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Effect of the anti-androgenic endocrine disruptor vinclozolin on embryonic testis cord formation and postnatal testis development and function

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Abstract

Vinclozolin is a systemic dicarboximide fungicide that is used on fruits, vegetables, ornamental plants, and turf grass. Vinclozolin and its metabolites are known to be endocrine disruptors and act as androgen receptor antagonists. The hypothesis tested in the current study is that transient embryonic exposure to an anti-androgenic endocrine disruptor at the time of testis determination alters testis development and subsequently influences adult spermatogenic capacity and male reproduction. The effects of vinclozolin on embryonic testicular cord formation in vitro were examined, as well as the effects of transient in utero vinclozolin exposure on postnatal testis development and function. Embryonic day 13 (E13, sperm-positive vaginal smear day = E0) gonads were cultured in the absence or presence of vinclozolin (50–500 μM). Vinclozolin treated gonads had significantly fewer cords ($P < 0.05$) and the histology of the cords that formed were abnormal as compared to vehicle-treated organs. Pregnant rats were exposed to vinclozolin (100 mg/kg/day) between embryonic days 8 and 14 (E8–E14) of development. Testis morphology and function were analyzed from postnatal day (P) 0, pubertal P20, and adult P60. No significant effect of vinclozolin on testis histology or germ cell viability was observed in P0 testis. The pubertal P20 testis from vinclozolin exposed animals had significantly higher numbers of apoptotic germ cells ($P < 0.01$), but testis weight was not affected. The adult P60 sperm motility was significantly lower in vinclozolin exposed males ($P < 0.01$). In addition, apoptotic germ cell number in testis of vinclozolin exposed animals was higher in adult P60 animals. Observations demonstrate that vinclozolin can effect embryonic testicular cord formation in vitro and that transient in utero exposure to vinclozolin increases apoptotic germ cell numbers in the testis of pubertal and adult animals. This correlated to reduced sperm motility in the adult. In conclusion, transient exposure to vinclozolin during the time of testis differentiation (i.e. cord formation) alters testis development and function. Observations indicate that transient exposure to an anti-androgenic endocrine disruptor during embryonic development causes delayed effects later in adult life on spermatogenic capacity.

Keywords: Vinclozolin; Testis; Sertoli; Endocrine disruptor; Embryonic development; Gametogenesis; Spermatogenesis; Anti-androgen; Apoptosis

1. Introduction

Endocrine disruptors are hormonally active environmental toxins that can influence normal sex determination [1,2], sperm viability [3] and estrous cycles [4]. There has been a decline in human adult sperm numbers [5,6], but the decline appears to be regional [7] suggesting the potential role of environmental factors. One of the potential environmental factors are endocrine disruptors. Endocrine disruptors are common pesticides (e.g. methoxychlor), fungicides (e.g. vinclozolin), and plasticizers (e.g. phthalates) utilized in our environment [8–10]. These compounds can affect physiological functions by acting as weak estrogenic, anti-estrogenic or anti-androgenic compounds [11,12]. Exposure to estrogenic or anti-androgenic endocrine disruptors at critical stages of development can promote reproductive failure in adult rodents [13–19].

Vinclozolin (3-(3-5-dichlorophenyl)-5-methyl-oxazolidine-2,4-dione) is a systemic dicarboximide fungicide registered for use on several fruits and vegetables, ornamental plant and turf grass [20]. Two hydrolytic degradation products of vinclozolin have been identified 2-[[(3,5-dichloropheniyl)-carboxymoyl]-2-methyl-3-butenoic

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acid (M1)] and 3′,5′-dichloro-2-hydroxy-2-methylbut-3-enanilide (M2) [21]. Vinclozolin and metabolites act as anti-androgens through actions as an androgen receptor antagonist or as a 5α-reductase inhibitor. The mechanism of anti-androgenic action of vinclozolin and metabolites have been elucidated [22]. Both vinclozolin and its metabolites act as androgen receptor antagonists rather than inhibitors of the 5α-reductase enzyme. Although the ability of vinclozolin to compete for androgen binding to the androgen receptor is weak ($K_i > 700$ μM) the two vinclozolin metabolites M1 and M2 are more effective antagonists with a K_i of 92 and 9.7 μM, respectively. Therefore, the metabolites are likely to be primary causal agents for the in vivo actions of the vinclozolin previously described.

Exposure of rats to 100 or 200 mg/kg/day vinclozolin between embryonic day (E)14 and postnatal day (P)3 causes external genitalia abnormalities at the neonatal stage such as reduction in anogenital distance (AGD) and retention of nipples in male pups. At puberty the male rats fail to achieve intromission and ejaculation due to cleft phallus with hypospadias. At 1 year of age these males demonstrate ectopic testis, vaginal pouch, epididymal granuloma and smaller or absent accessory sex gland and reduction of cauda epididymal sperm numbers [1]. Although embryos show sensitivity between E14 and E19, the most sensitive period appears to be E16–17 [23]. Peripubertal exposure to vinclozolin in male rats delays pubertal maturation and retards accessory sex gland and epididymal growth. However, the treatment was not as effective as the exposure at the perinatal stage in creating malformations in individuals [24].

