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Transgenerational Effect of the Endocrine Disruptor Vinclozolin on Male Spermatogenesis

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Abstract

The current study was designed to examine the actions of a model endocrine disruptor on embryonic testis development and male fertility. Pregnant rats (F0) that received a transient embryonic exposure to an environmental endocrine disruptor, vinclozolin, had male offspring (F1) with reduced spermatogenic capacity. The reduced spermatogenetic capacity observed in the F1 male offspring was transmitted to the subsequent generations (F2–F4). The administration of vinclozolin, an androgen receptor antagonist, at 100 mg/kg/day from embryonic day 8–14 (E8–E14) of pregnancy to only the F0 dam resulted in a transgenerational phenotype in the subsequent male offspring in the F1–F4 generations. The litter size and male/female sex ratios were similar in controls and the vinclozolin generations. The average testes/body weight index of the postnatal day 60 (P60) males was not significantly different in the vinclozolin-treated generations compared to the controls. However, the testicular spermatid number, as well as the epididymal sperm number and motility, were significantly reduced in the vinclozolin generations compared to the control animals. Postnatal day 20 (P20) testis from the vinclozolin F2 generation had no morphological abnormalities, but did have an increase in spermatogenic cell apoptosis. Although the P60 testis morphology was predominantly normal, the germ cell apoptosis was significantly increased in the testes cross

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sections of animals from the vinclozolin generations. The increase in apoptosis was stage-specific in the testis, with tubules at stages IX–XIV having the highest increase in apoptotic germ cells. The tubules at stages I–V also had an increase in apoptotic germ cells compared to the control samples, but tubules at stages VI–VIII had no increase in apoptotic germ cells. An outcross of a vinclozolin generation male with a wild-type female demonstrated that the reduced spermatogenic cell phenotype was transmitted through the male germ line. An outcross with a vinclozolin generation female with a wild-type male had no phenotype. A similar phenotype was observed in outbred Sprague Dawley and inbred Fisher rat strains. Observations demonstrate that a transient exposure at the time of male sex determination to the antiandrogenic endocrine disruptor vinclozolin can induce an apparent epigenetic transgenerational phenotype with reduced spermatogenic capacity.

Key words: Epigenetic, testis, gametogenesis, male infertility, antiandrogen, apoptosis

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Transgenerational" is defined as the transfer of heritable material from parents to offspring continuing through multiple subsequent generations, minimally through the F3 generation (Anway et al, 2005). It has been postulated that chemical, biological, and environmental toxicants may have the potential of inducing a transgenerational phenotype (DeRosa et al, 1998; Rakyan and Whitelaw, 2003). In order to transfer an induced phenotype from parents to offspring, the germ line genetic material must be altered via an epigenetic (eg, DNA methylation) or stable genetic alteration (ie, mutation, change in the DNA sequence) mechanism. Several causal factors, including irradiation, chemotherapy and environmental toxicants, have been argued to have the potential of altering the DNA via epigenetic or mutational mechanism(s) (Barber et al, 2002; Morris, 2002; Rakyan and Whitelaw, 2003). Although several induced phenotypes have been shown to propagate from pregnant females (ie, F0 generation) to subsequent offspring (Turusov et al, 1990; Zambrano et al, 2005), few have shown transgenerational effects (Anway et al, 2005).

A casual factor recently shown to induce a transgenerational phenotype is exposure to environmental endocrine disruptors (Anway et al, 2005). Endocrine disruptors are hormonally active environmental compounds that have been shown to influence both male and female reproductive development and function (Gray et al, 1994; Laws et al, 2000; Sharpe, 2001; Bayley et al, 2002). Endocrine disruptors such as pesticides (eg, DDT and methoxychlor) (Cummings, 1997), fungicides (eg, vinclozolin) (Kelce et al, 1994), insecticides (eg, trichlorfon) (Voccia et al, 1999), herbicides (eg, atrazine) (Cooper et al, 1999), and plastics (eg, phthalates) (Fisher, 2004) affect normal reproductive physiological development and functions by acting as weak estrogenic, antiestrogenic, or antiandrogenic compounds.

Vinclozolin (3-(3-5-dichlorophenyl)-5-methyl-oxazolidine-2, 4-dione) is a systemic fungicide registered for use on fruits and vegetables and commonly used in the wine industry (Kelce et al, 1994). Vinclozolin and metabolites, butenoic acid (M1) and enanilide (M2) derivatives, act as antiandrogens through actions on the androgen receptor (Pothuluri et al, 2000). Transient exposure of neonates to vinclozolin delays puberty and inhibits androgen-dependent male reproductive tract development (Gray et al, 1994). Embryonic exposure to vinclozolin influences male sexual differentiation and development as well as adult spermatogenesis (Wolf et al, 2000; Uzumcu et al, 2004). A previous study (Uzumcu et al, 2004) demonstrated that administrating 100 mg/kg/day of vinclozolin to pregnant rats during embryonic sex determination (embryonic day [E] 8–14 in the rat) reduced the spermatogenic capacity by decreasing germ cell survival in the subsequent F1 adult male offspring. Subsequently, vinclozolin exposure later in embryonic development (E15-postnatal day 0 [P0]) had no affect on adult spermatogenesis (Wolf et al, 2000; Uzumcu et al, 2004). In addition, Omezine and coworkers (Omezzine et al, 2003) demonstrated that embryonic exposure (E6-P0) of the

antiandrogen flutamide resulted in a similar reduced spermatogenic capacity in the subsequent F1 male offspring. Combined observations suggest that embryonic testis development is sensitive to androgen receptor signaling and can affect germ cell survival in the adult testis.

