ABSTRACT

WOLF, CYNTHIA JEAN. Mechanisms through which an environmental antiandrogen, vinclozolin, induces malformations of the male rat reproductive tract. (Under the direction of Gerald A. LeBlanc).

Androgens activate the androgen receptor (AR) to stimulate transcription of gene products that mediate local control of morphogenesis of the male reproductive tract. The fungicide vinclozolin (V) interferes with male reproductive tract development by antagonizing the AR. In this dissertation research, we investigated whether V also may elicit antiandrogenic effects by suppressing the AR or suppressing epidermal growth factor (EGF) and its receptor. EGF has been implicated as a local contributor to the androgen response.

Our first objective was to identify a dosing regime that would reliably interfere with masculine development of exposed male fetuses. Specific demasculinizing effects of V were also evaluated. Administration of V to the rat dam at 200 mg/kg from gestational day (GD) 14 - 19 induced a full array of antiandrogenic effects. Malformations, including cleft phallus, vaginal pouch and ectopic testes, were induced in 97% of the male offspring. V at 400 mg/kg increased the severity of these malformations without inducing overt toxicity.

Next, we used this dosing regime (200 mg/kg on GD 14 - 19) to test the hypothesis that co-administration of an androgenic competitor for the AR, testosterone propionate (TP), would antagonize V in vivo and ameliorate its effects. TP attenuated most antiandrogenic effects of V in the male offspring. V attenuated the masculinizing effects of TP in the female. The results indicate that V and androgen TP are competing
for occupancy of the AR and can attenuate the effects of the other.

We then investigated whether the EGF and the EGF receptor (EGFR) are employed downstream of the AR in androgen action, and are altered by V. First, we tested the hypothesis that EGF is a downstream agent of androgenic action by evaluating whether EGF co-administration during prenatal development would allow normal masculinization of V-treated fetuses. EGF at 20 or 100 µg/kg to the dam did not induce androgenic effects or attenuate antiandrogenic effects of V in the offspring.

We next tested the hypothesis that AR inhibition by V reduces EGFR expression in affected tissue. We analyzed EGFR expression levels in the fetal phallus exposed to V by immunohistochemistry. EGFR levels were not altered on GD 18 in the male phallus by V exposure. We concluded that EGFR levels do not play a role in initiation of the antiandrogenic effects of V.

Lastly, we hypothesized that AR expression is reduced by V in affected tissue immediately following exposure. We analyzed AR expression in weanling and adult sex accessory glands and in the fetal phallus of males exposed to prenatal V. We found that AR expression was not altered in the adult, weanling, or fetus by prenatal V. These results suggest that altered AR expression level is not a mechanism by which V induces antiandrogenic effects.

In summary, we have found that prenatal exposure to V produces a variety of permanent antiandrogenic effects in the male rat fetus. These effects can be attenuated with exogenous androgen treatment, which is consistent with the known ability of V to inhibit the AR. No evidence was generated to suggest that V also elicits antiandrogenic activity by suppressing AR levels, or suppressing EGF or EGFR levels. These results do
not disprove an association of EGF in AR action. Additional studies are required to
definitively establish any putative role of EGF in development of the male reproductive
tract.
MECHANISMS THROUGH WHICH AN ENVIRONMENTAL ANTIANDROGEN, VINCLOZOLIN, INDUCES MALFORMATIONS OF THE MALE RAT REPRODUCTIVE TRACT

by

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BIOGRAPHY

I was born on September 27, 1963, along with my twin sister, in St. Peter’s Hospital in New Brunswick, NJ to Paul and Jeanette Wolf. I spent my childhood in a public housing project on Bunns Lane in Woodbridge, NJ, as one of 5 children, playing and competing with neighborhood children and escaping with my father on weekends to the country to hunt. I attended schools in the Woodbridge Township school system and in Woodbridge Senior High School participated in Track and Field, the senior class play, and graduated in the top 10th percentile. The following year I attended Cook College, the agricultural school of Rutgers - The State University of New Jersey, with a major in Animal Science/ Pre-veterinary option. There I enjoyed various extracurricular activities including Rutgers Women’s Crew, intramural sports, the Veterinary Science Club, the Forestry and Wildlife Club, and Agricultural Field Day events. I graduated in the spring of 1985. After a period of self evaluation, I relocated to North Carolina and started graduate study at North Carolina State University. I received a Master of Science degree in Physiology in 1994. I obtained a position with the Environmental Protection Agency, committed to a relationship, and bought a house within a year. While employed, I enrolled in a PhD program in the Department of Environmental and Molecular Toxicology at NCSU. During this time, my beautiful daughter, Amanda, was born. I received a PhD degree in the fall of 2002. I plan to continue to work in reproductive toxicology at the EPA, and to be a good example to my daughter.
ACKNOWLEDGEMENTS

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I thank the EPA for allowing me the flexibility to pursue academic interests. I thank the current and past members of my committee, especially Dr. Gerald A. LeBlanc and Dr. L. Earl Gray, Jr. for giving me a chance, along with Dr. Russell Borski, and Dr. Stacy Branch, for also sticking through this with me for seven long years, and Dr. Sharon Meyer, Dr. Lee Robinette, and Dr. John Vandenbergh for their support and contributions. I also thank the technicians and students at the EPA for their assistance and support.
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INTRODUCTION

Vinclozolin is a fungicide widely known as an antiandrogenic environmental endocrine disruptor. This dissertation is concerned primarily with the endocrine disrupting, specifically antiandrogenic, quality of vinclozolin, and mechanisms by which it induces malformations of the reproductive tract in male rats during fetal development. To explain the need to study endocrine disrupters, an overview of endocrine disruption is provided. In order to study the mechanisms involved in the action of vinclozolin, basic information on androgens as well as antiandrogens, the hormonal regulation of sexual differentiation and mechanism of androgen action will be reviewed.

Endocrine Disruption

Endocrine disruption is a term applied to the mechanism of the effects of environmental pollutants on the endocrine system. This field of study evolved from evidence in nature that the reproductive systems of animals were being affected by chemicals in the environment. This realization started in 1947 with an observation as glaring as a decline in the population of birds and their errant or absent mating behavior (Broley, 1958). Other examples of adverse reproductive effects in wildlife later surfaced, including egg shell thinning (Broley, 1958; Hunt and Hunt, 1973), wasting syndrome in chicks, and female-female mating pairs in gulls (Hunt and Hunt, 1977). Comparisons were made between these effects in wildlife and effects in laboratory animals exposed to specific man-made chemicals. The egg shell thinning and wasting syndrome in chicks of
the 1960's and 70's looked suspiciously similar to the laboratory effects of DDT and
dioxin (Colburn et al., 1996). In addition, chemicals known to have adverse reproductive
effects in the laboratory were detected in the environments of those animals showing
impaired reproductive function. High levels of PCBs were detected in waters and fish
consumed by otters, eagles, gulls, and even women with reduced reproductive capacity
(Colburn et al., 1996). Then, laboratory investigation confirmed that DDT caused
“feminization” of chick embryos (Fry and Toone, 1981). The conviction that these
hormonally active chemicals found in the environment were the cause of the adverse
reproductive effects was also supported by evidence in the medical and research world.
In the 1940's and early 1950's, the estrogenic chemical diethylstilbestrol (DES) was given
to pregnant women for the purpose of reducing morning sickness. The drug was later
found responsible for effects in the sons and daughters of these women, including
undescended testes (Gill et al., 1979), abnormal development of the uterus, and cancers
of reproductive tract (Gunning, 1976; Herbst et al., 1979) and infertility (Palmer et al.,
2001). The DES debacle brought to everyone’s attention not only the idea that chemicals
ingested by the mother can have adverse effects on the offspring, as found with
thalidomide use, but that chemicals that mimic endogenous hormones such as estrogen
also can be harmful to humans, and adverse effects may be observable only later.
Environmental and medical data together heightened the concern for adverse reproductive
effects in humans by environmental chemicals. By 1979 the first workshop on this issue
was held, entitled Estrogens in the Environment (McLachlan, 1980). In 1991, an
international workshop called Wingspread re-evaluated claims and determined that
environmental pollutants were responsible for these reproductive problems in wildlife and that they posed a threat to humans as well (Colburn and Clement, 1992; Colburn et al., 1998). The report called for further research designed to detect environmental endocrine disrupting chemicals and analyze the risk of their effects in humans and wildlife. In 1996, under the Food Quality Protection Act and the Safe Water Drinking Act, Congress called for a committee, to establish a series of protocols for screening chemicals for hormonal activity and testing their activity in rodent models. This committee was the Endocrine Disruptors Screening and Testing Advisory Committee (EDSTAC), which gave rise to the Endocrine Disruptors Screening Program (EDSP; USEPA, 2002). This program is intended to aid in the assessment of chemicals for registration for use.

We continue to see adverse reproductive effects in wildlife suspected to be induced by environmental pollutants: in the masculinization and decline in population of mud snails near marinas (Smith, 1971), sudden decline of a seal population along the English coast after a chemical spill (Colburn, 1996), and the severely reduced penis size and decline in population of alligators in Lake Apopka, Florida, associated with high levels of DDE in their eggs and linked to a dicofol spill (Guillette et al., 1994). Concern has risen over an environmental cause for increased incidents of reproductive effects in humans, including reduced sperm counts (Skaakebak, 1972; Swan et al., 2000), cryptorchidism and the rise in incidences of hypospadias (Palouzzi, 1997; Chambers, 1999). Another factor that heightens concern is that these environmental endocrine disrupters are everywhere - in lakes and rivers, in toiletries, in medical devices, in
pesticides sprayed on lawns.

*Classes of Endocrine Disrupters*

Chemicals found in the environment that affect reproduction in animals were variably termed environmental estrogens, xenoestrogens, endocrine active compounds, environmental endocrine disrupters, or endocrine disrupting compounds (EDCs; McLachlan, 2000). These chemicals basically act either as hormone mimics, or agonists, or hormone blockers, or antagonists. They typically affect a sex steroid hormone pathway and can interfere with any component of the signalling pathway, usually the receptor, but including synthesis, metabolism or clearance of the hormone, and other hormone systems that regulate the hormone’s action. EDCs usually interfere with estrogen or androgen action, but include those that affect the action of any hormone, commonly thyroid hormones, progesterone or pituitary hormones.

Most of the chemicals initially found to have endocrine disrupting ability were estrogenic or perceived as so. Estrogenic EDCs mimic estrogen in the body and include DDT, PCBs, phenols such as bisphenol-A, alkylphenols such as nonylphenol, and some plant sources, or phytoestrogens, including genistein and coumestrol (McLachlan, 2000). Antiestrogens include some phytoestrogens such as indolo[3,2-b]carbazole (Liu et al., 1994), industrial organochlorines such as toxaphene (Bonefeld-Jørgensen et al., 1997), and may include the Ah receptor ligand, dioxin, the most common form being TCDD (Faqi and Chahoud, 1998). More recent study has revealed that chemicals in the environment also have antiandrogenic or androgenic activity.

*Androgens and Antiandrogens*
Androgenic chemicals are a smaller and more recently detected class of EDC. The masculinization of female mud snails was found to be attributable to tributyltin (TBT; Smith et al., 1981) due to its disruption of testosterone biotransformation and resultant accumulation of testosterone in the animal (Gooding et al., 2002). The masculinization of female mosquito fish and killifish are attributable to an as of yet unidentified androgenic factor in waters downstream of pulp mill effluent (Davis and Bortone, 1992; Parks et al., 2000). It has been hypothesized that plant sterols undergo transformation by bacteria to form testosterone or other androgenic compounds that could be responsible for masculinization of fish (LeBlanc, 1995; Guillette et al., 2000; McLachlan, 2000). Also, trenbolone acetate given to beef cattle to improve weight gain was shown to cause masculinizing effects including clitoral enlargement and increased AGD in female rat offspring (FDA summary, 2000) or enlarged clitoris and androgenic effects in cells of the vagina in heifers (Groot et al., 1989). It was shown that trenbolone is an AR agonist (Wilson et al., in press). The ability of androgens to masculinize the female mammalian fetus is an important aspect of one of the hypotheses of this dissertation.

More critical evaluation of endocrine disrupting effects revealed that some chemicals that had appeared estrogenic are actually antiandrogenic. DDT increased uterine weight in rodents and binds the estrogen receptor (Welch et al., 1969; vom Saal et al., 1995) indicative of estrogenic activity. However, DDT is metabolized in vivo to DDE, and p,p’-DDE was found to bind and inhibit the androgen receptor (AR; Kelce et al., 1995). In addition, p,p’-DDE administered to pregnant rats produces in the male
offspring effects indicative of impaired androgen action (Kelce et al., 1995). An antiandrogenic EDC was discovered, and opened minds to the idea that other chemicals previously thought to be estrogenic may be antiandrogenic. The micropenis in the Lake Apopka alligators was determined to be due to the antiandrogenic effects of DDE found in the eggs (Guillette et al., 1996). Phthalates, such as dibenzyl butyl phthalate (DBP) and benzylbutyl phthalate (BBP), found in plastics, were once considered to be estrogenic due to their estrogenic activity in vitro (Jobling et al., 1995), but were found to induce effects in vivo that were indicative of an antiandrogen, including reduced AGD, nipple retention, reduced sex accessory gland weights, reduced testis and epididymis weights, induced fluid-filled testes, and reduced sperm counts (Mylchreest et al., 1998, 1999; Gray et al., 2000). Investigation revealed that diethylhexyl phthalate (DEHP) reduced fetal testosterone synthesis and induced fetal Leydig cell hyperplasia and aggregation (Parks et al., 2000). Other chemicals that can be found in the environment that have been recognized as AR inhibitors include the pesticides procymidon (Ostby et al., 1999), fenitrothion (Tamura et al., 2001), vinclozolin (Gray et al., 1994; Kelce et al., 1994) and possibly linuron (Lambright et al., 2000). The antiandrogenic effects of these AR inhibitors include reduced weights of testosterone and dihydrotestosterone (DHT)-dependent tissues including the prostate, seminal vesicle, bulbourethral glands, the levator ani muscle, reduced AGD which is smaller in females than in males, presence of a vagina which is normally inhibited by DHT in males, ectopic testes, and improper development of the phallus manifested by a cleft with hypospadias. This dissertation focuses on further elucidating the mechanisms by which vinclozolin elicits these effects.
Vinclozolin

Vinclozolin is 3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione (Fig. 1), a fungicide manufactured by BASF and sold under the names Ronilan, Curalan and Ornilan (USEPA, 1998). As a pesticide, vinclozolin was subject to risk assessment for registration by the US Environmental Protection Agency. It was registered in 1981 for use on foods such as grapes, kiwi fruit, strawberries, lettuce, onions, turfgrass and ornamental plants (USEPA, 1998) but use on grapes and turfgrass in parks and schools has been terminated effective 1997 (USEPA, 2000). Vinclozolin has a half-life of 23 days when sprayed as Ronilan, a 50% mixture of vinclozolin, on soil, while 6-12% of the original compound is present after 12 months (Szeto et al., 1989a). On plant leaves, vinclozolin is detectable on the leaf as the parent compound but does not wash off (Szeto et al., 1989b), since it is more soluble in oil than in water (Szeto et al., 1989c). This implies that vinclozolin does not wash off easily from foods. Vinclozolin is unstable in methanol or in water and undergoes ester hydrolysis, causing its oxazolidine ring to open, and then undergoes decarboxylation to form the metabolite M1, or is attacked at another site to undergo amine hydrolysis, forming the metabolite M2, or at both sides of the nitrogen to form 3,5-dichloroaniline, or M3 (Szeto et al., 1989c). Vinclozolin can produce about 15 metabolites (USEPA, 1993). M1 and M2 are the most abundant metabolites, and both compete with synthetic androgen R1881 for binding the AR and inhibit the AR in vitro by competitive inhibition (Kelce et al., 1994). M2 is formed irreversibly following first-order kinetics, (Szeto et al., 1989a), and is the more potent AR-inhibitor, with a K_i of 9.65 μM, versus 92 μM for M2 (Kelce et al., 1994).
Vinclozolin administered to the rat inhibits androgen-dependent gene product C3 and increases testosterone repressed prostatic message (TRPM2), which confirms the mechanism of action is AR inhibition in vivo (Kelce et al., 1997). M1 and M2 were found in the serum of rats dosed with vinclozolin, which confirms the in vivo metabolism of vinclozolin (Kelce et al., 1994). Therefore, vinclozolin is readily metabolized to M1 and M2 in vivo and these metabolites competitively inhibit the AR in vivo. Neither vinclozolin, M1 or M2 inhibit 5α-reductase at any doses that elicit antiandrogenic effects in vivo, and only weakly at high doses (Kelce et al., 1994). The antiandrogenic activity of vinclozolin in rats was detected by investigators at BASF who observed areolae retention and reduction in AGD in male rats, among other signs (van Ravenzwaay, 1992). Vinclozolin administered to the pregnant rat at doses that did not affect the mother, 0, 100 and 200 mg/kg/day, from gestational day 14 to postnatal day 3 induced antiandrogenic effects in the male offspring at 100 and 200 mg/kg, including reduction in AGD from day of birth to puberty, reduction in ventral prostate and seminal vesicle weights, reduction in epididymal sperm, granulomas of the testes and epididymides, bladder stones, and induction of malformations of the genitalia that include a vaginal “pouch” at the base of the phallus without a complete vagina, ventrally cleft phallus, ectopic testes, and ventral prostate agenesis. AGD was permanently reduced by 200 mg/kg vinclozolin (Gray et al., 1994). It was later found that AGD is reduced and areolae are induced with perinatal exposure to vinclozolin at a dose as low as 3 mg/kg/day, and severity of the effect increases linearly with dose (Gray et al., 1999). The importance of this finding is that back extrapolation along this straight line predicts
an effect at any lower dose. Some toxic effects of vinclozolin have also been observed. Body weight was reduced in male offspring into adulthood also (Gray et al., 1994). Reduced liver and adrenal weights, and increased alanine aminotransferase (ALT, an indicator of liver toxicity) and bilirubin in the liver were reported in mice (USEPA, 1993). Vinclozolin can form an epoxide, dichloroanilide, and may have cancer initiating or promoting activity (Chiesara et al., 1982; Lioi et al., 1998). These effects were induced at doses higher than those used in reproductive studies, and this activity does not appear to be a component of the antiandrogenic effects seen in vivo.

Sexual Differentiation in Embryogenesis

To understand how vinclozolin induces antiandrogenic effects and to support further investigation into the mechanisms of action of vinclozolin, a basic understanding of sexual differentiation and timing of AR expression is needed. The mammalian fetus undergoes sexual differentiation in late gestation during the later stage of organogenesis. In the rat, most androgen-dependent processes begin on gestational day (GD) 14 (postcoital day 14; Clark et al., 1993; Husmann and McPhaul, 1991). Before differentiation begins, the reproductive tract is bipotentate. It contains both the Mullerian ducts, which potentially develop into the oviducts and uterus in the female, and the Wolffian ducts, which potentially develop into the components of the male reproductive tract. The genetic sex of the mammalian fetus determines the direction of development of the gonads - basically, to ovaries in the XX female and to testes in the XY male. Gonadal hormones in turn determine the phenotypic sex of the animal. In the absence of hormones, regardless of genetic sex, the reproductive tract develops into the female
phenotype, and in the presence of testicular androgens, develops into the male phenotype (Jost, 1953). Testosterone is produced by the fetal testes on GD 14 in the rat (Picon, 1976) and acts on AR of nearby Wolffian duct tissue to induce development into the epididymis, vas deferens, and seminal vesicle (Schultz and Wilson, 1974). The Mullerian ducts regress in response to another hormone from the testes, Mullerian inhibiting substance (MIS) or anti-Mullerian hormone (Josso et al., 1977). Testosterone also promotes growth of muscles including the levator ani /bulbocavernosus muscles which are attached to the penis (Tobin and Joubert, 1991), and is a major factor in mediating testicular descent (Elder et al., 1982; Levy and Husmann, 1995). Testosterone is reduced to dihydrotestosterone (DHT) by the enzyme 5α-reductase in some tissues including the Wolffian ducts and to a greater extent the urogenital sinus (UGS) tissue (Thigpen et al., 1993). DHT binds AR with 2X more affinity than that of testosterone (Luke and Coffey, 1994) to induce development and differentiation of urogenital sinus tissue to the prostate and the male phenotypical external genitalia (Imperato-McGinley et al., 1992) which involves fusion of genital folds to form a urethra, elongation of the penis, and development of a scrotum (Hughes et al., 2001). DHT also inhibits development of the mammary anlagen that form nipples in the rat (Goldman et al., 1976; Topper and Freeman, 1980; Imperato-McGinley et al., 1992). The bulk of sexual differentiation in the rat continues to roughly GD 21 (Clark et al., 1993), slightly after the peak in testosterone levels at GD 18 and 19 (Weisz and Ward, 1980), although development is not fully completed in many reproductive tissue until after puberty (Cunha, 1992).
The timing of expression of AR in each tissue determines the timing of the initiation of development of that tissue. AR are first expressed in the rat testes on GD 14-15 (Bentvelsen et al., 1995; You and Sar, 1998) and then appear in other parts of the Wolffian duct further from the testes in a gradient, decreasing with increasing distance from the testes (Bentvelsen et al., 1995). Therefore, the seminal vesicles acquire AR later than the epididymis and vas deferens (Bentvelsen et al., 1995). The urogenital tubercle and the mammary anlagen express AR at GD 14 (Bentvelsen et al., 1995; Imagawa et al., 1994) and the prostate at GD 16 (Bentvelsen et al., 1995). AR expression increases with time throughout the fetal period (Bentvelsen et al., 1995; You and Sar, 1998). The onset of AR expression in these tissues are coincident with onset of differentiation of these tissues, and thus their sensitivity to antiandrogenic action depends on timing of administration of the antiandrogen.

These masculine features that characterize the male phenotype are the result of T or DHT binding the AR. Improper function of the AR, such as mutation of the AR or inhibition of the AR, results in impaired development of androgen-dependent tissue (Wilson, 1992). Conversely, introduction of androgens to the female fetus causes retention of Wolffian duct tissue or development of other androgen-dependent tissue, since females have AR in their reproductive tissue (Greene et al., 1939; Bentvelsen et al., 1995). The determination of the normal phenotype correct for the genetic sex of the animals depends largely on the proper balance and timing of the hormones present in the fetus, a fact on which part of this dissertation is based.

*Mediation of Androgen Action: Local Growth Factors*
Developmental effects initiated by androgens are regulated by the entire signaling pathway(s) involved in androgen action. A more thorough look at what comprises “androgen action” provides information on the finer control of androgen-dependent sexual differentiation.

The AR is a 110 kD nuclear steroid receptor and a transcription factor. It is composed of the ligand binding domain, DNA binding domain, an amino terminus which contains a transcriptional activation site, and a hinge region between the ligand-binding and DNA-binding domains, which plays a role in nuclear transport (Bolander, 1994). Activation of AR occurs by binding of androgen to the AR with release of heat shock proteins (HSP) 70 and 90 from the AR, dimerization of the AR, transportation to the nucleus, subsequent binding of cofactors to the androgen-AR dimer complex, binding of this complex to the androgen response element (ARE) and gene activation (Keller et al., 1996; Hughes et al., 2001). However, activation of the AR can occur through non-ligand binding, by interaction with other transcription factors (Verhoeven and Swinnen, 1999) or by binding of other hormones such as glucocorticoids or estrogen (Keller et al., 1996). More than one ARE exists, a second promoter was found on the AR (Grossman et al., 1994) and various cofactors exist in various tissue. These variations may account for variation in the response to androgens among tissues, or between hormones with different affinity or activity (Keller et al., 1996; Haendler, 2002; Verhoeven and Swinnen, 1999).

AR action can be controlled by the availability of androgen. T and DHT are bound to a carrier protein, steroid hormone binding globulin (SHBG), in blood before being released to the AR-containing cell. Levels of SHBG could alter AR activation. T
and DHT can compete for binding at the receptor with other ligands, such as an AR-binding antiandrogenic pesticide, which could alter AR activation. AR action can be controlled also by the level of expression of AR. In this way androgens can execute variable responses in various tissue, or variable responses with time. AR expression can also be altered by other hormones such as estrogen (Prins and Birch, 1995) and AR can be downregulated by androgen itself (Quarmby et al., 1990). Transcriptional activity of the AR can be modulated by hormones such as FGF (Cronauer et al., 2000), FSH and cAMP (Blok et al., 1989; Lindzey et al., 1993).

Post-receptor mechanisms of androgen action can regulate the action of AR. Evidence suggests androgens initiate the physiological processes of sexual differentiation via local proteins transcribed by AR activation. Androgens in the fetal mesenchyme are responsible for initiating physiological processes such as morphogenesis, epithelial proliferation, epididymal coiling, ductal branching in the prostate, prevention of apoptosis in the Wolffian ducts (Cunha et al., 1992; Cunha, 1996; Hughes et al., 2001), and regression of mammary anlagen (Imagawa et al., 1994) before AR appear in the epithelium, where these processes are taking place. Tissue recombination experiments show that the mesenchymal AR of normal rats initiate morphogenesis in the Tfm epithelial layer, showing that AR is not solely or directly responsible for these changes, but that products that pass to other cells carry out AR action (Donjacour and Cunha, 1993). These agents of androgen action, or andromedins (Verhoeven and Swinnen, 1999), in fetal development have been identified as growth factors, including fibroblast growth factor (FGF), keratinocyte growth factor (KGF), and epidermal growth factor.
(EGF) and regulators of development include TGF-β (Cunha, 1996). EGF has been linked to androgen-dependent growth of components of the male urogenital tract, such as the testis, prostate, seminal vesicle, and external genitalia (Cunha, 1996; Culig et al., 1996; Liu et al., 1994; Gupta et al., 1996; Hayward et al., 1996; Thomson et al., 1997; Levine et al., 2000).

EGF is a 53 amino acid polypeptide that binds a tyrosine kinase receptor called the EGF receptor (EGFR; Bolander, 1994). EGF and the EGFR has been associated with the male and androgen-dependent processes. It is present in greater quantities in placentas of male fetuses than those of female (Brown et al., 1987) and plays a role in prostate growth and spermatogenesis (Cunha, 1996; Bartlett et al., 1990). EGF or the EGF receptor can be induced by androgen (Hiramatsu et al., 1988; Ravenna et al., 1995; Brass et al, 1995) and reduced by castration or antiandrogen (Nishi et al., 1996; Monti et al., 1997). EGF itself may have androgen activity. EGF induced the AR-regulated gene PSA in the absence of androgen in prostate cancer cells (Culig et al., 1995). Also, EGF co-administration was able to reverse flutamide-induced inhibition of testis descent (Cain et al., 1994). In addition, some studies have shown that androgen action is dependent on EGF. In one study, anti-EGF prevented Wolffian duct development in rats (Gupta, 1996), and in another study, the absence of EGF by sialoadenectomy inhibited androgen-dependent sex accessory gland development and sperm production (Liu et al., 1994).

Together, this body of evidence suggests that EGF may be a down-stream messenger of AR action. However, EGF was shown to induce testosterone in Leydig cells (Syed et al., 1991) and modulate transcriptional activity of AR or activate AR (Gupta, 1999; Culig et
al., 1995). Therefore, EGF may be a modulator of AR action. Nevertheless, these studies suggest the pathways are coupled.

This dissertation investigates the mechanisms through which vinclozolin alters male reproductive development, focusing on the AR and the involvement of EGF and its receptor. Most of the investigation into the mechanisms of vinclozolin have been performed using tissues from the pubertal or adult male exposed either perinatally or peripubertally. As discussed, the altered physiology of the rapidly developing fetus may cause the response to vinclozolin to vary as the mechanism of antiandrogenicity may be regulated in a different manner. This dissertation is concerned with the effects of vinclozolin on the male rat offspring exposed prenatally. The overall hypotheses are that vinclozolin alters sexual differentiation of the male rat by altering AR and EGFR levels in addition to inhibiting the AR, and that EGF or testosterone propionate can compensate for loss of androgenic activity by vinclozolin. The first chapter uses variable exposure times to find the period of sensitivity during fetal development to the effects of vinclozolin. The dose of vinclozolin required to elicit the effects in greater than 90% of the offspring is then determined for future mechanistic investigation in fetal tissue. The second chapter is a dose-response study of the effects in the female offspring of testosterone propionate (TP) administered during the same prenatal period. This was done to find a dose of TP that was potent enough to elicit androgenic effects while not eliciting toxicity in the dam or in litter size, i.e. suitable for co-administration with vinclozolin. The third and fourth chapters report the findings of a study designed to
address the hypothesis that co-administration of an androgen, TP, can attenuate the
effects of an AR-inhibitor, vinclozolin, *in vivo*, and vice versa. The fifth chapter
addresses the hypothesis that EGF is involved in androgen action, and therefore in the
antiandrogenic action of vinclozolin. In this chapter, EGF is co-administered with
vinclozolin to assess whether EGF can alleviate the effects of vinclozolin in the male
offspring. Also, tissue from adult and fetal rats exposed to vinclozolin during sexual
differentiation are analyzed for AR and EGFR expression levels, and testosterone levels.
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Figure Legend

Figure 1. Chemical structure of vinclozolin, 3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-2,4-oxazolidinedione.
Figure 1
Characterization of the period of sensitivity of fetal male sexual development to vinclozolin

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Abstract

Vinclozolin is a fungicide whose metabolites are androgen receptor (AR) antagonists. Previous work in our laboratory showed that perinatal administration of vinclozolin to rats results in malformations of the external genitalia, permanent nipples, reduced anogenital distance (AGD) and seminal vesicle, ventral prostate, and epididymal weights. The objectives of this study were to determine the most sensitive period of fetal development to antiandrogenic effects of vinclozolin and to identify a dosing regime that would induce malformations in all of the male offspring. Pregnant rats were dosed with 400 mg vinclozolin /kg/d on either GD 12-13, GD 14-15, GD 16-17, GD 18 -19, or GD 20-21, or corn oil (2.5 ml/kg) from GD 12 through GD 21 (experiment 1). All 2 day periods in which significant effects were produced were included in an extended dosing period, GD 14 through GD 19, in which pregnant rats were dosed with 200 or 400 mg vinclozolin/kg (experiment 2). In experiment 1, significant effects of vinclozolin were observed in rats dosed on GD 14-15, GD 16-17 and GD 18-19, while the most significant effects were observed in rats treated on GD 16-17. These effects include reduced AGD, areolas and nipples, malformations of the phallus and reduced levator ani/bulbocavernosus weight. In contrast, ventral prostate weight was reduced only in the GD 18-19 group. The expanded dosing regime (experiment 2) increased the percentage of male offspring with genital malformations (>92 %), and retained nipples (100 %), further reduced the weight of the ventral prostate and reduced the weight of the seminal vesicles. In addition, malformations were more severe and included vaginal pouch and ectopic/undescended testes. Ectopic/undescended testes was induced only in the 400 mg/kg group. These data indicate
that the reproductive system of the fetal male rat is most sensitive to antiandrogenic effects of vinclozolin on GD 16 and 17, although effects are more severe and 100 % of male offspring are affected with administration of vinclozolin from GD 14 through GD 19.

