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Interactive Effects of Vinclozolin and Testosterone Propionate on Pregnancy and Sexual Differentiation of the Male and Female SD Rat

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In mammals, androgens are essential in directing mammalian sexual differentiation of the male phenotype. Administration of testosterone during this period alters female development in a male-like direction, whereas exposure to an androgen receptor antagonist like vinclozolin (V) demasculinizes and feminizes the male offspring. In the current study, we administered V (gavage at 200 mg/kg/day) and/or testosterone propionate (TP, sc, at 1 mg/rat/day), alone and in combination to Sprague-Dawley (SD) rats on days 14 through 19 of pregnancy, to determine if V would antagonize the effects of TP in the female and, conversely, if TP would antagonize the effects of V in the male offspring. These doses of TP and V were selected because they significantly alter sexual differentiation in the majority of female and male rat offspring, respectively, without producing severe toxicity in the dam or offspring. The study design is a 2 × 2 factorial (7 dams per group) including vehicle control, V, TP, and V + TP groups. As expected, individually, both V and TP reduced maternal weight gain and the V + TP group was affected in a cumulative fashion. Litter size on postnatal day (PND) 2 was reduced only by V + TP, whereas pup body weight was reduced in all three treated groups, the effect of V + TP again being cumulative. In female offspring, TP-induced alterations (i.e., increased anogenital distance [AGD] and fewer nipples, vaginal agenesis, hydrometrocolpos, induced prostate and bulbourethral glands, and levator ani muscle tissues) were all reversed by coadministration of V. In male offspring, V-induced alterations were only modestly antagonized by TP. At the dosage levels used herein, V + TP-treated male offspring had less well-developed nipples as infants and adults and a lower incidence of ectopic testis than did the V group. However, V-induced changes in reproductive organ weights, AGD, atrophic testes, vaginal pouch, and agenesis of the sex accessory tissues were not antagonized by concurrent TP treatment in male offspring. We observed that the combination of V and TP, two chemicals with opposing endocrine action, antagonized one another during sexual differentiation, especially in the female offspring and induced cumulative effects on maternal and neonatal toxicity. We suspect that antagonism of V by TP would be enhanced in the male if lower dose levels of V were used, but then the antagonism of TP by V in the female would likely be attenuated.

Key Words: **sexual differentiation; endocrine-disrupting compounds; cumulative risk; androgen; antiandrogen.**

Humans and animals are exposed to combinations or mixtures of endocrine-disrupting compounds (EDCs) in the environment. Mixtures of EDCs have been found in human body fluids (Blount *et al.*, 2000; Younglai *et al.*, 2002), amniotic fluid (Foster *et al.*, 2000), and in children (Brock *et al.*, 2002) as well as in wildlife (Guillette *et al.*, 1999; LeBlanc *et al.*, 1995; Veith *et al.*, 1981; Vos *et al.*, 2000). It is therefore important to study the effects of combined exposure to environmental chemical contaminants, as mandated for pesticides under the Food Quality Protection Act (FQPA) in 1996 (United States General Accounting Office, 2000). A practical approach to studying the *in vivo* effects of combined exposure to EDCs is to simplify the model by studying the effects of chemicals that are well-characterized *in vivo* that alter sexual differentiation via a common mechanism or mode of action. In our laboratory, we have shown that combined exposure to two antiandrogens with either similar or dissimilar mechanisms of action, produces dose-additive antiandrogenic effects in the offspring (Gray *et al.*, 2001). In contrast, no work has been done to date to study the combined effects of an antiandrogen and an androgen. These two classes of EDCs are of relevant environmental concern, because androgens and antiandrogens are found in the environment. The current study focuses on the combined effects of an antiandrogen, vinclozolin (V) and an androgen, testosterone propionate (TP). V metabolites compete with testosterone (T) or dihydrotestosterone (DHT) for binding to androgen receptors (AR) (Kelce *et al.*, 1994) and inhibit androgen-induced gene expression (Kelce *et al.*, 1997) and reproductive development. Antiandrogenic effects of pre- or perinatal V exposure include induction of vaginal pouch, hy-

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pospadias, and nipple retention, reduced anogenital distance (AGD), reduced prostate weight, reduced testis and epididymal weights, and altered behavior in male offspring (Gray *et al.*, 1999; Hellwig *et al.*, 2000; Hotchkiss *et al.*, 2003; Wolf *et al.*, 2000). At high dosage levels, V has been shown to reduce maternal weight gain, litter size, and pup weight (Gray *et al.*, 1994; Hellwig *et al.*, 2000; Wolf *et al.*, 2000).