Embryonic testis development prior to and during sex determination (ie, E8–14 in the rat) appears to be the sensitive exposure period to antiandrogenic compounds (ie, vinclozolin) in the promotion of the reduced spermatogenic capacity in the adult male offspring (Uzumcu et al, 2004). The mechanism for a toxicant to influence embryonic development and transfer a disease state to an adult phenotype remains to be elucidated. The current study further investigates (Anway et al, 2005) the ability of the spermatogenic cell phenotype to be transferred to subsequent generations in both outbred and inbred strains of rats.

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In Vivo Vinclozolin Treatment

Timed pregnant rats (F0) were injected intraperitoneally with 100 mg/kg/day vinclozolin (n = 4, 99% pure; ChemService, West Chester, Pa) or vehicle (DMSO) as control (n = 4), from embryonic (E) E8 to E14 during gestation as previously described (Uzumcu et al, 2004). The number of treated F0 mothers was n = 4 for control and n = 4 for vinclozolin treatment for Sprague Dawley and n = 3 for control and n = 3 for vinclozolin-treated Fisher rats. The outbred Sprague Dawley (SD) and inbred Fisher (CDF) rat strains were used in separate experiments. Sibling sisters were used as control vehicle–treated and vinclozolin-treated F0 mothers to ensure that the genetic background of the control and vinclozolin generational animals were similar. F1 postnatal (P) P60 males and females from different litters of control and vinclozolin-treated groups were bred to generate the F2 generation. F2 generation rats were bred to generate the F3 generation, and the F3 generation was bred to give rise to the F4 generation. Breedings were carefully monitored to eliminate any sibling breedings and potential phenotypes as a result of inbreeding. For the outcross group (VOC), SD F2 males from the vinclozolin-exposed lineage were bred to SD wild-type control females. For the reverse outcross group (RVOC), SD F2 females from the vinclozolin-exposed lineage were bred with SD wild-type control males. Male SD rats were collected and analyzed at P60 for all generations, with the exception of the VOC and RVOC samples, which were collected and pooled for analyses from P60 and P90. The SD males collected at P20 were only from the F2 generation. The number of SD P60 males collected for replicates for each experiment (ie, n value) were: F1 (7 control, 7 vinclozolin), F2 (8 control, 17 vinclozolin), F3 (10 control, 8 vinclozolin), F4 (5 control, 5 vinclozolin), and outcross VOC (10 VOC), RVOC (6 RVOC), and wild-type as outcross controls (5 controls). The number of SD P20 males collected was 6 controls and 8 vinclozolin. The CDF male rats were collected and pooled from P80–P280 for analysis, with each vinclozolin and control generation rat being the same age at the time of analysis. The number of CDF males collected for replicates for each experiment (ie, n value) was: F1 (6 control, 10 vinclozolin), F2 (4 control, 5 vinclozolin), and F3 (11 control, 13 vinclozolin). It is important to note that only the original F0 pregnant rats were administered the vinclozolin treatment. The control and vinclozolin generational animals were all housed in the same room and rack in different cages with the same feeding and light conditions. Therefore the environmental conditions were exactly the same between control and vinclozolin animals. All animal procedures were approved by the Washington State University Animal Care and Use Committee.

Sperm Motility and Concentration Analyses

The sperm motility was determined using caudal epididymal sperm from the P60 males as previously

described (Uzumcu et al, 2004). Briefly, the epididymis was dissected free of connective tissue and a small cut made to the cauda. The tissue as placed in 5 mL F12 culture medium containing 0.1% BSA for 10 minutes at 37 °C. Fifty microliters was placed on a warm slide and gently cover-slipped. The specimen was immediately examined using phase contrast microscopy with 100x magnification. The sperm motility assays examined rapid progressive, slow progressive, and nonprogressive motility according to WHO category (Kvist and Björndahl, 2002). The ratio of motile sperm to the total number of sperm, including immotile sperm, was calculated. Approximately 50– 100 sperm were counted per microscopic field. The procedure was repeated at least twice, with a new specimen from the same epididymis. The average value was considered as percent motility for that rat and used as one replicate in statistical analysis. Epididymal sperm count was determined using the same epididymis according to a previously described method, with some modifications (Taylor et al, 1985; Uzumcu et al, 2004). Briefly, the epididymis that was placed in the 5 mL of culture medium was minced. The tissue pieces were removed, and the remaining sperm suspension was diluted with equal volume of 0.2% glutaraldehyde in 1x PBS to immobilize the sperm. Three independent sperm samples were counted using a hemacytometer. The counts were averaged and used as a replicate in statistical analysis. The control and vinclozolin generation analysis for an individual experiment were done at the same time. All analyses were done blinded, and different individuals were used for collection and counting.