Previous studies have primarily examined the effects of late embryonic and early postnatal exposure to vinclozolin [1,23]. The effect of exposure to vinclozolin during male sex determination and testicular cord formation has not been studied. The objectives of this study are to examine effects of vinclozolin on testicular cord formation using an embryonic gonad culture system and to examine the effects of transient in utero exposure to vinclozolin during testicular cord formation (E8–14) on postnatal testis development and function (i.e. day 0, 20, and 60). The P0 period reflects a rapid phase of testis growth and presence of only early stage germ cells. The P20 period reflects a mid-pubertal period after the onset of the spermatogenic process. The P60 period reflects on adult stage with complete spermatogenesis and fertility.

2. Materials and methods

2.1. Testis organ cultures

Sprague–Dawley rats from a timed pregnant rat colony were bred in the institutional vivarium at Washington State University. Institutional Animal Care and Use committee guidelines were approved and followed in all procedures. At 08:00 h on embryonic day 13 (E13; sperm-positive vaginal smear date = E0) gonads were dissected with the mesonephros attached. At this stage of embryonic development the average number of tail somites is 16–17. The organs were cultured in drops of medium on Millicell CM filters (Millipore, Bedford, MA) floating on the surface of 0.4 ml of CMRL 1066 media (Gibco BRL, Gaithersburg, MD) supplemented with penicillin–streptomycin, insulin (10 μg/ml), transferrin (10 μg/ml), 0.01% BSA and 350 μm L-glutamine [25]. Vinclozolin (100%; pure; AccuStandard Inc., New Haven, CT) or flutamide (2-methyl-*N*-[4-nitro-3-(trifluoromethyl)-phenyl]proponamide; Sigma, St. Louis, MO) were dissolved in vehicle dimethylsulfoxide (DMSO) or ethanol (EtOH) respectively, and used to treat one of the gonads and the contralateral gonad served as a control. Control organs were treated with vehicle control (final concentration of DMSO or EtOH were <0.1% (v/v)). The medium was changed on the second day of culture. E13 gonads and mesonephros were typically maintained for 3 days in culture at which point seminiferous cords were developed in the controls [26]. Images of whole organs were obtained using a digital image analysis system (Diagnostic Instruments Inc., Sterling Heights, MI). Cord number per testis was determined using whole organ images. Data was confirmed using hematoxylin and eosin (H&E) stained testis sections. Organ culture studies involved at least three separate experiments for each treatment group and each experiment contained six to eight pairs of male embryos. The sex of embryos was determined using polymerase chain reaction for Sry as previously described [26].

2.2. Hematoxylin and eosin staining and cord number analysis

Following imaging of the whole organs the tissue was fixed in Bouin's fixative (Sigma) for 2 h and stored in 70% ethanol until embedding in paraffin. Paraffin-embedded tissues were serially sectioned. The tissue sections closest to the largest cross-sectional area of the organs were used in the analyses. One of the serial sections from each experiment was stained with hematoxylin and eosin using standard procedures. Stained sections were imaged using light microcopy and used in confirming the cord number obtained in whole organ images.

2.3. In vivo procedures

Aged-matched timed-pregnant Sprague–Dawley rats were divided into control and treatment groups ($n = 9$–10) in five separate experiments. Treatment group rats were exposed to vinclozolin (99% pure; ChemService, West Chester, PA), from E8 to E14 during gestation. In preliminary experiments, additional sets of animals ($n = 2$) were injected with vinclozolin from E15 to E21. Control group dams were injected with vehicle (DMSO, volume was less than 100 μl). A normal gestation period in a rat is 21–23 days and was not affected by the vinclozolin treatment used (data not shown). The dose of vinclozolin was 100 mg/kg/day injected

in DMSO intraperitoneally. This does is higher than occupational exposure to vinclozolin, which is 25 mg/8 h of work [27]. However, this dose represents the low end of doses previously found to effect reproductive system of the offspring, but not to cause overt maternal toxicity [1]. Gonads were collected on postnatal day (P) 0, P20, and P60 from the male offspring. Testis weights were determined on a micro-analytical balance. All experiments were repeated a minimum of three times with total of 9–10 different exposed mothers. Approximately 10 males were collected for each time point for both control and treatment groups. P60 males from different litters of control and vinclozolin-exposed groups were utilized for breeding experiments to determine fertility. The testosterone radioimmunoassay (testosterone double antibody RIA kit, Diagnostic Systems, Webster, TX) was performed by the Center for Reproductive Biology Assay Core Laboratory. On P60 cauda epididymal sperm motility was determined using cauda epididymal sperm. Briefly, the epididymis dissected free of connective tissue and a small cut made to the cauda. The tissue as placed in 3–5 ml culture medium containing 0.1% BSA for 5 min 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 100× magnification. First, all the motile sperm including, rapid progressive, slow progressive and non-progressive were counted according to WHO category [28]. Percent ratio of the 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 once 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 [29]. Briefly, the epididymis was placed in 5 ml of culture medium and minced. The tissue pieces were removed and the remaining sperm suspension was diluted with equal volume of 0.2% glutaraldehyde in PBS to immobilize the sperm. Two to three independent sperm samples were counted using Makler Counting Chamber (Sefi-Medical Instruments; Haifa, Israel). The counts were averaged and used as a replicate in statistical analysis.