Androgenic compounds can disrupt sexual differentiation and reproductive function of female offspring when given perinatally. TP, methyl-testosterone, androsterone, and 17β-trenbolone, a growth-promoting food additive for cattle administered during the prenatal period, masculinize and defeminize female rats producing a partial male phenotype, as indicated by a longer AGD, absence of a vagina, and presence of male sex accessory tissues (Food and Drug Administration [FDA], 1996; Greene *et al.*, 1939; Swanson and Werff ten Bosch, 1965; Wilson *et al.*, 2002; Wolf *et al.*, 2002). Like V, androgens also can have adverse effects on pregnancy and pup viability. Testosterone, TP, or trenbolone acetate, given to pregnant female rats and sheep, was shown to reduce maternal gestational body weight, litter size, and body weight of offspring at high enough doses (DeHaan, *et al.*, 1987; Fritz *et al.*, 1984; Greene *et al.*, 1939; Wolf *et al.*, 2002).

In this study, we evaluated effects of coadministration of V and TP in the rat on reproductive development and hypothesized that each would attenuate effects of the other on androgen-dependent tissues in a sex-specific manner. V would antagonize the effects of TP in the female, while TP would antagonize the effects of V in male rat offspring. The design of the study is a 2 $\times$ 2 factorial with four groups: (1) Vehicle, (2) 1 mg/kg/d TP, sc, (3) 200 mg/kg/d V by gavage, and (4) 1 mg TP plus 200 mg/kg V. Dose selection was based on the results of two previous studies. In one, V administered to the dam on gestation days (GD) 14 through 19 at 200 mg/kg reduced the mean weights of sex accessory glands and induced malformations of the genitalia in 97% of the male offspring without inducing significant maternal of pup toxicity (Wolf *et al.*, 2000). V at 400 mg/kg was not selected because it significantly reduced maternal weight gain and body weight of adult male offspring. In another study, TP. administered sc on GD 14–19 at 0.5 mg/dam, induced rather minimal androgenic effects in females, whereas the dose selected for the current study of 1 mg TP/dam, was much more effective. Effects seen at 1.0, but not 0.5 mg TP/dam included permanently increased AGD, a full nipple and vaginal agenesis and hydrometrocolpos with slight but insignificant reduction in neonatal litter size, and delayed delivery (Wolf *et al.*, 2002). Higher doses of TP (2 mg/kg and above) significantly reduced maternal weight gain by 20 g, about 8% of maternal body weight, delayed parturition in half the dams, and reduced litter sizes. For the current study, we selected doses of V and of TP that would induce reproductive malformations in both male and female offspring, but not excessively toxic. We hypothesized that 1 mg/dam TP would attenuate the antiandrogenic effects of 200 mg/kg V in the male offspring, and, conversely, that V would attenuate the androgenic effects of TP in the female offspring. In addition, we hypothesized that the toxic effects of the combination of V and TP on maternal-weight gain and pup weight and viability would be cumulative.

MATERIALS AND METHODS

Animals. Timed pregnant SD rats arrived at the animal facility on gestation day (GD) 4 (GD 1 is considered as the day plug was observed) and were housed individually in polycarbonate cages (20 $\times$ 25 $\times$ 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 at 11:00 a.m.) 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 assigned to treatment groups in randomized, complete-block design (7 dams per treatment group). On GDs 14 through 19, dams were dosed once daily with corn oil (vehicle control; 2.5 ml/kg; by oral gavage), V (Reidl-de Haen, Germany, distributed by Sigma-Aldrich, lot # 9126X; 200 mg/2.5 ml/kg; oral gavage), TP (Sigma-Aldrich Co., St Louis, MO, lot #98H0566; 1 mg/rat, sc), or V + TP. Each dose of TP was on a per-rat basis without correction for body weight, as reported in the literature (Greene *et al.*, 1939; Rhees *et al.*, 1997; Swanson and Werff ten Bosch, 1965; Thornton and Goy, 1986; Tobin and Joubert, 1991) and following the protocol used in our previous TP study (Wolf *et al.*, 2002).

Maternal weight was monitored daily through the dosing period. Day of delivery was recorded and pups counted on GD 23 (postnatal day one [PND 1]). On PND 2, pups were counted, weighed, sexed if possible, and anogenital distance (AGD) measured. AGD was measured on each pup under a dissecting microscope fitted with an ocular micrometer reticle, with operator blinded to treatment group. On PND 14, pups were sexed again, and areolae were counted and scored on each pup, blind to treatment group. Areolae with and without a nipple were scored equally as "areolae." Each areola was scored on a scale of 1 to 3 on the basis of prominence, with 3 being most prominent. Scores for each of the 12 areola were then added together within a pup to produce a pup areola score for each pup, with a maximum possible score of 36 (12 areolae $\times$ maximum areola score of 3 = 36). Pup areolar scores were used for statistical analysis.