Testicular Histology and Cellular Apoptosis

Following the weight determination, testes were cannulated with Bouin fixative (Sigma, St. Louis, Mo), cut in half, and submerged in Bouin for 6–10 hours, then washed with 70% ethanol. Two cross sections from each testis were embedded in parafilm using standard procedures performed by the Center for Reproductive Biology Histology Core Laboratory. Paraffin-embedded tissues were serially sectioned. At least two nonserial sections were stained with hematoxylin and eosin (H&E) using standard procedures for morphological analyses. Apoptotic cells were detected on duplicate slides by TUNEL assay using a Fluorescein In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, Ind). The fluorescent cells in each testis cross-section were counted at 200x magnification. The average number of fluorescent germ cells per testis section from stages I–V, VI–VIII, and IX–XIV from duplicate slides from one animal (2 testis sections per slide) was used as a replicate in statistical analysis. Approximately the same number of tubules from stages I–V, VI–VIII, and IX–XIV were present in control and vinclozolin generation testis sections during the apoptosis analyses. The negative control sections where the terminal transferase enzyme was excluded from the assay showed no labeling (data not shown).

Homogenization-Resistant Spermatid Analysis

The number of homogenization-resistant spermatid heads was determined for F3 control (n = 3) and vinclozolin (n = 3) animals as previously described (Robb et al, 1978). Briefly, the right testis from control and vinclozolin generation animals was excised and tunica albuginea was removed and homogenized in 50 mL of saline-triton buffer (0.15 M NaCl and 0.05% (v/v) Triton X-100). Each elongated spermatid head resistant to the homogenization was counted using a hemacytometer. Each sample was counted 4 times and averaged. Data were represented as average spermatids per testis (Zirkin et al, 1989).

Radioimmunoassays

Serum and testicular fluid (TF) were collected according to previously described methods (Turner et al, 1984; Hill et al, 2004). All samples were stored at −80 °C until assay for testosterone. The serum and TF testosterone was determined by radioimmunoassay with a testosterone double antibody RIA kit (Diagnostic Systems, Webster, Tex) and was assayed by the Center for Reproductive Biology Assay Core Laboratory. The sensitivity of the assay was 10 pg/tube. Serum testosterone concentrations were

determined on all samples collected, whereas TF testosterone levels were measured on 3 control and 4 vinclozolin-treated SD P60 males from the F2 generation.

Statistical Analysis

The data from testis weights, apoptotic cell counts, testosterone assays, sperm motility, sperm count, and spermatid count were analyzed using SAS. The values were expressed as the mean ± SEM to account for sample and animal variation within a data set. Statistical analysis was performed, and the differences between the means of treatments and respective controls were determined using a paired Student's *t* test for single comparison made between control and vinclozolin generation animals. No multiple comparisons were made that would require an ANOVA, so the Student's *t* test was optimal. Experiments were repeated with 3–17 rats per experimental group. A statistically significant difference was confirmed at $P < .05$.

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The transient embryonic exposure to vinclozolin had no affect on the number of offspring born, male/female sex ratios, or pup mortality in any of the subsequent generations. Tables 1 and 2 show that the average litter size was constant between control and vinclozolin-treated generations in both rat strains. However, the total number of pups did decrease from F1 through F4 generation in both control and vinclozolin-treated SD generations. The male/female ratios per generation showed no difference between the control and vinclozolin-treated generation in the F1, F2, F4, vinclozolin outcross (VOC), and reverse vinclozolin outcross (RVOC), but did decrease from 1.3 in the control to 0.9 in the treated F3 SD generation (Table 1). Similar results were observed with the Fisher rats in the F2 vinclozolin generation (Table 2). These decreases are considered not physiologically significant and likely due to a small error associated with one of the samples. There was no difference in the pup mortality rate between control and vinclozolin-treated generations. The neonatal mortality rate was less than 1% for both control and vinclozolin-treated generations, and similar results were observed for Fisher strain rats as well.

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Table 1. *Sprague Dawley litter size and gender*

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Table 2. *Fisher litter size and gender*

The testis/body weight ratios between the control and vinclozolin generations were not statistically different (Figure 1). The testes/body weight ratios for the vinclozolin-treated generations F1, F2, F3, F4, VOC and the RVOC generations was 9.3 ± 0.2, 9.9 ± 0.2, 10.2 ± 0.5, 8.2 ± 0.3, 9.9 ± 0.4 and 9.8 ± 0.1, respectively. None of the adult P60 males tested had significantly reduced testis weights in the control or vinclozolin generations in Sprague Dawley rats (Figure 1A). Similar observations were made with Fisher rats, except the F1 generation had a statistically smaller

testis/body ratio (Figure 1B). The Fisher inbred strain did show some toxicology in testis weight in the F1, but not in the F2 or F3 generations. Combined observations suggest minimal toxicology of vinclozolin for any generation at the embryonic or early postnatal ages.

