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Vinclozolin—The lack of a transgenerational effect after oral maternal exposure during organogenesis

Steffen Schneider, Wolfgang Kaufmann, Roland Buesen, Bennard van Ravenzwaay*

Experimental Toxicology and Ecotoxicology, BASF SE Aktiengesellschaft, 67056 Ludwigshafen, Germany

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ABSTRACT

The purpose of the study was to investigate a possible transgenerational effect of the fungicide vinclozolin on the male reproductive system following oral exposure since this effect was reported by Anway et al. [Anway MD, Cupp AS, Uzumcu M, Skinner MK. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 2005;308(5727 (June 3)):1466–9] after intraperitoneal administration. Pregnant Wistar rats were dosed by oral gavage with vinclozolin 0, 4 or 100 mg/(kg bw day) on days 6–15 post coitum (p.c.). F1 male offspring was mated with untreated females to produce F2, which were then similarly mated to produce F3 offspring. F0 maternal treatment had no effect on mating and fertility indices or male offspring sexual development, mean sperm parameters, or histopathology of the sexual organs in F1, F2 or F3 males (at age 127–134 days). Apoptotic germ cell counts were statistically significantly lower in F1, F2 and F3 generations, however, control values showed a pronounced variance over time. Also, as anti-androgenic compounds are more likely to induce the opposite effect (increased apoptosis), this observation is not considered to be treatment related. Consequently, spermatogenesis was not affected by vinclozolin exposure in utero. As vinclozolin has been shown to induce clear anti-androgenic effects in offspring following treatment with 100 mg/(kg bw day) during entire gestation, the lack of effects in this study indicates that the window of sensitivity for anti-androgenic effects is from days 16–20 p.c.

No transgenerational effect on the male reproductive system was found. The NOAEL was >100 mg/(kg bw day) for fertility and reproductive performance, for systemic parental and developmental toxicity in F1, F2 and F3 males.

1. Introduction

Vinclozolin (3-[3,5-dichlorophenyl]-5-ethenyl-5-methyl-2,4-oxazolidinedione, C12–H9–Cl2–N–O3, 286 g/mol, CAS Number 50471-44-8, EINECS 256-599-6) is a nonsystemic dicarboximide fungicide which inhibits spore germination and is used for the control of several types of fungi in vines (such as grapes), vegetables, ornamentals, and also on turf grass. It is a crystalline solid at room temperature (melting point 108 °C) and is preferentially soluble in organic solvents (log *P*[octanol–water] = 3.0). The current ADI established by WHO for vinclozolin is 10 μg/(kg body weight day) (μg/(kg bw d)) based on a no observed adverse effect level (NOAEL) from a 24-month chronic and carcinogenicity study in rats of 1.4 mg/(kg bw d). The lowest NOAEL from reproduction studies is 4 mg/(kg bw d) (2-generation reproduction toxicity study in rats) [1].

Multigeneration studies in Wistar rats indicate that utero/lactational exposure results in demasculinized male offspring [2]. Effects include reduced anogenital distance and retained areolae at an oral dose of 1000 ppm (100 mg/(kg bw d)) and above, hypospadias, hypoplastic penis, reduced testicular size, aplasia/agenesia or reduced size of male accessory glands and, at 3000 ppm (307 mg/(kg bw d)), a vagina-like orifice. Similar male developmental effects were recently reported in a 2-generation reproduction toxicity study (continuous dietary exposure of F0 and F1 mating pairs and offspring) by Matsuura et al. [3]. Newborn F1 and F2 males had shortened anogenital distance and nipple/areola remnants at ≥40 ppm (2.29 mg/(kg bw d)), at weaning, F1 and F2 had decreased epididymis weights and morphological abnormalities of the external genitalia including cleft prepuce, penile hypoplasia, and vaginal pouch at 1000 ppm (58.65 mg/(kg bw d)). Interestingly, no effects on spermatogenesis were seen in either F0 or F1 males, although some F1 males at 1000 ppm were infertile. Since all infertile males

* Corresponding author at: GV/T-Z470, BASF SE, 67056 Ludwigshafen, Germany. Tel.: +49 621 60 56419.
E-mail address: bennard.ravenzwaay@basf.com (B. van Ravenzwaay).

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were affected by morphological abnormalities in the external genitalia, the authors concluded that this was the cause for the infertility in F1 males.