On PND 24, pups were weaned, counted, sexed, and AGD measured, using rotary dial micron calipers (Manostat) and with the operator blind to treatment group. Litter mates were distributed 2 or 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 one 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 every 2 to 3 days hereafter. Female offspring were euthanized after puberty if they developed a swollen abdomen (indicating hydrometrocolpos) or were in poor health.

On PNDs 90 through 94, female offspring were weighed and euthanized by CO_2 asphyxiation followed by decapitation, in compliance with U.S. EPA Institutional Animal Care and Use Committee (IACUC) standards, in blocked fashion by treatment group. Each carcass was shaved on the ventral side to view nipples, and necropsied. Malformations of the external genitalia and presence of internal reproductive structures were noted on all females. Malformations consisted of presence or absence of cleft phallus, vaginal orifice, vaginal thread, prostate, seminal vesicle, levator ani (LA), and the bulbourethral (BUG) gland. AGD and anovaginal distance (AVD; distance from anus to posterior edge of vagina) were measured with a rotary micron caliper. VGD was derived by subtracting AVD from AGD.

On PNDs 170 through 186, male offspring were weighed and necropsied in blocked fashion by treatment group. In order to avoid the effects of acute stress on serum steroid hormone levels, males were euthanized by decapitation without prior CO_2 asphyxiation, in accordance with U.S. EPA 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, selected randomly: malformations of the external genitalia including cleft phallus or prepuce, vaginal pouch, and ectopic testes; right testis weight and gross morphology, right epididymal weight and gross morphology, and seminal vesicle weight (plus coagulating glands and fluid); ventral prostate weight; glans penis weight; levator ani plus bulbo carvenosus (LABC) weight; and BUG weight. We assumed, based on earlier studies, that 3 animals per litter would be an adequate sample size to describe the effects of V on these organ weights. Necropsy was continued on every male offspring in the study (n = 143) for the following endpoints: malformations of the reproductive tract (agenesis or hypoplasia of the sex accessory tissues, LABC, testis, or epididymides) and external genitalia (hypospadias and vaginal pouch), right testis weight and gross morphology, and right epididymal weight, in order to assure that no malformations were present in these organs (as described above).

Fetal testosterone-level experiment. In a separate experiment, two groups of SD dams were received at the animal facility on two separate dates, each on GD 3, and were housed, one per cage. Conditions were the same as described in the *in vivo* section for each group. Fetus collection on GD 19, fetal homogenization, ether extraction for steroids and radioimmunoassays (RIA) (by Coat-A-Count kit #TKTT5, Diagnostic Products Company; Los Angeles, CA) were performed as previously described (Wolf *et al.*, 2002). Dams were dosed with either corn oil vehicle (control; n = 2 per group), V (200 mg/kg, n = 2 per set), or TP (1 mg/rat, n = 1 per set). Fetuses from a total of 4 control litters, 4 V litters, and 2 TP litters, each consisting of 10–12 fetuses, were used to determine testosterone concentrations.

Statistics. Data were analyzed on a litter-means basis by one-way analysis of variance (ANOVA) using a general linear models procedure (PROC GLM; due to the variable number of individuals per litter) with treatment as main effect, using SAS software (DEC versions 1.2–4 on U.S. EPA mainframe; SAS Institute, Inc, Cary, NC). AGD was analyzed with and without body weight as a covariate in male offspring, but not in female offspring, since normalizing an increased AGD to a reduced body weight would exaggerate the effect and bias the statistical significance. Sets of rats in the fetal testosterone level experiment were considered blocks, and 2-way ANOVA was performed by block. As no block effect was found, 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 the 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 (percentages with areolae, cleft phallus, undescended testes, etc.) were arcsine transformed to normalize the variance seen with binomial data, and analyzed by ANOVA as described above. Direct comparisons between two treatment groups (e.g., V versus V + TP for males or TP versus V + TP for females) were analyzed by t-test if the overall ANOVA was significant.

RESULTS

Maternal and Neonatal Data

Maternal weight-gain means during the dosing period in pregnant dams was reduced from 53 g in the control group to 35 g in the TP group ($p < 0.05$), 28 g in the V group ($p < 0.005$), and further reduced to 22 g in the V + TP group ($p < 0.0005$; Table 1). One control group dam was not pregnant. One dam in the TP group delivered late (GD 24). Live litter size on PND 1 (GD 23) was not measured since all dams had not finished delivering by the end of this day. Live litter size on PND 2 was not affected by either V or TP alone compared to controls (12–14 pups/litter in all groups), but was drastically reduced by V + TP (5.4 ± 2.3 pups/litter; Table 1). Whole-litter loss occurred in three litters in the V + TP group by PND 2. Viability of the offspring from PND 2 to weaning age was slightly reduced by V, but not by any other treatment.