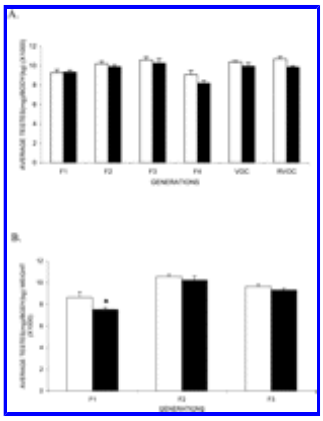

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Figure 1. Ratio of testes weights per body weight from adult (P60) male **(A)** Sprague Dawley and **(B)** Fisher rats from control and vinclozolin F1, F2, F3, and F4 generations, F2 generation male outcross (VOC) to wild-type females, and F2 generation female reverse outcross (RVOC) to wild-type male generations. Open bars (□) represent control and solid bars (■) represent vinclozolin generations. Statistically significant differences between control and vinclozolin generation rats are indicated by (*) for $P < .05$. Numbers of Sprague Dawley animals analyzed are F1 (n = 7 control, n = 7 vinclozolin); F2 (n = 8 control, n = 17 vinclozolin); F3 (n = 10 control, n = 8 vinclozolin); F4 (n = 5 control, n = 5 vinclozolin); and outcross VOC (n = 10), RVOC (n = 6) and wild-type as outcross controls (n = 5). Numbers of Fisher animals analyzed are F1 (n = 4 control, n = 10 vinclozolin); F2 (n = 4 control, n = 5 vinclozolin); and F3 (n = 11 control, n = 13 vinclozolin).

Previously, the inhibitory effects of vinclozolin on epididymal sperm number and motility used P60–P150 males (Anway et al., 2005). The epididymal sperm from only P60 males for F1, F2, F3, and F4 generations were collected and analyzed for percent forward motility and concentration. The sperm motility in the vinclozolin-treated F1, F2, F3, and F4 generations decreased 27%, 25%, 34%, and 43% compared to the controls, respectively. Although all the generations, F1–F4, showed a decrease in the average sperm motility, only the F1 and F4 were statistically significant. The VOC generation males also showed a significant decrease of 45% in sperm motility. However, the RVOC male rats did not show any significant decrease in epididymal sperm motility compared to wild-type rats. The reduction in sperm motility in the vinclozolin-treated generation males was paralleled by a reduction in epididymal sperm concentration (Anway et al., 2005). The sperm concentration in the P60 F1, F2, F3, and F4 vinclozolin-treated generations were decreased by 13%, 29%, 28%, and 22%. The outcrossed VOC rats also showed a statistically significant decrease of 33% in sperm number, whereas the RVOC generation was similar to its wild-type control. The decrease in sperm number and motility was more dramatic in older animals (Anway et al., 2005). Similar observations were made with Fisher rats with a decrease in epididymal sperm number and motility in the F1–F3 vinclozolin generations (data not shown). These epididymal sperm number and motility analyses have been previous reported (Anway et al., 2005).

A homogenization resistant spermatid analysis was performed to confirm that the testis spermatogenic cell number was decreased in vinclozolin generation animals and the potential causal factor in alterations in epididymal sperm numbers. The testis from F3 generation control animals had 470 ± 20 million spermatids per testis, while F3 vinclozolin generation animals had 336 ± 17 million spermatids per testis, which was statistically different ($P < .008$). In contrast, an F3 vinclozolin male that was found to not have a decreased epididymal sperm count also did not have a decreased spermatid per testis count (data not shown). Therefore, the decline in epididymal sperm count corresponded to a reduction in spermatid numbers per testis.

Testes of each male rat were collected, fixed, sectioned, and stained for morphological analysis. The testis morphology of the control SD P60 males was normal in all the generations (Figure 2A). The

testis morphology of the vinclozolin-treated SD P60 males was generally normal, with a percentage of the vinclozolin generation animals having morphological abnormalities in the testis. Approximately 15% of the vinclozolin generation animals had testes with more than one large vacuole within seminiferous tubules per testis cross section (Figure 2B). Furthermore, approximately 7% of the vinclozolin generation animals had testes that contained more than one seminiferous tubule with complete spermatogenic failure per testis cross section (Figure 2C and D). These seminiferous tubules were devoid of any advanced germ cell populations, with some tubules containing Sertoli cells only. When present, these abnormal tubules in testis cross sections made up 2%–12% of the total seminiferous tubules in the cross section. Morphological analyses of SD P20 testes from vinclozolin generations were similar to controls and did not show any abnormal morphology (Figure 2E and F). The testis morphology of the VOC rats was similar to the vinclozolin generations in that approximately 20% of the testis cross sections had more than one large vacuole and/or a lack of spermatogenesis within a tubule. In contrast, the RVOC testes were similar to controls and had no abnormalities (data not shown). In contrast to the SD rats, the vinclozolin-treated CDF rats showed no apparent increase in abnormalities in the morphology of the testis. Therefore, the outbred strain had a more dramatic phenotype.

