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Comparative anti-androgenic actions of vinclozolin and flutamide on transgenerational adult onset disease and spermatogenesis

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Abstract

Exposure of gestating female rats to the anti-androgenic endocrine disruptor vinclozolin has been shown to induce transgenerational adult onset disease phenotypes. The current study, was designed to compare the actions of vinclozolin to the known anti-androgenic compound flutamide. The gestating female rats were exposed to intraperitoneal injections during embryonic day 8–14 (E8–E14) to 100 mg/kg/day vinclozolin or flutamide at either 5mg or 20 mg/kg/day. As previously observed, vinclozolin induced a transgenerational testis phenotype of increased spermatogenic cell apoptosis and decreased epididymal sperm number. In contrast, the flutamide exposures resulted in a testis phenotype of increased spermatogenic cell apoptosis and decreased epididymal sperm numbers in the F1 generation only, and not the F2 and F3 generation adult males. Interestingly, some of the lowdose (5 mg/kg) flutamide F2 generation offspring developed spinal agenesis and supernumerary development (polymelia) of limbs. Although the actions of vinclozolin and flutamide appear similar in the F1 generation males, the transgenerational effects of vinclozolin do not appear to be acting through the same anti-androgenic mechanism as flutamide.

Keywords

Transgenerational; Epigenetics; Endocrine disruptor; Vinclozolin; Flutamide; Aging; Spinal agenesis; Polymelia; Spermatogenesis; Adult onset disease

1. Introduction

Exposure of a developing embryo to environmental factors or nutritional defects results in not only developmental abnormalities, but an increased propensity for adult onset diseases

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Conflict of interest

None of the authors have a financial or conflict of interest to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.reprotox.2008.07.008.

[1–4]. The mechanism for the fetal basis of adult onset disease is poorly understood [5]. Recently the ability of environmental compounds to promote transgenerational transmission of adult onset disease has been observed [1,6,7]. Exposing a pregnant female rat prior to and during embryonic sex determination to environmental endocrine disruptors, vinclozolin or methoxychlor, resulted in male offspring with reduced spermatogenic capacity [1]. The testis phenotype appears to be transgenerationally transmitted through F1–F4 generations by an epigenetic mechanism (i.e. DNA methylation) involving alterations of the male germ-line [1]. In addition to the testis abnormalities, age related adult onset diseases such as immune abnormalities, kidney disease, prostate lesions and cancer were also reported in the F1–F4 generation rats of 6–14 months of age [6].

The mechanism involved in this transgenerational adult onset disease phenotype appears to involve an epigenetic reprogramming of the male germ-line [1,4,6]. Prior to sex determination during embryonic development, the primordial germ cells migrate down the genital ridge and colonize the indifferent bipotential gonad [8,9]. As the primordial germ cells migrate down the genital ridge their genomic DNA becomes de-methylated [10,11]. Following gonadal sex determination, the germ cells DNA is re-methylated in a sex specific manner [12–14]. In the male, somatic cells in the developing gonad are required for normal germ cell development and DNA methylation [15,16]. Imprinted genes, H19 and Igf2, are differentially methylated in the male germ line during gonadal development [17]. Sex steroids can influence the methylation state of DNA sequences during normal tissue development, including that of imprinted genes [17–19]. The androgen receptor (AR) and estrogen receptor (ER) are present in the somatic cells (i.e. Sertoli and peritubular cells), as well as the developing germ-line stem cells, following sex determination. Although the testis does not produce steroids at this stage of development, estrogenic and androgenic substances have the ability to influence early testis cellular functions and differentiation [20]. Therefore, alterations in the epigenetic programming (i.e. DNA methylation) of the germ-line when the cell is establishing its methylation pattern following sex determination may be involved in the induction of epigenetic transgenerational disease states.

Vinclozolin (3-(3,5-dichlorophenyl)-5-methyl-oxazolidine-2,4-dione) is a systemic fungicide used on fruits and vegetables, as well as commonly used in the wine industry [21]. Vinclozolin and metabolites, butenoic acid (M1) and enanilide (M2) derivatives, act as antiandrogens through actions on the androgen receptor [22]. A number of other metabolites also exist that do not appear to have anti-androgenic activity (e.g. M3) [22]. Transient exposure of neonates to vinclozolin delays puberty and inhibits androgen dependent male reproductive tract development [23]. Embryonic exposure to vinclozolin influences male sexual differentiation and development, as well as adult spermatogenesis [24,25]. A previous study [6,24] demonstrated that administering 100mg/kg/day of vinclozolin to pregnant rats during embryonic sex determination (i.e. E8–E14) in the rat reduced the spermatogenic capacity by decreasing germ cell survival in the subsequent F1 generation adult male offspring. Subsequently, vinclozolin exposure later in embryonic development E15–postnatal day 0 (P0) had no effect on adult spermatogenesis [24,25]. In addition, Omezzine et al. [26] demonstrated that embryonic exposure (E6–P0) to the anti-androgen flutamide resulted in a similar reduced spermatogenic capacity in the subsequent F1 generation adult male offspring, however, no transgenerational transmission was reported. Combined

observations suggest that embryonic testis development is sensitive to androgen receptor signaling and can effect germ cell survival in the adult testis. In the current study, vinclozolin and flutamide were administered to pregnant female rats prior to and during gonadal sex determination, E8–E14, to determine if the transgenerational transmission induced by vinclozolin is acting through an anti-androgenic mechanism similar to flutamide in the developing embryonic testis.