Pup body weight on PND 2 was reduced in both male and female pups in the V, TP, and V + TP groups; the V + TP group were more severely affected (Table 1).

Female Offspring Data

Neonatal data: AGD, areolae, weaning. TP increased AGD in female offspring on PND 2 ($p < 0.0001$), and coad-

TABLE 1
Effects of Prenatal Vinclozolin (V), Testosterone Propionate (TP), or the Combination of the Two Chemicals (V + TP) on Pregnancy and Neonatal Health and Development

Endpoint	Treatment group			
	Control	TP	V	V + TP
Maternal weight gain (GD 14–19 (g)	53.1 ± 2.8	35.4 ± 2.6*	28.1 ± 8.4**	21.9 ± 4.1***
No. of dams delivered late (after GD 23)	0	1	0	0
Live litter size (PND 2)	13.2 ± 0.87	12.4 ± 0.57	14.1 ± 0.77	5.4 ± 2.27**
No. of whole litter loss by day 2/no. dams	0/7	0/7	0/7	3/7
No. of uterine implantation sites	13.8 ± 0.70	15.1 ± 0.77	15.1 ± 0.77	13.1 ± 1.12
Pup weight on PND 2 (male) (g)	7.97 ± 0.26	5.57 ± 0.19***	6.93 ± 0.26*	5.04 ± 0.29
Pup weight on PND 2 (female) (g)	7.60 ± 0.28	6.76 ± 0.26*	5.09 ± 0.19***	4.88 ± 0.10***
Litter size at weaning	13.2 ± 0.87	11.6 ± 0.37	11.3 ± 1.15	5.1 ± 2.12***
Viability, PND 2 to weaning (%)	100	93.6 ± 0.03	82.5 ± 0.09*	96.0 ± 0.02

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

*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$.

ministration with V reduced the TP-enhanced AGD to control values (Table 2). TP prevented development of areolae in roughly a third of female pups at PND 14 (Table 2). The number of areolae per female pup was reduced from 12 ± 0 to 3.7 ± 1.3 by TP and restored to the full complement by coadministration with V ($p < 0.0001$ compared to TP). Areola score was similarly reduced by TP treatment and restored by coadministration with V.

Body weight at weaning (PND 24) was significantly reduced in the TP group (Table 2). The body weight produced by V + TP was slightly but not significantly reduced ($p = 0.1432$).

Necropsy at adulthood. A total of 14 females from 4 litters from the TP group had died (5) or were euthanized (9) before necropsy due to poor condition resulting from reproductive tract malformations. Distended abdomen and vaginal agenesis were confirmed for 3 of the dead animals. Hydrometrocolpos, or distended uterus and vagina, resulting from vaginal agenesis, was confirmed in all 9 euthanized females and is likely responsible for all deaths prior to necropsy in the TP group. This result is not unusual for this dose of TP (Wolf *et al.*, 2002), but the dose was necessary in order to be sufficiently androgenic, and to counteract vinclozolin in the males.

Adult female offspring body weight was reduced by TP ($p < 0.01$), and V + TP ($p < 0.05$; Table 2). TP almost completely eliminated nipples on females but the number of nipples was completely restored by coadministration with V (Table 2). Agenesis or hypoplasia of the vaginal orifice was induced in 100% of the females by TP and was completely reversed by V + TP (Table 3). In addition, VGD, the distance between the genital papilla and the vaginal orifice, was reduced to one-third size by TP in the one female with a vaginal orifice in that group, and was attenuated by V + in the V + TP group. By contrast, cleft phallus was displayed in both the TP and V + TP groups, and the incidence was not different between the groups. Vaginal thread was displayed in the V + TP group but not in the TP group (these females had complete vaginal agenesis) (Table 3). Development of male organs VP, LA, and BUG was induced by TP and the incidence was attenuated by V in the V + TP group (Table 3).

Male Offspring Data

Neonatal data: AGD, areolae, weaning. AGD on PND 2 in male offspring was significantly reduced from control values by both TP ($p < 0.0002$) and V ($p < 0.0001$) alone. V + TP reduced AGD to the same extent as that by V (Table 4). When analyzed with body weight as a covariate, AGD was reduced only in those groups exposed to V (V and V + TP; both $p < 0.0001$).

V induced a full complement of areolae (12 ± 0) on 100% of the males, and TP coadministration (11.9 ± 0.1) did not attenuate this effect (Table 4). However, the prominence of areolae induced by V in males, measured by areola score, was attenuated by V + TP. Mean areola score in the V group was 30.4 ± 0.8 out of a possible score of 36, whereas areola score in the V + TP group was significantly lower, 23.8 ± 1.2 ($p < 0.005$ compared to V group). By weaning age (PND 24), body weight in male offspring was not affected by V, but was reduced in the TP and V + TP groups (Table 4).