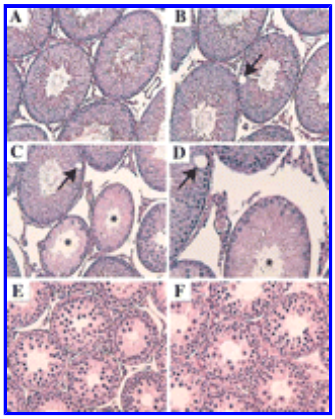

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Figure 2. Testis morphology of control P60 **(A)** and P20 **(E)** and vinclozolin P60 (**B**–**D**) and P20 **(F)** F2 generation SD rats. Testis cross sections stained with H&E. Arrows indicated large vacules and asterisks are in the lumen of seminiferous tubules that have degenerated and have no spermatogenesis. (**A**–**C**) Magnification 100x; (**D**–**F**) magnification 200x. Micrographs are representative of a minimum of 8 different analyses of P20 and 17 analyses of P60 F2 vinclozolin animals.

Testis cross sections from the P60 rats from each generation were analyzed for spermatogenic cell apoptosis. Figure 3 illustrates the apoptotic TUNEL positive labeling (yellow) in SD control and vinclozolin P60 F2 generation. The testis cross sections from the control samples had some labeled cells (Figure 3A). However, the vinclozolin generation testes cross sections had high levels of labeled cells (Figure 3B). The labeled apoptotic cells were identified as spermatogonial cells and spermatocytes within the seminiferous epithelium. Selected labeled germ cells were small and were located at the basement membrane of the seminiferous tubules (arrows), suggesting these could be spermatogonial cells. The majority of the labeled germ cells were large spermatocytes (arrowheads) and were located more towards the lumen (roman numeral) of the seminiferous tubules (Figure 3C and D). The identification of each germ cell type was verified by phase contrast microscopy of the tubule of interest (Figure 3D). The Sertoli and interstitial cells did not significantly label positive in either control or vinclozolin samples. The increase in apoptotic germ cells appeared to be stage-specific, with the highest level of labeled germ cells occurring in stages IX–XIV. Figure 3C and D illustrate a cross section of a seminiferous tubule at stage XI, with increased germ cell apoptosis and corresponding phase contrast images. This pattern of TUNEL positive germ cells was also observed in the testis sections from the treated CDF vinclozolin generations.

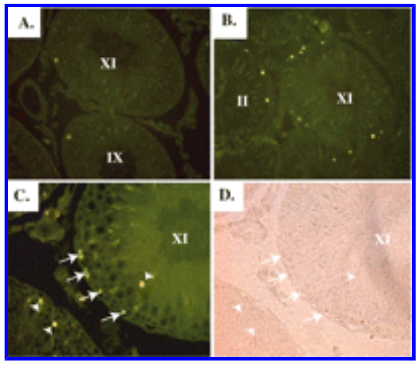

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Figure 3. Apoptosis (TUNEL) analyses in the testes P60 F2 generation SD rats from control **(A)** and vinclozolin **(B)** at 200x magnification and vinclozolin **(C)** and phase contrast **(D)** at 400x magnification. Apoptotic TUNEL positive cells labeled yellow. Arrows indicate possible spermatogonial cells along the basement membrane. Arrowheads indicate spermatocytes within the seminiferous tubule, and the lumen is depicted with a roman numeral indicating the approximate stage of the cycle of seminiferous epithelium. Micrographs are representative of a minimum of 10 different analyses of F2 vinclozolin generation animals.

The apoptosis from the SD P60 testes was counted (Figure 4). The data are presented as the number of tubules with more than four apoptotic germ cells at stages I–V, stages VI–VIII, and stages IX–XIV per testis cross section. The vinclozolin F1, F2, F3, F4, and VOC generations had a statistically significant increase of 26, 3.3, 9.3, 4.5 and 14-fold, in TUNEL positive germ cells at stages IX–XIV, respectively. The highest level of increase in spermatogenic cell apoptosis was at stages IX–XIV. The vinclozolin F1, F2, F3, and F4 generations at stages I–V had an increase of 2.3, 1.3, 3.3, 1.7, and 2.6, respectively (Figure 4B). However, only the increases in F1 and F3 generations were statistically significant. Stages VI–VIII did not have a significant increase in germ cell apoptosis in the vinclozolin generations. Approximately 90% of the testes analyzed from vinclozolin generations had an increase in TUNEL positive germ cells. The RVOC generation did not have an increase in germ cell apoptosis in any of the stages and was very similar to the wild-type controls. These data support the previous observation combining all tubule stages and ages 60–120 days (Anway et al, 2005). Analysis of Fisher rats also demonstrated a high rate of apoptosis in the stage IX–XIV tubules, in F1–F3 vinclozolin generations (Figure 5). The stage I–VIII only showed an increased apoptosis in the F1 vinclozolin animals, with no effect on spermatogenic cell apoptosis in F2 or F3 generations (Figure 5). The vinclozolin-treated CDF F1, F2, and F3 generations had 2.3, 1.9, and 1.9-fold increases in apoptotic spermatogenic cells, respectively. The apoptosis was also measured and quantified in the testis cross sections of the P20 Sprague Dawley males from the F2 generation (Figure 6). The testis cross section from the control P20 rats had few apoptotic germ cells. In contrast, the SD P20 testis cross section from the vinclozolin F2 generation had high levels of spermatogenic cell apoptosis (Figure 6A and B). The vinclozolin-treated SD P20 testes had a 1.8-fold increase in the number of apoptotic germ cells (Figure 6C).