2. Methods and materials

2.1. In vivo procedures

Gestating outbred Sprague–Dawley female rats were given intraperitoneal injections of 100 mg/kg/day of vinclozolin or flutamide 5mg/kg/day (T5), or 20 mg/kg/day (T20) from embryonic day 8 to 14 (E8–E14) of gestation (i.e. F0 generation), as previously described [27]. Sperm positive vaginal smear date is embryonic day 0. Gestating control females (i.e. F0 generation) received vehicle alone (i.e. sesame oil and DMSO). A minimum of 3 new lines (individual F0 injected females) were generated for each control, vinclozolin and T5 and T20 flutamide generations for these analyses. The F1–F3 generation animals derived from vinclozolin exposed F0 mothers are referred to as vinclozolin generation animals, from flutamide exposed F0 (5 mg/kg/day) females as T5 flutamide generation animals and exposed F0 (20 mg/kg/day) females as T20 flutamide generation animals. Animals from control F0 mothers are identified as control generation animals. Male rats from control, vinclozolin and flutamide generations were sacrificed at postnatal day 60–150 (P60–P150) for analysis. A smaller number of P160–P360 males were also collected for analyses. Some animals were euthanized at earlier ages (i.e. 2–36 days) due to development of abnormalities requiring euthanasia. F1 vinclozolin and flutamide generation males at postnatal day 60 (P60) were bred to P60 F1 vinclozolin or flutamide generation females from different litters to generate the F2 generation; F2 vinclozolin or flutamide males were bred to F2 vinclozolin females from different litters to generate the F3 generation. Rats from the control groups and flutamide exposed groups were bred in the same manner for all subsequent F1–F3 generations. No sibling breedings were used to avoid inbreeding artifacts. The flutamide generation animals and corresponding control generation animals were generated at different times than the vinclozolin generation animals and corresponding controls. The ages of the animals used for flutamide and vinclozolin experiments varied between experiments. All experiments had a corresponding control done at the same time and age. Some variability between different lines, ages and dates of the experiments exist to provide variability in measurements (e.g. TUNEL). The corresponding controls correct for this variation, but caution is needed when comparing control levels between experiments. All control and treated generation animals were housed in the same room in open rack cages with 14 h light and 10 h dark cycle, with ad libidum feeding and water, and maintained under identical conditions. The Washington State University Animal Use and Care Committee approved all procedures.

2.2. Sperm collection

Male rats from control, vinclozolin and flutamide generations were collected at P60–P360 for analyses as previously described [7]. Briefly, the caudal epididymis was dissected free of

connective tissue and the cauda was sliced in half. The tissue was placed in 5 ml of prewarmed F12 media containing 0.1% BSA for 10 min at 37 °C. Fifty microliters was placed on a pre-heated slide and cover-slipped. The specimen was immediately examined using phase contrast microscopy with 100× magnification. The sperm motility assays examined rapid progressive, slow progressive, and nonprogressive motility according to WHO category [28]. The ratio of motile sperm to the total number of sperm, including immotile sperm, was calculated. A minimum of 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 as previously described [7,24,29]. Briefly, the epididymis that was placed in the 5ml of culture medium was diced using a razor blade. The tissue pieces were removed, and the remaining sperm suspension was diluted with equal volume of 0.2% glutaraldehyde in 1 × PBS to immobilize the sperm. Three independent sperm specimens were analyzed using a hemacytometer. The counts were averaged and used as replicates in statistical analysis. Control, vinclozolin and flutamide exposed generational analysis for individual experiments were performed at the same time. All analyses were done blinded. The animal numbers for controls and for vinclozolin were (F1 9 and 9; F2 10 and 10; and F3 8 and 14); and for controls, T5 flutamide, and T20 flutamide (F1 7, 7 and 8; F2 14, 8 and 5; and F3 4, 11 and 4).

2.3. Histology

Tissues were fixed in Bouin's (Sigma, St. Louis, MO, USA) for 6 h, embedded in paraffin and sectioned. Sections were stained with hematoxylin and eosin (H&E) according to standard procedures. Multiple sections were obtained for each tissue for comparison to allow a representative histology to be selected. The Center for Reproductive Biology, Histology Core Laboratory assisted with these procedures.

2.4. Pathology

All pathology including X-ray analyses were done through the Washington State Disease Diagnostics Laboratory (WADDL) at Washington State University, as previously described [6].

2.5. Detection of cell apoptosis

Apoptotic cells in testis sections were identified with the Fluorescein In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN, USA) [7,27]. This system measures the fragmented DNA from apoptotic cells by enzymatically incorporating fluorescein-dUTP at the 3'-OH DNA ends using the enzyme terminal deoxynucleotidyl transferase using the principle of the TUNEL assay. Fluorescent apoptotic cells were imaged on a confocal microscope and number of apoptotic cells per testis cross-section determined. A minimum of $n = 6$ for vinclozolin, $n = 6$ for flutamide T5, $n = 5$ for flutamide T20 and $n = 8$ for controls for each generation were used, except for the F3 generation controls which was $n = 7$. All cross-sections used for TUNEL analysis had normal testis morphology. Tubule staging for localization of apoptotic cells was performed by two independent investigators for comparison, as previously described [7].