Necropsy at adulthood. Nipples were not found on males in the control or TP groups. V induced almost the full complement (mean = 11.7 ± 0.2 out of 12) of nipples on 100% of the males in the group. Coadministration with TP reduced the number of nipples per male to 9.5 ± 1.3 ($p = 0.051$ compared to V group) and the nipple score from 23.2 ± 1.6 in the V group to 14.5 ± 3.8 ($p < 0.005$; Table 5). V induced the genital malformations cleft phallus with hypospadias, and vaginal pouch. About 50% of the males from the V and V + TP groups displayed either ectopic right testes or fluid-filled descended right testes. About 20% of the males from these two groups also displayed atrophy of the right epididymis. The incidences of hypospadia and vaginal pouch were slightly but not signif-

TABLE 2
Androgenic Effects of Prenatal Testosterone Propionate Are Attenuated by Vinclozolin in Prepubertal Female Offspring

	Treatment group			
Endpoint	Control	V	TP	V + TP
No. of litters with females	7	7	7	3
AGD on PND 2 (mm)	1.73 ± 0.027	1.62 ± 0.042	2.51 ± 0.130**	1.62 ± 0.013[a]
AGD at adulthood (mm)	23.18 ± 0.36	22.17 ± 0.51	23.93 ± 0.79	22.41 ± 0.36
Pups with areolae (%)	100	100	66.50 ± 9.47**	100[a]
No. of areolae/pup	12.02 ± 0.02	11.98 ± 0.02	3.69 ± 1.30**	12.0 ± 0[a]
Pup areola score	34.92 ± 0.11	34.73 ± 0.47	4.45 ± 1.77**	33.77 ± 0.89[a]
No. of nipples at adulthood	12	12	0.762 ± 0.52**	12[a]
BW at weaning	62.8 ± 2.34	61.8 ± 2.46	51.7 ± 1.49*	56.2 ± 2.09

Note. Values are litter means ± SE. AGD, anogenital distance; PND, postnatal day; BW, body weight.
[a]Significantly different from TP group at $p < 0.0001$.
*$p < 0.05$, **$p < 0.005$ compared to control group.

TABLE 3
Masculine Development of the External Genitalia and Internal Reproductive Tract in Females Exposed Prenatally to Testosterone Propionate is Attenuated by Vinclozolin

	Treatment group			
Endpoint	Control	V	TP	V + TP
VGD (mm)	6.97 ± 0.45	6.37 ± 0.264	2.4[a]	4.8 ± 0.08**
Cleft phallus (%)	0	0	31.6 ± 11.7**	50 ± 25.2**
Vaginal agenesis (%)	0	0	94.4 ± 3.8**	0[b]
Vaginal thread (%)	5.6 ± 5.6	0	No data	20 ± 11.5*
Prostate (%)	0	0	100**	21.7 ± 11.75[b,**]
LA (%)	0	0	35.4 ± 16.9**	0[b]
BUG (%)	0	0	40.14 ± 16.7**	0[b]
BW at adulthood	290.8 ± 4.49	293.2 ± 5.32	252.5 ± 9.55**	263.8 ± 2.20*

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, bulbourethral gland; BW, body weight.

[a]Only one female in the TP group had a vaginal orifice from which to determine VGD or vaginal thread. No statistical analysis was performed.

[b]Statistically significant when compared to the TP group by at least $p < 0.05$ by Student's *t*-test.

*$p < 0.05$, **$p < 0.01$, compared to controls.

icantly reduced by coadministration with TP, and the incidence of ectopic testes was significantly reduced by TP (Table 5). In addition, a higher percentage of the V group displayed bilateral testicular ectopia than did the V + TP group (18% [7/39] versus 5% [1/21]), respectively. Ectopic undescended and fluid-filled testes in the V and V + TP groups were smaller and weighed less than normal testes (data not shown).

However, weights of the ventral prostate, seminal vesicle, LABC, and BUG, reduced by V, and V-induced VP and SV agenesis was not reversed by coadministration with TP (Tables 5 and 6). Right testis weight, including only grossly normally testes (excluding ectopic and fluid-filled atrophic testes), was not reduced by V, TP, or V + TP. Epididymal weight was reduced by V ($p < 0.01$) in the V and V + TP groups in males with normal testis size, indicating a direct effect of V on epididymal differentiation that was not attenuated by TP.

At adulthood (PND 170–186), male body weight was not affected by V or TP alone, but was reduced by V + TP (Table 6).

Fetal T Levels

TP at 1 mg/dam significantly elevated T levels in GD 19 female fetuses (0.0898 ng/fetus compared to 0.041 ng/fetus control value; $p < 0.005$), but not in GD 19 male fetuses (control value = 0.269). V had no effect on T levels in either sex (Table 7).