2.4. Testicular cell apoptosis

Following the weight determination, testes were fixed in Bouin's fixative (Sigma) for various length of time (1–6 h) depending upon the age of the animals, washed with 70% ethanol and stored until embedding. Paraffin-embedded tissues were serially sectioned. One of the serial sections from each testis was stained with H&E using standard procedures. One of the serial sections from each testis was used to detect the presence of apoptotic cells. To detect apoptotic cells a Fluorescein In Situ Cell Death Detection Kit was utilized as instructed by the manufacturer (Roche Applied Science, Indianapolis IN). This kit measures fragmented DNA from apoptotic cells by catalytically incorporating fluorescein-12-dUTP at the 3′ DNA end using the enzyme terminal deoxynucleotidyl transferase (TdT), which forms a polymeric tail using the principal of the TdT-mediated dUTP Nick-End Labeling (TUNEL) assay. The fluorescent cells in randomly selected 6–12 microscopic areas in each testis cross-section were counted at 100× magnification. The average number of fluorescent cells/microscopic area from one animal was used as a replicate in statistical analysis. The negative control sections where the TdT enzyme was excluded from the assay showed no staining. In positive control sections where artificial DNA break were introduced with DNase most cells showed fluorescent staining.

2.5. Statistical analysis

The data from cord number per testis, apoptotic cell numbers, serum testosterone, sperm motility and sperm count were analyzed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). The values were expressed as the mean ± S.E.M. Statistical analysis was performed and the difference between the means of treatments and respective controls were determined using a paired *t*-test. In vitro experiments were repeated for at least three times with six to eight testes/experiment. Data from one experiment averaged and used as one replicate for analysis. In vivo experiments were repeated five times, which included 10–12 individuals for each data point. Values were averaged from individuals from each experiment and used as one replicate in the statistical analysis. A statistically significant difference was confirmed at $P < 0.05$.

3. Results

3.1. Effect of vinclozolin and flutamide on seminiferous cord formation in organ culture

Embryonic day 13 (E13) testis organ cultures were treated with 50–500 μM vinclozolin. As a positive control, the anti-androgenic compound flutamide was also used. In all experiments the contralateral organ served as a control while the other gonad was treated. Control organs formed normal cords (Fig. 1A). The cord formation was similar in organs treated with 50, 100, or 200 μM vinclozolin (data not shown). The results from 100 and 200 μM treated groups were combined. The number and morphology of the cords from 100 to 200 μM vinclozolin treated organs were comparable to the control organs. The cord formation was effected by 500 μM vinclozolin. In 500 μM vinclozolin-treated organs, the cord number was reduced and the cords became less visible in whole-mount organ images (Fig. 1B). The effect of flutamide at 1 μM dose was more dramatic and treated organs showed no or minimal numbers of cords (Fig. 1C).