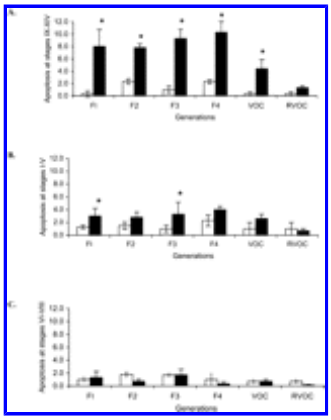

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Figure 4. Apoptotic germ cells from adult (P60) male Sprague Dawley rats from control (open bars, ☐) and vinclozolin (solid bars, ■) F1, F2, F3, and F4 generations, F2 generation male outcross (VOC) to wild-type females, and F2 generation female reverse outcross (RVOC) to wild-type male generations. Data presented as the number tubules with +4 TUNEL positive cells per testicular cross section at stages IX–XIV **(A)**, stages I–V **(B)**, and stages VI–VIII **(C)** of the cycle of the seminiferous epithelium. Statistically significant differences between individual controls and vinclozolin generation rats are indicated by (*) for $P < .05$. The numbers of animals analyzed are F1 (n = 3 control, n = 3 vinclozolin); F2 (n = 4 control, n = 4 vinclozolin); F3 (n = 4 control, n = 4 vinclozolin); F4 (n = 3 control, n = 3 vinclozolin); and outcross VOC (n = 4), RVOC (n = 3) and wild-type as outcross controls (n = 3).

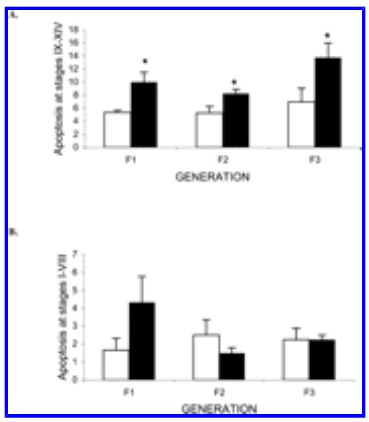

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Figure 5. Apoptotic germ cells from adult (P60) male Fisher rats from control (open bars, ☐) and vinclozolin (solid bars, ■) F1, F2, and F3 generations. Data presented as the number tubules with +4 TUNEL positive cells per testicular cross section at stages IX–XIV **(A)** and stages I–VIII **(B)** of the cycle of the seminiferous epithelium. Statistically significant differences between individual controls and vinclozolin generation rats are indicated by (*) for $P < .05$. The numbers of animals analyzed are F1 (n = 3 control, n = 3 vinclozolin); F2 (n = 4 control, n = 4 vinclozolin); and F3 (n = 4 control, n = 4 vinclozolin).

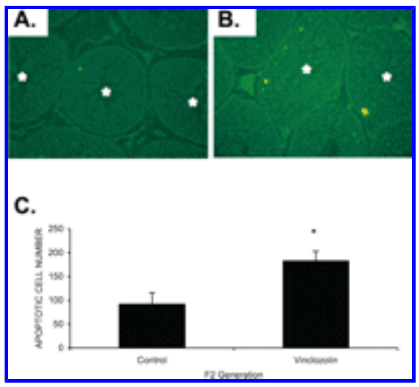

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Figure 6. Apoptosis (TUNEL) analyses in the testes P20 F2 generation of Sprague Dawley rats from control **(A)** and vinclozolin **(B)** at 2003 magnification. The lumen of the seminiferous tubule is indicated with an asterisk (*). **(C)** Presented as the total number of apoptotic germ cells from P20 male rats from control and vinclozolin F2 generation animals. The mean ± SEM is presented, and statistically significant differences between controls and vinclozolin generation rats are indicated by (*) for $P < .05$, F2 (n = 6 control, n = 8 vinclozolin).

The serum and testicular fluid testosterone levels were measured in the control and vinclozolin Sprague Dawley (SD) rat generations to determine if the vinclozolin exposure effected androgen production (Figure 7). The serum levels of testosterone in the vinclozolin F1, F2, F3, F4, VOC, and RVOC generations were 4.2 ± 0.8, 4.4 ± 0.6, 3.8 ± 0.8, 6.7 ± 2.0, 6.4 ± 1.2, and 5.8 ± 1.6, respectively. Testosterone levels were not different from their control values (Figure 7A). The testicular fluid was collected and analyzed in the control and vinclozolin F2 generation to measure the testis concentration of testosterone. As with the serum levels of testosterone, the testicular fluid level of testosterone in the vinclozolin F2 generation was 86 ± 16 and did not differ from the control value of 71 ± 12 (Figure 7B). Analysis of Fisher (CDF) rat control and vinclozolin generation males also demonstrated no effects on serum testosterone levels (Figure 8). The basal level of serum testosterone was lower in the CDF rats than SD, but no change in serum levels was observed. Therefore, the reduced spermatogenic capacity observed was not due to endocrine affects and a reduction in testosterone levels.