**Keywords:** vinclozolin, antiandrogen, androgen receptor, male reproductive development, critical period, levator ani, hypospadias, anogenital distance
Introduction

Endocrine disruptors, or EDCs (endocrine disrupting compounds), have been implicated in adverse effects on the populations and reproductive processes of wildlife such as egg shell thinning in eagles and gulls (Feyk and Giesy, 1998; Grier, 1982; Hunt and Hunt, 1973; Lundholm, 1984), female-female pairing of gulls and terns (Hunt and Hunt, 1973; 1977), imposex in gastropods (Gibbs et al., 1991), intersex in crustaceans near sewage discharge (Moore and Stevenson, 1991), small phallus in Florida alligators (Guillette et al., 1994), and cryptorchidism in the Florida panther (Facemire et al., 1995). In humans, trends in reproductive system abnormalities such as declining sperm counts and cryptorchidism (Carlsen et al., 1992), testicular cancer (Weir, et al., 1999), prostate cancer, and hypospadias have been linked to EDCs. Alterations in the human reproductive system continue to appear. Two surveillance areas in the United States reported a rise in the incidence of hypospadias in newborn boys from 1968 to 1993 (Dolk, 1999) and the incidence of testicular germ cell cancer is reported to have risen from 1964 to 1996 in Canada (Weir et al., 1999) and most other countries as well (Skaakebacke). EDCs, which can act as hormone mimics or antagonists to disrupt the reproductive system, can be especially injurious during gestation because of the undifferentiated state of the reproductive tract and the lack of compensatory homeostatic mechanisms. Vinclozolin is an EDC that acts as an antiandrogen, impairing development of the male rat reproductive system when administered during sexual differentiation.

Vinclozolin, or 3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione, is a fungicide used on fruits, vegetables, turfgrass and ornamental plants (USEPA, 1998).
Antiandrogenic effects of vinclozolin exposure have been found in our lab and by others in several mammalian species such as the rabbit, mouse, rat and dog (USEPA, 1993). Vinclozolin degrades in the environment or is metabolized in the body to several metabolites, two of which (M1 and M2) have been shown to competitively inhibit the androgen receptor (AR) \textit{in vitro} (Kelce et al., 1994; Wong et al. 1995). \textit{In vivo}, vinclozolin inhibits AR-dependent gene expression (Kelce et al., 1997) and produces a spectrum of anatomical defects. Administration of 50 and 100 mg/kg vinclozolin to rats on gestational day (GD) 14 through postnatal day (PND) 3 resulted in effects in the male offspring similar to those caused by flutamide, a well known AR antagonist. These effects include reduced anogenital distance (AGD), persistent nipples, cleft phallus, hypospadias, reduced weights of the ventral prostate, seminal vesicles and epididymis, and reduced sperm counts (Gray et al., 1994; 1999a). In addition, peripubertal administration of 100 mg/kg vinclozolin to male rats delays puberty and reduces weights of androgen-dependent tissues including the ventral prostate, seminal vesicles and epididymis (Monosson et al., 1999). Doses as low as 3 to 12.5 mg/kg administered perinatally reduce AGD and ventral prostate weight and induce areolas in males (Gray et al., 1999a). These responses in male offspring to low doses of vinclozolin emphasize the sensitivity of the developing fetus and pup to vinclozolin.

The heightened susceptibility of the fetus to antiandrogenic insult can be explained by the onset of AR expression during sexual differentiation, and the fact that normal AR function is essential for male reproductive tract differentiation. AR appear first in the testes at GD 14 (Bentvelsen et al., 1995) or GD 15 (You et al., 1998) and the mesenchyme of the fetal reproductive tract nearest the testes (Prins and Birch, 1995; Cooke et al., 1991;
Bentvelsen, et al., 1995), and the mesenchyme of the mammary anlage (Imagawa et al., 1994) at about GD 12 (mouse) or GD 14 (rat). The appearance of AR along the tract is coincident with the production of testosterone (T), by the testes and the onset of sexual differentiation of the reproductive tract (George and Wilson, 1994). Sexual differentiation is dependent on the androgens T or dihydrotestosterone (DHT), and on AR function. The role of androgens in male sexual development is made evident from human genetic and animal studies. Human males with mutations of the AR display androgen insensitivity syndrome, in which males have testes but display female-like external genitalia and breast development with variable development of internal male reproductive tract (Quigley et al., 1995). Males lacking 5α-reductase, which converts T to DHT, also display female-like genitalia but have normal Wolffian duct structures (Wilson et al., 1981). Similar results have been obtained in animal studies using antiandrogens that either inhibit the AR, such as flutamide or vinclozolin, or inhibit the 5α-reductase conversion of T to DHT, as with finasteride. The development of the Wolffian duct system, which includes the epididymides, vas deferens, and seminal vesicles, is much more affected by an AR inhibitor than by a 5α-reductase inhibitor (Imperato-McGinley et al., 1992), which illustrates T dependency in the Wolffian duct system. Testicular descent (Husmann and McPhaul, 1991a; Imperato-McGinley et al., 1992; Spencer et al., 1991) and levator ani weight (Breedlove and Arnold, 1983; Tobin and Joubert, 1991; van der Schoot, 1992) also appear to be mediated by T. In contrast, perinatal finasteride exposure did not affect the epididymis or vas deferens, and the incidence of undescended testes was much lower, although finasteride treatment greatly reduced the prostate weight, prevented closure of the penile folds as evidenced by a cleft
phallus, and allowed for development of nipples and a vaginal pouch (Clark et al., 1990; Imperato-McGinley et al., 1992), which reflects a dependency of the urogenital sinus, perineum and mammary anlage on DHT. Finasteride or flutamide treatment also induce hypospadias, microphallus and preputial adhesion in rodents and monkeys (Silversides et al., 1995; Prahalada et al., 1997).

We hypothesize that the development of any of these structures can be most effectively altered by an AR inhibitor such as vinclozolin at the time of onset of differentiation of each structure and coincident appearance of AR in the mesenchyme. Dose alone does not determine the poison. The timing, duration and dose of the antiandrogenic agent used will influence the severity and spectrum of effects. The objectives of this study were to establish the critical window(s) during fetal development within which male offspring are most sensitive to the effects of vinclozolin on the reproductive organs, and to characterize the spectrum and level of antiandrogenic effects obtained from these dosing regimes for use in future studies. In one experiment, we dosed rats in two-day windows on GDs 12 and 13, 14 and 15, 16 and 17, 18 and 19, and 20 and 21 using a high dose (400 mg/kg) of vinclozolin. In a second experiment, all affected stages were included in an extended dosing period in order to find a dose that would significantly affect all of the offspring. This dosing regime will then be used for future studies on the cellular and molecular effects of vinclozolin on male fetal tissue where it is crucial that every fetus be affected.

**Methods and Materials**
Two day window study (Experiment 1). Two sets of 90 day old female Long Evans hooded rats from Charles River Laboratories (Raleigh, NC) arrived at the animal facility on gestational day (GD) 3 (day of sperm positive smear = GD 1) on two separate dates. They were housed one per cage in polycarbonate cages (20 cm X 25 cm X 47 cm) with laboratory grade pine shavings as bedding in an atmosphere of 68-72°F, 40-50% relative humidity and a reversed light schedule (14 h light, 10 h dark: lights off at 11:00 am EST), and fed rat chow (Purina chow 5001) and water ad libitum. On GD 10, dams were weighed, weight ranked and randomly assigned to treatment groups that were equilibrated with respect to body weight. Dams were dosed with 400 mg/kg-bw vinclozolin (Crescent Chemical; Hauppauge, NY; lot# 10560) by oral gavage on GDs 12 and 13 (GD12-13; n = 6), 14 and 15 (GD14-15; n = 5), 16 and 17 (GD16-17; n = 5), 18 and 19 (GD18-19; n = 5), or 20 and 21 (GD20-21; n = 5), or with corn oil vehicle (Sigma; 2.5 ml/kg-bw;) on GD 12 through 21 (control; n = 6). On postnatal day (PND) 1 (day of delivery) pups were counted, weighed, and sexed, and anogenital distance (AGD) was measured in a blind fashion using a dissecting scope fitted with an ocular micrometer. On PND 13, male pups were checked for areolas, and a nipple or an areola was reported as an areola. Male offspring were weaned on PND 23 and litter mates were housed 2-3 per cage. Females were euthanized at weaning age. On PND 77-102, a portion of the rats (n= 73; all 28 from block 1, 44 from block 2) were sacrificed using decapitation, shaved for viewing nipples, and necropsied. Malformations of the genitalia, including cleft prepuce, cleft phallus, exposed os penis, hypospadias, vaginal pouch and undescended and ectopic testes, were identified and recorded. Vaginal pouch was recorded if severe, and ‘severe’ was characterized by a large, deep, unambiguous orifice at the base
of the phallus. The right testis and epididymis, the ventral prostate (VP), seminal vesicles (fluid-filled, + coagulating gland; SV) and levator ani with bulbocavernosus muscles (LA/BC) were weighed. Necropsy was performed as above on PND 175-176 (n= 35 animals, block 2) except that right testis and SV, which appeared unaffected at PND 70-102, were not weighed.

*Extended window study (Experiment 2).* Methods were the same as for the above experiment except for the following: The experiment was performed in one set (block); Dams were dosed with 200 mg/kg-bw vinclozolin (n = 10), 400 mg/kg-bw vinclozolin (n = 10), or corn oil vehicle (control; n = 10) on GD 14 - 19; Male offspring were weaned on PND 22; Males were sacrificed on PNDs 107 - 128 (n= 105); Testis and epididymis were not weighed but were observed for abnormalities; Serum was collected for radioimmunoassay of T levels by a method described previously (Kelce et al., 1991).

*Statistics*

Data were analyzed on a litter means basis using one-way ANOVA (experiment 2), or two-way ANOVA when the experiment was performed in 2 blocks (experiment 1), using general linear models procedure PROC GLM on SAS (Cary, NC) available through the USEPA network server. AGD and organ weights were analyzed with body weight as a covariate. When general differences (p< 0.05 in ANOVA) for treatment effects were found, specific differences between groups were analyzed with a two-tailed t test using least square means. Data given in percentages (areola, nipple and malformation data) were analyzed
after arcsin transformation of litter means. Fisher’s Exact test was also performed on categorical data (malformations, vaginal pouch and ectopic/undescended testes) on an individual basis.

Results

Experiment 1.

Pre-weaning litter data. Dystocia and late delivery occurred only in the treated groups, and the incidences were very low (Table 1). Live litter size and sex ratio were not affected by vinclozolin treatment. Pup weight was slightly reduced in only one of the treated groups, GD 20-21, although overall, pup weights did not significantly differ between control and vinclozolin treated groups. Pup survival to PND 13 or to weaning was low in all groups but was not significantly reduced by vinclozolin treatment. Litters not viable at weaning (n=3) occurred only in treated groups (Table 1) but these numbers appear too low to indicate a treatment effect.

Anogenital distance. AGD was significantly reduced on PND 1 in the GD 16-17 and GD 18-19 groups, although the greatest reduction was observed in the GD 16-17 group (Table 2, Fig. 1). The reductions in AGD were not due to a reduction in pup size since body weight was not reduced in these groups (Table 1) and AGD was found by statistical analysis not to be correlated with body weight (covariate F(2,16)=3.90; p< 0.06; Fig. 1, Table 2).

Areolas. Areolas in the offspring were most easily observed on PND 13. Areolas can appear occasionally in untreated male pups and were detected in the control group (23
This percentage, which is on a litter means basis, is deceptively high since only 2 /12 control pups had an areola but represented 2 / 5 of control litters. However, the percentage of pups with areolas increased significantly in the treated groups exposed on GD 14-15 and GD 16-17, and did not appear at all in the GD 12-13 or GD 20-21 groups (Table 2). The number of areolas per pup, out of a possible 12 areolas, also increased significantly in these groups from the control value (0.33), with the highest value of 8.84 areolas appearing in the GD 16-17 group (Table 2).

**Nipples.** Since untreated male rat pups can present areolas early in life but do not retain nipples, it was important to monitor the presence of nipples in adulthood. Nipples were observed on adult male offspring in the GD 14-15, GD 16-17 and GD 18-19 groups only. The percentage of males with nipples was significant in the GD 16-17 and GD 18-19 groups, while the highest incidence appeared in the GD 16-17 group. The number of nipples per animal was also highest in the GD 16-17 group (Fig. 1, Table 2).

**Malformations of genitalia.** Malformations of the external genitalia included cleft prepuce, incomplete preputial separation, cleft phallus with accompanying exposed os and hypospadias. These malformations were observed only in the GD 14-15, GD 16-17 and GD 18-19 groups. One animal from the GD 16-17 group displayed ectopic testis (Table 2). The incidence of malformations was significant only in the GD 16-17 group by both ANOVA (p< 0.005) and Fisher’s Exact test (12/24 individuals, vs. 0/17 individuals in control group; p< 0.001; Fig. 1; Table 2).

**Body weight.** Body weight was not significantly affected in any dose group (p > 0.85; Table 3).
Organ weights. Organ weight data was analyzed in two sets representing two age ranges. The testis, epididymis and seminal vesicle were not affected in any group. No agenesis of the VP or SV were found. The ventral prostate was slightly reduced only in the GD 18-19 group evaluated at age PND 77-102, and was not reduced in any group evaluated (no data for GD 18-19 group) at age PND 175-176. The weight of the levator ani/bulbo-cavernosus muscle was significantly reduced in the GD 14-15, GD 16-17 and GD 18-19 groups at PND 77-102, while the greatest reduction was obtained in the GD 16-17 group (Fig. 1, Table 3). The LA/BC weight was also reduced in the GD 16-17 group at PND 175-176. Reductions in VP and LA/BC weights were independent of body weight by covariate analysis on a litter means basis. Reductions in VP and LA/BC weights in the earlier, large age range were independent of age as determined by covariate analysis on an individual basis (VP, covariate p< 0.919; LA/BC covariate p< 0.617).

Experiment 2

Dam and pre-weaning pup data. No dams presented dystocia or delivered late. Dam weight gain through the dosing period (GD 14-GD 19) was not significantly reduced by vinclozolin treatment. Body weight was reduced from GD 14 to GD 19 in 1 dam in the control group, 2 dams in the 200 mg/kg group, and 1 dam in the 400 mg/kg group. Litter size and pup weight on PND 1 and pup survival to PND 13 and to weaning were not significantly reduced by vinclozolin treatment (Table 4).

Anogenital distance. AGD on PND 1 was significantly reduced by vinclozolin treatment at 200 and 400 mg/kg/day and was not related to pup weight (covariate F(2,16)=
The two vinclozolin treated groups were not significantly different from each other (Table 5).

**Areolas.** Presence of areolas was significantly increased in both treated groups. While only 7.2% of male offspring in the control group (n=5/34 individuals or 4/7 litters) displayed areolas on PND 13, nearly all the males in the vinclozolin treated groups displayed areolas. The mean number of areolas per pup was also significantly higher in the 200 mg/kg group and the 400 mg/kg group. The two treated groups were not significantly different from each other (Table 5).

**Nipples.** No adult control male had nipples while 100% of the treated males had nipples. The number of nipples per animal out of a possible 12 nipples was 9.5 in the 200 mg/kg group and 9.6 in the 400 mg/kg group, both significantly different from controls (Table 5).

**Malformations of the genitalia.** While no malformations were observed in the control group, virtually all animals in the vinclozolin treated groups displayed at least one malformation of the reproductive tract and this treatment effect was significant (Fig. 2, Table 5). Malformations included cleft prepuce, cleft phallus accompanied by exposed os and hypospadias, vaginal pouch, and ectopic and undescended testes. The degree to which each of these malformations manifested varied between individuals, and the incidence and severity of some malformations were higher in the 400 mg/kg than in the 200 mg/kg dose group. The incidence of severe vaginal pouch increased in a dose dependent fashion and was significant in both the 200 mg/kg and 400 mg/kg groups by Fisher’s Exact test (Fig. 2; Table 5) and in the 400 mg/kg group by ANOVA (F(2,18)=7.3; p< 0.005). Ectopic or
undescended testes occurred only in the 400 mg/kg group and was significant by Fisher’s Exact test (Fig. 2, Table 5). The abnormal testicular position found in this study was always near the inguinal region as opposed to the kidney area, but was outside the scrotum in a suprainguinal position either immediately rostral to the inguinal ring in the abdomen (undescended) or outside the abdominal muscle wall in the abdominal or pubic area immediately under the skin (ectopic).

**Body Weight.** Body weight at necropsy was slightly reduced in the 400 mg/kg dose group (p< 0.05; Table 5).

**Organ Weights.** Vinclozolin treatment at 200 and 400 mg/kg significantly reduced the weights of the seminal vesicles, ventral prostate and LA/BC by p< 0.0001 and there was no significant difference between the 200 and 400 mg/kg groups (Table 5). Also, ventral prostate agenesis occurred in one animal in the 200 mg/kg group and in one animal in the 400 mg/kg group. Significant reductions were independent of body weight as found by covariate analysis.

**Serum T levels.** Testosterone levels were not significantly altered by vinclozolin treatment (p<0.22 ; Table 5).

**Discussion**

*In utero* exposure to the AR-antagonist vinclozolin results in permanent modifications of androgen-dependent organs and structures of the male reproductive tract. In this study we sought to define the critical prenatal period during which vinclozolin exposure alters sexual differentiation and relate these effects to the onset of AR expression.
in the developing tissues as reported in the literature and to draw conclusions regarding the sensitivity of each tissue to vinclozolin. We identified GD 16-17 as the two-day period during gestation in which fetal males are most sensitive to insult from vinclozolin, and GD 14 - 19 as the entire sensitive period for the reproductive effects of vinclozolin in the male.

The endpoints most affected by 2-day dosing include AGD, presence of areolas and permanent nipples, weight of the levator ani/ bulbocavernosus muscles, and malformations of the external genitalia. These results not only show the critical 2 day period for sensitivity of sexual development to vinclozolin, but show that AGD, nipple retention and levator ani/ bulbocavernosus weight are sensitive indicators of antiandrogenicity. We also found that 200 mg vinclozolin /kg dam body weight administered from GD 14 through GD 19 is sufficient to significantly affect the reproductive development of every male rat fetus. In addition, a dose of 400 mg/kg vinclozolin administered in this time period is required to significantly induce ectopic and undescended testes. The fact that serum T levels in the adult were unaltered by prenatal vinclozolin treatment at a time when androgen dependent organs were reduced indicates that the decreased organ weights are not due to a decreased T level, but to an organizational effect during sexual differentiation.

The effects observed in this study can be related to the timing of appearance of AR in the male reproductive tract, and illustrate the dose dependent response of the tissues in the genital region.

The sensitive time frames found with 2-day AR inhibition by vinclozolin correlate with the onset of appearance of ARs in the mesenchyme of each tissue rather than the
epithelium. Appearance of ARs in the mesenchyme directs the development of reproductive tissues. Morphological development in the seminal vesicle, prostate and Wolffian duct begins after AR appear in the mesenchyme and before AR appear in the epithelial portion of these tissues, and epithelial proliferation and cytodifferentiation do not occur until testosterone binds mesenchymal cells nearest to the epithelial layer (reviewed by Cunha et al., 1992). Furthermore, mammary glands of the fetal mouse contain ARs in the mesenchyme surrounding the epithelial buds and not in the epithelium at the time of its responsiveness to testosterone and outgrowth of the primordial bud (Wasner et al., 1983).

AGD

AGD has been shown to be a sensitive indicator of antiandrogenicity. Doses as low as 3 mg/kg vinclozolin administered perinatally reduced the AGD on PND 2 in male rats (Gray et al., 1999a). AGD is used commonly as an indicator of antiandrogenicity and has been found by many to be sensitive with various types of antiandrogens (Clark et al., 1993; Ema et al., 1998; Gray et al., 1999b; Mylechreest et al., 1998; Ostby et al., 1999; You et al., 1998). In the current study, AGD proved a sensitive endpoint by its ability to be significantly reduced with only 2-day administration of vinclozolin and the significance of the reductions. Also, the temporal effect on AGD in our study correlates with the appearance of AR in the mesenchyme of the affected tissue. The urogenital tubercle, folds and swellings, which give rise to the phallus and perineum, first express mesenchymal ARs in the rat on GD 14 (Bentvelsen et al., 1995). AGD, a measurement in the perineal region, was first reduced with administration of vinclozolin on GD 14 - 15, and was further reduced on GD 16 - 17. The perineum on GD 18 - 19 still appeared responsive since AGD was
slightly reduced on GD 18-19.

**Nipples**

The appearance of nipples has also been reported in studies on antiandrogens (Gray et al., 1999b; Kelce et al., 1994; Mylchreest et al., 1998; You et al., 1998) as a definitive indicator of antiandrogenicity. Nipple retention was a sensitive endpoint to vinclozolin toxicity in this study, shown by its inducibility at several 2-day dosing periods and the great significance of the effect. Although many studies report only thoracic nipples, we observed nipples at every point along the ‘milk line’, or all 12 nipples. Male adult rats do not normally display nipples, and the presence of nipples indicates an interference of androgen action in this tissue. In the male rodent fetus, the mesenchyme of the mammary anlage contain ARs and respond to the rise in circulating testosterone, resulting in the condensation of the mesenchyme and disintegration and rupture of the epithelial stalk leading to the external skin (Topper et al., 1980). This response is to DHT rather than to T since 5α-reductase inhibition results in the presence of nipples in the male rat (Imperato-McGinley et al., 1992). Most investigation of mammary gland and nipple development has been conducted in the mouse where the onset of appearance of AR in the mammary anlage is on GD 12 (Wasner et al., 1983), which translates to GD 14 in the rat. Also, condensation of the mesenchyme of the mammary gland occurs at the time of testosterone production, or GD 14 in the rat. This time point correlates with the effects on nipple development in our study. Nipples were present in male adults after exposure to vinclozolin at GD 14 - 15 and increased in number, percent males affected, and significance at GD 16-17, when testosterone concentrations rise systemically.
Another significant finding is the sensitivity of the levator ani/bulbocavernosus muscle group to vinclozolin. The levator ani (LA) is sexually dimorphic at birth in rats and humans, which illustrates that the fetal period is the time of susceptibility to antiandrogenic insult. At GD 22 in the rat, the number of muscle units (MUs) is greater in the male than in the female (Tobin and Joubert, 1991) and the LA is larger in weight and cross sectional area in males (Jordan et al., 1997). The male rat LA muscle at PND 7 is highly immunoreactive to testosterone and contains a high number of AR (Jordan et al., 1997). This response is directly to testosterone and not DHT, as there is no 5α-reductase activity in this muscle. The onset of expression of AR in the fetal rat LA and reactivity of the fetal LA to androgens or antiandrogens has not been studied. We found the timing of effects in the LA/BC to be similar to that found in the nearby external genitalia. The LA/BC weight was significantly reduced was GD 14-15, and the reduction was even more significant at GD 16-17, while GD 18-19 dosing still resulted in a significantly reduced weight. These results provide indirect evidence that AR appear in the levator ani/bulbocavernosus muscles in the rat on GD 14 and increase in number and/or activity by GD 16-17.

Ventral Prostate

In the current study, ventral prostate weight was less sensitive to vinclozolin than the above endpoints. The prostate is derived form the DHT-dependent UGS. ARs first appear in the UGS in the rat on GD 16 but greatly increase in expression by GD 18 (Bentvelsen et al., 1995; Hayward et al., 1996), when rapid prostate development begins. In our study, the effect occurred with GD 18-19 dosing, which correlates with the rise in AR and rapid
proliferation of cells in the prostate rather than the onset of appearance of AR, and it is then that AR may be more functional.

External Genitalia

The external genitalia displayed abnormalities in response to vinclozolin exposure in this study consistent with the effects observed in other studies, and the temporal response to vinclozolin correlated with the appearance of ARs in the mesenchyme of the tissue. The mesenchyme of the external genitalia express AR on GD 14 and expression increases thereafter (Bentvelsen et al., 1995). These AR directly affect development of the penis since AR continue to be present in the penis of the immature and adult rat and are responsive to T (Rajfer et al., 1980). The incidence of malformations of the external genitalia occurred to a significant degree with GD 16 - 17 dosing. This response also occurs with 5α-reductase inhibitor finasteride treatment, and is most sensitive at this same gestational time point (Clark et al., 1990). As observed in the ventral prostate, the effect on the external genitalia correlated with the rise in AR rather than in the onset of appearance of AR.

The timing of appearance of AR in the Wolffian duct system could not be directly correlated with effects since much of the Wolffian derived structures were unaffected by two day vinclozolin dosing. Bentvelsen et al. (rat; 1995) and Cooke et al. (mouse; 1991) both observed a correlation between the onset of appearance of AR and the distance from the testes in the male reproductive tract - the more proximal to the testes, the earlier AR appeared in the mesenchyme. In the Wolffian duct system, AR appear first in the efferent ductules and epididymides around GD 14 (Bentvelsen et al., 1995) or GD 15 (You and Sar, 1998) in the rat, in the vas on GD 19 or 20, and in the seminal vesicles on GD 21
(Bentvelsen et al., 1995). Although vinclozolin administration included these time points, no effects were observed in the testes, epididymides, or seminal vesicles. Gray et al., (1994) observed that the Wolffian duct appeared more resistant to insult by vinclozolin and Imperato-McGinley et al., (1992) found that much higher doses of flutamide were required to affect the testes, epididymides and vas deferens.

Most of the aforementioned effects did not become more significant as the dose and duration of dosing were increased. Some effects, ectopic/undescended testes and vaginal pouch, were observed only when both the dose and duration of dosing were increased.

**Testicular Descent**

Ectopic and undescended testes were observed in our study to a significant degree only with GD 14 - 19 administration of vinclozolin and only in the 400 mg/kg group. Thus this T-dependent process (Imperato-McGinley et al., 1992) appears to be less sensitive to antiandrogenic insult than other events studied here. Testicular descent has been described in two stages, transabdominal and transscrotal, and both these events may be under androgenic control. However, factors such as intraabdominal pressure, suppression of the cranial suspensory ligament, neural control and hormones other than androgens may play roles in testicular descent (Levy and Husmann, 1995). The gubernaculum is at least partially responsible. ARs are present in the gubernaculum (Husmann and McPhaul, 1991b) and flutamide administered on GDs 15 to 17 prevented gubernaculum cord regression in the rat (Cain et al., 1995). The abnormal testicular position found in this study was always near the inguinal region, ie. the testes had accomplished transabdominal migration while transinguinal migration was interfered. It appeared more specifically that the positioning of
the gubernaculum was in the abdominal wall rather than in the scrotum. Anomalies such as these have been described in rodents after antiandrogenic treatment (Neuman et al., 1970; van der Schoot, 1992). Also, inguinal hernias were produced in wallabies treated with flutamide during sexual development (Lucas et al., 1997).

**Vaginal Pouch**

The fact that the presence of a vaginal orifice was observed only with extended dosing in experiment 2 and that the severity of this malformation increased with dose both illustrate the dose-dependency of this tissue on androgen action and its relative insensitivity to antiandrogenic insult. This finding is consistent with other AR-inhibitors. The AR-inhibitor flutamide demasculinized the external genitalia more completely than did the 5α-reductase inhibitor finasteride (Imperato-McGinley et al., 1992). Vaginal orifices appeared in rats after 12 day prenatal dosing using 10 mg flutamide per dam (van der Schoot, 1992) which is comparable in potency to the dosing regime used in our experiment 2. Although 25 - 100 mg/kg/d of procymidone significantly affected AGD and nipple formation, vaginal pouch was significantly affected only at 200 mg/kg from GD 14- PND 3 (Ostby et al., 1999). Also, while AGD was decreased and nipples were induced, vaginal pouches were not observed in male offspring exposed perinatally to p,p’-DDE (You et al., 1998b), nor in males treated with various antiandrogens that displayed hypospadias (Gray et al., 1999).

Extending the dosing period to include gestational days 14 through 19 while lowering the dose of vinclozolin by half did not increase the significance of the effect in the more sensitive endpoints but increased or produced the significance and incidence of others, including the reduced weight of the Wolffian duct derived seminal vesicle. The weight of
another Wolffian duct derivative, the epididymis, was reduced by vinclozolin administration at 200 mg/kg from GD 14 through PND 4 (Gray et al., 1999b) which suggests that the epididymis could be affected with the extended dosing regime in our study. The greater overall effect in androgen-dependent target tissues with the extended dosing period may show that effects are dependent upon the cumulative dose, or area under the curve (AUC), rather than the timing or concentration of the dose alone (eg., 6 days of 200 mg, or 1200 mg > 2 days of 400 mg, or 800 mg). In support of this theory, raising the dose level to 400 mg/kg vinclozolin for 6 days further affected development of the male genitalia. The effects may also be dependent on clearance of vinclozolin and its metabolites, requiring a continuous dose of the reversible AR-inhibitor over the course of a developmental event to ensure an effect in that organ. Since dosing on GD 12 - 13, a stage immediately before sexual differentiation and expression of AR, did not affect any tissues, clearance of vinclozolin and its metabolites from the rat may be within a day. In light of these two considerations, some antiandrogenic effects found in experiment 2 may actually be more significant if the dosing period were extended, or even moved to a later time frame.

This study illustrates the sensitivity of the fetus to vinclozolin as evident in the significance and spectrum of the effects. The tissues affected by vinclozolin are most sensitive at or soon after the time they are acquiring AR in the mesenchyme, and are further sensitive to the dose and duration of dosing during this sensitive period. Once ARs are located in the epithelium, malformations cannot be induced. Indeed, studies in which neonates are dosed with high levels of EDCs are unable to induce gross malformations (Prins, 1995). These effects and the timing of the effects are consistent with other studies.
(Husmann and McPhaul, 1991a; Silversides et al., 1995; Clark et al., 1990). The information obtained with the current study is invaluable to further investigation into the mechanisms of vinclozolin-induced antiandrogenicity in the developing fetus.
References


Gray, L.E., Wolf, C., Lambright, C., Mann, P., Price, M., Cooper, R.L., and Ostby, J. (1999b). Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p’-DDE, and ketaconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate)


in rat prostate lobes is altered after neonatal exposure to estrogen. *Endocrinol.* **136:**1303-1314.


van der Schoot, P. (1992). Disturbed testicular descent in the rat after prenatal exposure to
the antiandrogen flutamide. *J. Reprod. Fert.* **96:**483-496.


