day) was tested in tocopherol-stripped corn oil according to the same dosing regime. Again, the male tubercles appeared normal in both whole mount (Fig. 3-7) and section (3-8).

Effect of Finasteride – Dose Series in Tocopherol-Stripped Corn Oil

Analysis of male embryos from dams treated with 100, 125, 150 or 175 mg/kg/day of finasteride showed no defects in whole mount (Fig. 3-10). However, male embryos from dams treated with 200 mg/kg/day of finasteride did exhibit hypospadias, with the urethral opening extending about halfway down the tubercle. This was also confirmed by analyzing sectioned stained with hematoxylin and eosin (Fig. 3-11). In section, the urethral opening can be seen to open to the outside of the tubercle more proximally in section that in the corn oil control. Table 3-4 provides a summary of the number of embryos harvested as well as the anogenital distances. A linear regression of these average AGDs is shown in Figure 3-12. A significant trend is seen as AGDs decrease as the dose of finasteride increases (p = 0.039).
Figure 3-11. Finasteride dose series. Sections of a specimen from the highest dose and a stripped corn oil control specimen. Sections are oriented so that dorsal is at the top and ventral at the bottom of each. The 200 mg/kg finasteride specimen exhibits hypospadias.

Table 3-4. Results of finasteride dose response series

<table>
<thead>
<tr>
<th>Dose</th>
<th># of Litters</th>
<th>Average AGD (mm)</th>
<th>Genital Defects</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mg/kg/day</td>
<td>2</td>
<td>1.22</td>
<td>None</td>
</tr>
<tr>
<td>100 mg/kg/day</td>
<td>1</td>
<td>1.27</td>
<td>None</td>
</tr>
<tr>
<td>125 mg/kg/day</td>
<td>1</td>
<td>1.10</td>
<td>None</td>
</tr>
<tr>
<td>150 mg/kg/day</td>
<td>1</td>
<td>0.93</td>
<td>None</td>
</tr>
<tr>
<td>175 mg/kg/day</td>
<td>1</td>
<td>0.90</td>
<td>None</td>
</tr>
<tr>
<td>200 mg/kg/day</td>
<td>1</td>
<td>0.86</td>
<td>Hypospadias</td>
</tr>
</tbody>
</table>

Figure 3-12. Linear regression of average AGDs in finasteride treatment. There is a significant decrease in the average anogenital distances with regard to the increasing finasteride dose (p = 0.039).
**Effect of Vinclozolin and Possible Ameliorative Effect of α-Tocopherol**

The treatment groups of 150 mg/kg/day of Vinclozolin (V), 150 mg/kg/day of V plus 200 mg/kg/day of Vitamin E (E) and 150 mg/kg/day of V plus 500 mg/kg/day of E all produced litters exhibiting hypospadias at varying degrees. A graph representing the average ratings of the severities of hypospadias is shown as Figure 3-13. These ratings are based on the scoring system described in the methods section: an opening at the distal tip was given a rating of 0; an opening in the distal third of the tubercle was given a rating of 1; an opening in the middle third of the tubercle was given a 2; and an opening in the proximal third was given a rating of 3 (Fig. 2-1). A standard ANOVA was run to determine statistical significance. The only significant difference is seen when comparing the corn oil control group to the vinclozolin only group (p= 0.024). There are no differences between the corn oil group and the vinclozolin plus 200 or 500 mg/kg vitamin E groups (p = 0.054, p = 0.199). There are also no significant differences when comparing the vinclozolin only group to the vinclozolin with 200 or 500 mg/kg of vitamin E groups (p = 0.552, p = 0.346). There is no significant difference between the vitamin E supplementation groups either (p = 0.881).