Gray et al. [4] narrowed down the exposure period by administering vinclozolin in corn oil by gavage once daily on gestation day 14 through postnatal day 3 in rats at 0, 100, or 200 mg/(kg bw d). Male offspring exhibited dose-related incidences and severities of male reproductive tract malformations and renal system malformations, including hydroureter, hydrophrosis, and urinary bladder stones. Wolf et al. [5] characterized the period of sensitivity of fetal male sexual development to vinclozolin. They window-dosed (oral gavage, 200 and 400 mg/(kg bw d)) Long–Evans rats in 2-day periods from gestation day 12 through 21, and found the most sensitive period to be on gestation days 16 and 17, although severe effects were present and 100% of male offspring were affected with administration of vinclozolin from gestation days 14 through 19. Effects included dose-related incidences and severities of male reproductive tract malformations, retained nipples and reduced weight of musculi levator ani/bulbocavernosus and sexual accessory glands.

In one dose–response study [6], Wistar and Long–Evans rats were orally dosed with vinclozolin from gestation day 14 to postnatal day 3 at 0, 1, 3, 6, 12 or 200 mg/(kg bw d). The high dose (200 mg/(kg bw d)) was maternally toxic, and male offspring from both strains exhibited reduced anogenital distance; retained nipples/areolas; hypospadia; penile hypoplasia; development of a vaginal pouch; hypoplasia and chronic inflammation of the epididymidis, prostate, seminal vesicles, and coagulating glands; testicular tubule atrophy; and chronic inflammation of the urinary bladder. At 12 mg/(kg bw d), retained nipples/areolas were present in both strains in pre-weaning males but persisted only in Long–Evans rats. Long–Evans rats (but not Wistar) also exhibited a low incidence of hypoplasia of accessory sex organs. The NOAELs were therefore 12 mg/(kg bw d) in Wistar rats and 6 mg/(kg bw d) in Long–Evans rats (there were no effects in either strain at 1 or 3 mg/(kg bw d)). In another dose–response publication [7], maternal rats were dosed by gavage on gestation day 14 through postnatal day 3 with vinclozolin at 0, 100, and 200 mg/(kg bw d) (first study), with expected male offspring reproductive malformations observed at both doses. In a follow-up study, the doses were 0, 3.125, 6.25, 12.5, 25, 50, and 100 mg/(kg bw d). In offspring males, reduced anogenital distance was observed at ≥3.125 mg/(kg bw d), retained areolas were observed at ≥6.25 mg/(kg bw d), and ventral prostate weight was reduced and hypospadia observed at 50 mg/(kg bw d). Ectopic testes were only observed at 100 and 200 mg/(kg bw d). Effects on serum testosterone levels and spermatogenesis were only observed at ≥100 mg/(kg bw d).

The hypothesis that vinclozolin exposure produces androgenic receptor antagonism, as suggested by these studies, was supported by in vitro studies which indicate that, rather than vinclozolin itself, the two major vinclozolin metabolites bind to the androgen receptor [8] and, acting as anti-androgens, inhibit subsequent androgen receptor-dependent transcriptional activation [9]. Direct evidence identifying vinclozolin's MOA as androgenic receptor antagonism in vivo was provided by Kelce et al. [10] who showed that treatment of rats with vinclozolin resulted in changes in androgen-dependent gene expression in the prostate. However, in a recent publication, Molina-Molina et al. [11] showed that not only the two main metabolites, but also vinclozolin, exhibited a potent anti-androgenic activity in PALM cells, a stable prostatic cell line used to evaluate androgenic activities. Metabolite M2 was the most potent antagonist followed by vinclozolin itself and metabolite M1 (IC_{50} = 0.17, 0.3 and 53 μM for 0.2 nM R1881, respectively). These results are in agreement with those obtained by Wong et al. [9], who found that vinclozolin and its metabolites show antagonist effects (M2 > vinclozolin > M1) and with those obtained by van Ravenzwaay [2], who reported antagonist effects of vinclozolin on the androgen receptor in a MCF-7 cell line.

Uzumcu et al. [12] reported effects of vinclozolin on spermatogenesis of 20 and 60 days old male F1 offspring of Sprague–Dawley female rats after intraperitoneal administration of 100 mg/(kg bw d) on gestation days 8 through 14. The effects included significantly higher numbers of apoptotic germ cells in 20 and 60 days old male F1 offspring as well as significantly reduced sperm motility in 60 days old male F1 offspring.