Table 1. Dam Fertility and Preweaning Pup Weight and Viability (Experiment 1) Following Two-day Prenatal Administration of Vinclozolin (400 mg/kg). *

<table>
<thead>
<tr>
<th>Endpoints</th>
<th>Control</th>
<th>12-13</th>
<th>14-15</th>
<th>16-17</th>
<th>18-19</th>
<th>20-21</th>
</tr>
</thead>
<tbody>
<tr>
<td># dams assigned</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td># dams NP</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td># dystocia</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td># delivered late</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Live litter size at birth</td>
<td>13.2 ± 2.49</td>
<td>11.2 ± 6.87</td>
<td>13.3 ± 1.25</td>
<td>12.67 ± 1.75</td>
<td>8.0 ± 5.15</td>
<td>9.5 ± 5.0</td>
</tr>
<tr>
<td>Pup wt. at birth (g)</td>
<td>6.39 ± 0.16</td>
<td>5.69 ± 0.25</td>
<td>5.75 ± 0.21</td>
<td>5.95 ± 0.33</td>
<td>5.76 ± 0.26</td>
<td>5.19 ± 0.36</td>
</tr>
<tr>
<td>M/F sex ratio at birth</td>
<td>1.49 ± 0.44</td>
<td>1.47 ± 0.73</td>
<td>2.45 ± 0.62</td>
<td>0.75 ± 0.18</td>
<td>1.07 ± 0.24</td>
<td>0.92 ± 0.23</td>
</tr>
<tr>
<td>% pup survival to PND 13</td>
<td>52.8</td>
<td>48.1</td>
<td>66.7</td>
<td>87.1</td>
<td>55.8</td>
<td>52.0</td>
</tr>
<tr>
<td>% male survival to weaning (PND 23)</td>
<td>51.2</td>
<td>48.96</td>
<td>66.1</td>
<td>83.6</td>
<td>53.6</td>
<td>46.7</td>
</tr>
<tr>
<td>Pup wt. at weaning (g)</td>
<td>55.7 ± 3.82</td>
<td>59.5 ± 1.66</td>
<td>49.57 ± 2.04</td>
<td>54.4 ± 4.96</td>
<td>65.4 ± 5.60</td>
<td>52.9 ± 5.85</td>
</tr>
<tr>
<td># litters dead by weaning</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Note: NP = not pregnant.
* Significantly different at p< 0.005.
* Values are means ± SE unless otherwise described.
* Shaded areas denote a significant difference.
* Percentages are based on litter means.
Table 2. External Abnormalities in Male Offspring Induced by Two-day Prenatal Administration of Vinclozolin (400 mg/kg) (Experiment 1).a

<table>
<thead>
<tr>
<th>Endpoints</th>
<th>Control</th>
<th>12-13</th>
<th>14-15</th>
<th>16-17</th>
<th>18-19</th>
<th>20-21</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGD on PND 1</td>
<td>2.96±0.059</td>
<td>2.95±0.115</td>
<td>2.76±0.085</td>
<td>2.26±0.063</td>
<td>2.46±0.063</td>
<td>2.73±0.159</td>
</tr>
<tr>
<td>% male pups with areolas (PND 13)c</td>
<td>23.3±19.4</td>
<td>0</td>
<td>79.6±15</td>
<td>100±0</td>
<td>50 ±0.20</td>
<td>0</td>
</tr>
<tr>
<td># areolas/pup</td>
<td>0.33±0.211</td>
<td>0</td>
<td>4.86±0.986</td>
<td>8.84±0.683</td>
<td>1.625±0.851</td>
<td>0</td>
</tr>
<tr>
<td>% males with nipples (necropsy)</td>
<td>0</td>
<td>0</td>
<td>19.7±0.10</td>
<td>88.9±0.11</td>
<td>62.5±0.24</td>
<td>0</td>
</tr>
<tr>
<td># nipples/adult male</td>
<td>0</td>
<td>0</td>
<td>0.420±0.249</td>
<td>6.10±0.934</td>
<td>1.5±0.54</td>
<td>0</td>
</tr>
<tr>
<td>% males with malformations d</td>
<td>0</td>
<td>0</td>
<td>2.27±0.023</td>
<td>44.8±0.14</td>
<td>25±0.25</td>
<td>0</td>
</tr>
<tr>
<td># with hypospadias/# animals necropsied</td>
<td>0/17</td>
<td>0/11</td>
<td>0/38</td>
<td>10/24</td>
<td>1/9</td>
<td>0/9</td>
</tr>
<tr>
<td># ectopic-und testes/# animals necropsied</td>
<td>0/17</td>
<td>0/11</td>
<td>0/38</td>
<td>1/24</td>
<td>0/9</td>
<td>0/9</td>
</tr>
</tbody>
</table>

* p<0.05 ** p<0.01 *** p<0.001, ‡ p<0.0001.

a Values are given as litter means ± SE unless otherwise described.
b Shaded areas denote a significant difference from controls.
c Percentages are based on litter means.
d Malformations may include any of the following: incomplete preputial separation, cleft prepuce, cleft phallus, exposed os penis, hypospadias. See text for details of statistical analyses.
e Analyzed by Fisher’s Exact test.
Table 3. Effects of Two-day Prenatal Administration of 400 mg/kg Vinclozolin on Reproductive Organ Weights at Necropsy (Experiment 1) *

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Control</th>
<th>12-13</th>
<th>14-15</th>
<th>16-17</th>
<th>18-19</th>
<th>20-21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g) at necropsy (PND 77-102)</td>
<td>438.9 ± 27.15</td>
<td>458.0 ± 12.54</td>
<td>421.4 ± 10.67</td>
<td>430.0 ± 12.0</td>
<td>438.38 ± 19.4</td>
<td>433.06 ± 19.58</td>
</tr>
<tr>
<td>SV weight (g)PND 77-102</td>
<td>1.115 ± 0.10</td>
<td>1.285 ± 0.102</td>
<td>1.146 ± 0.026</td>
<td>1.065 ± 0.051</td>
<td>1.147 ± 0.169</td>
<td>1.030 ± 0.079</td>
</tr>
<tr>
<td>VP weight (g) PND 77-102</td>
<td>0.355 ± 0.030</td>
<td>0.389 ± 0.033</td>
<td>0.344 ± 0.022</td>
<td>0.307 ± 0.021</td>
<td>0.249* b</td>
<td>0.345 ± 0.012</td>
</tr>
<tr>
<td>LA/BC weight (g) PND 77-102</td>
<td>1.089 ± 0.06</td>
<td>1.116 ± 0.025</td>
<td>0.959 *</td>
<td>0.798 ‡</td>
<td>0.845 **</td>
<td>0.971 ± 0.031</td>
</tr>
<tr>
<td>Rt. Testis weight (g) PND 77-102</td>
<td>1.61 ± 0.069</td>
<td>1.667 ± 0.064</td>
<td>1.58 ± 0.055</td>
<td>1.687 ± 0.064</td>
<td>1.802 ± 0.07</td>
<td>1.711 ± 0.01</td>
</tr>
<tr>
<td>Rt. Epididymis weight (g) PND 77-102</td>
<td>0.490 ± 0.018</td>
<td>0.489 ± 0.03</td>
<td>0.476 ± 0.018</td>
<td>0.493 ± 0.016</td>
<td>0.473 ± 0.025</td>
<td>0.499 ± 0.015</td>
</tr>
<tr>
<td>VP weight (g) PND 175,176</td>
<td>0.473 ± 0.040</td>
<td>0.506 ± 0.049</td>
<td>0.459 ± 0.034</td>
<td>0.519 ± 0.040</td>
<td>ND</td>
<td>0.524 ± 0.069</td>
</tr>
<tr>
<td>LA/BC wt. (g) PND 175,176</td>
<td>1.341 ± 0.071</td>
<td>1.410 ± 0.087</td>
<td>1.157 ± 0.062</td>
<td>1.039 *</td>
<td>ND</td>
<td>1.131 ± 0.124</td>
</tr>
<tr>
<td>Rt. Epididymis wt. (g) PND 175,176</td>
<td>0.653 ± 0.018</td>
<td>0.627 ± 0.022</td>
<td>0.623 ± 0.016</td>
<td>0.657 ± 0.018</td>
<td>ND</td>
<td>0.668 ± 0.031</td>
</tr>
</tbody>
</table>

Note ND = no data (no animals alive in that group by PND 175)
* p< 0.05, ** p< 0.001, ‡ p< 0.0001
a Values are litter means ± SE.
bShaded areas denote a significant difference from controls.
Table 4. Dam Fertility and Preweaning Pup Viability (Experiment 2) Following Vinclozolin Administration from GD 14 to GD 19. *

<table>
<thead>
<tr>
<th>Endpoints</th>
<th>Control</th>
<th>200 mg/kg</th>
<th>400 mg/kg</th>
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</thead>
<tbody>
<tr>
<td># dams assigned</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td># dams NP</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td># dystocia</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td># delivered late</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Maternal wt gain through dosing period (g)</td>
<td>29.33 ± 6.18</td>
<td>20.88 ± 5.26</td>
<td>15.22 ± 7.44</td>
</tr>
<tr>
<td>Live litter size, day of birth</td>
<td>12.57 ± 0.84</td>
<td>12.67 ± 0.85</td>
<td>13.43 ± 0.61</td>
</tr>
<tr>
<td>Pup wt. at birth (g)</td>
<td>6.30 ± 0.16</td>
<td>6.056 ± 0.15</td>
<td>5.95 ± 0.21</td>
</tr>
<tr>
<td>M/F sex ratio</td>
<td>1.65 ± 0.48</td>
<td>1.32 ± 0.21</td>
<td>0.89 ± 0.16</td>
</tr>
<tr>
<td>% pup survival to PND13*</td>
<td>81.7 ± 0.06</td>
<td>87.8 ± 0.05</td>
<td>70.8 ± 0.13</td>
</tr>
<tr>
<td>% male survival to weaning (PND 22)</td>
<td>75.1 ± 0.11</td>
<td>88.2 ± 0.07</td>
<td>58.8 ± 0.11</td>
</tr>
<tr>
<td>Pup wt. at weaning (g)</td>
<td>52.8 ± 1.72</td>
<td>51.8 ± 2.43</td>
<td>51.7 ± 2.03</td>
</tr>
<tr>
<td># litters gone by weaning</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

* Note NP = not pregnant
* Values are means ± SE.
* Percentages are based on litter means.
Table 5. Malformations and Reduces Reproductive Organ Weights at PND 107-128 in Male Offspring Induced by Vinclozolin Administration from GD 14 to GD 19 (Experiment 2).

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Control</th>
<th>200 mg/kg</th>
<th>400 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGD (PND 1)</td>
<td>2.979 ± 0.288</td>
<td>2.23 ± 0.215</td>
<td>2.037 ± 0.182</td>
</tr>
<tr>
<td>% male pups with areolas (PND 13)c</td>
<td>7.2 ± 0.035</td>
<td>97.6 ± 0.024</td>
<td>100 **</td>
</tr>
<tr>
<td># areolas/male pup</td>
<td>0.19 ± 0.096</td>
<td>9.6 ± 0.355</td>
<td>10.81 ± 0.273</td>
</tr>
<tr>
<td>% males with nipples (adult)</td>
<td>0</td>
<td>100 **</td>
<td>100 **</td>
</tr>
<tr>
<td># nipples/male adult</td>
<td>0</td>
<td>9.49 ± 0.24</td>
<td>9.60 ± 0.71</td>
</tr>
<tr>
<td>% males with malformationsd</td>
<td>0</td>
<td>97.62 ± 0.02</td>
<td>92.86 ± 0.07</td>
</tr>
<tr>
<td># with ectopic/undescended testes / # necropsied (%)</td>
<td>0/36</td>
<td>0/42</td>
<td>5/28 * c</td>
</tr>
<tr>
<td># with severe vaginal pouch/ # necropsied (%)</td>
<td>0/36</td>
<td>15/42 ** c</td>
<td>15/28 ** c</td>
</tr>
<tr>
<td>VP weight (g)</td>
<td>.466 ± .026</td>
<td>.198 ± .03</td>
<td>.190 ± .040</td>
</tr>
<tr>
<td>SV weight (g)</td>
<td>1.471 ± .069</td>
<td>.874 ± .05</td>
<td>.714 ± .102</td>
</tr>
<tr>
<td>LA/BC weight (g)</td>
<td>1.110 ± .029</td>
<td>0.723 ± .025</td>
<td>0.663 ± .048</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>504.56 ± 12.14</td>
<td>490.66 ± 8.23</td>
<td>472.45 ± 6.97</td>
</tr>
<tr>
<td>Serum T</td>
<td>2.39 ± 0.227</td>
<td>2.53 ± 0.278</td>
<td>1.94 ± 0.247</td>
</tr>
</tbody>
</table>

* p< 0.05, ** p< 0.0001.

a Values are litter means ± SE. b Shaded areas denote a significant difference from controls.
c Percentages are based on litter means.
d Malformations include cleft prepuce, cleft phallus, exposed os penis, and hypospadias for all affected animals. See text for details of statistical analyses.
e Analyzed by Fisher’s Exact test.
Figure legends

Figure 1. *Experiment 1.* Summary of the effects observed in experiment 1 given in percentages, arranged by dose group to illustrate the overall window in which effects were found. Bars represent litter means. * percent reduction of AGD in males relative to average female AGD.

Figure 2. *Experiment 2.* Dose-dependent effects of vinclozolin administered from GD 14 to GD 19 on genitalia of male offspring. The incidences of malformations is equally significant at 200 and 400 mg/kg vinclozolin, although the incidence of certain types of malformations, severe vaginal pouch and ectopic/undescended testes, increases at the 400 mg/kg dose. Bars represent litter means. Significant differences were analyzed on an individual basis by Fisher’s Exact test. * p< 0.02, ** p< 0.0001.
Incidences of Abnormalities

Figure 1.
Percentage of Dose-dependent Malformations of the Genitalia Induced by Vinclozolin (GD 14 - 19)

Severe Vaginal Pouch

Ectopic and undescended testes

Figure 2.
Effects of Prenatal Testosterone Propionate on the Sexual Development of Male and Female Rats: A Dose-Response Study

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Abbreviated title: Prenatal TP dose-response

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ABSTRACT

Testosterone plays a major role in male sexual development. Exposure of females to testosterone in utero can induce masculine characteristics such as anovulation, increased anogenital distance (AGD), absence of nipples, retention of male-like tissues, and agenesis of the lower vagina. In addition, high levels of androgens during fetal development can lead to toxic effects such as reduced litter size and viability. The study of the effects of testosterone administration during sexual differentiation provides a foundation for understanding the effects of environmental androgens on fetuses, a sensitive subpopulation. In the current study, we investigated the ability of a range of concentrations of testosterone propionate (TP) administered prenatally to masculinize female and alter male offspring, and measured maternal and fetal T levels. Pregnant Sprague-Dawley rats were dosed by subcutaneous injection on gestational day (GD) 14 - 19 (GD 1= day of plug) with either corn oil (vehicle; 0.1 ml/rat) or with 0.1 ml of TP solution at 0.1, 0.5, 1, 2, 5, or 10 mg/0.1 ml. Parturition was delayed at 2, 5 and 10 mg TP, litter size was reduced at 5 and 10 mg TP and pup weight was significantly reduced in both sexes at 0.5 mg TP and higher doses. Viability of offspring was unaffected at any dosage level. Androgenic effects seen at 0.5 mg TP in females included increased AGD at weaning and adulthood, reduced number of areolas and nipples, cleft phallus, small vaginal orifice and presence of prostate tissue. This dose of TP elevated maternal T levels 10 X but had no effect on fetal T levels. At 1 mg TP and above, female AGD on postnatal day (PND) 2 (or postcoital (pc) day 24 [gestation length = 22½]) was increased; areolas and nipples were virtually eliminated; levator ani muscle, bulbourethral glands
and seminal vesicles (2 mg TP and above) were present; none of the females developed a vaginal orifice and many females in the 1 and 2 mg TP dose groups developed a greatly distended, fluid-filled uterus after puberty. Maternal T levels at 1 mg TP were elevated 30X, and female fetal T levels showed an 80% increase. Male offspring displayed a reduced AGD and body weight on PND 2 at 0.5 mg TP and higher doses. These effects were not evident by weaning and male offspring displayed no malformations. We conclude that gestational administration of 0.5 and 1 mg TP masculinizes female offspring without greatly affecting pup viability or pregnancy of the dam. This study provides a useful model for in utero testing of environmental androgens for their potential to induce developmental abnormalities.
Introduction

Sexual differentiation of mammals is dependent on the hormonal status of the fetus during a critical period of gestation. At this stage, the reproductive tract is bipotential and indifferent and development is acutely sensitive to androgens. Testosterone (T) and dihydrotestosterone (DHT) maintain the Wolffian duct system and promote growth and development of male sex accessory glands and external genitalia (Schultz and Wilson, 1974) whereas regression of the female reproductive tract is primarily dependent on Mullerian inhibiting substance (MIS; Josso et al., 1977). Females are also susceptible to the masculinizing effect of androgens since the female reproductive tissues contain functioning androgen receptors (AR; rat, Cunha, et al., 1991; Bentvelsen et al., 1995; human, Shapiro et al., 2000). Human females exposed prenatally to higher than normal levels of androgens, as in the case of drugs taken by the mother (Schardein, 1993), develop ambiguous internal and external reproductive organs, or as in the case of congenital adrenal hyperplasia, can develop male-like external genitalia visible at birth, including clitoromegaly and fused labia (Ammini et al., 1992; New and Wilson, 1999), and male play behavior (Berenbaum et al., 2000). Prenatal administration to the rat of exogenous androgens, such as T, methyltestosterone or testosterone propionate, induces in the female offspring male-like genitalia, increased anogenital distance, (AGD; Greene et al., 1939; Swanson and Werff ten Bosch, 1965; Kawashima et al., 1978; McCoy and Shirley, 1992; Rhees et al., 1997), delayed puberty, early constant estrus (Kawashima et al., 1978; McCoy and Shirley, 1992; Slob et al., 1983), delayed anovulatory syndrome (Swanson and Werff ten Bosch, 1965; Slob et al., 1983), and male-
like changes in brain nuclei (Ito et al., 1986). In other mammals, prenatal androgens increased AGD (mouse: Gandelman et al., 1979), and induced male-like sexual behavior (guinea pig: Phoenix et al., 1959; rhesus monkey: Goy et al., 1988) in the female.

In addition to their effects on sexual development, prenatal exposure to androgens can result in other adverse effects. High levels of T or other androgens administered to the dam can induce infanticide in rats (Rosenberg and Sherman, 1974) and mice (Mann and Svare, 1983) and can produce negative effects on the offspring such as low body weight (Slob et al., 1983; Fritz et al., 1984) and reduced litter size or low pup viability (Popolow and Ward, 1978; Rosenberg and Sherman, 1974; Fritz et al, 1984), and decreased reproductive capacity of the dam including delayed parturition and a greater number of resorptions (Greene et al., 1939; Swanson and Werff ten Bosch, 1965). In addition, women with hyperandrogenism often have low reproductive success (Redmond, 1995; Gustafson et al., 1996).

There is a growing awareness that androgenic chemicals are present in the environment. Female mosquito fish downstream of kraft pulp mill effluent were found with anal fins that had developed into a male-like gonopodium (Davis and Bortone, 1992) and female marine animals and bears have been found masculinized (Vos et al., 2000). More recently, the drug trenbolone acetate, given to beef cattle to improve weight gain and feed, was shown to cause masculinizing effects in female rat offspring including clitoral enlargement and increased AGD (FDA summary, 2000). The above information provides examples of how environmental androgens might alter development.
In light of this information, it is possible that the female reproductive system would be altered by environmental androgen exposure, especially during the prenatal period. The fetus is acutely susceptible to the effects of environmental endocrine disruptors (Gray et al., 1999; Wolf et al., 2000). Therefore, the study of the effects of prenatal testosterone propionate (TP) on male and female offspring will prove a useful model for the study of environmental androgens and allow us to identify sensitive endpoints to monitor in future studies. In the present study, we sought to characterize the effects of prenatal TP in the male and female rat and to define developmental endpoints that are sensitive to disruption of sexual differentiation by TP. In one experiment, presented first, we describe in vivo effects of various doses of prenatally administered TP in the adult offspring. In a second experiment, we measured the levels of T in the serum of the dam and in the gestational day (GD) 19 male and female whole fetuses after administration of TP to the dam to determine the levels in the fetus responsible for the physical effects of TP observed in the adult rat offspring. This information will be used to determine androgenic, non-toxic doses of TP suitable for co-administration with antiandrogens such as vinclozolin during the gestational period.
Methods

Study 1 - Dose-response adult offspring experiment

In vivo

28 timed-pregnant Sprague-Dawley rats (Charles River Labs; Raleigh, NC) were received on GD 3 and housed one per cage in polycarbonate cages (20 cm X 25 cm X 47 cm) with laboratory-grade pine shavings (heat-treated to remove resins) as bedding. They were acclimated to 68-74°F and 40-50% relative humidity on a reversed light schedule (14 h light:10 h dark; lights off 11:00 am E.S.T.). They were given Purina LabDiet 5008 (high energy diet for gestation and lactation) and tap water (Durham, NC municipal water, tested for pesticides and heavy metals) ad libitum. On GD 12, dams were weighed, weight ranked and randomly assigned to dose groups (4 dams per group) that were equilibrated with respect to body weight. On GD 14 - 19, rats were dosed daily by subcutaneous injection with 0, 0.1, 0.5, 1, 2, 5 or 10 mg TP (CAS# 57-85-2; lot# 98H0566) suspended in 0.1 ml corn oil. All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise noted. Doses used were on a per rat basis without correction for body weight in order to replicate methods used extensively by other investigators (Greene et al., 1939; Swanson and Werff ten Bosch, 1965; Kawashima et al., 1978; McCoy and Shirley, 1992; Rhees et al., 1997; Lee and Hutson, 1999).

Maternal weight was monitored throughout the dosing period. Day of delivery was recorded and GD 23 was designated postnatal day (PND) 1 for all litters, including those that actually delivered on a later day. On PND 2, pups were counted, weighed, sexed if possible (sexing of pups could not be reliably performed in the higher dose
groups), and anogenital distance (AGD) was measured. AGD was measured on each pup in a blind fashion using a dissecting microscope fitted with an ocular micrometer reticle. On PND 15, pups were reexamined for sexual phenotype, their sex confirmed or reassigned if necessary, and males and females were checked for areolas in a blind fashion. An areola or a nipple was considered an areola and areola counts were based upon the consensus of two technicians. Areolas were described as either faint, being smaller than normal, or normal, meaning prominent and easily identified.

On PND 22, pups were weaned, counted, sexed, and measured for AGD in a blind fashion using micron rotary dial calipers (Manostat). Litter mates were assigned an individual identification marking with picric acid stain, distributed 2 - 3 per cage, and housed under the same conditions as described for dams except that feed provided was switched to Purina LabDiet 5001 (standard rodent diet). Runts (3 animals) were weaned on PND 28, when they were large enough to reach the water dispenser. Dams were sacrificed 1 day after weaning (pup PND 23) by CO₂ asphyxiation followed by decapitation, and uterine implantation sites were counted by visual examination. Female offspring were checked for vaginal opening (VO) from PND 29-38 and male offspring were monitored for preputial separation (PPS) from PND 37-47 as indicators of puberty. From PND 88 to necropsy, females were sacrificed and grossly necropsied if they became bloated, weak and in poor health.

On PND 112 - 158, females (3 per litter where possible) were euthanized by CO₂ asphyxiation followed by decapitation, shaved on the ventral and lateral surfaces of the trunk for viewing nipples, and necropsied in blocked fashion by treatment group. Blood
was collected in sterile 13 ml vacutainer serum separation tubes (Becton Dickinson, Lincoln Park, NJ), centrifuged at 1000 x g at 8 °C for 15 minutes and serum collected and stored at -70 °C for subsequent measurement of estradiol (E₂) levels. Most of the endpoints measured that showed statistical significance at necropsy are summarized in Table 1. Phallus width and length, not included in table, were significant in some dose groups (measured on 3 per litter). Other endpoints, all of which were not significant, include weights of liver, right and left kidney, and paired adrenals (measured on 2 per litter), pituitary weight, and weight of filled or drained uterus (continued on 3 per litter). Uteri were classified as normal or having hydrometrocolpos, a condition marked by severe distention and fluid retention of both the uterus and upper vagina. Ovaries were observed fresh for the presence of corpora lutea (CL). Observation of bulbourethral glands (BUG) was included in the necropsy when this structure was noticed, after one female from each of the 0, 0.1, 0.5 and 1 mg TP dose groups had been necropsied. Considering the nonexistent incidence of BUG in the low dose groups, probably no BUG were overlooked.

A test was performed on 3 intact reproductive tracts in situ in the 2 and 5 mg TP dose groups. Tracts were selected from middle and high dose groups to represent severely affected urogenital tracts (2 and 5 mg TP group) and a tract with suspected hydrometrocolpos (2 mg TP dose group) for the determination of uterine fluid flow, as follows. Saline (~ 2 cc) was injected at a rate of approximately 0.5 cc/second into the uterus using a 3 cc syringe fitted with a 22 gauge needle, and the needle kept in place in the uterine wall during observation. The entire reproductive tract including the phallus
was viewed and any distention or leakage of fluid was noted and recorded.

Reproductive tissues (uterus, ovary, phallus, prostate, levator ani (LA), seminal vesicle (SV), BUG, any male structures, vaginal threads, pituitary) were stored in Bouin’s fixative for 24 hours and rinsed and stored in 70% ethanol for histological assessment (see histopathology section).

Males (2 per litter) were sacrificed by decapitation and necropsied on PND 161-172. Endpoints measured were weights of glans penis, pituitary, liver, right and left kidney, paired adrenals, right and left testes, left epididymis, right caput+corpus epididymis, right cauda epididymis, ventral prostate (VP), SV, and LA+bulbocavernosus (LA/BC). The remaining males were necropsied on PND 177 and 178 for one endpoint that showed a statistically significant difference, the glans penis weight, and these data represented all males, or 3-6 males per litter per dose group.

Radioimmunoassay

Adult female offspring serum from the necropsy was assayed for E2 using the E2 RIA kit #TKE21 (Diagnostic Products Company, Los Angeles, CA) with the supplied protocol.

Histopathology of female tissues

Reproductive tracts were dissected from the carcass intact with associated accessory organs (prostate, SV, phallus and LA) attached, fixed in Bouin’s solution for 24 hrs, rinsed with water and stored in 75% ethanol. Accessory organs were observed in situ and trimmed by the pathologist. All tissues [distended uteri (n = 5), malformed uteri (n = 3), BUGs (n=8) and ovaries, prostates, LAs, SVs, (1 per litter each)] were paraffin
embedded with VIP tissue processor, sectioned 3 microns thick to produce 1 section, 
stained with Harris hematoxylin and eosin Y (Anatach; Battle Creek, MI), and evaluated 
for pathologies and the identity of male organs by the pathologist. CL were identified in 
ovarian sections (1 section per ovary, 1 ovary per female, 1 female per litter) as large 
round masses of unorganized, luteal granulosa cells. Ovarian sections were scored for 
CL abundance and for antral follicle abundance in a blind fashion. Scoring system was 
designed as follows: no CLs (or antral follicles), score = 0; if 1 to 2, score = 1; if 3 to 6, 
score = 2; if 7 to 10, score = 3; if ≥11, score = 4.

**Study 2 - Maternal/fetal testosterone level experiment**

*In vivo*

Eight timed-pregnant Sprague-Dawley rats (Charles River Labs; Raleigh, NC) 
were received on GD 4 (GD 1 = day of sperm positive smear) and housed one per cage. 
Conditions were the same as described for the above experiment during the gestational 
period. On GD 13, dams were weighed, weight ranked and randomly assigned to 
treatment groups that were equilibrated with respect to treatment group. Dams were 
dosed on GD 14 - 19 by subcutaneous injection in the nape of the neck with 0.1 ml of 0 
(n= 3), 0.5 (n = 2) or 1 (n = 2) mg TP/0.1 ml corn oil dose solution. These doses were 
selected based on their masculinizing effects in the female offspring without any toxicity 
in the dam. On GD 19, one hour after dosing, dams were euthanized in blocked fashion 
by CO₂ asphyxiation followed by decapitation. Blood and fetuses were collected not 
sooner than one hour after dosing to allow TP to undergo distribution and metabolism 
and reach probable peak T levels in both dam and fetus, as TP has a longer half-life than
that of T (Sommerville and Tarttelin, 1983; Rhees et al., 1997). Each dam was euthanized and its fetuses collected before the next dam was euthanized. Fetuses were removed from the uterus, held on ice in a small plastic petri dish, sexed by opening of their abdominal wall under a dissecting microscope and viewing of internal reproductive organs, and saved in 15 ml plastic round-bottomed Falcon tubes (Becton-Dickinson; Lincoln Park, NJ). Fetuses were stored at -20° C for ~ 1 week until extracted and assayed for T levels. To avoid interference of the fetal carcass collection protocol, maternal blood was collected on a separate set of dams (n=6; 2 per dose group) at a later date. To avoid contamination of blood samples with the TP dose solution from the neck, blood was collected by cardiac puncture. Dams were maintained under the same housing conditions described above. Dams were selected for blood collection in blocked fashion by treatment group (1 representative of each dose group constitutes a block). Blood was collected by heart puncture while dam was under halothane anaesthesia and dams died by exsanguination. Maternal blood was centrifuged and serum was stored at -70 °C for 1 week until assayed for T levels. All chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO) unless otherwise noted.

Fetal testosterone extraction

T was extracted from fetal tissue as described previously (Parks et al., 2000). Fetuses were thawed and homogenized individually in 500 µl distilled deionized water with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). After homogenization, 2 ml ethyl ether (Fisher Scientific, Pittsburgh, PA) were added to each tube, tubes were vortexed for 30 seconds, and centrifuged at 2000 RPM (1000 x g) at 8°
C for 10 min. Following centrifugation, each tube was held one at a time in an acetone/dry ice bath until the bottom aqueous layer froze, and the supernatant (ether layer) was then transferred to a 12 X 75 mm glass tube. The ether extraction was performed twice. Glass tubes of ether extract were dried in a fume hood overnight. Tubes were stored for up to 2 weeks until analyzed by radioimmunoassay.

**Radioimmunoassay (RIA) of dams and fetuses**

Each tube of dried fetal extract was resuspended by vortexing for 30 seconds in 70 µl of 0 standard buffer provided in the Coat-A-Count Total Testosterone RIA kit #TKTT5 (Diagnostic Products Company; Los Angeles, CA). Fifty µl of the 70 µl fetal resuspension were transferred to the antibody-coated tubes in the RIA kit and T levels were determined according to the manufacturer’s protocol. Tubes were read for 1 min each in a gamma counter (CliniGamma 1272, LBK-Wallac, Finland). Counts are programmed to report a ng/ml value. This value was adjusted for volume of fetal extract by multiplying by 0.07 (70 µl/1 ml). Maternal serum was vortexed and 50 µl of straight and 50 µl of 5X diluted serum was assayed in duplicate by RIA for T following the DPC Total Testosterone RIA kit protocol.

**Statistics**

Data were analyzed by ANOVA on a litter means basis using the PROC GLM (general liner models) function on SAS (for Windows 95, version 3.0.554; Cary, NC). Weights and distance measurements (kidney to ovary distance, Kd-Ov; AGD, ano-vaginal distance, AVD; phallus length) were analyzed with and without body weight as a covariate. Percentage data (% late delivery, % with nipples) were performed with arcsine
transformation of the individual (dam) means or litter (pup) means. When significant differences were found for an main effect, a 2-tailed t test was used to test differences between treatment groups using least square means. Maternal endocrine data were log transformed to reduce heterogeneity of the variance. Uteri were classified as normal or having hydrometrocolpos both subjectively based on size and quantitatively based on weight. Counts and categorical data on malformations (cleft phallus, vaginal thread, absence of vaginal orifice, vaginal orifice-phallic cleft not separate, presence of prostate, SV, LA, BUG, hydrometrocolpos) and number of females dead before necropsy were analyzed on an individual basis using Fisher’s Exact test or Chi square as appropriate.