This trend can also be seen in the linear regression in Figure 3-14 using a mixed effects model. Each litter from the V 150 mg/kg plus E 0mg/kg, V 150 mg/kg plus E 200 mg/kg and V 150 mg/kg plus E 500 mg/kg treatment groups is plotted as an individual point. The regression shows a significant (p = 0.0175) slope with an $r^2$ value of -0.407. However, there is a notable litter from the V 150 mg/kg plus E 200 mg/kg group that had an average score (1.17) lower than that of the other litters in the group (2.00, 2.00 and 2.36). This is considered to be natural variation and was not removed from the analysis.
Figure 3-13. Average ratings of hypospadias severity. Bar graph showing average ratings measured in each treatment group with error bars. P values for ANOVA analysis are shown. A significant increase is seen in the scores of the vinclozolin alone group from the corn oil control, but this significance disappears in both vitamin E supplementation groups.

Figure 3-14. Hypospadias ratings for individual litters. The regression shows a significant (p = 0.0175) slope with an r^2 value of -0.407.
Also, histological data is shown in Figure 3-11. A specimen from each treatment group receiving each rating was chosen to show examples of morphology. For the corn oil group, there were only ratings of 0 and 1 and for the 500 mg/kg vitamin E group there were only ratings of 1 and 2.

Figure 3-15. Histological sections from vinclozolin and vitamin E series. Examples of each rating for each treatment group are shown. Sections are arranged from proximal to distal, with dorsal facing up and ventral facing down.
CHAPTER 4
DISCUSSION

Bisphenol A, Methoxychlor and p,p’-DDE Dose Response Series

My experiments with BPA, methoxychlor and p,p’-DDE produced no offspring with hypospadias, and only the p,p’-DDE dose response produced a marginally significant shortening of male anogenital distances. While these results are mostly negative, I do not think this proves that these compounds have no effect on sexual development. The dose response experiments were designed to mimic the most common means by which humans are exposed to these compounds, ingestion. However, using oral gavage in our experiments adds the complications of the time it takes for the compound to enter the mother’s blood stream and then be passed on to the embryos and the possible degradation or metabolism of the compound, which could lessen the effect. This is evidenced by the Material Data Safety Sheet (MSDS) for BPA, which states that the LD50 oral dose for mice is 2400 mg/kg, but the LD50 intraperitoneal dose is only 150 mg/kg. This suggests that these doses may have a more dramatic effect when administered differently, as with an intraperitoneal injection. Also, these compounds may have an effect at higher or lower doses than those tested in these studies.

I would also like to emphasize that the scale of this experiment was only one litter per treatment. This could mean that were these studies repeated with more litters, these anogenital distance averages could either stay insignificant or become significant. I emphasize this point because corn oil control males from three litters had anogenital distances ranging from 1.1 mm to 1.5 mm, so there is some variance involved among individuals.
Also, it is important to emphasize the strict parameters for these experiments which produced negative results. The only effects for which the embryos were examined were hypospadias and shortened anogenital distance. A lack of these effects does not necessarily mean that these compounds did not have some other effect(s) on sexual development. These compounds at these doses could be causing altered hormone levels, altered testis development or altered prostate development, for example. Therefore, I make the point that while almost all of these effects are negative, they do not prove that these compounds have no effect on sexual development.

The experiments using tocopherol-stripped corn oil produced similar results to those using unstripped corn oil, suggesting the tocopherols in the corn oil do not ameliorate or mask the effects of these compounds. One possible explanation for this is that the amount of tocopherols in corn oil is not enough to have an effect. Another is that the tocopherols in any amount do not have any effect, and this is tested directly with the supplementation experiments described later.