DISCUSSION

In most cases, combined exposure studies with chemicals that interfere with the androgen-signaling pathway have been performed using chemicals with similar mechanisms of action. Some of these studies provided results that were reasonably

TABLE 4
Effects of Vinclozolin on Sexual Development of Prepubertal Male Offspring are Attenuated by Testosterone Propionate

	Treatment group			
Endpoints	C	TP	V	V + TP
No. of litters with males	7	7	7	4
AGD on PND 2 (mm)[a]	4.04 ± 0.10	3.40 ± 0.10	2.18 ± 0.10**	2.07 ± 0.13**
No. of areolae/pup on PND 14	0.29 ± 0.16	0.19 ± 0.11	12 ± 0**	11.93 ± 0.10**
Pups with areolae (%)	12.27 ± 4.67	8.4 ± 4.012	100**	100**
Pup areola score	0.29 ± 0.16	0.19 ± 0.11	30.41 ± 0.84**	23.79 ± 1.20[b,**]
Body weight at weaning (g)	66.07 ± 2.75	56.36 ± 1.55*	65.69 ± 3.21	49.08 ± 6.09**[,b]

Note. Values are litter means ± SE, unless otherwise noted. AGD, anogenital distance; PND, postnatal day.

[a]Analyzed with body weight as a covariate.

[b]Significantly different compared to V group at $p < 0.005$.

*$p < 0.05$, **$p < 0.005$, compared to control group.

TABLE 5
Malformations in Male Offspring Induced by Vinclozolin are Partially Attenuated by Coadministration of Testosterone Propionate

Endpoints	Treatment groups			
	Control	TP	V	V + TP
No. of litters with males	7	7	7	4
% with nipples	0	0	100**	100**
No. nipples per male	0	0	11.68 ± 0.21**	9.5 ± 1.28[a]·**
Nipple score	0	0	23.2 ± 1.58**	14.5 ± 3.84[b]·**
% cleft phallus/hypospadia	0	0	92.8 ± 4.95**	75.0 ± 14.43**
% vaginal pouch	0	0	79.2 ± 12.0**	40.6 ± 22**
% ectopic left and/or right testes	0	0	61.3 ± 13.2**	16.7 ± 11.7**
% bilateral testicular ectopia	0	0	18% (7/39)**	16.7 ± 11.7[b]·*, 5% (1/21)
% right testis fluid-filled, descended, or ectopic (10/21)	0	0	49% (19/39)**	48%**
% fluid filled/flaccid right testis	0	0	13% (5/39)**	33% (7/21)**
Agenesis of VP	0/18	0/19	24% (5/21)*	42% (5/12)**
Agenesis of SV	0/18	0/19	14.3% (3/21)	8.3% (1/12)
Atrophy of right epididymis	0	0	20.5% (8/39)**	25% (5/21)**

Note. TP coadministration attenuated the effect of V on the number of nipples, nipple score, and incidence of ectopic, undescended testes. Values are litter means ± SE. VP, ventral prostate; SV, seminal vesicle.

[a]Statistically different from V group by $p = 0.051$ by Student's *t*-test.

[b]Statistically different from V group by at least $p < 0.05$ by Student's *t*-test.

*$p < 0.05$, **$p < 0.005$, compared to controls. Values in parentheses are numbers of affected males/number of males in group.

well predicted, as the effects were cumulative and appeared largely additive in nature (Gray *et al.*, 2001; Nelleman *et al.*, 2003). The current study extends the investigation to two EDCs that have opposing mechanisms of action: the antiandrogen V and the androgen TP. We predicted that each would antagonize the developmental effects of the other on androgen-dependent endpoints. The individual effects of the TP and V have been well characterized in previous studies *in vivo* and *in vitro* (Fritz *et al.*, 1984; Gray *et al.*, 1994; Greene *et al.*, 1939; Hellwig, *et al.*, 2000; Kelce *et al.*, 1994; Rhees *et al.*, 1997; Wolf *et al.*, 2000). These two chemicals act in an opposite manner via the same receptor and target tissues during sexual differentiation. We found that coadministration of V and TP to the pregnant rat from gestational day 14 through 19 resulted in the expected antagonistic effects on many androgen-dependent tissues in the developing fetus and caused cumulative adverse

TABLE 6
Reproductive Organ Weights at Necropsy in Males Exposed Prenatally to Vinclozolin Are Not Restored by Testosterone Propionate