Results

Study 1 - Dose-response in vivo

Preweaning maternal and pup data

Maternal weight gain during the dosing period decreased with increasing dose of TP and was significant in the 2, 5, and 10 mg dose groups (Table 3). One dam (in the 0.1 dose group) was not pregnant and was not included in the weight gain assessment. Late delivery, in which dams delivered all their pups by any day later than the expected GD 23, occurred at 1 mg TP and higher doses and was significant in the 5 and 10 dose groups (Table 2). Two dams who delivered late lost their entire litters within 2 days (2 days after late delivery = PND 5; Table 2). Two other treated litters consisted of only a few runts; 2 pups in a 2 mg group litter and 1 pup in a 5 mg group litter. Live litter size on
PND 2 was reduced in the 5 and 10 mg TP dose groups, and remnants of pups were found in nearly all treated group cages. Pup weight on PND 2 was significantly reduced at 0.5 mg and higher doses of TP, although this reduction was a transient effect. By weaning, PND 22, pup weight was not different in TP-exposed pups (Table 2). Although litter size was reduced at birth and weaning in the high dose groups (5 and 10 mg TP), viability to weaning was unaffected. Pups too small to reach water supply on PND 22 (runts) were weaned on PND 28.

AGD

On PND 2, sex was determined and AGD recorded for males and for females of each litter in the 0, 0.1 and 0.5 mg TP dose groups (Fig 1h). Sexing of the pups by AGD became more difficult and unreliable above the 0.5 mg dose. This dilemma is reflected in the AGD data shown in Figure 1. AGD measurements (AGDs) were obtained for each pup and all AGDs were grouped into ranges of AGDs. For each dose, AGD ranges were plotted along the X-axis and number of pups in each range was plotted along the Y-axis. The resulting line graphs then illustrate the frequency distribution of AGD measurements. In the control group (Fig. 1a), two populations of pups are displayed as two distinct peaks in the graph. One peak to the left represents a population of pups with smaller AGDs, identified as females, and the other peak represents a population of pups with larger AGDs, identified as males. With increasing dose of TP (Fig. 1b-g), both peaks move closer to the middle ranges and identification of the pups as male or female based on their AGD becomes more difficult and eventually impossible. At 0.5 mg TP, the mean AGD on PND 2 for males is significantly reduced (p < 0.05) while the female
AGD is unaffected \((F(2,8) = 0.88, p= 0.4496; \text{Fig. 1,h; Table 3})\). At 1 mg and higher doses, it is clear that both male and female AGDs were affected on PND 2, as the mean female AGD increased and the mean male AGD decreased.

At PND 22, AGD in males was not affected, while AGD in females was significantly increased at 1, 5 and 10 mg doses of TP (Table 3). With body weight co-variance analysis, AGD in females was significantly increased at 0.5 mg TP \((p < 0.05)\) and in all higher doses \((p \leq 0.0001; \text{Table 3})\). This effect persisted to necropsy (PND 112 - 158; Table 4) and is therefore permanent under the conditions of this study.

**Areolas**

Areolas were described in two degrees of severity; faint and normal. Normal was limited to only those areolas that were prominent. One hundred percent of females in the 0 and 0.1 mg dose groups displayed the full compliment of normal areolas (12). In the 0.5 mg dose group, although 100% of the females displayed areolas and most females displayed the full number of areolas \((\text{mean} = 11.9 \text{ areolas})\), many of these areolas were faint and the average number of normal areolas was significantly reduced \((\text{mean} = 8.6; \text{Table 3})\). In the 1 mg TP and higher dose groups, virtually all females lacked normal areolas (Table 3), and displayed only a few \((\text{mean} < 2)\) faint areolas if any. Areolas were not detected in any male offspring.

**Weaning, Puberty and Viability**

Viability from birth to weaning (PND 22) and body weight at weaning were unaffected in either sex by any dose of TP (Table 2).

The ages at PPS in all males and VO in females in the 0.1 and 0.5 mg dose groups
were unaffected by TP treatment (F(6,15) = 0.80; F(3,7) = 2.03, respectively; Table 3). However, inspection of females for VO revealed an absence of the vaginal orifice in 100% of females in the 1 through 10 mg TP dose groups (Fig. 3; Table 5), absence of the vaginal orifice in one female in the 0.5 mg dose group, and a vaginal orifice so small in one female in the 0.5 mg TP dose group VO could not be determined. Shortly after puberty, female offspring from the middle range of TP dose groups (in this case the 1, 2 and 5 mg TP groups) appeared to have distended abdomens and began dying (11 in the 1 mg group, 10 in the 2 mg dose group, and 1 in the 5 mg TP dose group died before scheduled necropsy). Thereafter, females with distended abdomens that appeared lethargic were sacrificed for gross necropsy (3 females in the 1 mg dose group, 1 female in the 2 mg dose groups; Fig. 2a). Necropsy of each female revealed an extremely large, distended, fluid-filled uterus and upper vagina (hydrometrocolpos), some uteri weighing as much as 94 grams, with no other gross abnormalities aside from the absence of a vaginal orifice (Fig. 3). This condition was suspected of being the cause of death of the females that had died.

**Female necropsy**

At necropsy of female offspring (PND 112 - 158), uterine weights varied greatly in the middle dose groups (1, 2 and 5 mg TP) due to the degree of uterine enlargement and fluid accumulation in some individuals (Fig. 2b; Table 4). Of the females sacrificed both before and during scheduled necropsy, 5 out of 9 females in the 1 mg group, 2 out of 10 females in the 2 mg group, and 1 out of 20 females in the 5 mg group displayed an abnormally distended, fluid-filled uterus, or hydrometrocolpos. No female from any
other dose group, including the 10 mg dose group, displayed such a condition. Females that had died before the necropsy period without being inspected are suspected of having had hydrometrocolpos.

Females that had no vaginal orifice did have a cervix and upper vagina. In the middle dose groups (1, 2 and 5 mg TP), the upper vagina ended blindly alongside the dorsal aspect of the urethra. In the higher dose groups (mostly 5 and 10 mg TP), the formation of the end of the upper vagina was difficult to determine visually, but the vagina was apparently continuous with and opened into the urethra, as explained subsequently. A test was performed on rats in two middle dose groups (2 and 5 mg TP) to determine whether the fluid in the uterus could escape. Saline injected into the uterus of one female in the 2 mg TP dose group did not escape but further distended the uterus (uterus initially appeared to be only slightly distended, not having hydrometrocolpos). Saline injected into the uterus of other females without hydrometrocolpos in the 2 mg (1 female) and 5 mg TP (1 female) dose groups passed into the bladder and exited via the urethra to the tip of the phallus, indicating that fluid in the uterus could escape in some females in the high dose groups with no vaginal orifice by exiting through the urethra. In addition to the hydrometrocolpos observed in the middle dose groups, 3 females in the high dose groups (1 in the 5 mg and 2 in the 10 mg TP dose group) had malformed uteri such that the ends of the horns were hard and curled or crumpled, or in one, the portion of the uterus in which the two horns meet had become hardened and enlarged.

Every female in the low dose groups (0, 0.1 and 0.5 mg TP) had nipples and had nearly all 12 nipples. However, at 0.5 mg TP the number of nipples was significantly
reduced to 11.25. At 1 mg TP and higher doses the percentage of females having nipples and the mean number of nipples per rat was drastically reduced to near zero (Table 4).

AGD at necropsy increased in a dose-dependent fashion and was significant (by covariate analysis with body weight) at 1 mg TP and higher doses (Table 4). Although AGD was not increased in the 0.5 dose group, many females displayed an array of genital malformations such as cleft phallus, vaginal thread, and a joined vaginal orifice-cleft phallus (Table 5). In the latter case, the perimeter of the vaginal orifice ran continuous with the cleft of the phallus so that the two were nearly indistinguishable. In those females that did have a complete vaginal orifice, the orifice appeared closer to the phallus than in control females, less well defined and smaller in diameter (not quantitated). The measurement of AVD (ano-vaginal distance; see Table 1) in the 0.5 mg dose group reflected this trend (p = 0.0532), and the difference between the AGD and the AVD, or the vaginal-genital distance (VGD), indicating the distance from the vaginal orifice to the phallus, was significantly smaller at 0.5 mg TP (p < 0.01; Tables 1, 4). These malformations were unique to the 0.5 mg dose group, with the exception of a partially cleft phallus which was observed in one animal in the 1 mg TP dose group (Table 5).

Phallus length was significantly increased in the middle dose groups (2 and 5 mg TP; Table 4). Phallus width was increased in the 1 and the 10 mg dose groups only, with or without body weight as a covariate - these results did not reveal a pattern and were variable, possibly due to body fat, and thus were considered less indicative of a response (Table 4). The internal shaft of the phallus appeared thicker and more developed, more masculine, with increasing dose of TP and included penile bulbs at their base to which
LA were usually attached.

Male structures such as prostate, SV, LA, and BUG or cowper’s glands, were present in females in the higher dose groups to a significant degree (Table 6, Fig. 4). Prostatic tissue appeared on the ventral side of the urethra close to the base of the bladder, a location in which it is found in the male, and its incidence was significant at doses as low as 0.5 mg TP. The prostate appeared to increase in size with increasing dose (not quantitated) and to acquire dorsolateral lobes. The SV appeared to be attached to either side of the cervical area of the uterus, and the BUG resided in a pocket within the perineal muscles at location corresponding to the location of the BUG in the male. The identity of these structures was confirmed histologically (1 per litter; Fig. 4c-f).

Some females in the 1 mg TP and higher dose groups also had the appearance of a gubernacular cord upon dissection, or at least the presence of a stream of fat and other connective tissue issuing out from an invagination in the muscle wall of the perineal region on either side of the upper vagina, an area corresponding to the scrotum.

The right and left kidney to ovary (Kd-Ov) distances and right and left vertical Kd-Ov (vtKd-Ov) distances were significantly increased in the two highest dose groups (5 and 10 mg; Fig. 5), indicating elongation of the ovarian ligament(s). In some cases, it appeared the ligament connecting the ovary to the dorsal body wall behind the kidney, or cranial suspensory ligament, was not just elongated but absent, as no tension could be produced upon pulling the ovary without tearing the fat lying between the ovary and kidney.

Paired ovary weight was slightly but significantly reduced at 10 mg TP when
normalized to body weight (p < 0.05), but otherwise not affected (Table 4). Upon gross
visual inspection at necropsy, ovaries from every female of each dose group appeared to
have CLs. CL score and antral follicle score in histological sections of ovaries (1 per
litter) was not different between dose groups (Table 6). Liver, right and left kidney,
paired adrenal gland, and pituitary weights were not affected by TP as compared to
controls (by covariate analysis with body weight; F(7,16) = 7.15, 3.68, 3.20, 1.14, and
1.78, respectively; data not shown).

E2 levels from female offspring at necropsy were unaffected (F(6,16) = 2.63;
Table 6). E2 levels and visual inspection of uteri and ovaries suggest females from every
dose group had estrous cycles.

Male necropsy

Male necropsy revealed only a reduction in glans penis weight, highly significant
in the middle dose groups (in this case 0.5, 1, and 2 mg TP) and slightly significant at 10
mg TP (Table 7), graphically approximating a U-shaped dose-response curve. No other
effect was found in the male offspring at necropsy (Table 7).

Study 2 - Maternal and fetal T levels

Maternal serum T levels (in ng/ml) increased in a dose dependent fashion and
were significantly elevated at 0.5 and 1 mg TP/dam (other doses were not analyzed). In
contrast, fetal T levels (in ng/fetus) rose significantly only in the female at 1 mg TP and
not at 0.5 mg TP, despite the increased T level in the dam at this dose (other doses not
analyzed). Male fetal T levels were not affected. The elevation in female fetal T levels
at 1 mg TP was increased to near normal male fetal T levels (Fig. 6).
Discussion

Presented herein is a comprehensive dose-response study of the reproductive and developmental effects of prenatal testosterone propionate (TP) exposure that provides a model on which to base testing studies for environmental androgens. We have also determined doses of TP that would effectively masculinize the female rat without inducing overt maternal or fetal toxicity.

A major outcome of this study is the identification of endpoints in the female sensitive to TP that can be used to detect in utero exposure to androgenic chemicals (Table 1). Endpoints in the female that were most sensitive to maternal subcutaneous TP administration, found at the 0.5 mg TP dose, include malformations of the external genitalia, inhibition of areolar and nipple development, and prostate development. Less sensitive endpoints, found in the middle dose ranges, include AGD, complete absence of nipples, complete absence of a vaginal orifice, precocious death and hydrometrocolpos, and LA development. Least sensitive endpoints, induced only at the 2, 5 and 10 mg TP dose levels, include SV and BUG development, and elongation of the ovarian ligament. In addition, our data revealed a remarkable inverted U-shaped dose-response curve for uterine condition which resulted in a similar level of mortality at the corresponding dose levels. A similar cascade of effects was induced by various doses of TP in the early study by Greene et al. (1939). In addition to those endpoints, we included AGD, areola and nipple count and incidence, and hormone levels, and we used a larger number of offspring for the study.
**Sensitive Endpoints in Female Offspring**

*Prostate*

Prostatic tissue was displayed in roughly half the female offspring at 0.5 mg TP, while 0.1 mg TP was without effect. It is possible that some effects, such as presence of prostate, could have been seen at 0.1 mg TP if a greater number of litters was used, however, determining a NOAEL was not the primary interest in this study. Earlier studies involving prenatal TP exposure have also reported presence of prostate tissue at doses lower than those capable of inducing development of other male sex accessory glands (Greene et al., 1939; Hamilton and Gardner, 1937). Development of the prostate is DHT-dependent (Schultz and Wilson, 1974; Imperato-McGinley et al., 1992) and the high induction of prostate tissue compared to seminal vesicle or other T-dependent Wolffian derived tissues may result from the greater potency of DHT as compared to T.

*Nipple Development*

Formation of the external portion of the mammary anlagen (i.e. the areola and nipple) during sexual differentiation in the rat is prevented by the presence of DHT (Goldman et al., 1976; Topper and Freeman, 1980; Imperato-McGinley et al., 1992; Imagawa, et al., 1994). The drastic reduction in areola or nipple number observed at 1 mg TP coincided with elevated T levels observed in the female fetus and thus T or its metabolites may have been responsible for the suppression of areola and nipple formation. The appearance and reduced number of areolas and nipples proved to be a sensitive endpoint for androgenicity in the female as evidenced by its significance at 0.5 mg TP and the drastic reduction at 1 mg TP. The presence of areolas in the preweaning
male rat is a sensitive indicator of antiandrogenicity as well (Clark et al., 1990; Gray et al., 1999; Ostby et al., 1999; Wolf et al., 2000).

External Genitalia

Another set of effects seen at the 0.5 mg TP dose is altered morphology of the genitalia. These effects include decreased size of the vaginal orifice, reduced distance from the phallus, absence of the vaginal orifice in one female in this dose group, and persistent cleaving of the phallus. These malformations have been reported previously in the rat prenatally exposed to TP (Swanson and Werff ten Bosch, 1965; Greene et al., 1939). These anatomical alterations are similar to the underdeveloped state of the reproductive tracts and urogenital sinus early in development. The vagina and urethra are nearer each other and open into the invaginated urogenital sinus, and the phallus is still cleft, or hypospadiac. We also observed the presence of vaginal thread, or persistent isthmus of tissue across the diameter of the vaginal orifice. The female cleft phallus and the vaginal thread have been associated with prenatal estrogen exposure (Vannier and Raynaud, 1980; Henry et al., 1984) and with TCDD or PCB exposure (Gray et al., 1997; Flaws et al., 1997; Wolf et al., 1999) as well. Cleft phallus is also an effect of antiandrogens in the male (Imperato-McGinley et al., 1992; Gray et al., 1994; Ostby et al., 1999; Wolf et al., 2000). Therefore, the presentation of cleft phallus and vaginal thread alone is not indicative of androgen action, but of endocrine disruption, and must be considered with other endpoints in order to identify the mode of action of the disrupter.

Anogenital distance
The effect of TP treatment on female AGD was permanent, being increased throughout life. The significant increase at weaning in the female at 1 mg TP, or at 0.5 mg TP when adjusted for body weight, attest to the sensitivity of this effect (Table 3).

**Internal Reproductive Effects**

**Female Urogenital tract**

A threshold for several developmental processes was apparent at 1 mg TP. The most unusual effect was hydrometrocolpos, or fluid retention and gross distention of the uterus and upper vagina, an effect associated with vaginal atresia (Fig. 3). This condition has been reported in humans at birth and was associated with vaginal atresia (Nguyen et al., 1984; Janus and Godine, 1986). Moderately masculinized female rats in early studies (Hamilton and Gardner, 1937; Greene et al., 1939) were found to have a greatly distended, fluid filled uterus. With moderate masculinization, development of the external portion of the vagina does not occur while the upper vagina ends blindly. With further masculinization, development of the urethra and associated genital ducts is directed by androgens in what can be considered a male-like fashion, joining with the urethra. Greene et al., (1939) showed with histology that the upper vagina of affected females was connected to the urethra via a fistula through which the contents of the uterus and vagina could flow freely. A similar fistula, between the vagina and the bladder, has also been reported in a human infant presenting absent vaginal orifice and mild hydrometrocolpos (Takeda et al., 1997). In the case of Greene et al., the uterus and upper vagina were not distended as the fluid can escape through this fistula and out the
urethra, to the tip of the masculinized phallus. Indeed, in our study, saline injected into the uterus in some females with vaginal atresia but no hydrometrocolpos exited through the urethra.

It appears that the females with hydrometrocolpos displayed estrous cyclicity despite the masculinization of their external genitalia. Estrous cyclicity is evidenced by the apparent uterine activity and the presence of CLs observed in fresh and histological sections of ovaries. Doses of prenatally administered TP that induce a similar degree of masculinization of the external genitalia in the rat do not alter the pattern of gonadotropin release (Swanson and Werff ten Bosch, 1965; Rhees et al., 1997). It also appears that females without hydrometrocolpos, specifically those in the higher dose groups, also displayed estrous cyclicity. Evidence of estrous cyclicity in these females is provided by the presence of CLs in fresh ovaries and the unaffected CL and antral follicle score from ovarian histological sections. In addition, serum E₂ levels and ovarian weights in adult female offspring were not significantly different among dose groups. The sensitive developmental period for masculinization of gonadotropin release is not the prenatal period but the early postnatal period (Rhees et al., 1997; Diaz et al., 1995). Prenatal androgen treatment is less effective than neonatal androgen treatment in inducing early androgen syndrome, an anovulatory syndrome (Swanson and Werff ten Bosch. 1965; Huffman and Hendricks, 1981; Slob et al. 1983). Neonatal androgen treatment can cause delayed anovulatory syndrome (DAS; Hendricks et al., 1977) characterized by lack of estrous cyclicity and reduced numbers of ovulatory follicles associated with reduced ovarian weight due to lack of CL in the adult female offspring, effects not observed in
The number of females in this study that died before necropsy by dose group is reflected by the number of females with hydrometrocolpos by dose group. Both plots depict an inverted U-shaped dose response curve. We hypothesize that the uteri of females in the middle dose groups were responding normally to circulating estrogens by cycling and producing fluid but that the fluid had no outlet for drainage and accumulated, leading to death. The inverted U-shape of this graph thus can be explained by a continuous increase in masculinization - the partial masculinization of the genitalia at middle dose groups, characterized by agenesis of a vaginal orifice with no outlet for drainage of uterine fluid, and a more complete masculinization of the genitalia at higher doses, characterized by a severely re-organized urogenital tract permitting drainage of uterine fluid and alleviation of the hydrometrocolpos. U-shaped dose-response curves generated from in vivo experiments are rare, but have been reported from studies on gestationally administered estrogens, DES and E₂, in mice (vom Saal et al., 1997). The issue of in vivo U-shaped dose response curves is currently under discussion (National Toxicology Program, 2000).

Elongation of ovarian ligament, as measured by kidney-to-ovary distance, occurred only in the high dose groups. This measurement was increased in a previous study in which females were exposed in utero to androgens (Lee and Hutson, 1999) and indicates masculinization, although only with high levels of androgen.

Male reproductive tissues in female offspring

The pattern or degree of induction of male reproductive tissues in females by
prenatal androgen exposure in this study closely follows that observed in past studies (Hamilton and Gardner, 1937; Greene et al., 1939). However, the induction of the LA and histology of these male tissues was not studied before. The LA is present in the male rat and is located in the perineal region attached to the penile shaft, but is not present in the normal female rat. LA development is T dependent (Tobin and Joubert, 1991). The LA has been induced in the female by neonatal T treatment in a previous study (Tobin and Joubert, 1991). In our study, the LA developed only in those females whose phallus had been sufficiently masculinized to form a penile shaft and penile bulbs from which the LA muscle is attached. The bulbocavernosus muscle (BC), another T dependent muscle of the male rat anatomically associated with the LA, did not appear in treated females at any dose. Thus, our study shows organization and development of the LA and BC muscles appear to be differentially regulated with the LA more sensitive to T than is the BC. This was shown elsewhere by differences in AR levels in these two muscles altered by castration (Antonio et al., 1999).

The SV appeared rudimentary in all but one observed cases. In previous studies, prenatal androgens induced well developed SV, but only at doses capable of inducing the vas deferens and epididymides as well (Wistar rat; Greene et al.1939). In the current study, vas deferens and epididymis were not observed in any female at any dose. Bulbourethral glands have been reported at doses that induce SV (Greene et al., 1939), as they were in the current study.

Masculine development of the phallus and internal penile shaft in the female offspring occurred in association with vaginal atresia. The increase in phallus length
itself at 2 and 5 mg TP, although not a robust effect, was indicative of masculinization. Phallus length was not increased at 1 or 10 mg TP, although this may be due to a lower number of individuals in these two dose groups.

*Male offspring*

The decrease in male AGD on PND 2 at 0.5 mg TP was not only a transient effect, but it was not significantly reduced at this dose when analyzed by co-variance with body weight. The body weight of all pups at 0.5 mg and higher doses of TP was reduced. However, the body weight of male pups on PND 2 did not further decrease in higher TP dose groups from that in the 0.5 mg TP dose group, while AGD did further decrease at 1 mg TP and again at 2 mg TP, indicating AGD is independent of body weight and the reduction in AGD is most likely a true effect. AGD could not be determined by sex at higher doses of TP and thus male AGD could not be analyzed by covariate analysis with body weight at these doses. Interestingly, decreased AGD can also be produced in the male offspring of antiandrogen-treated dams (Gray et al., 1994; Ostby et al., 1999; Wolf et al., 2000). However, these males also displayed nipples and other malformations not observed in our TP exposed males.

The only permanent effect seen in the male was a reduction in glans penis weight at 0.5, 1, 2, and 10 mg TP, but not at 5 mg TP. The graphical representation of these data approximates a U-shaped dose-response curve. Reduction in glans penis weight or phallus size is often associated with antiandrogenicity (Clark et al., 1990; Ostby et al., 1999; Prahalada et al., 1997), although it was reported in male offspring of dams fed androgenic Trenbolone acetate prior to pregnancy (FDA, 2000). However, this effect
could not be replicated in more recent studies by the authors of this manuscript (unpublished data). As this effect was the only permanent response seen in any of the androgen-dependent tissues in the male offspring, this response needs to be replicated before one can conclude that it is not spurious statistical vagary.

**Maternal Toxicity**

Adverse effects on reproduction were observed in the dam and neonates at high dose levels of TP. At the 2, 5 and 10 mg TP dose levels, significant adverse effects on maternal reproductive capacity were evident in the decreased weight gain through the dosing period, the delay in parturition and the decreased litter size. These are well known effects of pre- or perinatal androgen treatment (Greene et al., 1939; Swanson and Werff ten Bosch, 1965; Popolow and Ward, 1978; Rosenberg and Sherman, 1974; Fritz et al., 1984; Huffman and Hendricks, 1981; McCoy and Shirley, 1991; Rhees et al., 1997). The decrease in live litter size is due not to reduced number of implantation sites, but to late fetal resorption (Greene et al., 1939; our unpublished observations) and pup death at birth as evidenced by the pup carcass remnants on PND 1.

**T levels**

Fetal body T concentration was significantly increased at 1 mg TP in the GD 19 female fetus, the dose associated with the profound alterations in sexual development. In a similar study, Hotchkiss et al. (in review) found increased fetal T levels in GD 18 females exposed to 1.5 and to 2.5 mg/kg TP, doses similar to our 0.5 and 1 mg doses. Fetal T level was not elevated in the GD 19 female fetus in our study at 0.5 mg TP, a dose of TP that elicited androgenic responses in the female. However, maternal serum T
concentrations rose to 10 times normal levels in this dose group. The difference in T level elevation between dams and fetuses may be explained by the finding that T administered to the rat dam is not delivered to the fetus at equivalent levels, but is metabolized or blocked at the placenta (Vreeburgh et al., 1981). In addition, TP given to the rat dam is metabolized by the dam and placenta to other androgens, of which androsterone is the most abundant in the fetus, followed by 3α-androstanediol and epiandrosterone (Slob et al., 1983). Greene et al. (1939) showed that subcutaneous injection of androsterone to the dam can masculinize female rat offspring, thus androsterone may directly or indirectly be a hormone responsible for the malformations of the female genitalia, undetected by the T radioimmunoassay. However, androsterone has low affinity for the androgen receptor and may be converted in the fetus to a more potent androgen. T may also be metabolized to DHT, an androgen known to mediate the tissues and processes affected in the female offspring in this study. DHT levels in the fetus were not analyzed in this study, but will be addressed in future studies.

We have concluded that 0.5 mg and 1 mg TP given on GD 14 - 19 are effectively androgenic while not severely compromising to litter size, viability, or pregnancy and parturition, and illustrate sensitive endpoints to monitor for identification of androgenic chemicals.

It is noteworthy that few of the above endpoints presented herein are included in the standard multigenerational protocols. The standard multigenerational protocols do not include evaluation for the presence of nipples in male or female offspring or
examination of female offspring for ventral prostate, levator ani, or other male tissues. In addition, inclusion of AGD is restricted to the F2 generation only after an alteration of puberty is found in the F1 generation. We suggest consideration be given to including some of the more sensitive endpoints in the USEPA - Tier 2 testing phase for chemicals that display androgenic activity in Tier 1 screening.

Acknowledgments

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Table 1. Endpoints Monitored at Necropsy in Female Offspring Exposed to TP (GD 14 - 19), in order of sensitivity.

<table>
<thead>
<tr>
<th>LOAEL</th>
<th>Endpoint</th>
<th>Description</th>
<th>n # females</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 mg/rat</td>
<td>Number of Nipples</td>
<td>Number of nipples visible on rat after shaving</td>
<td>3 per litter</td>
</tr>
<tr>
<td>0.5 mg/rat</td>
<td>Cleft Phallus</td>
<td>Cleft or split through the dermis on ventral side of phallus</td>
<td>all on study</td>
</tr>
<tr>
<td>0.5 mg/rat</td>
<td>Vaginal Thread</td>
<td>Thread of tissue transecting the vaginal orifice</td>
<td>all on study</td>
</tr>
<tr>
<td>0.5 mg/rat</td>
<td>Vaginal-Genital Distance (GD)</td>
<td>AGD - AVD difference</td>
<td>3 per litter</td>
</tr>
<tr>
<td>0.5 mg/rat</td>
<td>Prostate</td>
<td>Presence of tissue similar in appearance and location to that in a male</td>
<td>all on study</td>
</tr>
<tr>
<td>1 mg/rat</td>
<td>Anogenital distance (AGD)</td>
<td>Distance (mm) from cranial edge of anus to base of phallus</td>
<td>3 per litter</td>
</tr>
<tr>
<td>0.5 mg/rat</td>
<td>Anovaginal distance (AVD)</td>
<td>Distance (mm) from anterior edge of anus to posterior edge of vaginal orifice</td>
<td>3 per litter</td>
</tr>
<tr>
<td>1 mg/rat</td>
<td>Vaginal Agenesis</td>
<td>Absence of lower vagina, or vaginal orifice (Vorifice)</td>
<td>all on study</td>
</tr>
<tr>
<td>1 mg/rat</td>
<td>Levator ani (LA)</td>
<td>Presence of tissue similar in appearance and location to that in a male</td>
<td>all on study</td>
</tr>
<tr>
<td>2 mg/rat</td>
<td>Bulbourethral glands (BUG)</td>
<td>Presence of tissue similar in appearance and location to that in a male</td>
<td>all on study</td>
</tr>
<tr>
<td>2 mg/rat</td>
<td>Seminal Vesicle (SV)</td>
<td>Presence of tissue similar in appearance and location to that in a male</td>
<td>all on study</td>
</tr>
<tr>
<td>5 mg/rat</td>
<td>Kidney to Ovary distance (Kd-Ov)</td>
<td>Shortest distance (mm) from edge of kidney to edge of ovary when pulled taught, side and downward movement is restricted (usually at an angle; see Fig. 6)</td>
<td>3 per litter</td>
</tr>
<tr>
<td>5 mg/rat</td>
<td>vertical Kidney to Ovary distance (vtKd-Ov)</td>
<td>Vertical distance (mm) from caudal tip of kidney to ovarian pelvis when ovary is pulled taught (will be a straight vertical line; see Fig. 6)</td>
<td>2 per litter (begun after 1 per litter)</td>
</tr>
<tr>
<td>10 mg/rat</td>
<td>Paired Ovary weight</td>
<td>-</td>
<td>2 per litter</td>
</tr>
</tbody>
</table>

LOAEL, lowest observable adverse effects level for each endpoint, in the current study.
Table 2. Effects of Increasing Doses of Testosterone Propionate Administered on GD 14 - 19 on Dam Fertility and Pup Weight and Viability.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>0</th>
<th>0.1</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal wt. gain (g)</td>
<td>50.78</td>
<td>55.26</td>
<td>35.45</td>
<td>36.45</td>
<td>31.0</td>
<td>33.68</td>
<td>26.03</td>
</tr>
<tr>
<td></td>
<td>± 3.14</td>
<td>± 4.67</td>
<td>± 4.45</td>
<td>± 4.63</td>
<td>± 2.25*</td>
<td>± 5.44*</td>
<td>± 4.17***</td>
</tr>
<tr>
<td># late/ # normal delivery (%)</td>
<td>0/4</td>
<td>0/3</td>
<td>0/4</td>
<td>1/4</td>
<td>2/4</td>
<td>3/4</td>
<td>3/4</td>
</tr>
<tr>
<td></td>
<td>(0)</td>
<td>(0)</td>
<td>(25)</td>
<td>(50)</td>
<td>(75)*</td>
<td>(75)*</td>
<td></td>
</tr>
<tr>
<td>mean # days late</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0.75</td>
<td>1</td>
<td>1.33</td>
</tr>
<tr>
<td># whole litter loss by PND 5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Live Litter size (PND2)</td>
<td>14.0</td>
<td>12.67</td>
<td>12.0</td>
<td>8.5</td>
<td>10.75</td>
<td>7.5</td>
<td>6.25**</td>
</tr>
<tr>
<td></td>
<td>± 0.58</td>
<td>± 0.67</td>
<td>± 0.91</td>
<td>± 2.90</td>
<td>± 2.46</td>
<td>± 1.89</td>
<td>± 2.14</td>
</tr>
<tr>
<td># uterine implants</td>
<td>15.25</td>
<td>14.0</td>
<td>15.5</td>
<td>15.5</td>
<td>15.0</td>
<td>13.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>± 0.43</td>
<td>± 0.82</td>
<td>± 1.12</td>
<td>± 1.80</td>
<td>± 2.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pup wt (g) PND 2 (m &amp; f)</td>
<td>7.43</td>
<td>7.89</td>
<td>4.76‡</td>
<td>5.23‡</td>
<td>4.83‡</td>
<td>4.97‡</td>
<td>5.26**</td>
</tr>
<tr>
<td></td>
<td>± 0.32</td>
<td>± 0.26</td>
<td>± 0.22</td>
<td>± 0.43</td>
<td>± 0.21</td>
<td>± 0.37</td>
<td>± 0.42</td>
</tr>
<tr>
<td>Pup wt at weaning (g) (male)</td>
<td>50.07</td>
<td>57.53</td>
<td>44.35</td>
<td>47.42</td>
<td>44.92</td>
<td>47.37</td>
<td>42.65</td>
</tr>
<tr>
<td></td>
<td>± 1.41</td>
<td>± 2.40</td>
<td>± 3.74</td>
<td>± 2.94</td>
<td>± 4.04</td>
<td>± 6.02</td>
<td>± 5.44</td>
</tr>
<tr>
<td>Pup wt at weaning (g) (female)</td>
<td>49.85</td>
<td>54.5</td>
<td>42.63</td>
<td>44.52</td>
<td>38.46</td>
<td>42.80</td>
<td>40.02</td>
</tr>
<tr>
<td></td>
<td>± 1.93</td>
<td>± 1.49</td>
<td>± 2.11</td>
<td>± 4.01</td>
<td>± 6.74</td>
<td>± 4.93</td>
<td>± 2.47</td>
</tr>
<tr>
<td>Litter size at weaning</td>
<td>13.5</td>
<td>11</td>
<td>10.75</td>
<td>8.00</td>
<td>8.25</td>
<td>7.25*</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>± 0.50</td>
<td>± 1.0</td>
<td>± 1.65</td>
<td>± 2.97</td>
<td>± 2.46</td>
<td>± 2.14</td>
<td>± 2.08</td>
</tr>
<tr>
<td>% Viability to weaning</td>
<td>96.7</td>
<td>86.5</td>
<td>88.2</td>
<td>73.2</td>
<td>73.3</td>
<td>87.5</td>
<td>74.1</td>
</tr>
</tbody>
</table>

Note. Values are litter means ± SE unless otherwise described. PND, postnatal day.