2.6. Statistical analysis

Statistical analysis for apoptotic cell numbers, caudal epididymal sperm motilities, concentrations and gross biological development used GraphPad Prism Version 3.0a for Macintosh, GraphPad Software, San Diego, CA, USA. Comparisons between two groups, mean±S.E.M., were performed using Student's *t*-test. Multiple comparison tests were performed using Dunnett's Multiple Comparison Test after a significant difference had been determined using one-way Analysis of Variance (ANOVA). In vivo experiments were repeated with 4–25 individual animals for each data point. Statistical significance for all analyzed groups was determined at $p < 0.05$.

3. Results

Gestating Sprague–Dawley rats were transiently exposed to daily intraperitoneal injections of the anti-androgenic endocrine disruptor vinclozolin or flutamide during embryonic gonadal sex differentiation, E8–E14. Males and females from different litters, F1 generation, were bred to generate the subsequent F2 generation and the F2 generation males and females were bred to generate the F3 generation. No sibling breedings were used to avoid any inbreeding artifacts. There were no effects observed in pup sex ratios, litter sizes or pup mortality in the F1–F3 vinclozolin and flutamide exposed generations compared to controls, data not shown.

In the adult P60–P150 males analyzed the body weight, testis weight, testis/ bodyweight ratios and serum testosterone levels for vinclozolin (Supplemental Table S1) and flutamide (Supplemental Table S2) were not statistically different when compared to control generations. Interestingly, there was a statistically significant increase in serum testosterone levels of the F1 flutamide T5 generation when compared to the control animals. This increase in serum testosterone was not observed in the F2 or F3 generations (Supplemental Table S2). Similar observations were made with older P160–P360 males from control and vinclozolin generation animals, while both T5 and T20 flutamide exposures caused alterations in testosterone levels in the older animals (Supplemental Tables S3 and S4).

Testis cross-sections from the P60–P150 rats from each generation were analyzed for spermatogenic cell apoptosis (Fig. 1). The apoptotic labeled cells were counted and graphed as apoptotic cells per testis cross-section (Fig. 2). The vinclozolin F1, F2 and F3 generations exhibit a statistically significant increase in spermatogenic cell apoptosis ($p < 0.05$) (Fig. 2A). In contrast only the F1 T5 and T20 flutamide generation males show a significant increase when compared to controls. Interestingly, the vinclozolin generation males were found to have the largest increase in spermatogenic cell apoptosis in stage 9–14 tubules, as previously described [7], while the flutamide T5 and T20 F1 generation males had the largest increase in spermatogenic cell apoptosis in stage 2–6 tubules (data not shown). Both the flutamide T5 and T20 F1 generation treatment groups have a statistically significant increase in apoptotic cells, but the T20 generation has a more dramatic increase (Fig. 2B). Neither the F2 or F3 flutamide generations had an increase in spermatogenic cell apoptosis. A similar effect was also observed in the testes of older males, P160–P360, where the flutamide testis samples only had an increase in spermatogenic cell apoptosis in the F1 generation males and not the F2 or F3 flutamide generation animals (data not shown).

Therefore, the vinclozolin and flutamide F1 generation males both have an increase in spermatogenic cell apoptosis, but only the vinclozolin has transgenerational effects in the F2 and F3 generation males. The basal control levels of the flutamide versus vinclozolin apoptosis were different due to the differences in time when the experiments were performed and ages of the animals as described in Section 2.

The epididymal sperm from P60 to P150 control, vinclozolin and flutamide F1–F3 generation males were collected and analyzed for percent forward motility and concentration. The sperm motility in the vinclozolin exposed F1–F3 generations had a statistically significant decrease compared to controls (Fig. 3A). The F1 flutamide generation had a statistical difference in sperm motility for the flutamide T20 animals when compared to controls (Fig. 3B), but no difference was observed in the F2 or F3 generations. These results were similar to the older P160–P360 males analyzed in the flutamide generations. Both the T5 and T20 flutamide exposed P160–P360 F1 generation males analyzed had a statistically significant decrease in sperm motility compared to the control generation (Supplemental Fig. S1). Although, the T5 flutamide F2 20 generation P160–P360 had a small but significant effect, the other F2 and F3 generation male sperm motility was similar to the controls.

The sperm concentration in the P60–P150 vinclozolin exposed F1–F3 generations had a statistically significant decrease compared to control generations (Fig. 4A). The T5 and T20 flutamide exposed F1 generation males analyzed had a statistically significant decrease (approximately 20%) in sperm number compared to the F1 control generation. Although the T5 flutamide F2 generation had a small (less than 10%) but significant decrease, the other F2 and F3 generations flutamide males had the same sperm concentration as controls (Fig. 4B). In the older P160–P360 flutamide generation males, the F1 generation had statistically significant decrease in sperm number, but no effect was observed in the F2 or F3 generations (Supplemental Fig. S1B).