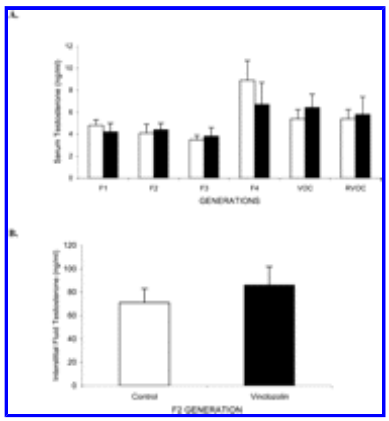

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Figure 7. Serum **(A)** testosterone levels from adult (P60) male Sprague Dawley rats from control and vinclozolin F1, F2, F3, and F4 generations, F2 generation male outcross (VOC) to wild-type females, and F2 generation female reverse outcross (RVOC) to wild-type males. **(B)** Testicular fluid (TF) testosterone levels from adult (P60) male rats from control and vinclozolin F2 generation. Open bars (☐) represent control and solid bars (■) represent vinclozolin generation animals. Serum analyses from F1 (n = 7 control, n = 7 vinclozolin); F2 (n = 8 control, n = 17 vinclozolin); F3 (n = 10 control, n = 8 vinclozolin); F4 (n = 5 control, n = 5 vinclozolin); and outcross VOC (n = 10), RVOC (n = 6) and wild-type as outcross controls (n = 5). TF testosterone analyses F2 (n = 3 control, n = 4 vinclozolin). The mean ± SEM is presented, with no statistical difference detected.

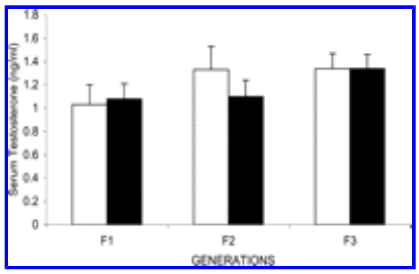

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Figure 8. Serum testosterone levels from adult (P60) male Fisher rats from control and vinclozolin F1, F2, and F3 generations. Open bars (☐) represent control and solid bars (■) represent vinclozolin generation animals. Serum analyses from F1 (n = 6 control, n = 10 vinclozolin); F2 (n = 4 control, n = 5 vinclozolin) and F3 (n = 11 control, n = 13 vinclozolin). The mean ± SEM is presented, with no statistical difference detected.

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The observations demonstrate a transgenerational effect of an endocrine disruptor on the testis of two strains of rats. The transient embryonic

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exposure from E8–E14 with vinclozolin reduced the spermatogenetic capacity of the adult male offspring (F1) (Uzumcu et al, 2004; Anway et al, 2005). This adult male phenotype was transmitted to the subsequent F2–F4 generations. An outcross (VOC) with males from the vinclozolin F2 generation bred to wild-type females produced male offspring that had reduced spermatogenic capacity, similar to the vinclozolin generations. A reverse outcross (RVOC) with a wild-type male bred to a female from the vinclozolin F2 generation resulted in male offspring that did not have the spermatogenic phenotype and were similar to controls. These data suggest that the male germ line is responsible for the transmission of the transgenerational phenotype. The current study supports the previous observation (Anway et al, 2005) with both an outbred Sprague Dawley (SD) and inbred Fisher (CDF) rat strains.

The spermatogenic cell defect was primarily localized to stage IX–XIV seminiferous tubules. This is a stage where spermatogenesis has advanced germ cell maturation and early spermatocyte development. Interestingly, both SD and Fisher rats had the same localization of spermatogenic cell apoptosis. Future studies with procedures such as cell sorting will help identify the most sensitive spermatogenic cell population. Epididymal sperm concentration and motility were also reduced in the vinclozolin generation animals for both strains of rats (Anway et al, 2005). The current study demonstrates that spermatid number per testis was also decreased in vinclozolin F3 generation animals. The inbred Fisher rats did show signs of greater toxicity to vinclozolin in the F1 generation males, with decreased testis/body weight and increased apoptosis in all stage tubules for the F1 vinclozolin males. However, this was lost in the F2 and F3 generations, which were similar to the SD rats.

The serum testosterone and testicular fluid testosterone levels did not change in any of the vinclozolin generation males. Therefore, the transgenerational phenotype and spermatogenic cell defect was not due to an endocrine defect. The transgenerational phenotype is likely more of a local molecular alteration in the germ line. Although the initial action of vinclozolin during embryonic sex determination is through an antiandrogenic mechanism, the adult phenotype and transgenerational transmission is presumed to be hormone-independent.