Anway et al. [13] also reported that a daily intraperitoneal exposure of gestating female Sprague–Dawley rats on gestation days 8–15 to 100 mg/(kg bw d) vinclozolin induced an adult phenotype in the F1 generation of decreased spermatogenic capacity (cell number and viability) and increased incidence of male infertility in males, at an age beyond 90 days. These effects were reported to be transferred through the male germ line to nearly all males of F2–F4 subsequent generations, suggesting an epigenetic alteration. In detail, reduced sperm numbers (by approximately 20%) and a reduced sperm forward motility (by about 25–35%) were noted for the F1–F4 generations. More than 90% of all males analyzed from all generations were reported to have the germ cell defect of a greater than two-fold increase in spermatogenic cell apoptosis. A total of 8% of the vinclozolin-exposed F1–F4 males developed complete infertility.

The aim of the study reported here was to investigate a possible transgenerational effect of vinclozolin through the male germ line, as it was reported by Anway et al. [13], on the integrity and performance of the male reproductive system following oral exposure. The reason for selecting the oral administration was that this route will provide the necessary and relevant data for risk assessment.

2. Materials and methods

2.1. Study design

Pregnant outbreed Wistar rats (F0 generation) were treated with doses of 0, 4 and 100 mg/(kg bw d) by oral gavage from gestation day 6 through gestation day 15. F1 animals were exposed to test item only during this limited time in utero, and F2 and F3 animals were not exposed to test item at all.

Twenty F0 females per group were used to generate the F1 offspring and 50 male pups per group were randomly selected to become F1 parental male animals to generate F2 pups with untreated female rats. Subsequent breeding was continued in the same manner for 2 generations. F1 and F2 generation male Wistar rats were observed for mating behavior and success as well as ability to generate healthy offspring. F1, F2 and F3 generation male gonads were examined on postnatal day 130 (sperm evaluation, detailed histopathology of testes, epididymides and accessory sexual glands, as well as quantification of apoptotic germ cells in the testes).

Reference was made to the 2-generation reproduction toxicity study defined in OECD test guideline 416 [14]. The study was conducted in accordance with Good Laboratory Practice guidelines and applicable animal welfare legislation [15,16].

2.2. Animals

F0 female time-mated outbreed Wistar rats (Crl:WI[Han]), aged about 10–12 weeks were delivered from Charles River Germany on day 0 p.c. (day 0 p.c. = detection of vaginal plug/sperm). All animals were free from clinical signs of disease. They were acclimatized to the test facility environmental conditions and diet from day 0 p.c. to the beginning of dosing on day 6 p.c.

This rat strain was chosen because of its general acceptance and suitability for embryotoxicity testing (including teratogenicity) and the availability of historical control data.

2.3. Housing

Parental adults were uniquely identified with an ear tattoo identification number, and were housed individually (except during mating) in stainless steel wire-mesh cages (DK III, Becker & Co., Castrop-Rauxel, Germany) or, in females from day 18 of pregnancy until day 21 of lactation, in Makrolon type M III cages containing nesting material (certified cellulose wadding, supplied by SSniff Spezialdiaeten GmbH, Soest, Germany). All live pups were identified by skin tattoo on day 1 postpartum and with picric acid marking of the fur between days 10 and 15 postpartum. The ani-

mal quarters were air-conditioned (20–24 °C, 30–70% relative humidity) with a 12 h light/12 h dark cycle; walls and floors were washed weekly. Certified feed (ground Kliba maintenance diet rat/mouse meal, Provimi Kliba SA, Kaiseraugst, Switzerland) was available *ad libitum* throughout the study, as was tap water (human drinking quality) in drinking bottles.

2.4. Treatment

Twenty F0 females per group received vinclozolin (BAS 352 F, 88/0375 batch N183, purity 99.1%) by oral (gavage) dosing daily from days 6 to 15 p.c. at a dose of 0 (control), 4 or 100 mg/(kg bw d) in vehicle (0.5% aqueous carboxymethylcellulose, 10 ml/kg bw; prepared fresh daily). Stability, homogeneity and actual content of dosing mixtures at the beginning and end of the dosing period were confirmed analytically.

The low dose of 4 mg/(kg bw d) was expected to be a NOAEL, and the high dose of 100 mg/(kg bw d) was expected to have effects on F1 spermatogenesis, based on previous studies [4,6,7].