The testis morphology in the vinclozolin and flutamide F1–F3 generations were investigated as previously reported [1,7]. The control vinclozolin and flutamide F1–F3 males all had complete spermatogenesis within the testis with approximately 15% of the males having few (<4) vacuoles within the seminiferous tubules per testis cross-section, data not shown. No major testis abnormalities were observed. As previously reported [6], P120–P360 males from vinclozolin F1–F3 generations had prostate and kidney abnormalities. In contrast, the flutamide generation males did not have similar pathologies, data not shown. Interestingly, low dose flutamide T5 F2 generation offspring developed 4 pups with spinal agenesis and 1 pup with supernumerary development (polymelia) of limbs (Fig. 5 and Supplemental Fig. S2). The percentage incidence was 5/128 or 3.9% of animals investigated. Both male and female sexes were represented. The phenotype was observed in 5 animals, which represented three out of four lines (i.e. F0 exposed females) from two separate experiments. It is unknown whether this phenotype was generated by males or female lines since additional litters from the male and female pairs, which initially generated the spinal agenesis and polymelia, did not result in additional pups with any abnormalities, data not shown. This spinal agenesis and polymelia phenotype only occurred in the F2 flutamide generation low dose T5 animals and not the high dose animals, nor the F1 or F3 generation

animals. The phenotype also was not detected in any of the F1–F3 vinclozolin generation animals. This F2 generation flutamide phenotype is not transgenerational, but appears to be due to the exposure of the F2 generation germ-line during exposure of the F0 gestating female. This appears to be due to effects of the low dose anti-androgenic activity of flutamide on the developing germ-line.

4. Discussion

Androgens have a critical role in male sex differentiation and the development and function of the testis [30]. Androgens are produced in the later stages of embryonic testis development by the developing Leydig cells and then postnatal androgen production is minimal [31,32]. In the early stages of male gonadal sex determination no androgen is produced by the developing testis, but androgen receptors are present in both the germ cells and somatic cells of the developing testis [33]. Previously, we have shown that the anti-androgenic endocrine disruptor vinclozolin can act on the E8–E14 developing testis to promote a transgenerational epigenetic adult onset disease phenotype [1,6]. Although this is not the normal period of androgen production and action in the embryonic testis, the presence of the androgen receptor suggests the capacity to respond to androgens and/or anti-androgenic factors [1]. The current study was designed to compare the actions of vinclozolin and the known anti-androgenic substance flutamide.

Late embryonic and early postnatal exposure to vinclozolin [21] has been shown to cause gonadal defects and male reproductive tract abnormalities [6,23,24]. Flutamide is a synthetic anti-androgenic compound shown to bind the androgen receptor and block androgen actions [26]. Late embryonic and early postnatal exposure to flutamide also can cause gonadal and reproductive tract abnormalities [26]. Observations presented demonstrate that both vinclozolin and flutamide had similar effects on the F1 generation males after E8–E14 exposure. The increased spermatogenic cell apoptosis, decrease in sperm number and motility was similar. One difference observed was the tubule stages with the highest increase in spermatogenic cell apoptosis. This was stage 9–14 for vinclozolin and 2–6 for flutamide. Therefore, the F1 generation phenotype appears to be due to anti-androgenic activities of the two compounds, but some differences exist. Although testis abnormalities were observed, no other major reproductive malformations such as cryptorchidism or hypospadias were observed with either vinclozolin or flutamide.

The decrease in sperm concentration (i.e. number) was approximately 20% and would not result in male infertility. The spermatogenic defect does suggest an abnormality in spermatogenesis, such that the mature sperm could be abnormal as well. The possibility that 5 mature sperm may have an increase in DNA fragmentation needs to be investigated in future studies. Although the spermatozoa in the lumens of the seminiferous tubules do not have an increase in apoptosis frequency (data not shown), a thorough analysis of mature sperm is required. Previously sperm motility has been shown to be reduced, but whether this correlates to DNA fragmentation is unknown.

The adult onset disease phenotype observed in the vinclozolin F1 generation males was also observed in the F2 and F3 generation males as well. The epigenetic transgenerational actions

of vinclozolin promotes a number of different adult onset disease states [1,6] that do not decline in frequency. The current study confirmed this vinclozolin induced transgenerational testis phenotype. In contrast, the flutamide F1 phenotype observed was not present in the F2 or F3 15 generation males. Neither dose of flutamide used caused transgenerational phenotypes. Observations demonstrate that the anti-androgenic compound flutamide does not promote the transgenerational phenotype, only an F1 generation phenotype. Therefore, the ability of vinclozolin to promote a transgenerational phenotype may not be due to anti-androgenic actions of the compound. If inhibition of the androgen nuclear receptor was critical for the transgenerational actions of vinclozolin, the flutamide should have similar actions. Observations suggest the F1 generation phenotype may be promoted through an anti-androgenic mechanism, but the transgenerational phenotype appears to be distinct.

Vinclozolin is metabolized into two major anti-androgenic compounds M1 and M2, along with a number of other metabolites not containing anti-androgenic activity [22]. The M3 metabolite is a product of further metabolism of vinclozolin, M1 and M2. Therefore, one potential mechanism for the transgenerational phenotype would be to have a non anti-androgenic metabolite be the active component. Alternatively, non-androgenic actions of metabolites such as M1 and M2 is also possible. In contrast, anti-androgenic actions unique from those of flutamide at cell surface receptors or alternative nuclear receptor actions also need to be considered. The specific active agent for vinclozolin and mechanism of action remains to be identified. Previously, the endocrine disruptor methoxychlor was also found to promote a transgenerational phenotype [1]. Methoxychlor metabolites and actions are distinct from vinclozolin suggesting different compounds and active metabolites may promote the transgenerational phenotype. Future studies are needed to identify what compounds and active agents are involved in the transgenerational actions of vinclozolin.