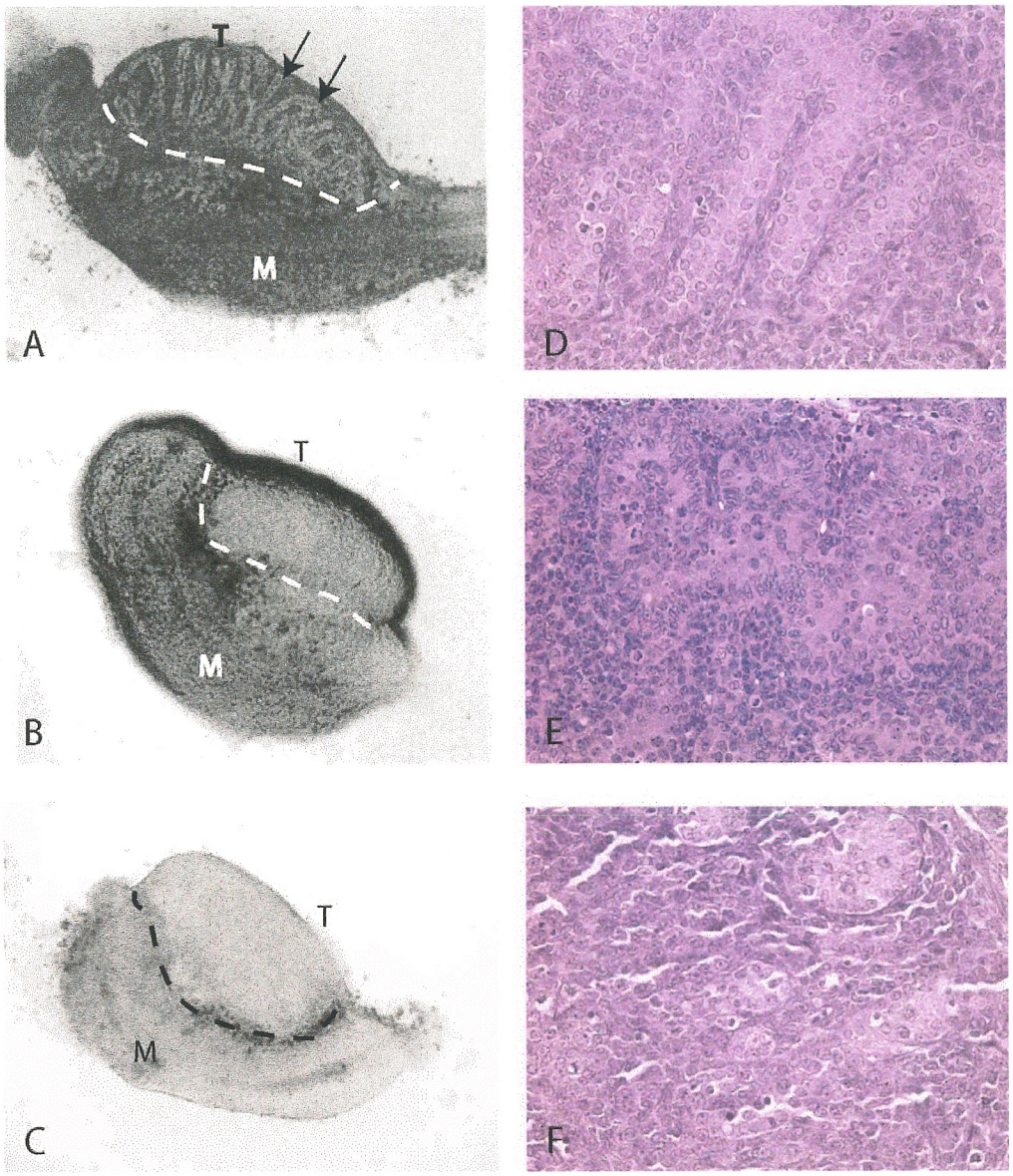


Fig. 1. Effect of vinclozolin and flutamide on embryonic (E) 13 testis organ culture. Whole-mount E13 organ cultures (A–C) that were treated with vehicle (A) 500 μM vinclozolin (B), or 1 μM flutamide (C). Testis and mesonephros are labeled (T) and (M), respectively. Arrows show the testicular cords. Hematoxylin and eosin stained sections from control (D), vinclozolin (E) or flutamide (F) treated organ cultures. Data are representative of at least three experiments for each treatment group with six to eight testes in each experiment.

Cord formation and morphology following treatment with vinclozolin or flutamide was analyzed using H&E stained sections. The histology of control organs showed distinct cords that were separated by intervening interstitium and most of the cords spanned the entire length of the organ (Fig. 1D). Sections from organs treated with 500 μM vinclozolin had cords that were few in numbers and these cord were not well separated by the interstitium and appeared to be enlarged or "swollen" (Fig. 1E). Sections from organs that were treated with 1 μM flutamide had patchy cords in limited areas (Fig. 1F). These data confirmed the observations from whole organ images and provided further insight into cord morphology in treated organs.

Cord numbers were analyzed using both whole-mount images and H&E stained sections. Analysis of cord number in control and treated organs indicated that 50 (data not shown), 100 or 200 μM vinclozolin treatment did not change the cord number formed ($P > 0.05$; Fig. 2). The 500 μM vinclozolin treatment reduced cord number ($P < 0.05$), but did not block cord formation (Fig. 2). In contrast 1 μM flutamide reduced the cord number dramatically ($P < 0.01$; Fig. 2). These data suggest that vinclozolin can effect testis cord formation, but only at the relatively high dose required to compete with the

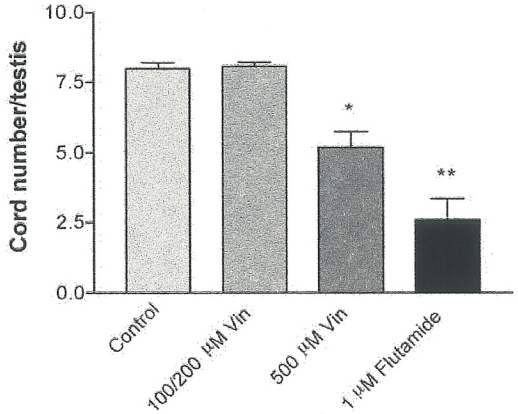


Fig. 2. Vinclozolin reduced cord number in E13 organ culture. The number of cords was determined using whole-mount and hematoxylin and eosin stained sections. Organs were incubated in the absence (control) or presence of 100–200 μM or 50 μM vinclozolin (Vin) or 1 μM flutamide. (*) and (**) represent statistically significant difference as compared to the respective control testes at $P < 0.05$ and $P < 0.01$, respectively. Data represents mean ± S.E.M. from organs and sections described in Fig. 1.

androgen receptor (i.e. K_i: 700 μM). In contrast, flutamide at relatively low dose inhibited cord formation.

3.2. Effect of in utero exposure to vinclozolin between E8 and E14

To follow-up the above in vitro observations, the in vivo effects of vinclozolin on testis development and function were assessed. Pregnant rats were transiently exposed to 0 or 100 mg/kg/day vinclozolin at the time of male sex determination and testicular cord formation between E8 and E14. Exposure of pregnant mothers to vinclozolin did not cause any overt toxicological effect on the mother or offspring. The vinclozolin did not significantly alter total body weight (BW), testis weight (TW) on postnatal day (P) 0, 20, or 60. At P0, BW (gram, mean ± S.E.M.) for control and vinclozolin groups was 6.92 ± 0.25 and 7.37 ± 0.35, respectively. The anogenital distance was assessed at P0, which was almost identical for control and treated groups. At P20, the ratio of TW (mg) to BW (g) for control and treated groups were 4.63 ± 0.12 and 4.49 ± 0.30, respectively. On P60, TW/BW for control and treated groups were 8.93 ± 0.39 and 9.54 ± 0.36, respectively. Vinclozolin treatment did not affect normal testicular descent. In preliminary experiments vinclozolin treatment between E15 and E21 also did not alter AGD, testis weight, body weight on P0, 20, or 60 (data not shown). The data showed that the gross morphology of the testis and external genitalia was not significantly altered by the vinclozolin treatment.