2.5. Mating

For mating, 50 F1 males per dose group were randomly selected prior to weaning on day 21 postpartum with at least one animal from each available litter ("parental males"). From the resulting F2 offspring, 1–2 males per litter were selected to get 50 males for mating.

Starting 10 weeks post-weaning, the selected F1 and F2 parental males were mated with nulliparous, untreated females of the same strain and age. Pairs were cohabitated overnight until positive mating (sperm in vaginal smear = day 0 p.c.), for a maximum of 2 weeks. Positively mated F1 and F2 females were allowed to deliver and rear their F2 and F3 pups.

2.6. Observations

Cageside examinations were conducted at least once daily. Body weights were measured in F0 females at 1–3 day intervals throughout pregnancy. Food consumption and body weights were measured weekly in F1 and F2 parental males and in mated females during gestation and lactation.

F1, F2 and F3 offspring were examined as soon as possible on the day of birth (day 0 postpartum) to determine the sex and number of liveborn and stillborn per litter. Pup clinical signs and macroscopic changes were checked at least once daily throughout lactation.

All F1, F2 and F3 litters were randomly culled on day 4 postpartum to 4 male and 4 female pups where possible. Pups were weighed on the day after birth (day 1 postpartum) and on days 4, 7, 14 and 21 postpartum. To quantify sexual maturation, male preputial separation was assessed daily from day 40 postpartum in the selected parental F1 and F2, and in all surviving F3 offspring ($n = 50$ per dose).

2.7. Post-mortem

At 127–134 days of age, parental F1 and F2 and adult F3 males were fasted for 16–20 h, weighed, and sacrificed by decapitation under isoflurane anesthesia. After exsanguination, gross pathology was assessed, with special attention to the reproductive organs.

Testes, epididymides, cauda epididymidis, prostate, and seminal vesicles including coagulation glands were weighed. Immediately afterwards, motility of sperm from the right cauda epididymidis of all parental males was microscopically quantified (in randomized order) according to Slott et al. [17]. Mean number of homogenization-resistant testicular spermatids and caudal epididymal sperm and percentages of morphologically abnormal sperm were quantified based on Feuston et al. [18].

Histopathology of one half of the left testis, the left epididymis, seminal vesicles, coagulation glands and prostate (all fixed in Bouin's solution) was assessed in hematoxylin–eosin stained Paraplast sections.

Apoptosis was quantified in the other half of the left testis (fixed in 4% buffered formaldehyde solution) with the In Situ Cell Death Detection Kit, AP (Roche Diagnostics, Mannheim, Germany). This kit uses TUNEL immunohistochemistry (terminal deoxynucleotidyl transferase fluorescein-dUTP Nick End Labeling) to label the characteristic DNA strand breaks in apoptotic cells by terminal deoxynucleotidyl transferase (TdT), which catalyzes polymerization of labeled nucleotides to free 3′-OH DNA ends; the incorporated fluorescein is detected by sheep anti-fluorescein antibody Fab fragments conjugated with alkaline phosphatase (AP). After substrate reaction, stained apoptotic cells were quantified by light microscopy.

F0, F1 and F2 dams were sacrificed after their pups were weaned (day 21 postpartum) and discarded without further examinations. Mated females which produced no litter were discarded after examination of their stained uteri for evidence of early resorptions [19].

Surplus male F1, F2 and F3 pups culled on day 4 postpartum or sacrificed on day 21 postpartum as well as all stillborn and subsequent decedent males were examined externally, eviscerated and their organs assessed macroscopically. In case of any notable findings at necropsy or in the daily clinical observation, grossly abnormal organs/tissues were preserved for histopathological examination if necessary. Female F1, F2 and F3 pups culled on day 4 postpartum as well as all stillborn pups and subsequent mortalities were examined as for male pups, but female pups sacrificed on day 21 postpartum were discarded without further examination.

2.8. Reproductive parameters

Reproductive performance of parental males was summarized by dose group using the following indices:

- male mating index (%): number of males with confirmed mating (=vaginal sperm or pregnancy in cohabited female) × 100/number of males placed with females;
- male fertility index (%): number of males proving their fertility (=parturition or presence of embryos or fetuses in utero in cohabited female) × 100/number of males placed with females;
- female fertility index (%): number of females pregnant (=embryos or fetuses in utero or giving birth) × 100/number of females placed with males;
- gestation index (%): number of females with liveborn pups × 100/number of females pregnant;
- livebirth index (%): number of liveborn pups × 100/number of liveborn + stillborn pups;
- viability index (%): number of pups alive on day 4 postpartum (preculling) × 100/number of liveborn pups;
- lactation index (%): number of pups alive on day 21 postpartum × 100/number of pups alive on day 4 postpartum (postculling).