The critical developmental period involved in the induction of the transgenerational phenotype involves the epigenetic reprogramming of the male germ-line during gonadal sex determination [1,6]. The current and previous studies have used daily IP injections during E8–E14 of embryonic development. Although IP injection is not the standard toxicology administration used such as gavage, vinclozolin is primarily being used as a pharmacologic agent to induce the transgenerational phenotype and study the mechanism involved. The dose of vinclozolin used is higher than anticipated in the environment, such that no conclusion on the toxicology can be made. Future studies now in progress are needed to assess dose curve effects and relate this to environmental levels of vinclozolin. However, the ability of an environmental compound to promote an epigenetic transgenerational adult onset disease phenotype is an important phenomenon to characterize and clarify the mechanisms involved.

Interestingly, flutamide promoted a unique F2 generation phenotype of approximately a 4% rate of spinal agenesis and abnormal limb development. These are serious birth defects that occur in the human population as well [34], and appear to be derived from teratogenic effects of environmental factors [35]. Although flutamide did not promote a transgenerational epigenetic phenotype similar to vinclozolin, it did promote this F2 generation phenotype, that was not observed in either the F1 or F3 generations. In addition, only the low dose flutamide exposure had the F2 generation phenotype. When a gestating F0

generation female is exposed to an environmental compound the F1 generation embryo is directly exposed, as well as the F2 generation germ-line that is within the F1 generation embryo [4]. A direct exposure of the F1 generation germ-line appears to be the mechanism involved in this low dose flutamide F2 generation phenotype. The observations presented provide one of the best examples of a direct exposure of an F1 generation embryo causing an F2 generation disease state. This appears to be a teratogenic action of the flutamide and not involving an epigenetic transgenerational mechanism. The actions of compounds such as flutamide on the germ-line can now be further investigated as a potential causal factor in these types of birth defects. Since a number of anti-androgenic endocrine disruptors exist, the correlation of these factors and the birth defects of spinal agenesis and limb abnormalities can now be investigated. The levels of flutamide used in the current study were also high. Therefore, potential non-androgenic actions of flutamide also need to be considered. What degree F2 phenotypes of endocrine disruptors are due to germ cell exposure and teratogenic actions needs to be assessed.

In summary, the epigenetic transgenerational actions of vinclozolin on adult onset disease [1,6] does not appear to involve an anti-androgenic mechanism similar to flutamide. Alternative metabolites and mechanisms now need to be considered. Although flutamide did not promote a transgenerational phenotype, it did promote the F1 generation phenotype and unique direct germline effect to generate F2 generation abnormalities. The role of anti-androgenic compounds in the induction of these types of pathologies now needs to be considered. Further elucidation of the actions of vinclozolin in the induction of the epigenetic reprogramming of the male germ-line to generate transgenerational adult onset disease now needs to consider alternate or non antiandrogen mechanisms as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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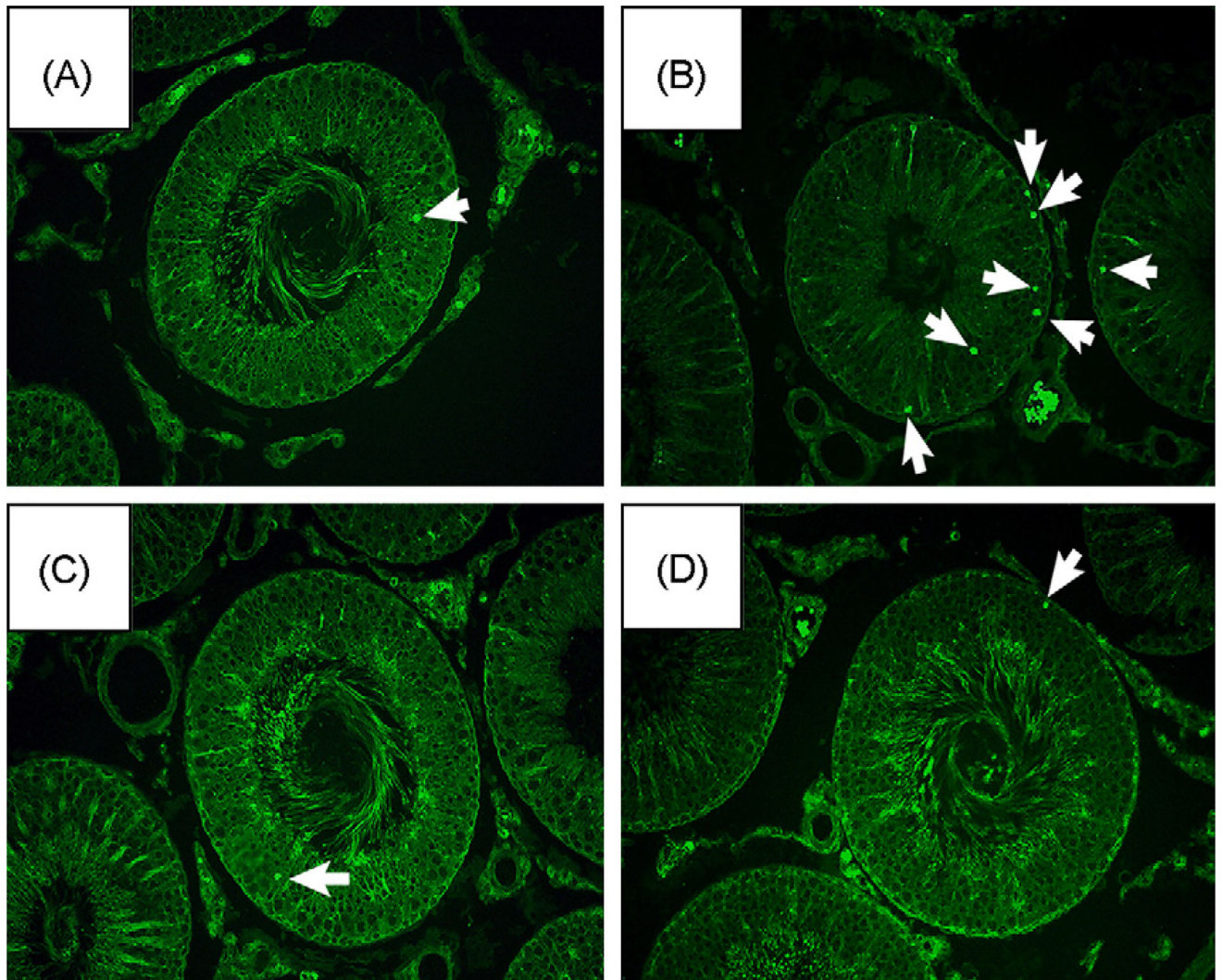