3.3. Effect of vinclozolin on spermatogenic cell apoptosis

To assess whether transient in utero exposure to vinclozolin affected the postnatal testis, effects on germ cell apoptosis were assessed in P0, P20, and P60 animals. There was a negligible level of apoptosis in P0 control testis and the vinclozolin treatment did not change the level of apoptosis (data not shown).

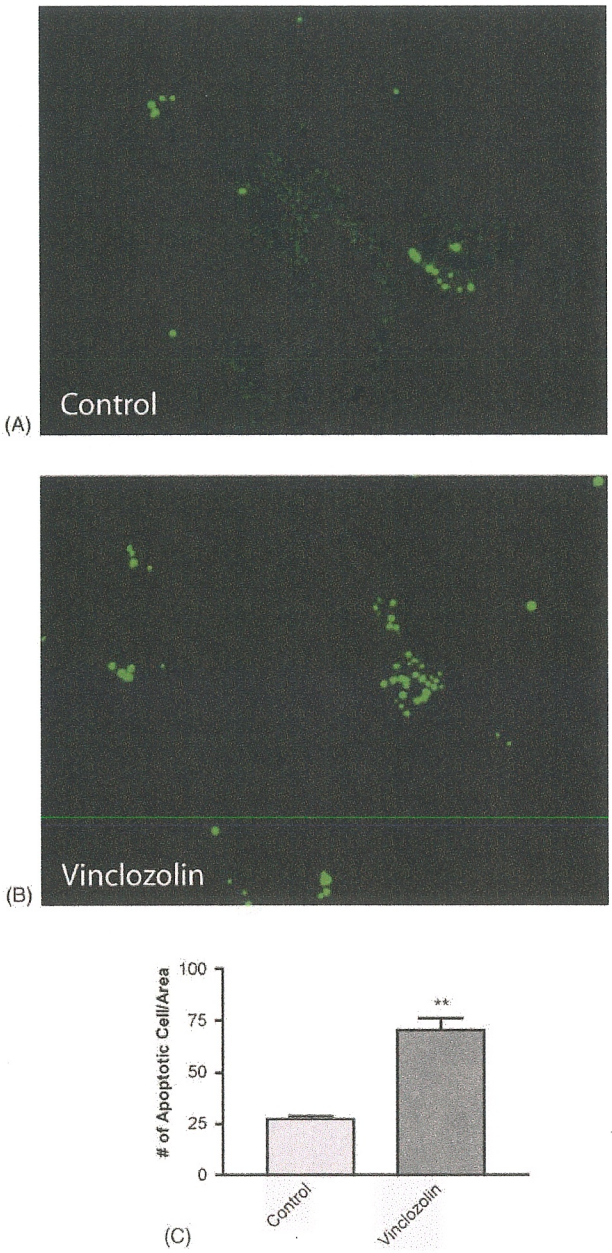
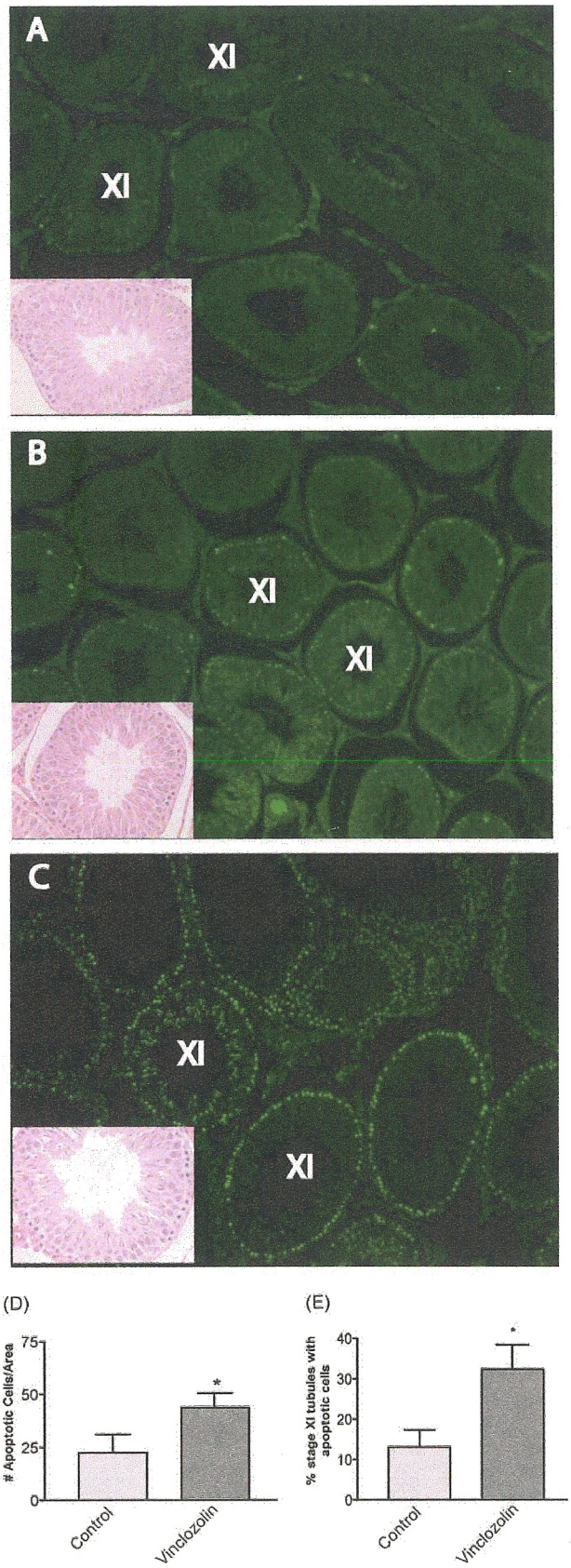