2.9. Statistical analysis

The experimental unit of analysis was the parental animal or litter, i.e. one individual per litter was taken for examinations of the F2 and F3 generations. In order to have consistent statistics, F1 generation littermates were not considered as the extent was limited to generally only 2–3 littermates.

Dunnett's test [20,21] was used for simultaneous comparison of all dose groups with the control group in food consumption (g/parental animal), body weights and body weight change (parental animals and litters), number of cohabitation days to positive mating, duration of gestation, number of pups delivered per litter, and age at preputial separation.

Kruskal–Wallis (two-sided) tests followed if significant by pairwise Wilcoxon tests [22–24]. Wilcoxon tests were also used to assess group differences in terminal parental body weights and parental and pup absolute and relative organ weights, proportion of pups per litter with necropsy observations, proportion of males with histopathology observations, total spermatids/g testis or cauda epididymides, and % sperm motility, for which variable Bonferroni–Holm adjustment [25] was applied.

Fisher's Exact test [24], one-sided, was used for pairwise comparison of each dose group with the control for mating, fertility and gestation indices, females with liveborn, stillborn and with all stillborn pups, livebirth index, pups stillborn, pups dead, pups cannibalized, pups sacrificed moribund, viability and lactation indices, number of litters containing pups with necropsy findings, and age at preputial separation, and number of animals with >4% abnormal sperm.

3. Results

Vinclozolin had no adverse effects in treated F0 dams; reproduction indices (fertility, gestation, livebirth, viability and lactation) were unaffected by treatment (Table 1). There were no treatment-related clinical signs (the only signs in the study were transient skin lesions or chromodacryorrhea in a total of 3 parental F1 and F2 and F3 males without relation to treatment), and premating food consumption and body weight gain of parental males did not differ between groups (data not shown). At F1 pup necropsy on days 4 and 21 postpartum, incidence of macroscopic findings did not differ between groups (data not shown). Isolated findings included dilated renal pelvis (total 2, 2 and 4 pups in control, 4 and 100 mg/kg groups) and small testis (total 4, 0 and 2 pups in 0, 4 and 100 mg/kg groups).

One F1 male selected for mating was found dead in week 6 of the premating period; it had no remarkable macroscopic necropsy findings, and no pathology of the sex organs. There were no other unscheduled post-weaning deaths in this or any other generation. F1 male development (body weight and age at preputial separation) was unaffected by treatment, as was reproductive performance.

Table 1
Reproduction data, F0 females/gestation and lactation data, F1 litters

	Dose group (mg/(kg bw d))		
	0	4	100
Female fertility index (%)	95	100	95
Duration of gestation (days, mean ± S.D.)	21.4 ± 0.5	21.6 ± 0.5	21.6 ± 0.5
Food consumption, gestation days 0–20 (g/(dam day), mean ± S.D.)	18.6 ± 1.4	18.7 ± 1.4	18.6 ± 1.3
Body weight gain, gestation days 0–20 (g, mean ± S.D.)	106.5 ± 8.3	110.7 ± 11.2	109.0 ± 11.0
Gestation index (%)	100	100	100
Number of litters	19	20	19
Pups delivered per litter (mean ± S.D.)	8.6 ± 1.2	9.3 ± 1.6	9.3 ± 1.8
Livebirth index (%)	98	100	99
Sex ratio of live newborns (% male)	50.6	51.1	51.4
Pup weight day 1 postpartum (g, mean ± S.D./litter)	6.2 ± 0.5	6.4 ± 0.5	6.5 ± 0.6
Maternal food consumption, lactation days 1–14 (g/(dam day), mean ± S.D.)	35.9 ± 11.3	38.6 ± 11.0	39.1 ± 11.0
Maternal body weight gain, lactation days 1–21 (g, mean ± S.D.)	30.4 ± 7.7	29.1 ± 8.9	28.1 ± 9.9
Viability index (survival days 0–4 postpartum) (%)	98	100	99
Lactation index (survival days 4–21 postpartum) (%)	100	100	100