Fig. 1. Spermatogenic cell apoptosis (TUNEL) analyses of 100-day-old F3 generation testis cross-sections from control (A and C) vinclozolin (B) and flutamide 20 mg/kg/day dose (D) 400× magnification. (A) TUNEL F3 control; (B) TUNEL F3 vinclozolin; (C) TUNEL F3 control; (D) TUNEL F3 flutamide 20mg dose. Arrows indicate apoptotic cells.

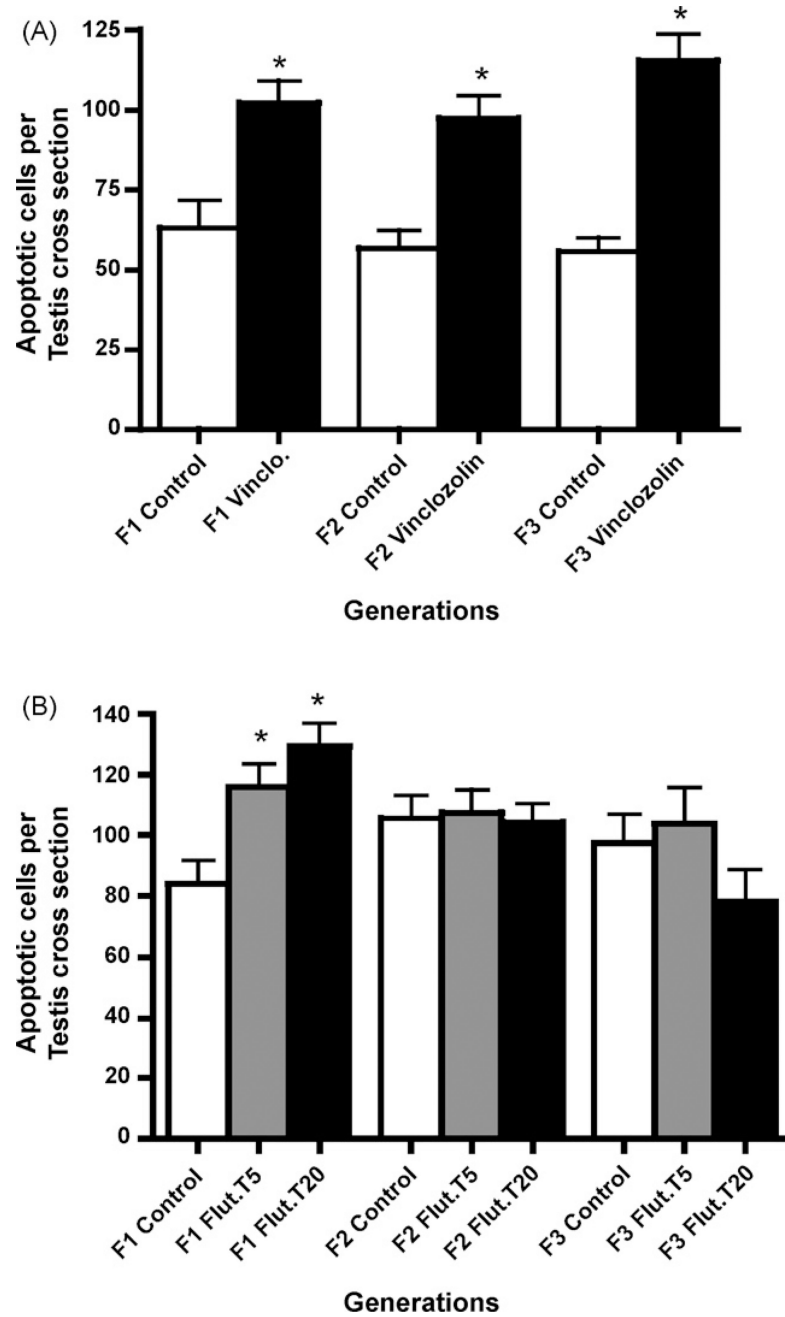


Fig. 2. Spermatogenic cell apoptosis in P60–150 control and vinclozolin (A) and flutamide (B) rats in the F1, F2, and F3 generations. Statistically significant differences between control and treated generations are indicated by (*) for $p < 0.05$.