Fig. 3. Effect of transient in utero vinclozolin treatment on germ cell apoptosis in postnatal day 20 testis. Apoptotic cells (green fluorescent) shows DNA fragmentation that was demonstrated using TUNEL assay. Representative sections from control testis (A) and vinclozolin (100 mg/kg/day) treated testis (B). The original magnifications are 200×. The graph (C) presents mean ± S.E.M. of apoptotic germ cell number/microscopic area (100× magnification) in control and vinclozolin-treated P20 testis sections. (**) represents statistically significant difference as compared to control at $P < 0.01$. Data obtained using sections from each three to four pairs of testis in two separate experiments.



At the mid-pubertal P20 period, the testis sections from vinclozolin-exposed individuals (Fig. 3B) showed more apoptotic germ cells compared to control sections (Fig. 3A). The number of the apoptotic cells increased more than twice in vinclozolin treated group (Fig. 3C) that was statistically significant ($P < 0.01$). At the adult P60 period, the testis sections from vinclozolin exposed offspring had higher apoptotic germ cell numbers (Fig. 4B) than the control sections (Fig. 4A). The increase in the individual apoptotic germ cell number was significant (Fig. 4D; $P < 0.05$). In addition, tubules at spermatogenic stages XI and XII of the seminiferous cycle in vinclozolin-treated group showed increased number of apoptotic cells (Fig. 4C). The percentage of stages XI and XII tubules that shows more than 10 apoptotic germ cells in tubules was determined. Stages XI and XII tubules with more than 10 apoptotic germ cells per tubule increased more than three times in vinclozolin treated group (Fig. 4E; $P < 0.05$). The effect of vinclozolin treatment appears to be stage specific. In preliminary experiments, the rats that were exposed to vinclozolin at a later stage of pregnancy, such as E15–21, did not show a significant increase in apoptosis in either P20 or P60 animals (unpublished observation).

3.4. Effect of vinclozolin on serum testosterone

To assess the effect of vinclozolin on serum testosterone (T), T levels were determined at P0, P20, and P60. At the P0 and P20 periods, levels of serum T were lower than the assay detection limit of the assay (1 ng/ml). In the P60 adult animals, the serum T levels were slightly higher in the control group, but the difference was not statistically significant ($P > 0.05$; Fig. 5). The larger variation is attributed in part due to the durinal variations related to the time of the serum collection. Both the T levels in control and vinclozolin-treated animals were within the normal range for the adult rat.

3.5. Effect of vinclozolin on cauda epididymal sperm motility, sperm count and fertility

To assess whether increased spermatogenic cell apoptosis affected sperm motility, sperm motility was measured using

Fig. 4. Effect of transient in utero exposure to vinclozolin on germ cell apoptosis in postnatal adult day 60 testis. Apoptotic cells (green fluorescent) show DNA fragmentation that was demonstrated using TUNEL assay. Representative sections from control (A) and vinclozolin treated animals (B). Sections from 100 mg/kg/day vinclozolin treated group showed excessive apoptotic germ cells in spermatogenic stages XI and XII tubules (C). Stages XI and XII tubules were marked. The insets are representative from H&E stained serial sections that were used for staging. The original magnifications for sections with TUNEL staining and H&E staining are 100× are 400×, respectively. The graphs represent mean ± S.E.M. of all apoptotic cells/microscopic area (D) and of percentage of stages XI and XII tubules with more than 10 apoptotic cells (E) from control and vinclozolin treated animals. (*) represents statistically significant difference as compared to control at $P < 0.05$. Data obtained using sections from each three to four pairs of testis in two separate experiments.

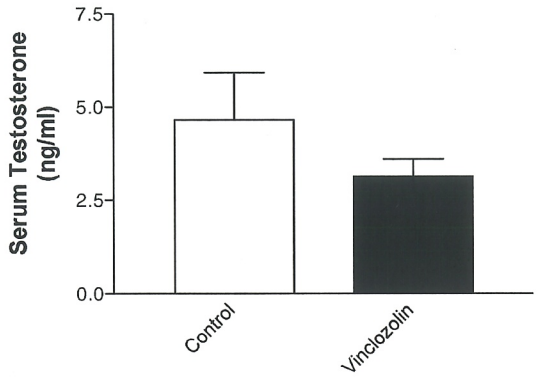


Fig. 5. Serum testosterone levels (ng/ml) from control and vinclozolin-treated P60 animals. The mean ± S.E.M. from 6 control and 10 vinclozolin-treated animals is presented. No statistical difference between control and treatment group was found at $P > 0.05$.

cauda epididymal sperm. The vinclozolin treatment reduced the sperm motility significantly (Fig. 6A; $P < 0.01$). Epididymal sperm count was also assessed. Although epididymal sperm numbers were reduced, the difference was not statistically significant (Fig. 6B; $P > 0.05$). Preliminary analysis of the male reproductive tract demonstrated no gross effects on the epididymis, seminal vesicles or prostate.