* p < 0.05, ** p < 0.01, *** p < 0.005, ‡ p < 0.0001, all compared to control values.
Table 3. Effects of Increasing Doses of Testosterone Propionate Administered on GD 14 - 19 on Androgenic Endpoints during Prepubertal Development.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>0</th>
<th>0.1</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGD on PND 2 (male)</td>
<td>3.80 ± 0.06</td>
<td>3.89 ± 0.21</td>
<td>2.92 ± 0.26 *</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AGD on PND 2 (female)</td>
<td>1.72 ± 0.099</td>
<td>1.51 ± 0.11</td>
<td>1.64 ± 0.107</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>AGD on PND 22 (male)</td>
<td>16.88 ± 0.38</td>
<td>17.34 ± 0.73</td>
<td>15.12 ± 1.14</td>
<td>16.25 ± 0.46</td>
<td>15.60 ± 0.92</td>
<td>16.28 ± 0.68</td>
<td>15.77 ± 0.58</td>
</tr>
<tr>
<td>AGD on PND 22 (female)</td>
<td>10.30 ± 0.28</td>
<td>10.44 ± 0.59</td>
<td>10.70 ± 0.33</td>
<td>12.32 ± 0.61 *</td>
<td>11.85 ± 0.99</td>
<td>13.81 ± 0.27 ***</td>
<td>13.81 ± 0.41 ***</td>
</tr>
<tr>
<td>% Areolas (female)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>50.0 ± 0.19 *</td>
<td>25.0 ± 0.19 ***</td>
<td>26.2 ± 0.14 ***</td>
<td>30.0 ± 0.15 ***</td>
</tr>
<tr>
<td># Areolas/ pup (all)</td>
<td>12.03 ± 0.03</td>
<td>12.0 ± 0</td>
<td>11.92 ± 0.08</td>
<td>1.25 ‡ ± 0.66</td>
<td>0.65 ‡ ± 0.58</td>
<td>0.52 ‡ ± 0.29</td>
<td>0.53 ‡ ± 0.29</td>
</tr>
<tr>
<td># Normal Areolas/ pup</td>
<td>12.03 ± 0.03</td>
<td>12.0 ± 0</td>
<td>8.61 ** ± 1.87</td>
<td>0 ‡</td>
<td>0 ‡</td>
<td>0.095 ‡ ± 0.095</td>
<td>0 ‡</td>
</tr>
<tr>
<td>Age at VO</td>
<td>34.74 ± 0.48</td>
<td>34.56 ± 0.78</td>
<td>34.05 ± 0.34</td>
<td>NV</td>
<td>NV</td>
<td>NV</td>
<td>NV</td>
</tr>
<tr>
<td>Age at PPS</td>
<td>43.62 ± 0.43</td>
<td>42.64 ± 1.83</td>
<td>45.11 ± 0.40</td>
<td>44.62 ± 1.37</td>
<td>45.39 ± 0.31</td>
<td>44.36 ± 1.52</td>
<td>44.28 ± 0.53</td>
</tr>
</tbody>
</table>

Note: Values are litter means ± SE. AGD, anogenital distance; PND, postnatal day; ND, no data - sexes could not be distinguished; VO, vaginal opening; PPS, preputial separation; NV, no vaginal orifice.

- One rat in the control group had 13 nipples.
- Values in brackets represent significance when analyzed by covariate analysis with body weight.
- * p < 0.05, ** p < 0.01, *** p < .001, ‡ p ≤ 0.0001, all compared to control values.
Table 4. Effects Found at Necropsy (PND 112 - 158) in Females Offspring Exposed to Testosterone Propionate on GD 14 - 19.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>0</th>
<th>0.1</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Nipples</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0 ‡</td>
<td>8.33 ‡</td>
<td>5.00 ‡</td>
<td>6.67 ‡</td>
</tr>
<tr>
<td></td>
<td>± 0.08</td>
<td>± 0.08</td>
<td>± 0.08</td>
<td>± 0.08</td>
<td>± 0.08</td>
<td>± 0.07</td>
<td>± 0.07</td>
</tr>
<tr>
<td># Nipples/adult fem.</td>
<td>12.0</td>
<td>11.92</td>
<td>11.25 *</td>
<td>0 ‡</td>
<td>0.17 ‡</td>
<td>0.08 ‡</td>
<td>0.27 ‡</td>
</tr>
<tr>
<td></td>
<td>± 0.08</td>
<td>± 0.08</td>
<td>± 0.08</td>
<td>± 0.08</td>
<td>± 0.17</td>
<td>± 0.08</td>
<td>± 0.27</td>
</tr>
<tr>
<td>Phallus Length a</td>
<td>3.53 ± 0.26</td>
<td>3.05 ± 0.14</td>
<td>3.04 ± 0.07</td>
<td>3.61 ± 0.31</td>
<td>4.69 ***</td>
<td>4.10 *</td>
<td>3.48 ± 0.25</td>
</tr>
<tr>
<td>Phallus Width a</td>
<td>4.49 ± 0.08</td>
<td>4.02 ± 0.12</td>
<td>4.90 ± 0.13</td>
<td>5.17 *</td>
<td>4.96 ± 0.13</td>
<td>4.73 ± 0.29</td>
<td>5.28 ** ± 0.12</td>
</tr>
<tr>
<td>AGD a</td>
<td>24.12 ± 0.38</td>
<td>23.71 ± 0.46</td>
<td>23.41 ± 0.63</td>
<td>25.94 *</td>
<td>26.69 **</td>
<td>29.52 ‡</td>
<td>31.38 ‡ ± 2.12</td>
</tr>
<tr>
<td>AGD-AVD difference</td>
<td>7.47 ± 0.61</td>
<td>7.66 ± 0.70</td>
<td>4.43 ± 0.61 **</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Uterine wt (filled) (mg)</td>
<td>646.92 ± 45.27</td>
<td>594.03 ± 89.76</td>
<td>455.24 ± 96.07</td>
<td>28,633.6 b</td>
<td>8624.90 ± 7927.7</td>
<td>10,632.5 ± 10,209.2c</td>
<td>361.13 ± 96.05</td>
</tr>
<tr>
<td>Uterine wt (drained) (mg)</td>
<td>535.23 ± 30.46</td>
<td>518.87 ± 49.01</td>
<td>355.40 ± 34.00</td>
<td>1347.46 ± 553.36</td>
<td>1469.44 ± 939.5</td>
<td>1234.27 ± 1029.7</td>
<td>423.13 ± 87.13</td>
</tr>
<tr>
<td>Ovary wt a (paired) (mg)</td>
<td>102.97 ± 6.00</td>
<td>102.92 ± 7.06</td>
<td>83.01 ± 7.32</td>
<td>70.72 ± 2.22</td>
<td>81.57 ± 6.61</td>
<td>84.72 ± 13.71</td>
<td>82.93 * ± 23.65</td>
</tr>
<tr>
<td>Body Weight (g)</td>
<td>332.09 ± 17.88</td>
<td>342.44 ± 19.15</td>
<td>293.69 ± 11.14</td>
<td>290.43 ± 36.13</td>
<td>320.67 ± 20.89</td>
<td>319.69 ± 33.08</td>
<td>350.83 ± 25.38</td>
</tr>
</tbody>
</table>

Note Values are litter means ± SE, 2-3 individuals per litter. AGD, anogenital distance; AVD anovaginal distance (see text for description).

a Analyzed by co-variate analysis with body weight.

b Value represents mean of 4 individuals from one litter, therefore one litter mean and no SE.

c One individual had a filled uterus wt of 92,046.0 mg. The mean filled uterus weight of the other individuals on a litter means basis was 466.5 mg.

* p < 0.05 ** p < 0.01, *** p < 0.0005, ‡ p < 0.0001, as compared to controls.
Table 5. Malformations in Female Offspring Exposed to 0.5 mg or 1 TP/dam on GD 14-19.

<table>
<thead>
<tr>
<th>Endpoints</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td># Cleft phallus/ # necropsied (%)</td>
<td>0/28</td>
<td>15/26 (57.7) **</td>
<td>1/6 (16.7)</td>
</tr>
<tr>
<td># VOr in cleft/ # necropsied (%)</td>
<td>0/28</td>
<td>7/26 (26.9) * a</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.004</td>
<td></td>
</tr>
<tr>
<td># Vaginal thread/ # necropsied (%)</td>
<td>0/28</td>
<td>7/26 (26.9) *</td>
<td>0/6</td>
</tr>
<tr>
<td># absent VOr/ # weaned females (%) b</td>
<td>0/28</td>
<td>1/26 (3.85)</td>
<td>19/19 (100) **</td>
</tr>
</tbody>
</table>

* Note Percentages are on an individual basis. VOr, vaginal orifice.

a The 7 with VOr in cleft were not necessarily the same 7 that had vaginal thread. Animals displaying one particular malformation often, but not always, displayed another malformation.

b Data collected on PND 29-38 while checking for VOp.

* p = 0.004, ** p < 0.0001, all as compared to control values. Analyzed by Fisher’s Exact test.
Table 6. Presence of Male Reproductive Organs and CL and Antral Follicles in Females Exposed to Testosterone Propionate on GD 14 - 19.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>0</th>
<th>0.1</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td># females</td>
<td>28</td>
<td>16</td>
<td>26</td>
<td>6</td>
<td>9</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>necropsied</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td># with Prostate</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>5</td>
<td>6</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>(%)a</td>
<td></td>
<td></td>
<td>(53.8) **</td>
<td>(83.3) **</td>
<td>(66.7) **</td>
<td>(100) **</td>
<td>(100) **</td>
</tr>
<tr>
<td># with SV (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(11.1)</td>
<td>(95.0) **</td>
<td>(100) **</td>
</tr>
<tr>
<td># with LA (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td>(%)a</td>
<td></td>
<td></td>
<td></td>
<td>(16.7)</td>
<td>(33.3)</td>
<td>(100) **</td>
<td>(87.5) **</td>
</tr>
<tr>
<td># with BUG (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>(%)b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(33.3)</td>
<td>(10.0)</td>
<td>(25.0)</td>
</tr>
<tr>
<td>CL score</td>
<td>3.5 ± 0.58</td>
<td>3.67 ± 0.58</td>
<td>3.5 ± 0.58</td>
<td>3.5 ± 0.71</td>
<td>3.5 ± 1.00</td>
<td>3.25 ± 0.96</td>
<td>2.667 ± 1.53</td>
</tr>
<tr>
<td>Antral score</td>
<td>2.75 ± 0.96</td>
<td>3.67 ± 0.58</td>
<td>3.75 ± 0.50</td>
<td>3.5 ± 0.71</td>
<td>2.75 ± 1.26</td>
<td>3.5 ± 0.58</td>
<td>3.67 ± 0.58</td>
</tr>
<tr>
<td>E2 levels at</td>
<td>67.97 ± 8.0</td>
<td>33.61 ± 3.47</td>
<td>51.91 ± 9.79</td>
<td>26.29 ± 9.35</td>
<td>35.58 ± 9.52</td>
<td>66.22 ± 6.18</td>
<td>41.19 ± 16.15</td>
</tr>
<tr>
<td>necropsy (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note* Percentages are on an individual basis, not a litter means basis. SV, seminal vesicle; LA, levator ani; BUG, bulbourethral gland.

*a* Identity of organs confirmed by pathology at 1/litter.

*b* Identity of every BUG confirmed.

*c* Scores based on CL or antral follicle abundance in ovarian sections (see text for details). Values are means ± SD.

*d* Overall F value not significantly different.

Data analyzed by Fisher’s Exact test unless otherwise noted.

* p < 0.05, ** p < 0.0001, compared to control values. † p = 0.0103 by Pearson’s Chi square test, not different by Fisher’s Exact.
Table 7. Minimal effect of exposure to TP on GD 14 - 19 on reproductive organs of male offspring at necropsy (PND 161-178)

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>0</th>
<th>0.1</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>613.26 ± 15.70</td>
<td>656.37 ± 25.23</td>
<td>571.87 ± 12.10</td>
<td>566.06 ± 26.77</td>
<td>552.66 ± 23.08</td>
<td>578.11 ± 68.85</td>
<td>571.00 ± 14.87</td>
</tr>
<tr>
<td>Rt. testis (g)</td>
<td>1.802 ± 0.058</td>
<td>1.847 ± 0.043</td>
<td>1.671 ± 0.029</td>
<td>1.688 ± 0.063</td>
<td>1.665 ± 0.090</td>
<td>1.712 ± 0.153</td>
<td>1.673 ± 0.109</td>
</tr>
<tr>
<td>Rt. caput epi.</td>
<td>0.329 ± 0.019</td>
<td>0.349 ± 0.013</td>
<td>0.299 ± 0.004</td>
<td>0.322 ± 0.008</td>
<td>0.307 ± 0.017</td>
<td>0.299 ± 0.008</td>
<td>0.305 ± 0.014</td>
</tr>
<tr>
<td>Rt cauda epi. (g)</td>
<td>0.329 ± 0.020</td>
<td>0.338 ± 0.016</td>
<td>0.307 ± 0.013</td>
<td>0.287 ± 0.007</td>
<td>0.320 ± 0.018</td>
<td>0.344 ± 0.018</td>
<td>0.301 ± 0.007</td>
</tr>
<tr>
<td>VP (g)</td>
<td>0.579 ± 0.045</td>
<td>0.685 ± 0.040</td>
<td>0.603 ± 0.032</td>
<td>0.715 ± 0.021</td>
<td>0.649 ± 0.076</td>
<td>0.640 ± 0.044</td>
<td>0.697 ± 0.071</td>
</tr>
<tr>
<td>SV (g)</td>
<td>1.900 ± 0.155</td>
<td>2.026 ± 0.199</td>
<td>1.698 ± 0.119</td>
<td>1.626 ± 0.088</td>
<td>1.705 ± 0.077</td>
<td>1.811 ± 0.096</td>
<td>1.714 ± 0.025</td>
</tr>
<tr>
<td>LA/BC (g)</td>
<td>1.344 ± 0.069</td>
<td>1.393 ± 0.078</td>
<td>1.214 ± 0.041</td>
<td>1.172 ± 0.034</td>
<td>1.205 ± 0.042</td>
<td>1.338 ± 0.078</td>
<td>1.262 ± 0.042</td>
</tr>
<tr>
<td>Glans penis (mg)</td>
<td>125.1 ± 1.48</td>
<td>118.9 ± 1.90</td>
<td>111.32 ± 1.45**</td>
<td>111.4 ± 1.69**</td>
<td>110.99 ± 1.73**</td>
<td>122.08 ± 1.67</td>
<td>117.69 ± 1.68*</td>
</tr>
</tbody>
</table>

*Note* Values represent litter means ± SE. epi., epididymis; VP, ventral prostate; SV, seminal vesicle; LA/BC, levator ani/bulbocavernosus muscle group.

* p < 0.05, ** p < 0.0001, compared to control values.
Figure legends

Figure 1. Effect of testosterone propionate administered prenatally (gestational days 14 through 19) on anogenital distance (AGD) in 2 day old pups. a-g: Frequency distribution histograms of AGD in pups. Solid line in each graph represents the distribution of measurements for the dose group represented, dashed line represents control group distribution for comparison. a) control b) 0.1 mg TP, c) 0.5 mg TP, d) 1 mg TP, e) 2 mg TP, f) 5 mg TP, g) 10 mg TP; h) Mean AGD ± SE for the lower dose groups, in which sexing of pups was possible. AGD was significantly reduced in male pups in the 0.5 mg TP dose group. * p < 0.05.

Figure 2. Effects of prenatal testosterone propionate (TP; gestational days 14-19) on a) hydrometrocolpos and mortality b) uterine weight at necropsy (PND 112 - 158). H, hydrometrocolpos. * p < 0.05, ** p < 0.01, *** p < 0.0001, compared to controls; analyzed using Fisher’s Exact test.

Figure 3. Severe distention and fluid retention of the uterus (hydrometrocolpos) with vaginal atresia in postpubertal females exposed prenatally to middle range doses of testosterone propionate (TP; gestational days 14 - 19). Female was exposed prenatally to 1 mg testosterone propionate and sacrificed at postnatal day 74 after presenting a distended abdomen. The uterus was filled with brown, viscous, odorous fluid.

Figure 4. Development of male organs in females prenatally exposed to 10 mg TP (gestational days 14 - 19). o, ovaries; u, uterus; b, bladder; p, prostate; sv, seminal vesicles;
ph, phallus; la, levator ani; c, colon.  a) Note normal appearance of uterus and follicles in ovaries indicating cyclicity, with male reproductive organs attached to tract. Seminal vesicles were found attached to sides of uterus at cervical area; prostate tissue formed on ventral and sometimes lateral sides of urethra at base of bladder. Such affected animals also had no vaginal orifice and commonly developed the levator ani muscle (b) attached to the penile bulbs of the inner shaft of the penis.  c-f) Photomicrographs of sections of male reproductive organs found in testosterone propionate-exposed (gestational days 14 - 19) females. c) seminal vesicle from a female in the 10 mg TP dose group, d) prostate from a female in the 0.5 mg TP group, e) bulbourethral gland from a female in the 2 mg TP dose group, f) levator ani muscle from a female in the 2 mg TP dose group. Magnification 250X. Bar in each photo represents 100 μm.

Figure 5. Kidney to ovary distance, or ovarian ligament length, in females exposed prenatally to testosterone propionate (TP; gestational days 14 - 19). Increased ovarian ligament length is an indicator of androgenic activity. Diagram illustrates the distances measured between the kidneys and ovaries. Data are displayed below diagram. Values are litter means ± SE.  

\[ \begin{align*}
\text{Kd-Ov} & \text{, kidney to ovary distance; vtKd-Ov, vertical kidney to ovary distance (see methods for detailed description of measuring technique).} \\
\text{\{\^{\dagger}\}} p < 0.05, \text{\{\^{\ast}\} } p < 0.01, \text{\{\^{\ast\ast}\} } p < 0.0005, \text{\{\^{\ast\ast\ast}\} } p \leq 0.0001, \text{ all as compared to controls.}
\end{align*} \]

Figure 6. T levels in dams and fetuses at gestational day (GD) 19, after subcutaneous administration of 0.5 or 1 mg TP/dam (GD 14 - 19). a) T levels in dam serum are increased in a dose-dependent fashion at 0.5 mg (p < 0.05) and 1 mg (p < 0.01) TP/rat. n = 2 dams per
treatment group; Values represent litter means ± SE. Analyzed using log transformed data due to variability among litters. b) T levels in fetal homogenates increased only at 1 mg TP/dam in female offspring (p < 0.05). n = 2 litters in control group, 3 litters each in 0.5 mg TP and 1 mg TP groups, mean litter size = 13 fetuses. Values represent litter means ± SE. * p < 0.05, ** p < 0.01.
Figure 1
Figure 2
Figure 3

uteras

bladder

(no vaginal orifice)
Figure 4
Figure 5. Kidney to Ovary Distances at Necropsy in Females Exposed to Testosterone Propionate on GD 14 - 19.

<table>
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<th>Endpoint</th>
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<th>0.5</th>
<th>1</th>
<th>2</th>
<th>5</th>
<th>10</th>
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<td>24.82 ± 1.88</td>
<td>22.32 ± 0.68</td>
<td>19.51 ± 5.51</td>
<td>25.66 ± 1.80</td>
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<td>Rt. vtKd-Ov distance</td>
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<td>11.16 ± 3.34</td>
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<td>Lt. vtKd-Ov distance</td>
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<td>13.53 ± 4.73</td>
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<td>14.8 ± 3.17</td>
<td>14.22 ± 3.17</td>
<td>36.03 ± 3.76</td>
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</tbody>
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Figure 5
Figure 6
Interactive Effects of Vinclozolin and Testosterone Propionate on Sexual Development of Male Sprague-Dawley Rats

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keywords: antiandrogen, androgen, sexual differentiation, fetal development, endocrine disrupter, chemical mixtures

abbreviated title: Vinclozolin and TP in male development

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Abstract

Male sexual development is directed primarily by androgens, and the absence of androgens or the dysfunction of the androgen receptor during sexual differentiation in the mammalian fetus produces the female phenotype. In previous studies in our laboratory, administration of vinclozolin (V), a competitive inhibitor of the androgen receptor, to rat dams on gestational day (GD) 14 - 19 compromised masculine development of male offspring, resulting in vaginal pouch, cleft phallus, nipples, and reduced prostate, seminal vesicle, glans penis and levator ani weights. Administration of testosterone propionate (TP) to dams during gestation did not affect males but induced masculine development in females. Each of these chemicals produced adverse effects on pregnancy. In the current study we investigated the effects of the combination of V and TP on maternal and neonatal health and on sexual differentiation of the male Sprague-Dawley rat. We hypothesized that TP antagonizes the effects of V on male sexual differentiation, but that they add to each other’s adverse effects on pregnancy and neonatal health.

Dams were dosed daily on GD 14 – 19 with corn oil vehicle (Control; 2.5 ml/kg-bw; oral gavage), V (200 mg/kg/2.5 ml; oral gavage), TP (1 mg/0.1 ml/rat; sc), or V+T. Dams and their offspring were monitored from GD 14 through adulthood. V and TP each reduced maternal weight gain, although V+TP further reduced maternal weight gain. Litter size on postnatal day (PND) 2 was reduced significantly by V+TP. Pup body weight was reduced by V alone and more significantly by TP alone or V+TP. V induced nipples, ectopic testes, vaginal pouch and cleft phallus in the offspring, while V+TP attenuated these responses. However, while V reduced weights of the ventral prostate, seminal vesicle, levator ani and bulbourethral glands, V+TP did not attenuate these responses. We have observed that the combination of two EDCs with opposing action, V
and TP, antagonize the effects of the other on some indices of androgen-dependent sexual
development, and may have additive effects or non-interactive effects on both pregnancy
and other indices of androgen-dependent development.
Introduction

Humans may come in contact with endocrine disrupting compounds (EDCs) that affect the reproductive system through sources such as the use of plastics, personal care products, pharmaceuticals or exposure to agrochemicals. Considering the wide variety of sources of EDC exposure, it is realistic to assume humans are exposed to combinations or mixtures of EDCs. Certainly, mixtures of endocrine disrupting compounds have been found in human body fluids (Blount et al., 2000; Foster et al., 2000; Younglai et al., 2002) as well as in wildlife (LeBlanc et al., 1995; Guillette et al., 1999; Veith et al., 1981; Vos et al., 2001) and many of these were associated with altered reproductive function or morphology.

Although the effects of individual EDCs have been well characterized in laboratory studies, the effects of combinational exposure on humans and wildlife are difficult to predict by data generated from laboratory studies on individual chemicals. Furthermore, most laboratory studies that have been performed on chemical mixtures involved only in vitro studies, and in vivo effects cannot be predicted accurately by the results of in vitro assays, given the complex physiology of organisms. The inherent complications that arise from risk assessments of combinational exposure to EDCs have been discussed at international conferences (discussed in Carpy et al., 2000) and the need for in vivo research on combinational exposure agreed upon. Some work has been done in vivo to assess the effects of combinational exposure to environmental EDCs. Results sometimes contrasted with predictions, and only supports the need for further research. Estrogenic compounds (You et al., 2002), and persistent organic pollutants (TCDD and DDE; Loeffler and Peterson, 1999) were used in concert to reveal effects in vivo that contrast with the effects of these chemicals in vitro. Another aspect to consider in risk
assessment is the identification of the populations most at risk. The developing fetus, being more sensitive to the endocrine disrupting and toxic effects of EDCs to begin with, may more readily exhibit any additive effects of combinational exposures to chemicals. Combinational exposure to two antiandrogenic EDCs with the same mechanism of action, vinclozolin and procymidon, or dissimilar mechanisms of action, procymidon and dibutyl phthalate (DBP), were performed on pregnant rats and produced additive antiandrogenic effects (Gray et al., 2001). Combinational exposure studies that are designed to ultimately protect human populations need to include these susceptible populations.

Antiandrogens found in our environment include the group of plasticizers called phthalates, the herbicide linuron, and the pesticides vinclozolin, procymidon and fenitrothion. Vinclozolin (V) is a competitive inhibitor of the AR that binds reversibly to the AR (Kelce et al., 1994). Antiandrogenic effects of V include induction of vaginal pouch, hypospadias, and ectopic testes, nipple retention, reduced anogenital distance (AGD) or reduced weight of prostate, seminal vesicle and levator ani/bulbocavernosus muscle group, and at extended dosing periods at higher concentrations, reduced testis or epididymis weights (Gray et al., 1994; Hellwig et al., 2000; Wolf et al., 2000). V can also be deleterious to pregnancy and pup health, as it has been shown to reduce maternal weight gain, litter size, and pup weight (Hellwig et al., 2000; Gray et al., 1994; Wolf et al., 2000).

Several reports suggest androgens exist in the environment. Female mosquito fish downstream of pulp mills have been found masculinized (Howell et al., 1980; Parks, et al., 2000). Trenbolone acetate, which is fed to beef cattle for growth and weight gain and is found in feed lot run-off, was shown to masculinize rodents, dogs (FDA, NADA
summary, accessed 2001; Wilson et al., submitted), fish (Ankley et al., 2002), sheep and
cattle (DeHaan, et al., 1990; Groot et al., 1989). Androgens also bind the AR reversibly
and competitively, but activate rather than inhibit the AR. Androgenic compounds can
disrupt sexual differentiation and reproductive function, exacting effects mostly in the
female. Androgens such as testosterone propionate (TP), methyltestosterone and
androsterone administered during the prenatal period masculinize the internal and
external genitalia of female rats to produce a male phenotype (Greene et al., 1939; Wolf
et al., 2002). However, androgens, like antiandrogens, can have adverse effects on
pregnancy at high concentrations. Testosterone, trenbolone acetate, or TP, given to
mother animals was shown to reduce maternal gestational body weight, litter size and
body weight of offspring (Greene et al., 1939; Fritz et al., 1984; DeHaan, et al., 1987,
1990; Wolf et al., 2002). Should an animal be exposed to the combination of V and TP,
each of these two chemicals could antagonize the action of the other in vivo, since the
actions and effects of TP and V are in direct contrast with one another.

In this study, we evaluate the reproductive and developmental effects of co-
administration of V and TP, chemicals that use the same mode of action, binding the AR,
with opposing actions at the AR. We base this study on the results of two previous
studies in which the characteristics and effective dose levels of each compound, V and
TP, have been identified (Wolf et al., 2000; 2002). We hypothesize that TP co-
administration will antagonize the antiandrogenic effects of V in the male offspring, and
will together augment the toxic effects of each compound.

**Materials and Methods**

**Animals**
Timed pregnant Sprague-Dawley rats arrived at the animal facility on gestational day (GD) 4 (GD 1 = day of plug) and were housed individually in polycarbonate cages (20 cm X 25 cm X 47 cm) with laboratory-grade pine shavings, heat-treated to remove resins, as bedding. They were acclimated to an atmosphere of 68-74°F, 40-50 % relative humidity and a reversed light schedule (14 h light :10 h dark; lights off 11:00 am E.S.T.) They were given PMI® LabDiet® 5008 (Purina Mills Inc., Brentwood, MO) and tap water (Durham municipal water, tested for pesticides and heavy metals every 4 months) ad libitum. On GD 12, dams were weighed, weight ranked and 28 dams randomly assigned to treatment groups (7 dams per treatment group) that were equilibrated with respect to body weight. On GD 14 through GD 19, dams were dosed once daily with corn oil (vehicle, control; 2.5 ml/kg, by oral gavage), vinclozolin (V; Reidl-de Haen, Germany, distr. by Sigma-Aldrich, lot # 9126X; 200 mg/kg, by oral gavage), testosterone propionate (TP; Sigma-Aldrich Co., St Louis, MO, lot #98H0566; 1 mg/0.1 ml/rat, by subcutaneous injection), or V+TP. Dose of TP was on a per rat basis without correction for body weight as reported in literature and in the previous TP study (Chapter 2).

Maternal weight through the dosing period was monitored. Day of delivery was recorded and pups counted on GD 23 (PND 1). On PND 2, pups were counted, weighed, sexed if possible, and measured for anogenital distance (AGD). AGD was measured on each pup in a blind fashion using a dissecting microscope fitted with an ocular micrometer reticle. On PND 14, pups were sexed by palpation of testes and males were checked for areolae count and scored in blind fashion. A nipple or an areola was considered an areola. Areola count was by consensus of two technicians. Each areola was scored on a scale of 1 to 3 on the basis of prominence, with 3 being most prominent (Fig. 1). Scores for each of the 12 areolae were then added together within a pup to
produce a “pup areola score” with a maximum possible score of 36 per pup (12 areolae X maximum areola score of 3 = 36). Pup areola scores were used in statistical analysis.

Eye opening was also monitored on PND 14. The number of individual eyes that were open in each litter were counted.

On PND 24, pups were weaned, counted, sexed, and measured for AGD in blind fashion using rotary dial micron calipers (Manostat). Litter mates were distributed 2 - 3 per cage, given an ear punch identification for treatment group, assigned an individual identification marking with picric acid stain, and provided Purina LabDiet® 5001 and tap water ad libitum. Dams were euthanized 1 day after weaning (pup PND 25) by carbon dioxide asphyxiation followed by decapitation and uterine implantation sites were counted by visual examination. Viability and general health of offspring were monitored.

On PND 170 - 186, male offspring were weighed and necropsied in blocked fashion by treatment group. Males were euthanized by decapitation without prior CO₂ asphyxiation in accordance with USEPA IACUC standards. Each carcass was shaved on the ventral side to view nipples, and then necropsied. The following endpoints were monitored on 3 males per litter: malformations of the external genitalia including cleft phallus or prepuce, vaginal pouch, ectopic testes; right testis wt; right epididymis wt; full and expressed seminal vesicle weights; ventral prostate weight; glans penis weight; levator ani weight; and bulbourethral gland (BUG) weight. Necropsy was continued on every male offspring in the study (n = 143 total) for the following endpoints: malformations, right testis and epididymis weight. Undescended testes were not included in testis weight measurements.