Transgenerational transmission of such a phenotype requires either an epigenetic phenomenon involving DNA methylation or stable alterations involving a DNA sequence mutation (Rakyan and Whitelaw, 2003). The frequency of the phenotype observed in this study based on the apoptotic TUNEL labeling analyses was approximately 90%. This frequency is substantially higher than the mutational rate (less than 1%) observed in the irradiation-induced mutational analyses previously described (Barber et al, 2002). The DNA sequence mutation rate of a hot spot unstable DNA sequence may reach 1%–5%, but the general DNA sequence mutation rate is less than 0.001% (Barber et al, 2002). This suggests that an epigenetic mechanism involving DNA methylation appears to be responsible for the transgenerational phenotype. Recent reports have led to the idea that an epigenetic reprogramming of the DNA methylation state in the germ line is possible (Rakyan and Whitelaw, 2003; Anway et al, 2005). Recently we have reported evidence for a transgenerational epigenetic effect of endocrine disruptors on specific genes in the male germ line (Anway et al, 2005).

Gonad formation in embryonic development is initiated when primordial germ cells (PGCs) migrate from the hindgut into the genital ridge prior to E12 in the rat. During PGC migration the DNA in the PGCs undergo a demethylation process that is completed prior to colonization of the early gonad (Durcova-Hills et al, 2001; Hajkova et al, 2002). In the rat, sex determination and testis development occurs between E11 and E15 and is initiated by the differentiation of precursor Sertoli cells in response to the testis determining factor Sry. In the gonads during sex determination, E11–E15, the germ cells undergo a remethylation process involving sex-specific determination of the germ cells. This

remethylation is dependent on the direct interactions with the somatic cells of the gonad (Reik and Walter, 2001; Hajkova et al, 2002). The aggregation of the precursor Sertoli cells, PGC, and migrating mesonephros cells (precursor peritubular myoid cells) promotes testis morphogenesis and cord formation. Testis morphogenesis (ie, cord formation) is completed prior to E14 in the rat. The androgen receptor (AR) and estrogen receptor-beta (ERß) are present in Sertoli cells, precursor peritubular myoid cells and prespermatogonial cells at the time of cord formation (E14). Although the testis does not produce steroids at this stage of development, estrogenic and androgenic substances appear to have the ability to influence early testis cellular functions. Future studies with defined antiandrogenic substances (ie, flutamide) are now needed to assess the role of the endocrine disruptor versus potential toxicology of the vinclozolin.

Sex steroids, estrogen and androgens, have the ability to influence the methylation state of DNA sequences (Sasaki et al, 2000; Rosinski-Chupin et al, 2001; Kumar and Thakur, 2004; Szabo et al, 2004). Although many reports on sex steroids influencing the methylation state of DNA involve the onset of cancers, such as uterine (Sasaki et al, 2000), prostate (Tekur et al, 2001), and breast (Leu et al, 2004), sex steroids can influence the methylation state of DNA during normal tissue development, including imprinting of genes in the germ line (Rosinski-Chupin et al, 2001; Kumar and Thakur, 2004; Szabo et al, 2004). Imprinted genes, H19 and Igf2, are differentially methylated in the male germ line during gonadal development (Szabo et al, 2004). Szabo et al (2004) proposed that Igf2 and H19 DNA sequence methylation patterns were influenced by steroid receptor binding to the specific sequences. This suggests that an environmental endocrine disruptor could influence the germ line methylation pattern during sex determination and differentiation. This hypothesis is further supported by the actions of diethylstilbestrol (DES), an environmental synthetic estrogen, on uterine tissue (Li et al, 2003a,b). The administration of DES to neonatal mice decreased the methylation state of the c-fos gene approximately 30% in uterine tissue, as well as inducing reproductive tract abnormalities and increased incidents of cancers (Li et al, 2003a,b).

In summary, observations provide evidence that an endocrine disruptor exposure results in a transgenerational effect on spermatogenic capacity and testis function. The embryonic exposure to vinclozolin resulted in a reduced spermatogenetic capacity in the male offspring, and this phenotype was transmitted to the subsequent generations, F2–F4. The potential of such endocrine disruptors to alter the epigenetics and impact evolutionary biology and disease states has been discussed (DeRosa et al, 1998; Walter and Paulsen, 2003; Anway et al, 2005; Guerrero-Bosagna et al, 2005). The observations reported support that fetal exposure to environmental toxicants (ie, endocrine disruptors) increases the potential of adult disease states. The epigenetic transgenerational actions previously shown (Anway et al, 2005) provide a potential mechanism for the fetal basis of adult disease (Basha et al, 2005; Heindel, 2005). For example, environmental toxicants have been speculated as a potential cause for the reported regional specific decline in adult sperm numbers (Swan et al, 1997; Sharpe, 2001). This report suggests that fetal exposure to endocrine disruptors could have the potential to reduce sperm number. Although the dose of vinclozolin used in the current in vivo study was lower than previously used (Gray et al, 1994; Kelce et al, 1994; Wolf et al, 2000), the exposure levels in these studies were higher than anticipated environmental exposures, and thus the impact on human populations remains to be elucidated in further toxicology studies. Independent of the environmental toxicology impacts, the epigenetic transgenerational phenotype observed is anticipated to have a significant impact on the area of andrology and requires further investigation.

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