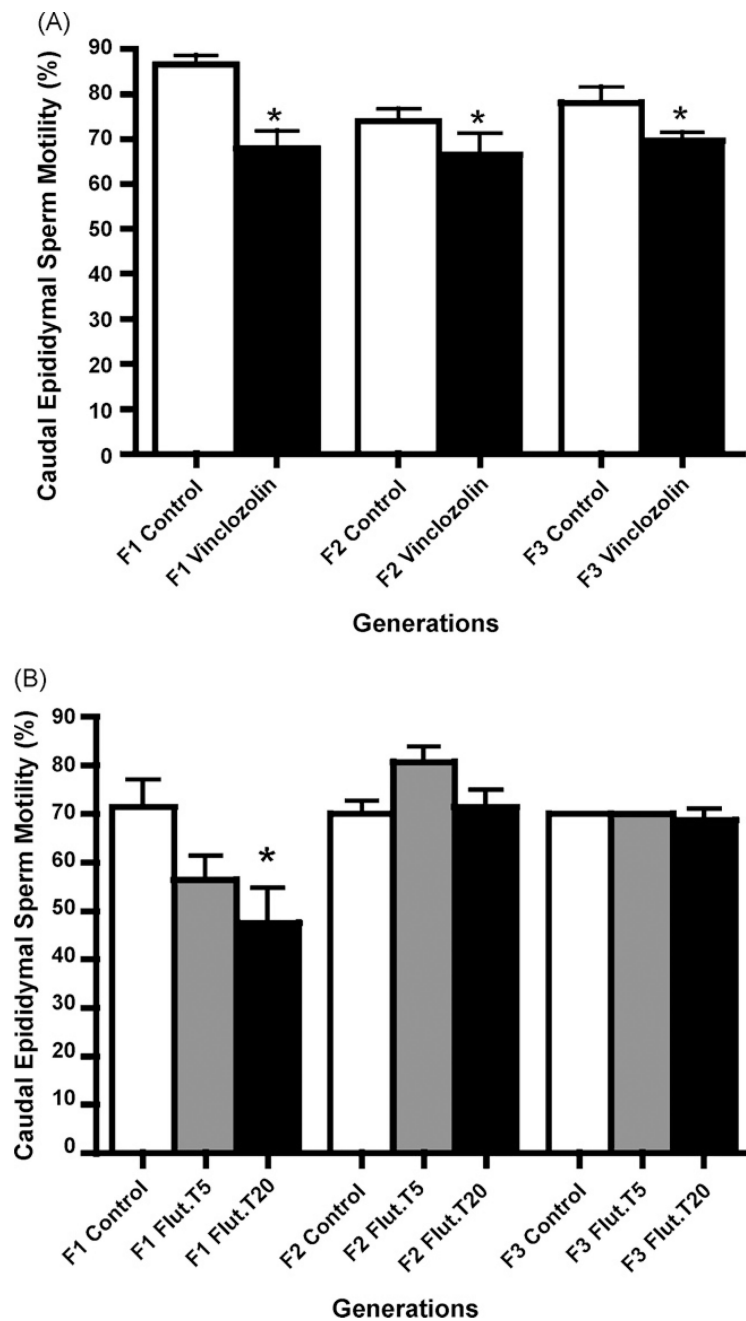


Fig. 3. Caudal epididymal sperm motility in P60–P150 control and vinclozolin (A) and flutamide (B) rats in the F1, F2, and F3 generations. Statistically significant differences between control and treated generations are indicated by (*) for $p < 0.05$. The n value for each bar ranged between 10 and 25 animals.