Statistics
Data was analyzed on a litter means basis by one-way analysis of variance (ANOVA) using general linear models procedure (proc glm; due to the variable number of individuals per litter) using SAS software (HERLSAS DECversion 1.2-4 on USEPA LAN terminal; SAS Institute Inc, Cary, NC). When general differences for treatment effects were found in overall ANOVA (p < 0.05), differences between treatment groups were analyzed with a two-tailed t test using least square means. Counts were converted to percentages. Percentage data on litter means (% eyes open, % cleft phallus, % undescended testes, etc.) were arcsine transformed to normal distribution for analysis by ANOVA as described above. Organ weights and AGD were also analyzed by co-variate analysis by body weight. Agenesis data was analyzed by two tailed Fisher’s Exact test.

Results

Maternal and Litter Effects

Maternal weight gain was reduced in the TP group to 66 % of control value (p < 0.05), in the V group to 52.9 % of the control value (p < 0.005), and further reduced in the V+TP group to less than half the control value (41.3%; p < 0.0005; Table 1). One control group dam was not pregnant. This animal was not used in the data analysis for weight gain.

One dam in the TP group delivered later than PND 1. On the day of birth (PND 1), pup count was incomplete since not all dams finished delivering by the end of the day. Litter size on PND 2 was not affected by either V or TP alone (12.4-14 pups/litter in all groups), but was drastically reduced by V+TP to 6.50 pups/litter (p < 0.001; Table 1). The reduction in litter size was due to pup loss after the implantation period, since the number of uterine implantation sites in all groups was not different. Whole litter loss
occurred in three litters in the V+TP group by PND 2. At weaning (PND 23), litter size remained significantly lower in the V+TP group. Viability of the offspring to weaning age was only slightly reduced by V, but not by any other treatment.

Pup body weight on PND 2 was slightly reduced by V (p< 0.05), but greatly reduced by TP (p< 0.0001). Pup body weight in the V+TP group was reduced to a weight slightly less than but similar to that in the TP group (Table 1).

**Neonatal development**

AGD on PND 2 in male offspring was significantly reduced from control values by both T (p < 0.0002), V (p < 0.0001) and V+TP (p < 0.0001) without adjustment for body weight. When analyzed with co-variance analysis by body weight, AGD was reduced only in those groups exposed to V (V and V+TP; both p < 0.0001) and not by TP, since TP exposed pups were smaller (Table 4).

Areolae do not develop in male rats due to inhibition by prenatal androgenic activity, and virtually no areolae developed on control or TP exposed males (Table 2). One areola was found on only one pup in each of the control or TP groups (which appears inflated on a litter means basis; control mean = 12.3 %; Table 2) but a single, temporary areola on one control male is not unusual for this sample size. In contrast, the full compliment of areolae (n = 12 areolae) developed on every male in the V and the V+TP groups. Although the V and V+TP groups did not differ from each other in areola count, areola score reflected a difference in the prominence of the areolae between the V and the V+TP groups (Table 2). Mean areola score for males in the V group was 30.4 out of a possible score of 36 (p < 0.0001), while areola score for males in the V+TP group was significantly lower, 23.8 (p < 0.0001 compared to V group), showing a reduction of the prominence of V-induced areolae by co-administration of TP.
Eye opening, or the percent of all eyes in a litter open, is a landmark of development. Eye opening was severely reduced on PND 14 by TP (Table 2), but restored to control values by V+TP. Percent eyes open was somewhat but not statistically reduced by V as well.

By weaning age (PND 24), body weight in male offspring was not affected by V, was slightly reduced in the TP group (p < 0.05), and was further reduced in the V+TP group (p < 0.005; Table 2).

Necropsy

Nipples were not found on males in the control or TP group. Nipples had developed on all males in the V group and the number of nipples per male rat in this group was almost the full compliment, 11.68 out of 12 (Table 3). V+TP did not reduce the percentage of males with nipples, but reduced the number of nipples per male to 9.5 (p < 0.005 compared to V group) and the nipple score from 23.21 in the V group to 14.5 in the V+TP group (p < 0.005). V-exposed male offspring displayed the malformations cleft phallus with hypospadias, vaginal pouch, and ectopic testes, and the incidences of each of these endpoints were reduced in the V+TP group (Table 3). At adulthood (PND 170 - 186), male body weight was not affected by V or TP alone, and was only slightly affected by V+TP (p < 0.01; Table 4). Weights of ventral prostate, seminal vesicle, LA/BC, BUG and glans penis were significantly reduced from control values to the same extent by either TP or V+TP. Weights of these organs in the V+TP group were not significantly different from those in the V group (Table 4). When adjusted to the reduced body weight in the V+TP group, means and statistical significance did not differ when the V+TP group was compared to the control or to the V group. Weights of these organs were measured on a smaller sample size than that used for measuring malformations,
because a number of males in the V and the V+TP groups displayed agenesis of the VP or SV. Agenesis induced by V was not reversed by V+TP, but in the case of VP agenesis, was enhanced.

**Discussion**

This study addresses the issue of exposure to combinations of environmental endocrine disrupters in sensitive populations such as the pregnant female and the developing fetus. The study of chemical mixtures and risk assessments thereof is infinitely complicated. Therefore it is most informative to use a systematic approach and begin with simple combinations of chemicals, chemicals that are likely to interact in a predictable manner and those most likely to be encountered in combination in the environment. Vinclozolin (V) metabolites and testosterone propionate (TP) metabolites (T and DHT) both bind the AR reversibly. V metabolites inhibit while TP metabolites activate the AR. Both types of endocrine disrupters are found in the environment, especially in the agricultural setting, and both androgens and antiandrogens are used medicinally. We investigated the effects of combinational exposure to V and TP on pregnancy and on sexual differentiation of the male rat fetus. Our primary goal was to determine the ability of TP to antagonize the effects of V in the male rat. We have found that, in general, co-administration of V and TP to the pregnant rat from gestational day 14 through 19 results in additive adverse effects on maternal and neonatal health, while TP antagonizes only some of the effects of V in the developing male fetus.

**Toxicity in Dams and Neonates**

Effects on maternal weight and litter size were greatest in the V+TP group, suggestive of additivity or synergy, but not antagonism. Litter size was reduced only by
the combination of V + TP. This effect resembles synergy but when considering the
dose-response effects of each compound as defined in previous studies, these results
actually indicate potentiation, in which one compound has no effect itself but augments
the effects of the other (Cassee et al., 1998). Higher doses of TP can reduce live litter
size to the extent seen in the current study (Wolf et al., 2002), while V given for a longer
duration (GD 14 through PND 3) or at a higher dose did not reduce litter size (Gray et al.,
1994; Wolf et al., 2000). Co-administering the two chemicals at the doses used in the
current study produced the effect of TP seen at a higher dose. Thus the interaction of V
and TP on litter size is potentiation. Why V would not alleviate the effects of TP is not
understood, but suggests a more complex regulatory mechanism of AR action, and an
activity of AR binding that is in addition to the androgenic/antiandrogenic activity.

TP also caused adverse effects in the offspring as evidenced by reduced body
weight and delayed eye opening. The reduction in PND 2 body weight in the V+TP
group is attributable to TP since body weight means in the two groups did not differ. The
weight reduction caused by prenatal TP persisted through weaning age. Reduced body
weight, dystocia, and decreased milk production have been reported as an effect of
androgens previously (DeHaan et al., 1990; Swanson and Werff ten Bosch, 1965). The
decrease in milk production may have compounded the already reduced body weights of
the pups at birth, causing the reduced body weight to persist to weaning. Eye opening in
male offspring as determined by one day observation was delayed by TP. The reduction
in pup body weight together with delayed eye opening may indicate a delay in
development, although it does not appear to be a factor in the assessment of androgenic
developmental processes.

Interaction between V and TP in Offspring
It was presumed that TP has no effect alone on males, as shown in a previous study (Wolf et al., 2002), and that any effect in these androgen-dependent tissues would be due to V, and alleviation of these effects by TP. Some antiandrogenic effects of V were attenuated by TP, although some were exacerbated, and some did not demonstrate any interaction between V and TP.

*TP attenuates effects of V*

TP clearly antagonized V in some tissue. TP attenuated the prominence of areolae, the number of nipples, and the incidence of malformations of the external genitalia, including cleft phallus, vaginal pouch, and ectopic testes induced by V. The use of an areola score of prominence was valuable in detecting a difference between groups that would have been missed at this early age by conventional endpoints (i.e. number of areola). These tissues are mediated by dihydrotestosterone (DHT), produced from T by 5α-reductase. DHT binds the AR with at least 2X more potency than T (Luke and Coffey, 1994), so in these tissues, the more potent DHT may be able to elicit more androgenic activity from the AR and thus attenuate the effects of V. However, the presence of 5α-reductase in some tissue cannot always predict the interaction of V by TP, since the reduction in prostate weight, a DHT-mediated tissue, was not attenuated by TP.

*TP enhances the effects of V*

V+TP reduced testis and epididymis weights further than V alone. High doses of prenatal V can reduce testis and epididymis weight (Gray et al., 1994). High doses of prenatal TP has not been shown to affect testis weight, here or previously (Wolf et al., 2002). Thus this indicates potentiation, or the ability of TP to enhance the effect of V on the testis. In addition, V induced agenesis of the VP, as observed in a previous study (Gray et al., 1994), and V+TP exacerbated this effect rather than reversed it. We
observed a phenomenon in the interaction of V and TP in male sexual development that seems paradoxical, as these chemicals are known to have opposing action at the AR. It is not understood why TP did not rescue or antagonize the effects of V on these androgen-dependent tissue. These data suggest V and TP may act in the same manner on some target tissue, suggesting a possible alternate mechanism of each chemical, or of the AR.

*Lack of Interaction between V and TP*

In the seminal vesicle, ventral prostate, levator ani, and BUG, V+TP reduced weights no further or less than did V. The incidence of agenesis of the VP and SV in the V and the V+TP groups are not different from each other nor high enough to have influenced the organ weight data. The reason for the lack of attenuation in the organ weights is not understood. However, the mechanism of steroid hormone receptor binding involves many cofactors, and variation in the composition of cofactors between various androgen-dependent tissue could be responsible for the variation in response to the combinational effects of TP and V.

AGD is an endpoint that was not expected to respond in the same way as other tissues to co-administration with TP, since in our previous study, TP itself reduced AGD in males (Wolf et al., 2002), as does V. The reason for this apparent antiandrogenic effect of TP is not understood. In the current study, no interaction of TP and V on AGD was observed. The absolute mean AGD in the TP group was lower than that in the control group, but this reduction was due to reduced body weight.

*Dose*

In contrast to these results, the results of the same dosing regimen on female offspring clearly illustrated antagonism between TP and V in all endpoints (Wolf et al., submitted). The inability of V and TP to antagonize all of each other’s effects in the
male compared to the female could be due to the doses used of the compounds, V apparently more potent than TP. Using other doses of V or TP could not be justified. The previous studies on V and TP indicated that higher doses of TP would have produced more toxicity and fewer offspring, and lower doses of V would not have elicited sufficient effects for study alone or in combination with another EDC. Regardless of some shortcomings, it was advantageous to have observed incomplete attenuation of V-induced effects by TP in the male as it allowed us to see the difference in the responsiveness of the tissues to the androgen and the antiandrogen. This difference in response is the most confusing outcome of this study but a phenomenon that alerts us to unexpected results of combinational exposures. Although we hypothesized that the androgen TP would attenuate the effects of the antiandrogen V, we did not observe that in every tissue. The reason for predominance of V or of TP in various tissues is not understood. An explanation for this difference in response among the various androgen-dependent tissue cannot be elucidated from this study. We are aware that different tissue may contain different coregulators of androgen action and different concentrations of enzymes and substrates, which may act differently in the face of V or TP, but an explanation is not available at this time.

**Summary**

This study alerts us to several important concepts. Most readily apparent is that the action of two compounds that act in opposite ways at a common target, the AR, do not necessarily manifest as attenuation of the effects of one compound by the other *in vivo*. Secondly, we saw that the interpretation of the effects of combinational exposure requires knowledge of the dose-response relationships of each chemical. This is required
in order to attribute an effect found only with a combination of compounds to one or to
the other compound, or to both. In addition, we showed that other more critical
endpoints such as nipple or areola score rather than number may be required in the
testing of EDCs to reveal subtle effects of exposure. This would be most useful in
detecting interactions, or characterizing more distinguishing dose-response relationships
of one chemical. Lastly, the fact that both these chemicals, V and TP, have similar
effects on some androgen-dependent tissues, and both affect processes or tissues not
classically identified as androgen-dependent, suggest that they each possess another
mechanism of action. Whether this alternate mechanism of action is common to both
chemicals or involve the AR cannot be determined by this study.

This study is testimony to the belief that in vivo effects cannot be accurately
predicted by *in vitro* data, or data on each individual compound.

**Acknowledgements**

The author gratefully acknowledges Joseph Ostby, Jonathan Furr, and Andrew
Hotchkiss for their assistance with animal handling and data collection.
References


Table 1. Effects of Prenatal Vinclozolin, Testosterone Propionate, or the Combination of the Two Chemicals on Pregnancy and Neonatal Health and Development

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Treatment Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Maternal weight gain (g)</td>
<td>53.08 ± 2.82</td>
</tr>
<tr>
<td></td>
<td>*</td>
</tr>
<tr>
<td>No. Dams delivered late</td>
<td>0</td>
</tr>
<tr>
<td>Live litter size (PND 2)</td>
<td>13.17 ± 0.82</td>
</tr>
<tr>
<td>Whole litter loss by day 2</td>
<td>0</td>
</tr>
<tr>
<td>No. Uterine implantation sites</td>
<td>13.83 ± 0.703</td>
</tr>
<tr>
<td>Pup weight on PND 2 (male) (g)</td>
<td>7.966 ± 0.260</td>
</tr>
<tr>
<td>Litter size at weaning</td>
<td>13.2 ± 2.14</td>
</tr>
<tr>
<td>% Viability</td>
<td>100</td>
</tr>
</tbody>
</table>

Note: Values are litter means ± SE, unless otherwise noted. Live litter sizes include those litters with live litter size of 0 on PND 2. PND, postnatal day.

* Values are means ± standard deviation

* p < 0.05, ** p < 0.005, *** p < 0.0005, † p < 0.0001
Table 2. Effects of Vinclozolin on Sexual Development of Prepubertal Male Offspring are Attenuated by Testosterone Propionate.

<table>
<thead>
<tr>
<th>Endpoints</th>
<th>Treatment group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>AGD on PND2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.042 ± 0.10</td>
</tr>
<tr>
<td>No. Areolae/pup on PND 14</td>
<td>0.29 ± 0.16</td>
</tr>
<tr>
<td>% pups with areolae</td>
<td>12.27 ± 4.57</td>
</tr>
<tr>
<td>Pup Areola score</td>
<td>0.29 ± 0.16</td>
</tr>
<tr>
<td>% Eyes Open on PND 14</td>
<td>60.0 ± 0.127</td>
</tr>
<tr>
<td>Body weight at weaning</td>
<td>66.07 ±2.75</td>
</tr>
</tbody>
</table>

Note: Values are litter means ± SE, unless otherwise noted. NSD, not statistically different.

<sup>a</sup> Analyzed with body weight as a co-variate.

<sup>b</sup> Significantly different at p < 0.005 compared to V group.

<sup>c</sup> see text in Materials and Methods and Figure 1 for description of areola score.

* p < 0.05, ** p < 0.005, ***p < 0.0005, † p ≤ 0.0001 compared to control group.
Table 3. Malformations in Male Offspring Induced by Vinclozolin are Often Attenuated by Co-administration of Testosterone Propionate.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Treatment Group</th>
<th>Control</th>
<th>TP</th>
<th>V</th>
<th>V+TP</th>
</tr>
</thead>
<tbody>
<tr>
<td>% with nipples</td>
<td></td>
<td>0</td>
<td>0</td>
<td>100 †</td>
<td>100 †</td>
</tr>
<tr>
<td>No. nipples per male</td>
<td></td>
<td>0</td>
<td>0</td>
<td>11.68 ± 0.21 †</td>
<td>9.5 ± 1.28 †</td>
</tr>
<tr>
<td>Nipple score</td>
<td></td>
<td>0</td>
<td>0</td>
<td>23.2 ± 1.58 †</td>
<td>14.5 ± 3.84 †</td>
</tr>
<tr>
<td>Percent cleft phallus</td>
<td></td>
<td>0</td>
<td>0</td>
<td>92.8 ± 4.95 †</td>
<td>75.0 ± 14.43 †</td>
</tr>
<tr>
<td>Percent ectopic testes</td>
<td></td>
<td>0</td>
<td>0</td>
<td>65.48 ± 12.9 ***</td>
<td>17.48 ± 11.8 a</td>
</tr>
<tr>
<td>Percent vaginal pouch</td>
<td></td>
<td>0</td>
<td>0</td>
<td>79.17 ± 11.96</td>
<td>40.63 ± 22.46</td>
</tr>
</tbody>
</table>

Note: Values are litter means ± SE. VP, ventral prostate; SV, seminal vesicle.

* Statistically different compared to V group by at least p < 0.05.

b Significantly different compared to V group at p = 0.082.

*p < 0.05, ** p < 0.01, ***p < 0.005, † p < 0.0001 compared to controls.
Table 4. Reproductive Organ Weights at Necropsy in Males Exposed Prenatally to Vinclozolin are Not Restored by Testosterone Propionate.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>TP</th>
<th>V</th>
<th>V+TP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventral prostate wt (g)</td>
<td>0.814 ± 0.047</td>
<td>0.710 ± 0.042</td>
<td>0.151 ± 0.048</td>
<td>0.196 ± 0.076</td>
</tr>
<tr>
<td>Seminal vesicles - full wt (g)</td>
<td>1.955 ± 0.040</td>
<td>1.788 ± 0.094</td>
<td>0.869 ± 0.112</td>
<td>0.836 ± 0.218</td>
</tr>
<tr>
<td>LA+BC wt (g)</td>
<td>1.339 ± 0.025</td>
<td>1.297 ± 0.042</td>
<td>0.566 ± 0.048</td>
<td>0.640 ± 0.043</td>
</tr>
<tr>
<td>paired BUG wt (g)</td>
<td>0.184 ± 0.014</td>
<td>0.187 ± 0.007</td>
<td>0.058 ± 0.021</td>
<td>0.062 ± 0.035 **</td>
</tr>
<tr>
<td>Glans Penis wt (mg)</td>
<td>123.5 ± 1.80</td>
<td>118.82 ± 2.59</td>
<td>73.41 ± 3.64 †</td>
<td>75.47 ± 4.39 †</td>
</tr>
<tr>
<td>Right testis wt (g)</td>
<td>1.769 ± 0.044</td>
<td>1.735 ± 0.038</td>
<td>1.621 ± 0.041</td>
<td>1.312 ± 0.177 **b</td>
</tr>
<tr>
<td>Rt epididymis wt. (g)</td>
<td>0.6586 ± 0.0167</td>
<td>0.6384 ± 0.0112</td>
<td>0.4809 ± 0.0553*</td>
<td>0.3835 ± 0.1304 **</td>
</tr>
<tr>
<td>Agenesis of VP</td>
<td>0/18</td>
<td>0/19</td>
<td>5/21 *</td>
<td>5/12 **</td>
</tr>
<tr>
<td>No. agenesis / No. animals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agenesis of SV</td>
<td>0/18</td>
<td>0/19</td>
<td>3/21</td>
<td>1/12</td>
</tr>
<tr>
<td>No. agenesis / No. animals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW</td>
<td>621.84 ± 11.32</td>
<td>598.03 ± 23.32</td>
<td>599.45 ± 11.83</td>
<td>558.26 ± 20.34 **</td>
</tr>
</tbody>
</table>

Note: Values are litter means ± SE. Wt, weight; LA+BC, levator ani + bulbocavernosus muscles; BUG, bulbourethral glands.

a Absolute means and statistical analysis reported in table. See text for analysis relative to body weight.

b Statistically different compared to V group at p < 0.05.

* p < 0.05, ** p < 0.005, † p < 0.0001 compared to controls.
Figure Legend

Figure 1. Scoring system for areolae on PND 14, based on prominence or development. a) areola with a score of 1, note the area in center with less hair and slightly darker skin; b) areola with a score of 2; c) areola with a score of 3, note the darker areolar region and the nipple bud. Areola scores for each areola of a possible 12 areolae on a pup were added together to generate a pup areola score with a possible value of 0 - 36. Litter mean pup areola scores for each sex were used in statistical analysis. A similar system was used for nipple score.
Vinclozolin Reverses Effects of Testosterone Propionate on Sexual Development of Female Sprague-Dawley Rats

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keywords: antiandrogen, androgen, masculinization, fetal development, endocrine disrupter, chemical mixtures

abbreviated title: Vinclozolin and TP in female development

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This manuscript is being submitted to Toxicological Sciences and is in the format required of that journal.
Abstract

The presence of androgens during fetal sexual differentiation in the mammal regardless of genetic sex induces the male phenotype. In previous studies in our laboratory, administration of testosterone propionate (TP) to rat dams on gestational days (GD) 14–19 resulted in female offspring with male reproductive organs including prostate, seminal vesicles, bulbourethral glands, and levator ani, and male–like external genitalia. Vinclozolin (V), a competitive inhibitor of the androgen receptor, administered to the dam had no effect in the female offspring but inhibited masculine development in male offspring that resulted in vaginal pouch, cleft phallus, ectopic testes, and reduced weights of prostate, seminal vesicle, glans penis and levator ani/bulbocavernosus muscle. In the current study we investigated the ability of V to antagonize the action of TP and thus attenuate the effects of TP in the female offspring.

Dams were dosed daily on GD 14–19 with corn oil vehicle (Control; 2.5 ml/kg-bw; oral gavage), V (200 mg/kg/2.5 ml; oral gavage), TP (1 mg/rat/0.1 ml; sc), or V+T. Dams and their female offspring were monitored from GD 14 through adulthood. Neonatal body weight was slightly reduced by V alone and was further reduced by TP alone and V+TP. TP increased AGD, reduced the number of areolae and nipples, induced cleft phallus, vaginal agenesis, prostate, bulbourethral glands and levator ani muscle, while V co-administration significantly attenuated these effects. We conclude that 200 mg/kg V fully attenuates the effects of 1 mg TP in the female rat when co-administered during sexual differentiation.
Introduction

The reproductive tract of the mammalian fetus is bipotentate and undergoes sexual differentiation in the late period of organogenesis. The reproductive tract will differentiate into the female phenotype in the absence of hormones, and the male phenotype in the presence of androgens. The female fetus expresses androgen receptors (AR; Cunha et al., 1991) and will also respond to androgens by developing Wolffian ducts structures and masculinized urogenital sinus, or external genitalia. Studies using androgens such as testosterone propionate (TP), methyltestosterone and androsterone administered during the prenatal period showed masculinization of the internal and external genitalia of female rats characteristic of the male phenotype. These characteristics include a larger AGD, lack of nipples or a vagina, and the presence of prostate, seminal vesicles, bulbourethral glands and levator ani muscle (Greene et al., 1939; Swanson and Werff ten Bosch, 1965; Wolf et al., 2002). Masculine development of the reproductive tract including the external genitalia is also observed in humans with congenital adrenal hyperplasia (CAH; New and Wilson, 1999). Masculine development in females can occur in response to environmental androgens as well. Female mosquito fish downstream of pulp mills have been found masculinized (Howell et al., 1980; Parks, et al., 2000). Trenbolone acetate, which is fed to beef cattle for growth and weight gain and is found in feed lot run-off, was shown to masculinize female rodents, dogs (FDA, NADA summary, accessed 2001; Wilson et al., submitted), fish (Ankley et al., 2002), sheep and cattle (DeHaan, et al., 1990; Groot et al., 1989) when administered during gestation. However, androgens, like antiandrogens, can have adverse effects on pregnancy. Testosterone, TP, or trenbolone acetate given to mother animals was shown
to reduce maternal gestational body weight, litter size and body weight of offspring (Greene et al., 1939; Fritz et al., 1984; DeHaan, et al., 1987, 1990; Wolf et al., 2002).

Antiandrogens also exist in the environment and it is possible that humans or wildlife can be exposed to an environmental androgen and an environmental anitandrogen simultaneously. Certainly exposure to environmental pollutants is on a multiple rather than a singular basis. One environmental antiandrogen that has been extensively studied is the fungicide vinclozolin (V), whose metabolites competetively inhibit the AR and prevent the AR from binding DNA (Kelce et al., 1994). V has been shown to be antiandrogenic in vivo, reducing weights of the ventral prostate, seminal vesicle, levator ani /bulbocavernosus muscles, reducing AGD, and inducing nipple development, vaginal pouch, hypospadias, cleft phallus and ectopic testes in the male offspring exposed pre- or perinatally (Gray et al., 1994; Hellwig et al., 2000; van Ravenzwaay et al., 1992; Wolf et al., 2000).

Exposure to combinations of chemicals and the risk assessments of such exposures is a complicated task and is a subject of much attention (Carpy et al., 2000). It is difficult to predict and difficult to determine from the produced effects what the interaction between two or more chemicals in the body may be. The usual prediction is that each chemical will exact the same effect on a target with other chemicals present that it would if it were administered by itself, and that two chemicals that act in the same fashion would compound to produce additive effects (Carpy, et al. 2000). Conversely, two chemicals with opposing action would also have contrasting actions in concert and thus antagonize each other, resulting in less of an effect of either chemical. However, these interactions cannot be accurately predicted in vivo given the complex nature of the animal model, thus application of combinations of chemicals to the animal is necessary.
It is in the interest of human health and safety to perform such combined exposure studies on EDCs, since androgens and antiandrogens exist in the environment, and populations such as the fetus undergoing sexual differentiation would be susceptible to such exposures.

The current study is a combinational dosing study based on the results of two previous studies. The androgenic effects of prenatal TP have been evaluated in the female (Wolf et al., 2002), while antiandrogenic effects of V administered during the same dosing period have been characterized in the male (Wolf et al., 2000). In this study, we evaluate the developmental effects of co-administration of these two chemicals that target the same receptor, the AR, with opposing actions. Co-administration of a competitive AR inhibitor should antagonize the action of androgens in the developing female fetus and alleviate the masculinizing effects. We hypothesize that V would antagonize the androgenic effects of TP \textit{in vivo} in the female rat offspring.

**Materials and Methods**

*Combinational dosing study*

Methods and materials used for animal dosing, husbandry, and monitoring of dams and pups up to and including weaning are described previously (Wolf et al., submitted). Female offspring were euthanized on an individual basis after puberty if they developed a swollen abdomen or were in poor health.

On PND 90-94, female offspring were weighed and euthanized by CO$_2$ asphyxiation followed by decapitation, in compliance with USEPA International Animal Care and Use Committee (IACUC) standards, in blocked fashion by treatment group. Each carcass was shaven on the ventral side to view nipples, and necropsied.
Malformations of the external genitalia and presence of male internal reproductive structures were noted. Malformations include presence or absence of cleft phallus, vaginal orifice, vaginal thread, prostate, seminal vesicle, levator ani, bulbourethral gland. Anogenital distance (AGD), anovaginal distance (AVD; distance from anus to posterior edge of vagina) and phallus length were measured with a rotary micron caliper. AVD was subtracted from AGD to produce a variable called VGD, or vaginal-genital distance. Ovaries and filled and drained uterus were weighed.

_Fetal Testosterone Measurements_

In a separate experiment, two sets of Sprague-Dawley dams were received at the animal facility on two separate dates, each on GD 3, and housed one per cage. Conditions for each set of rats were the same as described in the previous section. Fetus collection on GD 19, fetal homogenization, ether extraction for steroids and RIA by Coat-A-Count kit #TKTT5 (Diagnostic Products Company; Los Angeles, CA) were performed as previously described (Wolf et al., 2002) with the following exceptions. Dams were dosed with either corn oil vehicle (control; n = 2 per set), V (200 mg/kg, n = 2 per set), or TP (1 mg/rat, n = 1 per set). Serum was not collected from dams. A total of 4 control litters, 4 V litters, and 2 T litters, each consisting of 10 - 12 fetuses each, were analyzed.

_Statistics_

Data was analyzed on a litter means basis generally by one-way analysis of variance (ANOVA) using general linear models procedure (proc glm; due to the variable number of individuals per litter) using SAS software (HERLSAS DECversion 1.2-4 on USEPA LAN terminal; SAS Institute Inc, Cary, NC). Sets of rats used to measure
testosterone levels were considered blocks, and 2-way ANOVA was performed by block. No block effect was found, and data were pooled and all litters were used in the litter means in a one-way ANOVA. When general differences for treatment effects were found in overall ANOVA (p < 0.05), differences between treatment groups were analyzed with a two-tailed t test using least square means. Percentage data on litter means (% pups with areolae, % with prostate, etc.) were arcsine square root transformed to reduce heterogeneity of the variances, and analyzed by ANOVA as described above. Post-hoc analysis of data on percent eyes open was performed using a one-tailed t test.

**Results**

**Neonatal development**

Female offspring were not affected by V treatment in any endpoint other than pup weight.

AGD on PND 2 was increased only by TP, independent of body weight (p < 0.0001; Table 1). AGD was analyzed independent of body weight since body weight was reduced and any co-variate analysis would reflect a falsely enhanced increase in significance. AGD in the V+TP group was near the control value, suggesting V attenuated the effect of TP on female AGD at PND 2 (Table 1).

TP prevented development of areolae in roughly a third of female pups at PND 14 (Table 1). The number of areolae per female pup was reduced by TP (n = 3.7) and restored to the full compliment by V+TP (n = 12; p < 0.0001 compared to TP). Areola score (see methods) was similarly reduced by TP and restored by V+TP.

Eye opening was severely reduced on PND 14 by TP and V+TP (Table 1). The combination of V+TP does not appear to have restored the percentage of eyes open as
observed in the males (Wolf et al., submitted). These slight differences between males and females in the response to V or V+TP may not be significant since eye opening was monitored only on one day, and eye opening should not vary between the sexes.

Body weight at weaning (PND 24) was significantly reduced in the TP group only (Table 1). The body weight in the V+TP group was somewhat greater than that in the TP group, but not significantly different from that in the TP (p = 0.1432) or control groups.

**Necropsy**

A total of 14 females from 4 litters had died before necropsy, all from the TP dose group. In 3 cases, a distended abdomen was associated with the death. We believe all females that died prior to necropsy in the TP group were afflicted with hydrometrocolpos, which is not unusual for this dose of TP, as observed in the previous study of TP (Wolf et al., 2002).

Body weight of females at 90-94 days of age remained significantly reduced only by TP (p < 0.001; Table 2). Nipples on females were virtually eliminated by TP (p < 0.0001) but completely restored by the combination of V+TP (Table 1).