**Finasteride Dose Response Series**

Administering finasteride in these experiments resulted in shortened anogenital distances as doses of finasteride increased, and administering a dose of 200 mg/kg induced hypospadias. These results are in line with the study cited earlier where rabbits dosed with 10 mg/kg of finasteride during gestational days 19 through 28 were born with shortened anogenital distances and hypospadias (Kurzrock 2000), suggesting a conserved need for a functional 5-α reductase type II enzyme for normal genital development. Since 5-α reductase type II converts testosterone to the more potent androgen dihydrotestosterone, this also suggests that dihydrotestosterone is essential for genital development. The clinical syndrome of 5-α reductase deficiency was first
described more than thirty years ago (Imperato-McGinley et al. 1974) and patients with this condition exhibit ambiguous genitalia with a clitoral-like phallus, penile hypospadias or pseudovaginal perineoscrotal hypospadias. It is, however, surprising and puzzling that the knockout 5-α reductase type II or 5-α reductase type II plus type I mice exhibit normal genitalia, although they note an altered prostate phenotype (Mahendroo et al. 2001). This is difficult to reconcile, but my experiment with 200 mg/kg of finasteride raises the possibility that a functional 5-α reductase type II enzyme is necessary for normal genital development not only in rabbits and humans but also in mice.

**Vinclozolin with Vitamin E Supplementation**

The treatments with vinclozolin were all at doses of 150 mg/kg of vinclozolin, since a fellow member of the lab, postdoctoral researcher Dr. Zhengui Zheng, had administered vinclozolin at this dose and had observed hypospadias in almost all male offspring (Z. Zheng, personal communication). Since this was a known and predictable effect of an EDC in our lab, I chose to use this dose to test the possible ameliorative effect of α-tocopherol (vitamin E). After performing these experiments, I realized that the range of hypospadic phenotypes was wide, so a rating scale was devised to quantify the severities of these phenotypes (Fig. 2-1). When comparing the average scores of the treatment groups, the only statistically significant difference is between the corn oil control group and the vinclozolin only (no vitamin E supplementation) group. The fact that the vitamin E supplementation groups (both 200 and 500 mg/kg) are not significantly different from either the corn oil control group or the vinclozolin only group. I interpret these as intermediate groups in this analysis, with scores in between those of the corn oil and vinclozolin groups. This is interesting because these vitamin E supplementation groups are not different from the corn oil group as the vinclozolin only
This suggests an ameliorative effect, and were more litters added with future experiments to increase the sample size, a statistically significant difference would be more likely to emerge if it does exist. A result that would be the most supportive of my hypothesis would be if the vitamin E supplementation groups were significantly different from the vinclozolin only group but not the corn oil group.

Also of note was the 200 mg/kg vitamin E litter (seen in Figure 3-14) which had a lower score than the other litters within the group (1.17 versus 2.00, 2.00 and 2.36). I had initially thought this litter to be an outlier and that the mother had somehow not received the appropriate dose of vinclozolin. After re-analyzing this litter, I can say that the dose was probably administered correctly. This litter has males with scores ranging from 0.5 to 3, with a fair amount showing defects. This lead me to conclude that this is most likely biological variation in these individuals’ susceptibility to the treatment and may seem like less of an outlier if more experimental groups (litters) were added to these studies.

Based on the results presented here, I propose that additional experiments should be performed to increase the number of experimental groups (litters) for each treatment. It would also be warranted to add treatment groups with higher doses of vitamin E in order to determine whether the effects of EDCs can be altered and whether such alterations are statistically significant. I believe that if the vitamin E is indeed ameliorating the effect of the vinclozolin then it is doing so by reversing the oxidative stress caused by the vinclozolin on the Leydig cells of the testes, allowing them to produce testosterone at higher levels than if they had only been exposed to vinclozolin. This, however, will have to be tested in future studies by examining the testes of treated
males as well as hormone levels. Also, it would be interesting to analyze gene expression levels, as with quantitative real-time PCR, to see if genes known to be important in genital development are upregulated or downregulated in response to exposure to vinclozolin and whether or not these levels are altered by vitamin E supplementation.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Lori Boger received a Bachelor of Science majoring in biology from Nicholls State University in Thibodaux, Louisiana in 2007. Here she received the TOPS Honors Award Scholarship, the Louisiana Board of Supervisors Academic Scholarship, and the Burt Wilson Outstanding Honors Biology Student Award. Lori was also the recipient of the Dr. Linton E. Grinter College of Medicine Graduate School Fellowship and the Alumni Graduate Program Award while she attended the University of Florida.