Table 2
Gestation and lactation data, F2 litters

	Dose group (mg/(kg bw d))		
	0	4	100
Female fertility index (%)	96	98	94
Duration of gestation (days, mean ± S.D.)	21.9 ± 0.3	22.0 ± 0.4	21.9 ± 0.4
Gestation index (%)	100	100	100
Number of litters	47	46	47
Pups delivered per litter (mean ± S.D.)	11.2 ± 2.0	11.8 ± 1.7	11.4 ± 2.3
Livebirth index (%)	99	98	99
Sex ratio of live newborns (% male)	44.7	49.5	47.6
Pup weight day 1 postpartum (g, mean ± S.D./litter)	6.4 ± 0.6	6.2 ± 0.4	6.1 ± 0.5
Viability index (survival days 0–4 postpartum) (%)	99	100	99
Lactation index (survival days 4–21 postpartum) (%)	99	100	100

Gestation and lactation parameters for the resulting F2 litters were not affected by treatment (Tables 2 and 4a).

Relative to body weight, the weight of the epididymides, prostate and seminal vesicles were comparable across groups. At necropsy, reduced size of testes (uni- as well as bilateral findings) and epididymides were noted in a total of 3/50 F1 offspring at 4 mg/kg, reduced size of testes were noted in 2/50 and reduced size of epididymides were noted in 4/50 F1 offspring at 100 mg/kg (Table 4a). From these animals, in 2/50 males at 4 mg/kg and 2/50 males at 100 mg/kg the reported macroscopical findings were correlated to diffuse tubular degeneration in the testis, oligozoospermia/aspermia in the epididymis, markedly reduced homogenization-resistant spermatid/sperm counts and infertility. 1/50 males at 4 and 100 mg/kg each exhibited a tubular degeneration in the left testis and oligozoospermia in the left epididymis, which were not accompanied by changes in spermatid/sperm counts from the right testis/epididymis and infertility. 1/50 males at 100 mg/kg had a pyogranulomatous orchitis/diffuse tubular degeneration in the left testis which was not accompanied by histopathological changes in the epididymis or changes in spermatid/sperm counts and infertility. All described findings were seen in animals of different litters, none of the individual incidences was statistically significantly different to control (Wilcoxon test, one-sided).

Mean testis spermatid and epididymis sperm counts did not differ between groups. Mean % abnormal sperm also did not differ statistically between groups. The variance of the 100 mg/kg group was higher than the other groups, due to one male with 97% abnormal sperm (mainly head abnormalities and headless) which was

Table 3
Gestation and lactation data, F3 litters

	Dose group (mg/(kg bw d))		
	0	4	100
Female fertility index (%)	100	100	98
Duration of gestation (days, mean ± S.D.)	22.0 ± 0.3	21.9 ± 0.5	21.8 ± 0.4
Gestation index (%)	100	100	100
Number of litters	50	49	49
Pups delivered per litter (mean ± S.D.)	12.0 ± 1.9	12.0 ± 1.9	11.9 ± 2.3
Livebirth index (%)	100	99	99
Sex ratio of live newborns (% male)	49.0	48.5	55.3
Pup weight day 1 postpartum (g, mean ± S.D./litter)	6.1 ± 0.4	6.2 ± 0.6	6.1 ± 0.5
Viability index (survival days 0–4 postpartum) (%)	99	100	98[a]
Lactation index (survival days 4–21 postpartum) (%)	99	100	100

[a] The number of pups surviving to day 4 was significantly ($p \leq 0.01$) lower in the 100 mg/kg group than controls, due to one litter in which 3 pups were stillborn, 5 died and the remaining 6 were cannibalized on day 1 postpartum (3 of the day 1 deaths had no milk in the stomach; no other abnormalities were detected). Such litter losses occur in untreated control animals in this laboratory and this is therefore considered fortuitous, not treatment-related.