Endpoints	Treatment groups			
	Control	TP	V	V + TP
Ventral prostate (g)	0.814 ± 0.047	0.710 ± 0.042	0.151 ± 0.048**	0.196 ± 0.076**
Seminal vesicles (g)	1.955 ± 0.040	1.788 ± 0.094	0.869 ± 0.112**	0.836 ± 0.218**
LABC (g)	1.339 ± 0.025	1.297 ± 0.042	0.566 ± 0.048**	0.640 ± 0.043**
Paired BUG (g)	0.184 ± 0.014	0.187 ± 0.007	0.058 ± 0.021**	0.062 ± 0.035**
Glans penis (mg)	123.5 ± 1.80	118.8 ± 2.59	73.4 ± 3.64**	75.5 ± 4.39**
Right testis wt (g) (not including ectopic or atrophic tissue weights)	1.77 ± 0.04	1.74 ± 0.04	1.74 ± 0.04	1.72 ± .02
Epididymal (mg) (only including wt from rats with normal testes)	657 ± 17	632 ± 10	565 ± 18*	562 ± 9*
Body (g)	621.84 ± 11.32	8.03 ± 23.32	599.45 ± 11.83	558.26 ± 20.34**

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

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

[b]Statistically different from V group at $p < 0.05$ by *t*-test.

*$p < 0.05$, **$p < 0.005$, compared to controls.

TABLE 7
Testosterone Levels in Male and Female Fetuses on Last Day of Dosing, GD 19

Endpoints	Treatment groups		
	Control	V	TP
Male GD 19 fetus (ng/fetus)	0.2687 ± 0.04	0.2151 ± 0.04	0.2464 ± 0.064
Female GD 19 fetus (ng/fetus)	0.0412 ± 0.007	0.0334 ± 0.007	0.0898 ± 0.011*

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. GD, gestational day.

*$p < 0.005$ compared to controls.

effects on maternal and neonatal health. The antagonism of TP by V in the female offspring was more evident that the antagonism of TP by V in the male offspring. We suspect that the effects of TP would be more robust if we used lower doses of V than that employed here. In such a case, however, it is likely that the antagonism of TP by V in the female would be attenuated.

Maternal and Neonatal Alterations

As expected, the combination of V + TP induced cumulative or additive reductions in maternal weight gain, litter size, and pup weight. The reduction in maternal weight gain by V + TP can be attributed to both V and to TP, as each of these chemicals alone can reduce maternal weight gain (Gray *et al.*, 1994; Wolf *et al.*, 2000, 2002). Both TP and V alone reduced pup weight, but TP is often associated with more drastic reductions in weight of the offspring. Androgens, including testosterone and TP, administered to the mother, have been associated with dystocia, reduced body weight of the offspring, decreased milk production (DeHaan *et al.*, 1990; Swanson and Werff ten Bosch, 1965), and a reduction in placental weight (Slob *et al.*, 1973). It is possible that metabolites of testosterone, including other androgens or estrogens, could be partially responsible for some of the adverse effects on pregnancy and the reproductive development of the offspring. Estrogens are capable of inducing dystocia, reduced weight gain, and fetotoxicity (Bartholomeusz *et al.*, 1999; Zimmerman *et al.* 1991). However, these effects could be androgen-induced, since nonaromatizable androgens such as androsterone and dihydrotestosterone can reduce number of litters born and pup viability (Greene *et al.*, 1939). It is known that testosterone does not cross the rodent placenta well, but is metabolized to weaker androgens that do reach the fetus and they can masculinize and defeminize female reproductive development (Vreeburg *et al.*, 1981). For this reason, it would be useful to obtain fetal female steroid hormone levels on several estrogens and androgens in addition to testosterone (shown in Table 7).

Female Offspring—V Reverses Effects of TP

The effects of combined exposure to V + TP in the female offspring clearly show antagonism between the two compounds on androgen-responsive tissues. TP induced longer AGDs, and prostate, LA, and BUG-tissue development caused vaginal agenesis and reduced the number of areolae and nipples. V coadministration completely abolished the presence of LA and BUGs, reduced the incidence of prostate tissue by 80%. Coadministration also restored the vaginal orifice, areola and nipple counts, and female-like AGD, and eliminated the display of hydrometrocolpos. With regard to the female, estrogens derived from aromatization of androgens could have been responsible in part for the induction of hypospadias, as very high doses of estrogens *in utero* also can induce this effect (Henry *et al.*, 1984). However, many of the effects observed in the current study in the TP group, such as induction of male-typical tissues, can be induced by nonaromatizable androgens such as DHT but not by estrogens (Schultz and Wilson, 1974).

Male Offspring—Various Responses to V and TP

Administration of V from GD 14 to 19 at 200 mg/kg, in the current study, produced a spectrum of malformations and other effects similar to those reported in our previous studies (Gray *et al.*, 1994; Wolf *et al.*, 2002) that used the same dose of V from GD 14 to PND 3. In the current study TP coadministration only partially attenuated the effects of V on some tissues and not at all for other tissues. TP partially antagonized the effects of V on the prominence of the areolae and nipples in the male, testis descent. hypospadias, and vaginal pouch. However, TP treatment had no effect on the response to V in tissues like seminal vesicle, ventral prostate, levator ani, and BUG and AGD.