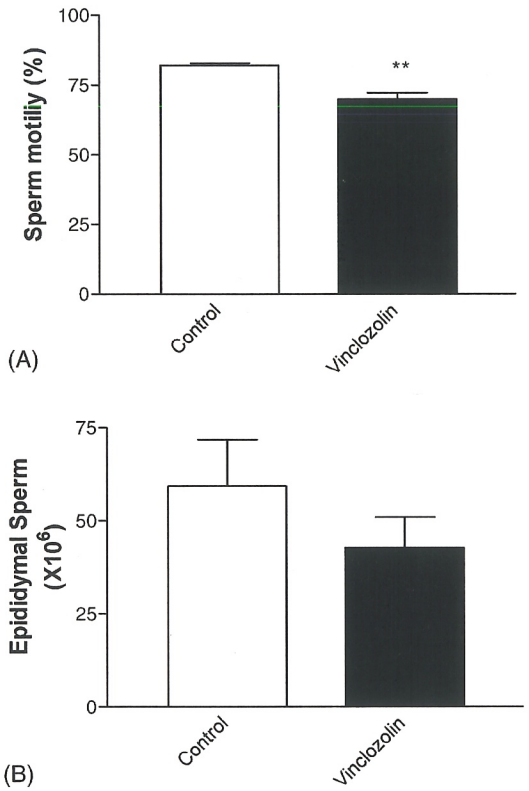


Fig. 6. Effect of in utero vinclozolin exposure on cauda epididymal sperm motility (A) and epididymal sperm number (B) in P60 animals. (**) represents statistically significant difference as compared to control at $P < 0.01$. The sperm motility is expressed as percent motile and sperm counts as $\times 10^6$. The data represent mean ± S.E.M. from at least 10 animals from 5 experiments for sperm motility and 6 animals from 3 experiments for sperm count.

To assess whether the reduced sperm motility affected male fertility, treated males were mated overnight with treated proestrous females. Nine out of twelve matings in treated males showed sperm-positive vaginal smear on the following morning. In general, fertility of the vinclozolin-treated males was comparable to the control males in breeding experiments. Three treated males did not show positive mating, however, two of the females used had irregular estrous cycle. The reduction in sperm motility appears not to dramatically reduce the fertility in the adult males.

4. Discussion

The primary objective of this study was to investigate the effects of an anti-androgenic compound on embryonic testis cord formation and subsequent testis development and function. Although low doses of vinclozolin treatment did not show apparent major effects, 500 μM vinclozolin interfered with normal cord formation. Flutamide was used as a positive control and 1 μM flutamide inhibited cord formation confirming a previous report from the laboratory [30]. Seminiferous cord formation occurs on embryonic day 13 (E13) in the rat and is the initial morphological event in testis development and male sex determination. Sertoli cell differentiation and mesonephric cell migration into the testis are critical events for normal cord formation and appear to be primarily under the control of the sex determining gene Sry [31,32]. Local growth factors play a role in testis differentiation downstream to Sry [33,34]. The current study has shown that vinclozolin causes abnormal testicular cord formation in vitro and that transient in utero exposure to vinclozolin at the time of cord formation interferes with normal testicular development and function in the adult.

The expression of the androgen receptor (AR) is first detectable in embryonic rat testis at E14 [30,35] that is about the same time the embryonic testis starts to produce testosterone [36,37]. The androgen receptor is also localized in the mesenchymal cells of the mesonephros [35,38] that migrate and form certain cell lineages in the gonad including peritubular myoid cell precursors. Mesonephros cell migration is essential for testicular cord formation [39] and the migrating cells contain the AR. Androgen has a role in cell migration in other systems. Androgen stimulates human prostate cancer cell migration which is mediated by the PI3K signaling pathway and MMP-2. Androgen stimulated prostate cell migration can be blocked by specific inhibitors of MMP-2 or PI3K [40]. Whether inhibition of androgen receptor by vinclozolin interferes with the mesenophros cell migration in the testis is currently not known. However, similar to prostate cells, mesonephric cell migration during testis cord formation requires both the PI3K signaling pathway [41] and MMP actions since inhibition of either blocks mesonephric cell migration [41,42]. The exposure period utilized was

E8–E14 and previous observations demonstrate that the AR is expressed in the genital ridge and mesonephros during the early part of this exposure and AR is expressed in the germ cells, Sertoli cells and migrating peritubular cells at the later stages of the exposure [30,35–38]. Therefore, the actions of vinclozolin could be at the level of both the germ cells and somatic cells. The effects observed suggest an abnormal programming of germ cell differentiation that then manifests in the adult spermatogenic and sperm defects.