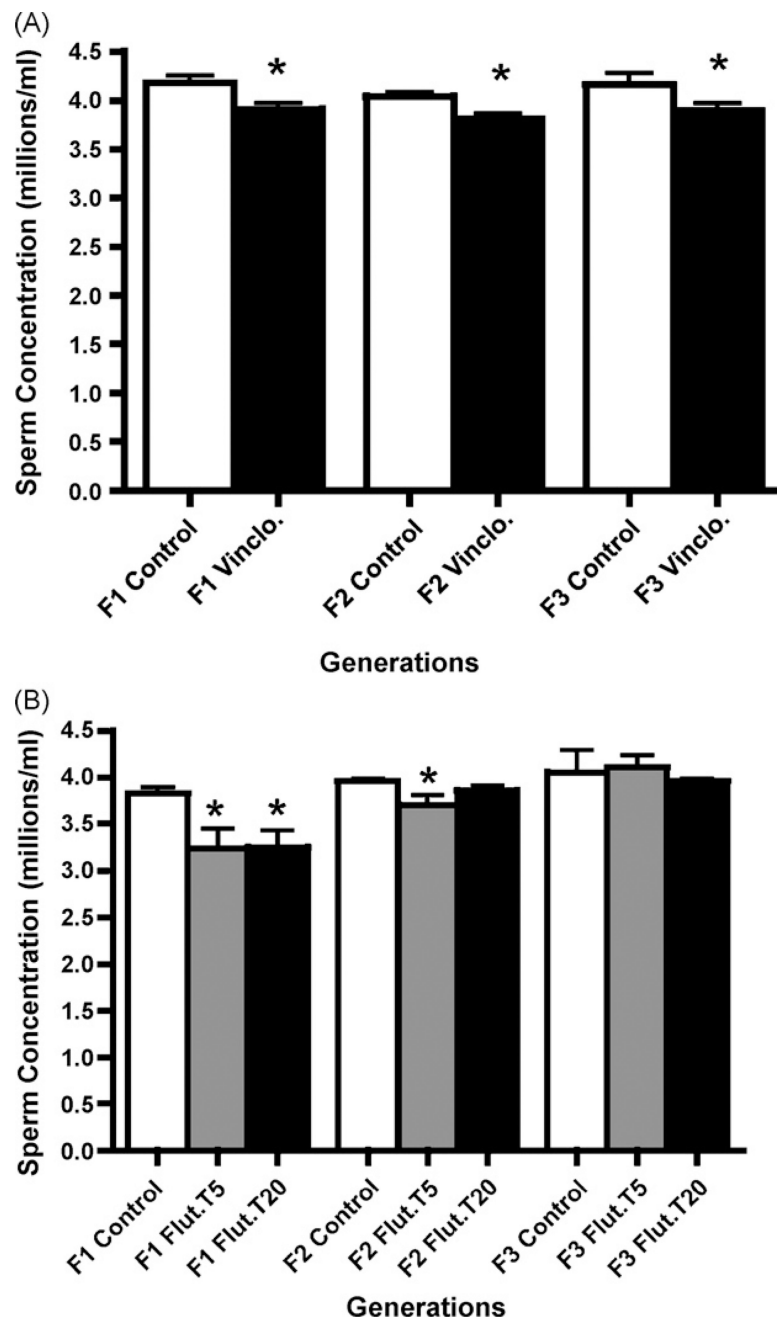


Fig. 4. Caudal epididymal sperm concentrations in P60–P150 control and vinclozolin (A) and flutamide (B) rats in the F1, F2, and F3 generations. Statistically significant differences between control and treated generations are indicated by (*) for $p < 0.05$. The n value for each bar ranged between 10 and 25 animals.

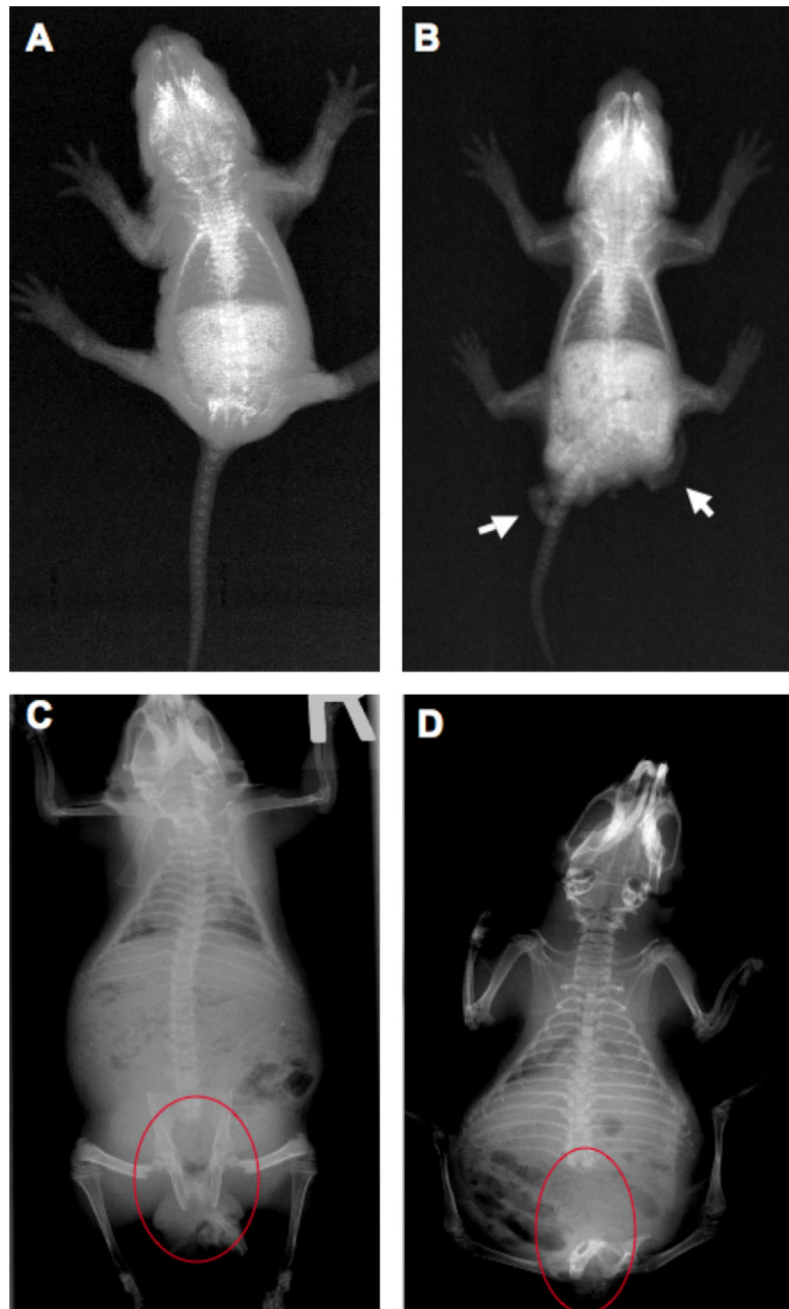


Fig. 5. Morphogenic analysis (X-ray imaging) of F2 flutamide generation T5 animals with skeletal abnormalities. Control animal (A) supernummary development (polymelia) of limbs (B), spinal agenesis of lumbar vertebrae, tail vertebrae, pelvis (C and D). Red circles denote area of spinal agenesis and white arrows identify additional limbs. Both male and female sexes represented. Phenotype observed in (5/128) animals represented in three out of four lines from two separate experiments.