The various endpoints of the external genitalia varied in their responses (Table 2). Agenesis of the vaginal orifice occurred in nearly 100% of the females exposed to TP, but vaginal development was normal in those exposed to V+TP. VGD, the distance between the sex papilla and the vaginal orifice, was reduced by TP, and this effect was attenuated by V+TP. Development of male organs, VP, LA and BUG, was induced by TP and attenuated by V+TP (Table 2). Induction of LA and BUG by TP was completely abolished by co-administration with V. Interestingly, phallus length was significantly increased by TP when adjusted to body weight, but was reduced to below control length
by V+TP. In contrast, vaginal thread was not induced by V or TP but was detected in 20% of the females exposed to V+TP. Cleft phallus was not found in control females but occurred in approximately a third of the female offspring exposed to TP and in yet a greater proportion exposed to V+TP. Also, AGD was no longer affected by any treatment independent of body weight at necropsy age, and increased by TP only when adjusted to the reduced body weight in this group (statistics not shown; Table 2).
Discussion

This study addresses the complicated issue of combined exposure to environmental endocrine disrupters in sensitive populations such as the developing fetus. We addressed these issues with an environmental endocrine disrupting chemical, V, and a representative of an environmental androgen, TP, that act at the same receptor and target tissue with opposing action. They also represent chemical classes humans could be exposed to, as androgens and antiandrogens are used in agriculture and medicinally. We investigated the effects of exposure to the combination of V and TP during gestation on sexual differentiation of the female rat. Our primary goal was to determine the ability of V to antagonize the effects of TP in the female rat offspring. We have found that V clearly attenuated all effects of TP monitored in the female offspring at the doses used in this study.

Health and development of female pups

TP adversely affected the developing female pup, apparent in the reduced body weight and delayed eye opening in both the TP and the V+TP groups. Together these endpoints may indicate a delay in overall development, as suggested by previous studies on early androgen exposure (Slob et al., 1983, Fritz, et al., 1984, Popolow and Ward, 1978; Wolf et al., 2002). V itself also produced a reduction in body weight on day 2, although this effect was transient, and no other index of general development was adversely affected by V. A delay in development by TP could explain some other adverse effects on androgen-dependent development, such as areolae development. However, the reduction in number of areolae does not appear to be due to a delay in development, since the two endpoints are not always correlated. In the V+TP group, the number of areolae was normal while percent eye opening was apparently reduced.
The effects of combined exposure to V and TP in the female offspring clearly show antagonism between the two compounds on androgen-responsive tissues. While TP induced effects that were found in the previous dose-response study (Wolf et al., 2002), including induction of prostate, levator ani (LA), and bulbourethral glands (BUGs), reduction in the number of areolae and nipples, lack of a vaginal orifice, and increased AGD in females independent of body weight, V co-administration completely abolished the presence of LA and BUGs, reduced the incidence of prostate tissue 80%, restored the vaginal orifice, areola count, nipple count, and female-like AGD, and reversed the increased phallus length. These results clearly show antagonism between V and TP and were expected. Interestingly, phallus length in the V+TP group was reduced to below control length. A dominant effect of a more potent V may explain this result, although the fact that V alone had no effect does not substantiate this explanation.

In contrast, the incidence of some malformations of the external genitalia induced by TP were increased by V+TP rather than attenuated, and some malformations were only observed with combinational exposure to V and TP. For instance, incidence of cleft phallus induced by TP alone was increased by the combination of V+TP, and vaginal thread not observed in any female in the V or TP groups was observed in a significant proportion of females in the V+TP group. These results may appear to indicate an additive or synergistic interaction. However, investigation into the dose-response relationship of these chemicals reveals attenuation of the masculinizing effects of TP by V. In a previous TP dose-response study (Wolf, et al., 2002), cleft phallus and vaginal thread were unique to the 0.5 mg TP dose group. This is because the phallus at higher dose groups was masculine and at lower doses was feminine, while intermediate doses of
TP at 0.5 produced an intermediate stage of development characterized by cleft phallus and vaginal thread (Fig. 1). In the current study, few females in the 1 mg TP group had cleft phallus because most phalluses were so masculinized they were no longer cleft (Table 3). Co-administration of V caused the phalluses to be less masculine, partially affected, and cleft. Thus, these morphological abnormalities that were produced only by the combination of V+TP in this study illustrate not synergy, but antagonism between V and TP.

Fetal T levels

The elevation in testosterone levels in the female GD 19 fetus and not male fetus concurs with the previous TP dose-response study (Wolf et al., 2002). This elevation in T levels in females correlates with the many androgenic effects of TP at the 1 mg dose level and apparently is responsible for these effects. T levels in the male fetus appeared to be somewhat elevated, but was not significant as it was in females since the proportionate increase over basal levels was less than that in females.

Summary

We found in this study that 200 mg/kg V can antagonize 1 mg/dam TP and alleviate its effects on all anatomical measures of androgenicity monitored in female rat offspring. We are alerted by this study to some important concepts to consider in combination exposure studies. Firstly, that an organism with no apparent alteration to its reproductive system is not necessarily free of endocrine disrupting contaminant exposure, but could have been exposed to and affected by two chemicals that act in opposing fashion. The result of this exposure may manifest itself instead in whole body toxicity or subtle effects on other systems of the body, or in no obvious way at all. Therefore, it is crucial to include a variety of endpoints in the risk assessment of any combination of
chemicals, even when focusing on one functional system. Secondly, caution must be taken when interpreting the effects of combined exposures observed *in vivo*, especially confusing or unexpected effects, to consider the dose-response relationship of each compound. The combined action of two compounds at a given dose together may produce effects similar to one or each compound at a higher, or even a lower, dose, as illustrated by the effects of V+TP compared to those of TP on the morphology of the external genitalia. These cautions were illustrated by this study and support the view that combinational dosing studies provide valuable information to aid in the task of protecting human health from environmental pollutants.

**Acknowledgements**

The author gratefully acknowledges Joseph Ostby, Jonathan Furr, and Andrew Hotchkiss for their assistance with animal handling and data collection, Dr. Louise Parks, Christy Lambright and Dr. Vickie Wilson for their assistance with fetus collection, fetal testosterone extraction, and RIAs.
References


Table 1. Androgenic Effects of Prenatal Testosterone Propionate Are Attenuated by Vinclozolin in Prepubertal Female Offspring

<table>
<thead>
<tr>
<th>Endpoints</th>
<th>Control</th>
<th>V</th>
<th>TP</th>
<th>V+TP</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGD on PND 2</td>
<td>1.73 ± 0.027</td>
<td>1.62 ± 0.042</td>
<td>2.51 ± 0.130†</td>
<td>1.62 ± 0.013†</td>
</tr>
<tr>
<td>AGD at adulthood</td>
<td>23.18 ± 0.36</td>
<td>22.17 ± 0.51</td>
<td>23.93 ± 0.79</td>
<td>22.41 ± 0.36</td>
</tr>
<tr>
<td>Percent pups with areolae</td>
<td>100</td>
<td>100</td>
<td>66.50 ± 9.47‡</td>
<td>100</td>
</tr>
<tr>
<td>No. of Areolae/pup</td>
<td>12.02 ± 0.02</td>
<td>11.98 ± 0.02</td>
<td>3.69 ± 1.30‡</td>
<td>12.0 ± 0</td>
</tr>
<tr>
<td>Pup Areola score</td>
<td>34.92 ± 0.11</td>
<td>34.73 ± 0.47</td>
<td>4.45 ± 1.77‡</td>
<td>33.77 ± 0.89</td>
</tr>
<tr>
<td>% Eyes Open on PND 14</td>
<td>64.0</td>
<td>48.2</td>
<td>24.8 *</td>
<td>25.8 *</td>
</tr>
<tr>
<td>No. of Nipples at adulthood</td>
<td>12</td>
<td>12</td>
<td>0.762 ± 0.52‡</td>
<td>12</td>
</tr>
<tr>
<td>BW at PND 2</td>
<td>7.60 ± 0.28</td>
<td>6.76 ± 0.26*</td>
<td>5.092 ± 0.19†</td>
<td>4.88 ± 0.097†</td>
</tr>
<tr>
<td>BW at weaning</td>
<td>62.8 ± 2.34</td>
<td>61.8 ± 2.46</td>
<td>51.7 ± 1.49*</td>
<td>56.15 ± 2.09</td>
</tr>
</tbody>
</table>

Note: Values are litter means ± SE. AGD, anogenital distance; PND, postnatal day; BW, body weight.

* significantly different compared to TP group at p < 0.0001.

* p ≤ 0.05, ** p < 0.005, † p ≤ 0.0001 compared to control group.
Table 2. Masculine Development of the External Genitalia and Internal Reproductive Tract in Females Exposed Prenatally to Testosterone Propionate is Attenuated by Vinclozolin

<table>
<thead>
<tr>
<th>Endpoints</th>
<th>Treatment Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>VGD</td>
<td>6.97 ± 0.45</td>
</tr>
<tr>
<td>Phallus length</td>
<td>4.53 ± 0.107</td>
</tr>
<tr>
<td>Cleft phallus (%)</td>
<td>0</td>
</tr>
<tr>
<td>Vaginal agenesis (%)</td>
<td>0</td>
</tr>
<tr>
<td>Vaginal thread (%)</td>
<td>5.6 ± 0.056</td>
</tr>
<tr>
<td>Prostate (%)</td>
<td>0</td>
</tr>
<tr>
<td>LA (%)</td>
<td>0</td>
</tr>
<tr>
<td>BUG (%)</td>
<td>0</td>
</tr>
<tr>
<td>BW at adulthood</td>
<td>290.76 ± 4.49</td>
</tr>
</tbody>
</table>

Note: Values are litter means ± SE. Phallus length adjusted to body weight with covariate analysis. AGD, anogenital distance; VGD, vaginal-genital distance; LA, levator ani; BUG, bulbo urethral gland; BW, body weight.

a only one female in this treatment group had a vaginal orifice from which to derive VGD.

b Statistically significant when compared to the TP group by at least p < 0.05.

* p < 0.05, ** p < 0.01, † p < 0.0001 compared to controls.
Table 3. Testosterone Levels in Male and Female Fetuses on Last Day of Dosing, GD 19.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Treatment group</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>V</td>
<td>TP</td>
</tr>
<tr>
<td>Male GD 19 fetus (ng/fetus)</td>
<td>0.2687 ± 0.04</td>
<td>0.2151 ± 0.04</td>
<td>0.2464 ±0.064</td>
</tr>
<tr>
<td>Female GD 19 fetus (ng/fetus)</td>
<td>0.0412 ± 0.007</td>
<td>0.0334 ± 0.007</td>
<td>0.0898 ± 0.011 *</td>
</tr>
</tbody>
</table>

*Note: Values are litter means ± SE, of 2 blocks of the experiment (C and V, n=2 litters; TP, n=1 litter each), for a total of 4 control, 4 V-treated, and 2 TP-treated litters. * p < 0.005 compared to controls.*
Figure Legend

Figure 1. Continuum of increasing masculine development of the phallus and surrounding perineal tissue. The phenotype on the left is female and on the right is male. Increasing levels of androgen push the morphology of the genitalia along the continuum to the right, and antiandrogens push the morphology along the continuum toward the left. A combination of androgens and antiandrogens, whether in the male or female developing rat, produces a morphology resembling those in the middle of the continuum.
Figure 1
Investigation into the Involvement of EGF, EGF Receptor, and Androgen Receptor in the Disruption of Male Sexual Development by Vinclozolin

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NC State University
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Abstract

Prenatal exposure to vinclozolin (V) causes antiandrogenic effects in male offspring via androgen receptor (AR) inhibition. AR inhibition may lead to a decrease in AR expression. The role that other hormone systems, such as growth factors, may have in androgen action are not fully elucidated. Epidermal growth factor (EGF) may be regulated by AR and play a role in masculine development. Here we investigate the ability of EGF co-administration to reverse the antiandrogenic effects of V. We also investigated whether V-induced effects initiated during fetal development are mediated by reduced AR and EGF receptor (EGFR) expression levels. To address the first hypothesis, pregnant Long Evans Hooded rats were dosed on GD 14-19 with either corn oil vehicle (Control; 2.5 ml/kg, orally), V (200 mg/kg, orally), EGF (20 μg/kg, orally) or V+EGF (experiment 1), or with corn oil, V, EGF (100 μg/kg, sc), V+EGF, or V + testosterone propionate (TP; V+T, 2 mg/rat, sc; experiment 2). Androgen dependent endpoints were monitored in the male offspring. To address the second hypothesis, tissue from weanling and adult males were analyzed for AR levels. In addition, Sprague-Dawley rats were dosed on GD 14-18 with corn oil (control) or V and fetal phalluses were collected on GD 18 for immunohistochemical and densitometric analysis. We found that EGF did not alter the effect of V on male sexual development, V did not alter AR expression in maturing male offspring, and V did not alter AR or EGFR expression in fetal phalluses, although AR and EGFR expression tended to be lower in phallus sections of females as compared to males. We conclude that effects of V induced during fetal development are not dependent on EGF or EGFR expression levels, and are not related to AR expression levels. Other local mediators of AR-dependent development may have a larger role in antiandrogenic effects.
Introduction

The male reproductive tract develops in late gestation in response to androgens and functional androgen receptors (AR). While the complete mechanisms involved in androgenic action in the reproductive tract has yet to be elucidated, much of the basic signal and effect mechanism is understood. Testosterone (T) from fetal testes binds and activates the AR of Wolffian duct tissue to promote development of the epididymis, vas deferens, and seminal vesicle. T also promotes growth of muscles including the levator ani/bulbocavernosus muscles which are attached to the penis (Tobin and Joubert, 1991). In some tissues, T is reduced by 5α-reductase to dihydrotestosterone (DHT), which binds the AR with higher affinity than T (Luke and Coffey, 1994). DHT binds AR in urogenital sinus tissue to induce differentiation and development of the prostate and the external genitalia, and in the mammary anlagen to inhibit development of external nipples in the rat (Imagawa et al., 1994). Disruption of this system by environmental chemicals is a cause for concern for human health. Vinclozolin (V) is a fungicide used on foods and turfgrass which has antiandrogenic activity (USEPA, 1993; Gray et al., 1994). It binds and inhibits the AR in human and rat tissue in vitro (Kelce et al., 1994; Wong et al, 1995) and in the rat in vivo (Kelce et al., 1997). V administered to the rat on gestational day 14 - 19 induces malformations in the male including cleft phallus, vaginal pouch, nipples, and reduced weights of the ventral prostate, seminal vesicle, levator ani/bulbocavernousus muscle group, and bulbourethral glands (Wolf et al., 2000). A defect in humans that resembles cleft phallus in the rat is hypospadias. Hypospadias, or the opening of the urethra at a location other than the tip of the penis, is displayed in boys at birth and its incidence is increasing in the US (Palouzzi et al., 1997). The cause of
Hypospadias most often involves antiandrogenic action (Baskin, 2000), and could be linked to environmental antiandrogens (Chambers et al., 1999).

Although androgenic and antiandrogenic effects in mammals have been thoroughly studied and are clearly defined, the mechanisms of androgen action are complex. AR interacts with various coactivators, corepressors, androgen response elements, hormone systems and signal transduction pathways, and triggers various gene products (Haendler, 2002). Growth factors and their receptors have been implicated in androgen action and male reproductive tract development. EGF in particular is suspected to have a major role in androgen action (Cunha et al., 1995; Tanji et al., 2001). Much of this work focuses on the prostate gland and carcinogenesis (Sherwood and Lee, 1995; Culig, et al., 1996; Thomson and Cunha, 1999), although EGF has been linked to androgen-dependent growth of other components of the male urogenital tract, such as the testis, seminal vesicle, and external genitalia (Liu et al., 1994; Gupta et al., 1996; Hayward et al., 1996; Thomson et al., 1997; Levine et al., 2000). Some studies have shown that androgen action is dependent on EGF. In one study, anti-EGF prevented Wolffian duct development in rats (Gupta, 1996), and in another study, the absence of EGF by sialoadenectomy inhibited androgen-dependent sex accessory gland development and sperm production (Liu et al., 1994). Also, Cain et al. (1994) showed that EGF co-administration was able to reverse flutamide-induced inhibition of testis descent. Some studies show more directly that EGF has androgen activity. One study showed that EGF induced the AR-regulated gene PSA in the absence of androgen in prostate cancer cells (Culig et al., 1995). EGF (Hiramatsu et al., 1988; Ravenna et al., 1995) and EGF receptor (Brass et al, 1995) has been shown to be induced by androgen and reduced by...
antiandrogen (Nishi et al., 1996; Monti et al., 1997) and may therefore be downstream of AR.

Receptors of the androgen and growth factor systems may also be a point in the signal transduction pathway that regulates the androgen signal. The receptors may be upregulated or downregulated as an effect of antiandrogenic action, or may be the initial regulator of the altered androgen signal. It was shown that 100 mg/kg V administered for 30 days to peripubertal rats reduced AR binding in nuclear receptors in association with the reduced weight of the ventral prostate and epididymis (Monosson et al., 1997). Reduction in AR expression in association with antiandrogen exposure in the testes was shown by immunohistochemistry by another investigator (DDE; You et al., 1998). It has not been determined whether AR expression is reduced in the adult male or in the fetal male in response to prenatal exposure to V. In this study, we are interested in androgen and EGF receptor levels in tissues affected by antiandrogenic action. In the adult male offspring, ventral prostate and seminal vesicle are affected and tissue is plentiful for bioassay. In the fetus, the ventral prostate and the seminal vesicle are not sufficiently developed to bioassay for study, although the phallus, or sex papilla in the fetus, is present throughout sexual differentiation. The phallus is greatly affected by V, development of the sex papilla into the male phallus is dependent on androgen, and functional AR are expressed in the papilla at GD 14 and increase in quantity thereafter (Bentvelsen et al., 1995; Murikami, 1987). Furthermore, EGF receptors are also present in the reproductive tissue of the fetus (Gupta and Jaumotte, 1993; Bossert et al., 1990), although an effect of gestational antiandrogen treatment on fetal EGFR has not been shown.
This study is designed to investigate the AR and post-AR mechanisms of V action in disruption of male sexual development utilizing *in vivo* and *in vitro* approaches. We have hypothesized that EGF and the EGFR are post-AR mediators of androgen action. We propose that EGF co-administered with V during gestation should restore androgen action and thus attenuate the antiandrogenic effects of V in male offspring. We also propose that V treatment results in downregulation of AR and EGFR in target tissues at the time of insult.

**Materials and Methods**

*In vivo Co-administration Study*

*Experiment 1*

Twenty six timed-pregnant Long Evans Hooded rats from Charles River Laboratories (Raleigh, NC) arrived at the animal facility on gestational day (GD) 3 (GD 1 = day of plug). Animals were weighed and then housed 1 per cage in polycarbonate cages in an atmosphere of 68-74° F, 30-50 % humidity, and a reversed light cycle of 14 hr dark:10 hrs light (lights off at 11 am EST). Dams were given Agway 3000 rat chow and tap water *ad libitum*. On GD 12, dams were weighed, weight ranked and 24 dams randomly assigned 6 per treatment group equilibrated with respect to body weight. On the mornings of GD 14 - 19, dams were weighed and dosed by oral gavage with either corn oil vehicle (control; Sigma, St Louis, MO; 2.5 ml/kg), vinclozolin (V; Reidel-deHaen, distr. by Crescent Chemicals; 200 mg/kg), epidermal growth factor suspended in 10% ethanol in corn oil (EGF; Sigma, St Louis, MO; 20 μg/kg) or V + EGF. Dams were monitored on GD 22 and 23 for delivery of offspring. Dams were sacrificed at weaning (PND 23) and uterine implantation sites counted.
On postnatal day (PND) 1 (=GD 23), pups in each litter were counted, weighed individually and anogenital distance (AGD) measured on each pup in blind fashion using a dissecting microscope fitted with a micrometer ocular reticle. Sex was determined by AGD. Pups were monitored and counted every few days thereafter until weaning. Areolae on each pup were counted on GD 15. An areola or a nipple constituted an “areola” on this day. Offspring were weaned on PND 23. Offspring were weighed, sexed and distributed 2-3 per cage by sex. On PND 24, females were sacrificed and males were observed for malformations of the genitalia. After observing malformations, males in EGF and V+EGF groups were sacrificed. Males in control (n=3 litters) and V (n= 3 litters) groups were kept to PND 84-90 for collection of ventral prostate (VP), seminal vesicles (SV) for immunohistochemical analysis and a portion of VP saved for AR binding assay. All animals were euthanized by CO₂-induced anaesthesia followed by decapitation as per IACUC guidelines.

Experiment 2

On a separate date, thirty three pregnant Sprague-Dawley rats arrived GD 3 at the USEPA animal facility. They were weighed upon arrival and housed and fed as described for Experiment 1. On GD 10 dams were weighed, weight ranked, and assigned to treatment groups equilibrating for body weight among groups. Treatment groups were as follows: Control (corn oil vehicle 2.5 ml/kg-bw by oral gavage; n = 8), V (vinclozolin, 200 mg/kg by oral gavage, n = 8), EGF (epidermal growth factor in PBS, 100 µg/kg by sc injection; n = 5), V+EGF, and V+TP (V + testosterone propionate, 2 mg/0.1 ml corn oil/rat by sc injection; n = 5). Dams were dosed on GD 14 - 18 once daily as per treatment group. On GD 18, 3 control and 3 V-treated dams were sacrificed for collection of fetuses for assaying testosterone (T) levels (see next section). On GD 19, the
remaining dams were dosed. Corn oil and testosterone propionate (lot # 109C-0316) were obtained from Sigma (St Louis, MO), V was obtained from Crescent Chemicals (Islandia, NY; lot # 10560), and EGF was tissue culture grade mouse EGF obtained from Harlan Bioproducts (Indianapolis, IN).

Pups were counted on day of delivery (GD 23 or postnatal day (PND 1). AGD was measured on PND 2 in a manner described in Experiment 1. Areolae were counted as described in experiment 1. Males in Control and V groups (n= 1 per 3 litters) were necropsied on PND 21 for collection of VP and SV for immunohistochemical analysis. All other offspring were monitored for malformations of the external genitalia after puberty and then sacrificed by animal facility staff by CO₂ asphyxiation.

**Fetal carcass and testis T levels**

On GD 18, 3 control and 3 V-treated dams from experiment 2 were euthanized in blocked fashion by treatment group by CO₂ anaesthesia followed by decapitation. Fetuses (5 males per litter) were collected on GD 18 as described previously (chapter II) except that testes were first excised from male fetuses before the carcass was stored, and the paired fetal testes were stored in 12 X 75 glass tubes in -20° C until assayed. Fetuses were homogenized, steroids extracted and radioimmunoassayed for testosterone (T; Coat-A-Count kit #TKTT5; Diagnostic Products Company, Los Angeles, CA) as previously described (chaper II) except that maternal blood was not collected. Testes were homogenized and T extracted as previously described (Parks et al., 2000). Briefly, fetal testes were thawed in 100 µl distilled deionized water, crushed, 1 ml ether added, and tubes vortexed. Samples were frozen in a dry ice/acetone bath until the lower aqueous portion froze. Organic layer (supernatant) was poured into clean glass test
tubes, and dried overnight. The residue was resuspended and analyzed for T (Coat-A-Count kit #TKTT5, DPC).

**AR binding assay**

A total of 22 control and 14 V-treated prostates from experiment 1 were used in 3 sets of assays to determine AR number by scatchard analysis in AR competitive binding assays. Binding assay was performed at 4° C as previously described (Kelce et al., 1994; Lambright et al., 2000). Briefly, untreated prostates were thawed, homogenized on ice in low salt TEDG buffer at 10 ml per gram tissue, and centrifuged at 30,000 X g to separate the cytosolic (supernatant) and nuclear (pellet) androgen receptors. The pellet was resuspended in high salt TEDG buffer at 10 ml per gram tissue, and centrifuged as above to produce a supernatant that contained the nuclear fraction. 300 µl aliquots of pooled prostate extract were competed with increasing concentrations (0.5 - 20 nM) of ^3^H-R1881 (total binding), and with increasing concentrations of ^3^H-R1881 with 100 fold excess radioinert R1881 (non-specific binding), and incubated for 20 hours. Free and bound steroid receptors in each sample were separated by hydroxylapatite (HAP). HAP suspension was added to each tube at 500 µl HAP:100 µl sample, tubes were vortexed, centrifuged at 600 X g, supernatant aspirated, pellet washed in TRIS buffer 3X, bound ligand eluted from the receptor with ethanol extraction, and radiolabelled ligand in each tube quantified on a scintillation counter. Specific binding for each concentration was determined by subtracting the non-specific binding from the total binding and plotted to produce a binding curve with which to compare the sample values. Prostates from experimental animals were processed as above, and a 300 µl aliquot of each prostate sample nuclear extract added to tubes with 20 nM ^3^H-R1881 or with 20 nM ^3^H-R1881 + excess radioinert R1881, and the assay performed as described above.
**Immunohistochemistry of adult VP and SV**

Portions of VP and SV were immersed in O.C.T. compound (TissueTek for Sakura Finetek USA, Inc., Torrance, CA), snap frozen in liquid nitrogen or on dry ice, and stored in -70°C. Blocks of each sample were thawed to -25°C in the cryostat microtome, sectioned 8 um thick, and sections laid on a warmed positively-charged glass slide. Each slide contained control and treated sections, and a section for negative control. Immunohistochemistry was performed following the protocol in the Vector ABC kit #4001 (Burlingame, CA) and reagents from the kit were used unless otherwise noted. Sections were fixed in a drop of 4% paraformaldehyde (JT Baker, Phillipsburg, NJ) with 10% sucrose for 8 min, rinsed with PBS, treated with 2% Triton (EM Sciences, Gibbstown, NJ) for 10 min in a humidity chamber to porate cells, blocked with 5% normal goat serum (NGS; Vector Laboratories), incubated with primary antibody (PA1-111, Affinity Bioreagents, LaJolla, CA) overnight at 4°C. The next morning, slides were rinsed in fresh PBS and then 1% NGS, blocked as before, and incubated with biotinylated secondary antibody provided in the kit for 1 hour. Slides were then rinsed with PBS followed by 1% NGS 10 min, and incubated with ABC reagent 1 hour, rinsed afterward with PBS and then stained with drops of 3,3'-diaminobenzidine (DAB; Sigma, St. Louis, MO) solution. AR was localized by detection of a brown product from peroxidase reaction with DAB substrate. Reaction was arrested by immersion of slides in PBS, and sections were dehydrated in 70-95-100% ethanol, 100% xylene. Slides were mounted under a coverslip with Permount (Fisher Scientific, Fair Lawn, NJ).

**Fetal Receptor Analysis Study**

*Animals*
Eight 90 day old timed-pregnant Sprague-Dawley rats from Charles River Laboratories (Raleigh, NC) arrived on gestational day (GD) 3 at the animal facility. They were housed individually in polycarbonate cages with laboratory grade pine shaving bedding and acclimated to a reverse light cycle (14 hrs light:10 hrs dark; lights off at 11am EST), a temperature of 72-74° F, and 30-50% humidity. They were fed LabDiet® 5008 (PMI Nutrition International, Brentwood, MO) and tap water ad libitum.

On GD 12 dams were weighed, weight ranked, and assigned randomly to the treatment groups and equilibrated with respect to mean body weight. On GD 14 through GD 18, dams were dosed once daily by oral gavage with either corn oil vehicle (Control; 2.5 ml/kg) or vinclozolin (V; 400 mg/kg) and body weight was monitored. Test compounds were obtained from Sigma-Aldrich (St. Louis, MO).

On GD 18, 1 hour after dosing, 6 dams (3 Control and 3 V treated dams) were sacrificed in blocked fashion by treatment group. Each dam was euthanized by CO₂ asphyxiation followed by decapitation as per IACUC guidelines, and fetuses were removed from the dam, held on ice, and dissected before the next dam was sacrificed. Fetuses were sexed and a phallus sample isolated from 3 male and 3 female fetuses from Control litters, and 3 male fetuses from V litters. Samples from each litter were fixed for 1 hour in 3% paraformaldehyde (J.T. Baker, Phillipsburg, NJ) on ice, rinsed briefly in PBS and dehydrated in cold 30%-50%-70% ethanol series. Samples were stored in 70% ethanol at 4° C until embedded (1 - 2 weeks).

*Histology and Immunohistochemistry*

Phallus samples were dehydrated in a cold 95% - 100% ethanol series. Ethanol was replaced with paraffin by soaking in 100% xylene (Fisher Scientific, Springfield, NJ) 30 min 2X, followed by 50:50 xylene:paraplast™ (Sherwood Medical, St. Louis, MO).
7.5 min 2X, and 100% paraplast+® 30 min 3X at 60-65° C. Samples were embedded in rubber flat molds (Ladd Reserach Industries, Inc., Williston, VT) to facilitate orientation, and the slightly hardened paraffin blocks made from these molds inserted into gelatin capsules size “00” (Electron Microscope Sciences, Ft. Washington, PA) filled with melted paraffin for enlarging the block for use in the microtome. Blocks were sectioned, at a location within the phallus common to all samples, 6 μm thick with a glass knife on a Leica RM 2135 (Leica, Deerfield, IL) microtome and dried onto warmed gelatin-coated slides over a drop of autoclaved dd water. Sections were distributed to slides in a matrix fashion with one representative from each litter and each treatment group represented on each slide. Three treatment groups (control male, control female, vinclozolin male) of 3 litters each and 3 samples per litter for each antibody were used for immunoassay.

Standard immunohistochemical techniques were used to process slides. Endogenous peroxidase activity was quenched in 0.3% H2O2 in methanol for 20 min and rinsed in 100% PBS for 30 min. Nonspecific binding sites were blocked with serum for 20 min before incubation with primary or secondary antibody. Serum used for each assay was made from the species used to make the secondary antibody (for androgen receptor, AR, 5% normal goat serum, Vector Laboratories, Burlingame, CA; for EGF receptor, EGFR, 5% normal rat serum, Jackson ImmunoResearch Labs, West Grove, PA). Sections were incubated with primary antibody overnight at 4° C (for AR, 4 μg/ml polyclonal rabbit anti-rat, PA1-111A, Affinity Bioreagents, LaJolla, CA; for EGFR, 1:400 diluted F-4 clone, raised in mouse against synthetic peptide homologous to human and rat EGFR, Sigma, St.Louis, MO). Sections were incubated with biotinylated secondary antibody for 20 minutes at room temp (for AR, goat anti-rabbit IgG provided in Vectastain standard ABC kit, Vector Laboratories; for EGFR, biotinylated rat anti-
mouse IgG, diluted 1:200, Jackson ImmunoResearch Labs). ABC reagent (Vectastain Elite kit, Vector Laboratories) was applied for 20 minutes. Sections were then rinsed and receptors localized with 3,3'-diaminobenzidine (DAB; Sigma, St. Louis, MO) until staining was visualized, approximately 4 minutes. A brown color, not present in sections with no antibody, was indicative of receptor protein. Incubation and reaction times were tightly monitored to maintain identical conditions for each set of slides in the assay. Sections were then dehydrated in xylene and a coverslip mounted on each slide with Permount (Fisher Scientific, Fair Lawn, NJ). Slides were allowed to dry overnight before imaging.