The effects of vinclozolin on cord formation were interesting even though it was moderate. The cords that were formed in vinclozolin treated testis showed incomplete division between the individual cords and the cords were fused to each other. Similar abnormalities appear in the E13 testis organ cultures that are treated with PDGF receptor specific inhibitors, AG 1295 or AG 1296 [26]. PDGFR-α knock out mice have abnormal cord formation similar to the inhibitor treated rat embryonic testis [34]. A defective cell migration has been suggested as a mechanism for abnormal cord formation in the receptor null-mice [34]. The current observations combined with these reports suggest abnormal cell migration may be a potential mechanism for partial inhibition in cord formation in vinclozolin treated testis, and supports the possible role of androgens in mesonephric cell migration.

Since the vinclozolin was effective only at relatively high doses to cause abnormal cord formation, this might be due to the high K_i value of the vinclozolin [22]. Therefore, we tested vinclozolin using in vivo experiments to examine potential actions of vinclozolin metabolites, butenoic acid and enanilide, which have lower K_i values (92 and 9.7 μM). The pregnant rats were exposed to 100 mg/kg/day vinclozolin between E8 and E14, a period that covers the critical time of testis differentiation. Exposure of rats to 100 or 200 mg/kg/day during E14 to P3 results in external genital abnormalities such as reduced anogenital distance, retained nipples, cleft phallus and hypospadias in neonatal males [1]. The exposure to higher dose (400 mg/kg/day) for shorter period (2 days) is effective in variable degrees on external genitalia abnormalities between E14 and E19 [23]. However, the most sensitive period is E16–17. Exposure between E12 and E13 had no overt effect in external genitalia formation. In the current study, exposure to 100 mg/kg/day between E8 and E14 also showed no significant effect on external genitalia (i.e. anogenital distance) or other gross developmental or physiological parameters (e.g. body weight, testis weight or serum testosterone). However, transient exposure caused changes in testicular development and function such as increased spermatogenic cell apoptosis and reduced sperm motility.

Apoptosis is a part of the normal spermatogenesis and lack or reduction of apoptosis results in abnormal spermatogenesis [43]. A minimal degree of apoptosis takes place at the first week of birth [43]. We have also observed a minimal degree of apoptotic cells in the P0 testis and the vinclozolin treatment did not alter the low germ cell apoptosis at P0 (data not shown). A high level of apoptosis takes place at the time of the first wave of the spermatogenesis that peaks around the third week of age [43]. This reduction in the number of germ cells may reflect an adjustment to the available Sertoli cell number since the number of the germ cells that a Sertoli cell can support is limited [44]. Damaged germ cells are also selectively eliminated by apoptosis [44]. The vinclozolin treated animals had more apoptotic germ cells as compared to control mid-pubertal animals at P20. Observations suggest an increase in germ cell abnormalities in the vinclozolin treated animals. Increased apoptosis of individual germ cells was also observed in vinclozolin exposed animals in the adult P60 age. In addition, there was increased apoptosis of elongating spermatids at the stages XI and XII of the spermatogenic cycle. This stage of seminiferous tubules was also sensitive to endocrine disrupters in animals that are exposed to transient methoxychlor in utero [19].

Sperm motility was reduced in the vinclozolin treated group. The reduction did not affect the overall fertility. At adult stage (P60–70), vinclozolin treated males were capable of producing offspring with normal litter size and pup weight. Similar results were observed in previous studies using mice [45]. Exposure to 100 mg/kg/day vinclozolin between E10 and E18 caused a reduction in spermatogenesis in F1 males without affecting male fertility. In agreement with the previous study, the current study suggests that 100 mg/kg/day vinclozolin between E8 and E14 treatment does not significantly affect male fertility. The reduction in sperm motility is likely due in part to abnormal spermatogenesis in the testis. However, reduced motility may also reflect effects of vinclozolin on the epididymis and male reproductive tract. Although no gross morphological abnormalities were observed, more thorough analysis is required to clarify potential actions of vinclozolin on epididymal sperm maturation.

Exposure to vinclozolin (100–200 mg/kg/day) from E14 to P3 [1] or exposure to higher doses (400 mg/kg/day) for short duration during later stage of pregnancy (E14–19; [23]) causes overt effects in external genitalia. In this study, the vinclozolin treatment did not cause an overt effect on testis weight, total body weight and external genitalia morphology at all stages examined. The vinclozolin treatment also did not cause a significant change in serum testosterone levels that is in agreement with the lack of overt endocrine effects in treated animals. The difference in results between the current study and these previous studies is primarily attributable to the differences in the period of embryonic exposure. The period of sex determination and testis morphogenesis (E8–14) is likely more sensitive to effects on the testis and spermatogenic defects. The late embryonic exposure is likely more sensitive to reproductive tract abnormalities due to these organs developing during this period of development.

In summary, treatment with the anti-androgen vinclozolin altered in vitro cord formation in the embryonic testis. More importantly, transient in utero exposure at E8–14 to vinclo-

zolin caused a delayed stage specific germ cell apoptosis potentially due to increased germ cell abnormalities. These observations suggest the possibility that the exposure to vinclozolin during testis differentiation alters programming of germ cells and/or Sertoli cells, which cause germ cell apoptosis and reduced sperm motility later in adult life. Although a reduction in sperm motility and an increase in germ cell apoptosis was observed in the adult P60 males, male fertility was similar to the control animals. Future studies will examine the mechanistic aspects of the effects of the antiandrogenic compound vinclozolin on male reproduction.

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