One might expect that the ability of TP to counter the effects of V would be inversely related to the ED_{50} of the androgen-dependent tissues (Gray *et al.*, 1999) (Fig. 1). If this were true, however, then it would be easier to protect against the effects of V with TP on ectopic testes, and hypospadias (higher dose effects), than for infant male areolar/nipple retention (a low-dose effect). Obviously, this was not the case. Another variation in response to the combined effects of TP and V between tissues could arise from unique differences between tissues at the cellular and molecular level. These differences include variations in testosterone, 5α-reductase, aromatase; blood flow; in the composition of cofactors for the AR (Gao *et al.*, 2002; Yeh *et al.*, 1999); the composition of other factors that regulate androgen action; or metabolic enzymes that change the levels of each metabolite, and thus the potency, of V.

Male vs. Female Sensitivity

A sex difference of the offspring in response to V, TP, and their combination, is apparent when comparing the responses in the female to those in the male offspring. The ability of V to

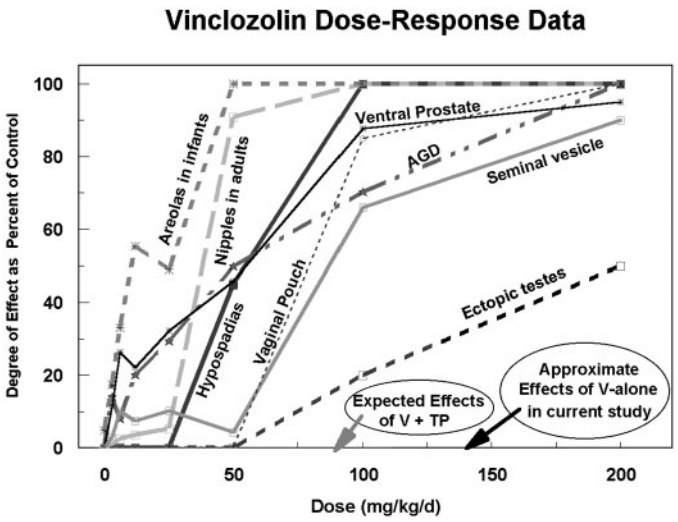

FIG. 1. Dose-response relationships for antiandrogenic effects of vinclozolin on various tissue in male offspring, modified from Gray *et al.,* (1994, 1999) who administered vinclozolin (V) by gavage from day 14 of pregnancy until day 3 of lactation to the dam. In the current study, V was administered at 200 mg/kg on GD 14–19 in the current study. The arrow at the right of the figure along the *X* axis indicates the overall effect of V by itself seen in the current study, relative to what was seen in the earlier study which used a longer dosing period. The arrow in the middle of the figure on the *X* axis indicates the potential attenuating action of co-administration of 1 mg TP on the androgen-receptor mediated effects of V. This was what one might expect to see if TP attenuated all the effects of V.

attenuate almost all the effects of TP in the female is in contrast with the lack of ability of TP to attenuate most of the effects of V in the male. This difference could be due to the doses of the compounds used, the dose of V used herein, was apparently more potent as an AR antagonist than the dose of TP was as an AR agonist. However, we could not have used a higher dose of TP due to its maternal toxicity, nor could we have used a much lower dose of V, as it would not have induced prominent malformations in male offspring.

T was elevated in the female fetus at a dose level that produced reproductive tract malformations. As expected, T levels were not affected by V in either the male or the female. We did not expect T levels to be reduced because V is an AR antagonist but does not inhibit testicular T synthesis or turnover. In fact, in the pubertal and adult male rat, administration of V results in an increase in serum T and LH (Kelce *et al.*, 1997; Monosson *et al.*, 1999) due to inhibition of negative feedback of T on the hypothalamic-pituitary axis. However, we did not expect to see this effect either in V-treated fetal male rats, because the testis is not regulated by LH at this age when T synthesis is constitutive. Hence, V treatment did not result in an increase in fetal T levels during sexual differentiation.

Summary

We have shown that combined exposure to an androgen and an antiandrogen can produce complex results, despite the thorough characterization of the effects of each chemical individually in earlier studies. Whereas some responses were expected, as in the attenuation of androgenic effects in the female and the cumulative toxicity to dams and neonates, the lack of attenuation of the effects of V by TP in some tissues in the male was not expected. Better prediction of the effects of combined exposure requires additional research using lower dosage levels of V combined with TP and mechanistic research to elucidate the cellular and molecular basis for tissue specific responses to androgens and antiandrogens, individually and as mixtures.

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