Image Analysis

Images of each section were captured under a bright field microscope (Olympus BH2) with a CoolSnap (RS Photometrix) monochrome cooled CCD camera with high resolution (1390 X 1040 pixels). Images were acquired with ImagePro Plus® camera and software (MediaCybernetics, Silver Spring, MD) in 12Bit Grayscale with an intensity range of 0 - 4095 and saved in TIFF format. Each image was given a code such that the operator analyzed images blind to identity. Saved images were analyzed in ImagePro Plus. Illumination conditions were kept constant across slides as change in illumination conditions can alter intensity. Illumination conditions were measured in the imaging program using histographical representation of the light intensity range of a blank area of one slide, and this area of the slide was used as a reference standard. Illumination conditions were kept constant across slides by matching histograms of a blank area of each slide to the reference standard. Uneven illumination attributable to microscope or lenses that could distort intensity levels of staining was adjusted for using background correction in ImagePro Plus. Calibration of the system was performed to establish the
black and incident light level values and to spatially calibrate the images with a stage micrometer.

Morphological and densitometric characteristics of sections were identified for each antibody, and endpoints and intensity thresholds were defined based on these characteristics and programmed into an environment file. Environment files which established guidelines, limits and functions were used to analyze images for densitometric and morphometric characteristics in a standardized fashion across sections from each antibody. Morphological and staining characteristics such as area, mean density, number of objects, were measured and reported by the program as data.

AR was evaluated in the epithelial layer of the epidermis and of the urethral groove (term applied to the seam of the urogenital fold and the urethral lumen, previously; Baskin, 2000) by mean density and area in each section, and in the mesenchyme by number of objects, number of objects in proportion to area of interest (AOI), mean area of objects in one section, summed area of objects, and mean density of the objects. “Objects” was the term applied by the program to discreet, darkly stained areas, and were considered AR-positive cells. EGFR was evaluated in the epithelium of the epidermis and of the urethral groove by mean density and area, and again in these regions by a separate characteristic we termed “high expression”, or staining of aggregated cells at a higher threshold of intensity, quantitated by summed area, and area in proportion to total epithelial area. Data were exported to and saved in a spreadsheet for statistical analysis.

Statistics
Identity of each section representing each individual was decoded and data was sorted. Values were averaged by litter by treatment group, and litter means were averaged in each treatment group for each antibody evaluated. Data were analyzed by ANOVA on SAS software on the LAN at the USEPA and when a general difference was found for an endpoint, differences between groups were analyzed by a post-hoc least square means procedure.

Results

*In vivo Co-administration Study*

**Maternal and Litter effects**

*Experiment 1 - V +/- EGF at 20 μg/mg*

Maternal weight gain was slightly reduced in groups that were treated with V (V and V+EGF; Table 1) and pup body weight was reduced in the V group on PND 1 by \( p < 0.05 \) (data not shown). Parturition was not delayed. Litter size and viability to weaning was not affected by any treatment (Table 1) despite that one litter in the V group was dead by PND 3.

*Experiment 2 - V +/- EGF at 100 μg/kg, V+T*

Maternal weight gain was significantly lower in the V+TP group, but not in any other group (Table 1). Dam weight was significantly reduced on GD 18 and 19 in the V+TP group, suggesting dams may have resorbed pups late in gestation. The number of uterine implantation sites did not differ among treatment groups (data not shown). Litter size was drastically reduced in the combination groups, V+EGF and V+TP (Table 1). Many litters in the V+T group were found dead on PND 1, and only one litter containing 2 pups was viable in this group. The percent viable litters by PND 2 was 60% (3 of 5
litters) in the V+EGF group and 20% (1 of 5 litters) in the V+T group. No pups in the V+TP group remained at weaning.

**Offspring - Experiment 1**

AGD in females on PND 1 was not affected by any treatment (data not shown). AGD in males on PND 1 was reduced by V as observed previously (Gray et al., 1994; Wolf et al., 2000), and to the same extent by V+EGF, but not by EGF alone (Fig. 1a). Thus the observed reduction of AGD in the combinational dosing group was attributed to V and EGF had no effect or interaction with V. We observed the same phenomenon in areola count. Areolae were induced by V as expected, and the number of areolae per pup was increased to the same extent by V or V+EGF, and not by EGF alone (Fig. 1c). Malformations observed at weaning including cleft phallus, vaginal pouch, and ectopic testes were induced in 100% of males by V and by V+EGF (Fig. 1d), and was attributed to V. EGF had no effect alone on androgen dependent tissues and no interaction with V. The lack of effects precluded keeping animals to adulthood.

**Offspring - Experiment 2**

Results of AGD analysis was identical to the results in experiment 1 (Fig. 1b). In addition, the V+TP group did not differ from controls. Areola count and eye opening were similar to Experiment 1 (data not shown). The V+T group contained only one litter, which was small in number and underdeveloped and therefore not able to be included in areola and eye opening assessment. Only one litter remained in the V+EGF group after PND 2 as well. The litter in the V+EGF group contained 12 males and was monitored for ectopic testes in light of findings by Cain et al. (1994). In this group, incidence of ectopic testes was lower compared to that observed with the antiandrogen alone (Fig. 2).
No other effect of V on androgen-dependent tissue was ameliorated by co-administration with EGF in that litter.

**In Vitro Analysis of AR in Co-administration Study**

**AR binding in ventral prostates**

AR binding and therefore AR quantity in ventral prostates of PND 55 male offspring tended to be somewhat lower in each of 3 trials but not significantly altered by prenatal exposure to V (Fig. 3).

**T levels in Fetal carcass and testes**

Prenatal V did not significantly alter testosterone levels in GD18 fetal carcass homogenates, nor in fetal testis homogenates (Fig. 4), as expected.

**Immunohistochemistry in adult VP and SV**

No difference in AR number or distribution was observed in PND 84 VP or SV by subjective assessment of immunohistochemically stained sections (Fig. 5), nor in PND 21 VP or SV (data not shown) which looked very much the same as those for PND 84. We did observe a reduction in the number and size of lumens within the prostate gland, or a reduction in the epithelium:stroma ratio in one severely affected animal on PND 21 (data not shown). However, the incidence and sample size was low and we were unable to measure or define this effect well enough to determine whether it was indeed an effect.

Regardless, this pilot study did not give any indication of finding a difference in AR number in the VP or SV of prepubertal or postpubertal rats.

**Receptor analysis study**
AR and EGFR were expressed in cross sections of the GD 18 fetal phallus and each receptor appeared to be expressed in its unique pattern (Fig. 6). These patterns were measured and quantitated by image analysis as described in Materials and Methods and Fig. 7 (mean density in epithelium not shown).

The number of AR as determined by the mean density of staining appears to be slightly reduced in the epidermis and in the urethral groove of the phallus in females compared to males, but not affected by V in males, although none of the data was significant (Fig 8). The quantity of AR in the mesenchyme of the phallus as determined by the summed area of all objects appeared to be slightly reduced in females as compared to males, but again was not significantly different (Fig 8). The number of AR, or AR-positive cells, in the mesenchyme as determined by the number of objects again appeared slightly reduced in females as compared to males but was not significant. In V-treated males, the quantity or number of AR in the mesenchyme as determined by summed area of objects or by number of objects is not different from that of control males and appears to be more variable (Figs. 9a,b). The variability was not due to the one outlier that contained a far greater number of objects that other sections in the group (Fig. 9c), since omitting the outlier did not reduce the standard error of the mean of number of objects in the V group (Fig. 9b). We determined that we observed greater variability in the number of AR-positive cells in the mesenchyme of the fetal phallus exposed to V.

The number of EGFR as determined by mean density of staining in the epithelial layer of the epidermis was significantly reduced in females compared to males (p < 0.05), but was not affected by V (Fig. 10). Mean density of staining in the urethral groove was slightly but not significantly reduced in females compared to males, but not affected by V
in males. The quantity of EGFR in these epithelial regions as determined by summed area of higher expression (HE) or by the ratio of HE area in the epithelium to the total area of the epithelium was not altered in females compared to males or by V in males (Fig. 10).

This study showed a consistent slight but in most cases insignificant reduction in AR and EGFR at GD 18 in female phalluses compared to males, but no effect of V.

**Discussion**

This study was designed to determine whether EGF and the EGF receptor are employed in AR action, and whether AR expression levels are suppressed by V to mediate antiandrogenic effects. Specifically, we addressed the hypothesis that EGF co-administration would rescue some androgen dependent tissues from antiandrogenic effects of vinclozolin (V). Also, we investigated the hypothesis that AR inhibition by V during fetal sexual differentiation will alter AR levels in adult and fetal tissue and EGFR levels in the GD 18 fetal phallus. We found no evidence to support these hypotheses, suggesting alternate candidates for regulation of AR action should be considered.

We found that while V induced the expected antiandrogenic effects in both Long Evans and Sprague-Dawley rats, oral or subcutaneous administration of EGF at 20 or 100 \( \mu g/kg \) had no detectable effect on pregnancy or sexual development, and co-administration of EGF did not attenuate the effects of V on the androgenic endpoints AGD, areola development, and genital malformations. Since no effects were observed in the offspring by EGF administration and few effects were observed by EGF co-administration, we have little evidence that EGF crossed the placenta and reached the fetus. Therefore we cannot draw conclusions from this part of the study.
The concentration of EGF selected was based on similar concentrations used by other investigators (100 μg/kg, Liu et al., 1994; 200 μg/kg, Cain et al., 1994) administered subcutaneously that produced effects, although most were in the mouse. EGF is reported to have a difficult time passing the placenta (Eaton et al, 1987; DiAugustine et al., 1987) and concentrations may need to be higher to have an effect on the offspring. In one study, 500 μg/kg EGF administered to the rat dam accelerated sexual development in female offspring (da Silva et al., 1991). However, a higher concentration of EGF may not have been tolerated in combination with V in our study since the combination resulted in increased mortality. EGF may increase the transfer of other chemicals through the placenta (Mimouni et al., 1991) and thus may have accelerated the amount of V or its metabolites in crossing the placenta, to elicit increased toxicity.

In contrast, we cannot reliably conclude that EGF does not play a role in V action. The one endpoint in which EGF may have attenuated the effect of V was testis descent, although statistical evaluation was not possible. Attenuation of antiandrogen-induced cryptorchidism, or undescended testes, by prenatal EGF co-administration was demonstrated previously in the mouse (Cain et al., 1994). Their study used 200 ul/kg EGF, but also failed to demonstrate attenuation of any direct effect of EGF or other antiandrogenic effect by co-administration of EGF. It appears that testis descent is the only process or tissue associated with androgen action that is dependent on EGF. But testis descent is under the control of many other factors (Levy and Husmann, 1995; Prahalada, 2002) and so the role of EGF in testis descent is not necessarily dependent upon androgen action. The outcome of this portion of the study gives no indication that androgen-dependent development is affected by or dependent upon EGF.
Our *in vitro* analysis of adult offspring did not find alteration of AR expression in the ventral prostate (VP) or seminal vesicle (SV) after prenatal exposure to V, using either receptor binding assay or immunohistochemistry. These results are in contrast to the reduction in AR expression in the epididymis (Kelce et al., 1997; Monosson et al., 1999) or in the prostate (Monosson et al., 1999) of weanling rats immediately after postnatal exposure to V. However, our analysis was done in the maturing rat long after prenatal exposure to V. By this time, the hormone-receptor system may have equilibrated after continued growth of the sex accessory glands with continued androgen production that maintains their AR level (Hayward et al., 1996). The reduced lumen development, or increased stroma:epithelia ratio, in one VP sample may be a true effect since Cunha et al. (1992) also found increases the mesenchyme:epithelial ratio in the prostate with inhibition of mesenchymal AR. The reason we saw this in one animal and not others may be because this animal was more affected. We allow that reliability of the immunohistochemical analysis in this experiment is questionable due to the small number of samples and the lack of sophisticated means of quantitating the density of staining available at the time. Nonetheless, these data suggest that permanent alterations in AR levels are not responsible for the reduction in VP or SV weight by prenatal V. Rather, receptor levels may be altered transiently, immediately following exposure to V. Hence, we monitored AR and EGFR levels in the fetal phallus at the time of exposure.

Analysis of the fetal phallus revealed a slight decrease in AR and EGFR expression in the phallus of GD 18 females as compared to males, appropriate for this gestational age, but no effect of V on AR or EGFR expression in the male. Fetal tissue may not respond to V in the same way as tissue of mature males. AR in the fetus are increasing each day of development (Bentvelsen et al., 1995) and this process may
overcome downregulation by an AR inhibitor. Also, castration of neonatal rats did not alter AR expression in the prostate (Husmann et al., 1991), whereas castration of adult rats does alter AR expression in various tissue, which suggests AR expression is regulated by variable factors dependent on age. Given the results of AR analysis in fetal and mature tissue, perhaps AR is downregulated in older rats after more recent exposure to antiandrogen, as in other studies (You et al., 1998; Monosson et al., 1999). Based on these results, we conclude that antiandrogenic effects in male offspring by prenatal V are not due to any organizational effect on the AR. We also have no evidence to suggest that V alters EGFR as part of the mechanism of action in abnormal development of the phallus.

The lack of effect of V in the fetal phallus is not necessarily due to failure of the agent to reach the fetus, since all male offspring in a cohort litter of a pilot run displayed cleft phallus, and a previous study indicated that virtually all male offspring exposed in utero to 400 mg/kg V have cleft phallus (Wolf et al., 2000). Rather, the lack of effect of V at GD 18 may be explained by a timepoint too early for sexual dimorphism, or development of a permanent cleft. Several studies describe the normal phallus as open, or cleft, in early development, and undergoing a drawing together of the folds, fusion of the folds, and subsequent removal of the urethral seam (Baskin, 2000; Kim et al., 2001; Kurzrock et al., 2000) that involves apoptosis and cell migration in the rat (Baskin, 2000). Morphology of the fetal phallus cross-section in our study has the same appearance described in these studies at the same stage of development. It appears that the folds come together in the V treated phallus, as in the untreated, but that the signals to eliminate the urethral seam are not given or followed, hence fusion does not occur. The seam may open with continued growth of the phallus, to appear as a cleft past GD 18.
Furthermore, it is not until GD 22, after the age we monitored, that AR number becomes sexually dimorphic by, higher in males than in females (Bentvelsen et al., 1994). Our findings are consistent with these previously defined aspects of development and AR expression in the phallus. Determining whether EGFR is affected by AR inhibition would be better addressed at later time points with a time-course study.

T levels in the fetus and in the fetal testis were unaffected by V. As T is pre-receptor in the androgen signalling pathway, we did not expect it to be affected by AR inhibition. This supports the assertion that V is an AR inhibitor. The elevation of T in an earlier study by 100 mg/kg V peripubertally after 30 days of dosing (Monosson et al., 1999) was presumed to be due to inhibition of pituitary AR thereby blocking the negative feedback loop of the HPG axis, ultimately causing more T production. The fetal exposure to V in this study was of a shorter duration and at a time of fetal development in which functional AR in the pituitary or hypthalamic-pituitary-gonadal axis is questionable.

Together, these results indicate that neither AR nor EGFR expression levels are affected in the fetus or mature rat by prenatal administration of the AR inhibitor V and thus that these receptor levels are not responsible for the altered development of androgen-dependent tissue. It is more likely that reduced AR activity would reduce expression of androgen-dependent genes and their products, and apparently, EGFR is not one of those. It is also possible that parallel systems that interact and regulate AR action, such as coactivators of AR, are affected by AR inhibition rather than AR itself. Future investigation will address these possibilities.
Acknowledgements

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References


**Table 1. Maternal and Neonatal health is affected only by the combination of V and Testosterone Propionate (2 mg/kg) but not by V, EGF, or V+EGF**

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Treatment Group</th>
<th>C</th>
<th>V</th>
<th>EGF</th>
<th>V+EGF</th>
<th>V+TP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Weight Gain GD 14-19 (g) Exp. 1</td>
<td></td>
<td>49.82 ± 3.45</td>
<td>39.52 ± 10.23 *</td>
<td>58.73 ± 5.55</td>
<td>37.28 ± 10.85 *</td>
<td>_</td>
</tr>
<tr>
<td>Maternal Weight Gain GD 14-19 (g) Exp. 2</td>
<td></td>
<td>60.34 ± 8.20</td>
<td>52.72 ± 14.65</td>
<td>54.52 ± 14.32</td>
<td>53.66 ± 13.03</td>
<td>28.28 ± 7.22 **</td>
</tr>
<tr>
<td>Litter size - Exp. 1</td>
<td></td>
<td>12.5 ± 1.50</td>
<td>14.2 ± 1.17</td>
<td>12.8 ± 1.67</td>
<td>12.67 ± 1.49</td>
<td>_</td>
</tr>
<tr>
<td>Litter size - Exp. 2</td>
<td></td>
<td>14.2 ± 1.47</td>
<td>13.2 ± 1.47</td>
<td>11.2 ± 3.12</td>
<td>4.2 ± 6.49 **</td>
<td>0.4 ± 0.8 **</td>
</tr>
<tr>
<td>Pup weight PND 1 - Exp. 1</td>
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<td>6.40 ± 0.168</td>
<td>5.75 ± 0.184 *</td>
<td>6.59 ± 0.168</td>
<td>6.44 ± 0.155</td>
<td>_</td>
</tr>
<tr>
<td>Pup weight PND 2 (m) - Exp. 2</td>
<td></td>
<td>7.71 ± 0.26 **</td>
<td>6.29 ± 0.26</td>
<td>7.66 ± 0.26 **</td>
<td>5.96 ± 0.58 **</td>
<td>4.65 ± 0.58 **</td>
</tr>
</tbody>
</table>

*Note:* Values are litter means ± SE

* p < 0.05, ** p < 0.01
Figure Legends

Figure 1. Lack of ability of EGF to alter antiandrogenic effects of V in male offspring. Pregnant rats were dosed on gestational day (GD) 14-19. a) In experiment 1 in Long Evans Hooded rats, 200 mg/kg V reduced AGD, but co-administration of 20 μg/kg EGF did not alter the outcome. b) In experiment 2 in Sprague-Dawley rats, 200 mg/kg V reduced AGD in pups, but co-administration of 100 μg/kg EGF or of 2 mg/rat TP did not alter the outcome. c,d) In experiment 1, V induced areolae and malformations of the external genitalia including cleft phallus, vaginal pouch and ectopic testes in male offspring, while co-administration with EGF did not alter the outcome. These results are representative of the results in experiment 2 as well. AGD, anogenital distance; PND, postnatal day; C, control; V, vinclozolin; EGF, epidermal growth factor; TP, testosterone propionate. * p< 0.01.

Figure 2. Percent incidence of ectopic testes in male offspring exposed to 200 mg/kg V or V + 100 μg/kg EGF on GD 14-19. V induced >70% ectopic testes in male offspring, while co-administration of EGF with V induced a much lower incidence of ectopic testes (21%). Control males had no incidence of ectopic testes (not shown). Only one litter of 12 males remained at adulthood in the V+EGF group, and statistics could not be performed. V, vinclozolin; EGF, epidermal growth factor; GD, gestational day.

Figure 3. Quantitation of androgen receptor (AR) by competitive receptor-binding analysis in ventral prostates of PND 55 male offspring exposed to corn oil (Control) or 200 mg/kg V on GD14-19. In each of 3 assays, the amount of AR in fmol did not differ
between Control and V prostates. The same pattern exists in each assay. Block differences prevented combining data from 3 assays. Fmol, femtamoles; AR, androgen receptor; V, vinclozolin.

Figure 4. Testosterone levels in male GD 18 fetuses and testes after exposure to corn oil (Control) or 200 mg/kg V on GD 14-18. a) Although fetal body T levels appear slightly reduced in the V group, this reduction was not significant. b) V did not alter T levels in the testes of these fetuses. n = 3 litters per treatment group. T, testosterone; GD, gestational day.

Figure 5. Immunohistochemical analysis of AR in PND 84 ventral prostates and seminal vesicles of male offspring of dams exposed to corn oil or 200 mg/kg V on GD 14-19 shows no difference was observed between control and V-treated groups. a) Ventral prostate of adult control male offspring. b) Ventral prostate of adult male offspring exposed to 200 mg/kg V on GD 14-19. c) Negative control section of adult ventral prostate, no primary antibody. d) Seminal vesicle of adult control male offspring. e) Seminal vesicle of adult male offspring exposed to 200 mg/kg V on GD 14-19. f) Negative control section of adult seminal vesicle. n = 3 litters per treatment group. Bar represents 10 µm for each photograph.

Figure 6. Expression of AR and EGFR visualized by immunohistochemistry with DAB staining in sections of gestational day (GD)18 phalluses. a) Staining pattern obtained with this androgen receptor. b) Staining pattern obtained for EGF receptor. Receptor
staining patterns and morphology of phallus are representative of both control and V group sections.

Figure 7. Gray scale images of GD 18 phallus sections of control or 200 mg/kg V-exposed fetuses used in image analysis, showing immunohistochemical localization of AR (a), and EGFR (c), with image analysis overlay (b,d). b) AR expression was quantitated by defining an area of interest (AOI; green line) and programming the ImagePro Plus program to count the number of objects of a given density or higher (red spots). Mean density of staining of the epithelial layer in each the epidermis (large arrow in a) and urethral groove (small arrows in a) was also quantitated for AR and EGFR (not shown, see text). d) EGFR high expression (HE) was quantitated by defining an intensity level higher than that for epithelium, representing high expression, and having the ImagePro Plus program highlight those areas in red and measure that area.

Figure 8. Quantitation of AR by density and area in GD 18 phallus sections of control or 200 mg/kg V-exposed fetuses, using image analysis (described in Fig. 7 and text). a) Mean density of AR staining in the epidermis (outer edge of phallus section not including urethral lumen or seam) was slightly but not significantly reduced in females, and was not affected in males by V-treatment. b) Mean density of AR staining in the urogenital fold seam and urethral lumen was slightly reduced in females but was not affected in males by V treatment. c) Mean area of each object in the mesenchyme appeared slightly reduced in females and was not affected in males. Variability of “object” data increased in the V group. d) Summed area of all objects in the mesenchyme appears somewhat
reduced in females. V treatment does not appear to have increased or decreased the summed object area in males, but to have induced variability of the response.

Figure 9. Quantitation of AR by number of objects, or AR-positive cells, in the mesenchyme of the GD 18 phallus of control or 200 mg/kg V-exposed fetuses, using image analysis and densitometry (as described in text or Fig. 7). Number of objects appears slightly reduced in the female compared to the male, but mean number of objects do not differ between control and V-treated males (a,b). V appears to have induced variability in the number of objects, or AR-positive cells, regardless of whether all data points are included (a) or an outlier is omitted (b). c) Frequency distribution of the number of objects in control male mesenchyme and in V-treated male mesenchyme, showing the outlier, to the far right. The number of sections (X-axis) that had a number of objects that fit a given range (Y axis), is plotted for the control group (back) and V-treated group (front). Sections in the control group are clustered between 0 and 450 objects. Sections in the V group are similarly clustered, but one section contained a number of objects in a much higher range, 750-800.

Figure 10. Quantitation of EGFR by mean density and area of staining in the GD 18 phallus of control or 200 mg/kg V-exposed fetuses. Sections were analyzed as described in text or Fig. 7. a) Mean density of staining in the epidermis is significantly reduced in the female compared to the male. b) Mean density of staining in the urethral groove appears slightly but not significantly reduced in the female. c) Total area of high expression (HE) was very variable in the female, but when considered proportional to the total epithelial area (d), was typical of the other characteristics - slightly but not
significantly reduced. Density of staining in V-treated males did not differ from control males in any location or from any perspective (a-d).
Figure 1
Incidence of Ectopic Testes in Mature Males

![Graph showing incidence of ectopic testes in mature males.]

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Percent</th>
</tr>
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<tbody>
<tr>
<td>V</td>
<td>80 ± 10</td>
</tr>
<tr>
<td>V + EGF</td>
<td>20</td>
</tr>
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n litters = 5 for V, 1 for V + EGF

Figure 2
Figure 3
Figure 4
Figure 8.
Figure 10
SUMMARY AND CONCLUSIONS

The possibility that congenital alterations of the male reproductive tract may be due to *in utero* exposure to environmental endocrine disrupting chemicals (EDC) is a concern today (Baskin et al., 2001). The purpose of this research was to characterize the effects of *in utero* exposure to vinclozolin, an environmental EDC, and investigate some of the possible mechanisms of action employed by vinclozolin to produce malformation of the external genitalia. First, we established a dosing regime that would induce malformations in all male offspring, and then used that dosing regime to investigate the mechanisms by which vinclozolin induces malformation of the external genitalia. Specifically, we sought to determine whether 1) co-administration of the androgen testosterone propionate can ameliorate the effects of vinclozolin, 2) a local mediator of androgen action, epidermal growth factor (EGF), can induce androgenic effects or co-administration can ameliorate the effects of vinclozolin, 3) androgen receptor (AR) number or testosterone levels are altered in the fetus or the adult by prenatal vinclozolin exposure, 4) EGF receptor (EGFR) levels are altered in the fetus as part of the mechanism of vinclozolin antiandrogenicity. This research revealed that the male rat fetus is most susceptible to the antiandrogenic effects of vinclozolin between gestational days 14 and 19 and that altered testosterone levels or number of AR is not a mechanism by which vinclozolin induces its effects. In addition, there was no evidence to suggest that EGF or its receptor is a necessary component of the mechanism of action of vinclozolin.
In the first study, successive 2-day windows of exposure to vinclozolin from gestational 12 to 21 in Long Evans Hooded rats revealed that gestational day 16-17 is the time fetuses are most susceptible to the effects of vinclozolin, and that greater than 90% of the male offspring have malformations of the genitalia when exposed to 200 mg/kg vinclozolin from GD 14-19. The most sensitive endpoints of prenatal vinclozolin exposure were reduced AGD, presence of areolae, reduced levator ani/ bulbocavernosus weight, and cleft phallus. These results laid the foundation for investigation into the mechanisms of action of vinclozolin in the fetus in the next set of experiments.

A dose-response study of testosterone propiontate found that androgenic effects, including development of prostate tissue, are seen in female offspring starting at 0.5 mg TP/dam in Sprague-Dawley rats. A greater number of androgenic effects, inducing regression of nipples and induction of male sex accessory glands and a levator ani muscle, were observed at 1 mg TP/rat/day without adverse effects to the dam or litter size. We predicted 1 mg TP/dam would be potent enough to antagonize the effects of vinclozolin in the male offspring. This experiment also revealed some unexpected malformations in the female including hydrometrocolpos, a condition described in humans (Nguyen et al., 1984; Janus and Gondine, 1986). This experiment provides a useful foundation for the study of androgenic chemicals \textit{in vivo}. In addition, the results of this and the first study provide the information on dose and duration of dosing required for testing the hypothesis that the effects of vinclozolin can be attenuated by co-administration of androgen.

In the third experiment, reported in chapter III and IV, co-administration of
vinclozolin and TP revealed that the AR agonist and the AR antagonist are functionally antagonistic in their effects on androgen-dependent tissue, while their toxicity is additive. Vinclozolin antagonized the effects of TP in the female offspring, and TP antagonized the effects of vinclozolin in the male offspring in most cases. The lack of ability of TP to antagonize some effects of vinclozolin in the male offspring was probably due to the strength of the doses of each chemical that were used. The varying degree to which TP attenuated the effects of vinclozolin between different tissue illustrates the varying responsiveness of some male reproductive tissue to the effects of TP. This study shows that T can compete with vinclozolin or its metabolites for binding the AR in the fetus and attenuate its effects. This is possible since binding of V, M1 or M2 to the AR is reversible, and this study illustrates that characteristic in vivo. This study also shows that the vinclozolin induces the same effects in the Sprague-Dawley as well as the Long Evans Hooded rat, so its effects are not strain-specific.

In the next study, EGF was co-administered with prenatally administered vinclozolin to test the hypothesis that EGF could mimic androgen and thereby attenuate the effects of vinclozolin, much as TP did in the previous study. The effects of vinclozolin were not attenuated by co-administration of EGF. In addition, EGF alone did not induce any discernable effects in the offspring of either sex. Since no effects of EGF were observed and EGF was not assayed in the fetuses, we had no indication that EGF was able to cross the placenta and reach the fetus. Also, a high degree of mortality was observed with combination dosing that precluded collection of enough data for statistical analysis. Therefore, any results from this experiment are inconclusive. It is worth
mentioning, however, that the incidence of ectopic testes was lower in the group exposed to vinclozolin and EGF than in the group exposed to vinclozolin alone. This may indicate that EGF can attenuate the incidence of ectopic testes, and may play a role in testis descent, a finding offered by other investigators (Cain et al., 1994; Siow and Fallat, 1997). More recently, other growth factors including insulin-like 3 (Insl3) a hormone in the insulin-relaxin superfamily secreted from Leydig cells, were found in the gubernaculum and to play an important role in testis descent (Kubota et al., 2001).

The next experiment tested the hypothesis that AR and EGFR expression levels are altered as part of the mechanism by which vinclozolin induces cleft phallus. To test this hypothesis, we monitored the fetal phallus at the time of exposure to vinclozolin. We did not find that AR or EGFR expression levels were altered by vinclozolin in the developing male phallus at the time of exposure, although adults exposed to this prenatal dosing regime display cleft phallus. In addition, AR and EGFR levels tended to be lower in female than in male phalluses, but not statistically different. This finding agrees with data produced by others for this age, GD 18, in the rat phallus (Bentvelsen et al., 1994). Sexual dimorphism of androgen receptor expression occurs after GD 18, although antiandrogenic treatment by GD 18 can induce cleft phallus. Therefore, cleft phallus can be initiated by V on or before GD 18, and critical signaling components mediating phallus development afterwards, such as perhaps EGFR, may be interrupted in the following days. We conclude that the number of AR or EGFR on GD 18 is not a mechanism by which vinclozolin induces cleft phallus and that other mechanisms are being employed at this time to induce cleft phallus in these male offspring.
Analyses of AR protein expression in the prostates and seminal vesicles of adult and weanling male offspring also did not show alteration in expression levels with prenatal vinclozolin exposure. Therefore, the lack of alteration of AR levels in the fetus after vinclozolin exposure remains in the adult, despite the antiandrogenic effect present. Although the AR is inhibited during fetal differentiation, its expression is not altered immediately or after the animal matures. Testosterone levels were also not altered in the adult or the fetus exposed prenatally to vinclozolin. Together these results suggest post-receptor mechanisms mediate antiandrogenic effects of V.

The results of these studies show that the mechanism by which vinclozolin induces cleft phallus and reduces organ weights in male rats does not depend on alteration of testosterone or AR expression levels. Two attempts were made to show post-AR or simultaneous involvement of EGF or its receptor in vinclozolin action. The results of co-administration of EGF were inconclusive, but analysis of fetal phallus tissue demonstrated no effect of V on EGFR expression. One could expect AR-dependent genes and their products to be altered by AR inhibition. Our research did not fully investigate the signaling pathway inactivated by AR inhibition by vinclozolin through time and so involvement of EGFR at a later time cannot be ruled out. Future investigation may focus on later timepoints, or redirect focus from EGF to other mediators and agents of androgen action discovered by genomic analysis of affected tissues. Recently, a gene, EphrinB2, has been found to be involved in the induction of hypospadias, a malformation in the human equivalent to cleft phallus in the rat. This gene is often associated with morphogenesis, usually of vascular and neural tissue
(Nakamoto, 2000). Genetic analysis may be a route to discovery of factors that are required in androgen-related processes in future investigation.

The research findings reported in this dissertation support the findings of others on the antiandrogenic effects and mechanisms of action of vinclozolin. In addition, this research adds to this body of information by having found the critical stages of susceptibility of the fetus to vinclozolin. The interactive effects of the androgen, TP, and vinclozolin add to the data on exposure to mixtures of endocrine disrupting compounds, which aid the prediction of effects by similar EDCs.
REFERENCES