Table 4a
Sexual development and necropsy data, F1 males

	Dose group (mg/(kg bw d))		
	0	4	100
Number of male offspring/litters evaluated	50/19	50/20	50/19
Age at preputial separation (days, mean ± S.D.)	43.3 ± 1.9	44.1 ± 2.0	42.9 ± 2.5
Weight at preputial separation (g, mean ± S.D.)	177 ± 11	177 ± 12	178 ± 14
Male mating index (%)	98	96	100
Male fertility index (%)	94	94	94
Cohabitation days to positive mating (mean ± S.D.)	2.9 ± 1.7	2.5 ± 1.1	2.4 ± 1.8
Relative weight: testis (% term. body weight, mean ± S.D.)	0.988 ± 0.077	0.952 ± 0.171[a]	0.975 ± 0.152
Relative weight: epididymis (% term. body weight, mean ± S.D.)	0.308 ± 0.02	0.3 ± 0.04	0.296 ± 0.041
Relative weight: cauda epididymidis (% term. body weight, mean ± S.D.)	0.119 ± 0.011	0.113 ± 0.018	0.113 ± 0.019
Relative weight: prostate (% term. body weight, mean ± S.D.)	0.308 ± 0.046	0.314 ± 0.064	0.314 ± 0.059
Relative weight: seminal vesicles (% term. body weight, mean ± S.D.)	0.316 ± 0.054	0.309 ± 0.058	0.323 ± 0.051
Macro/histopathology of testis			
Reduced size/diffuse tubular degeneration; bilateral	0	2	2
Reduced size/diffuse tubular degeneration; unilateral	0	1	1
Calcification/orchitis; unilateral	0	0	1
Macro/histopathology of epididymis			
Reduced size/oligospermia (aspermia); bilateral	0	2	2
Reduced size/oligospermia (aspermia); unilateral	0	1	1
Reduced size/no findings; bilateral	0	0	1

[a] $n = 49$; #161 testis was not weighed because of premature death.

Table 4b
Sperm evaluation, F1 males

	Dose group (mg/(kg bw d))		
	0	4	100
Sperm parameters, number evaluated	50	47[a]	49[b]
Testis spermatid count (M/g)	108 ± 16	108 ± 15	109 ± 16
Cauda epididymidis sperm count (M/g)	538 ± 122	577 ± 125	546 ± 114
% abnormal sperm	1.5 ± 1.2	1.4 ± 0.9	3.8 ± 13.7[c]
Number of animals with >4% abnormal sperm	1	0	2
% motility (mean ± S.D.)	86 ± 8.9	86 ± 7.5	83 ± 11.9
Number of animals with sperm count <12 M/g testis or ≥90% abnormal morphology	0/50	2/50	2/50

[a] #156 and #196 did not have enough sperm for analysis, #161 died prematurely.
[b] #222 did not have enough sperm for analysis.
[c] High variance due to #238 with 97% abnormal sperm, mainly head abnormalities and headless.

also affected by reduced size of epididymis and oligozoospermia (see above) (Table 4b).

F2 male development (body weight and age at preputial separation) was unaffected by treatment, as was reproductive performance. Gestation and lactation parameters for the resulting F3 litters were not affected by treatment (Tables 3 and 5a). Mean body weight-related testis and cauda epididymidis weights were 4–6% lower than controls in both treated groups ($p \leq 0.05$), however, fur-

Table 5a
Sexual development and necropsy data, F2 males

	Dose group (mg/(kg bw d))		
	0	4	100
Number of male offspring/litters evaluated	50/46	50/46	50/45
Age at preputial separation (days, mean ± S.D.)	41.5 ± 1.5	41.7 ± 1.6	41.2 ± 1.4
Weight at preputial separation (g, mean ± S.D.)	168 ± 12	170 ± 13	167 ± 12
Male mating index (%)	100	98	100
Male fertility index (%)	94	94	94
Cohabitation days to positive mating (mean ± S.D.)	2.4 ± 1.1	2.6 ± 1.1	2.6 ± 1.2
Relative weight: testis (% term. body weight, mean ± S.D.)	1.013 ± 0.092	0.953 ± 0.147*	0.97 ± 0.08*
Relative weight: epididymis (% term. body weight, mean ± S.D.)	0.307 ± 0.024	0.292 ± 0.041	0.296 ± 0.026
Relative weight: cauda epididymidis (% term. body weight, mean ± S.D.)	0.117 ± 0.011	0.111 ± 0.019*	0.112 ± 0.014*
Relative weight: prostate (% term. body weight, mean ± S.D.)	0.328 ± 0.056	0.318 ± 0.05	0.321 ± 0.053
Relative weight: seminal vesicles (% term. body weight, mean ± S.D.)	0.322 ± 0.056	0.309 ± 0.044	0.321 ± 0.053
Macro/histopathology of testis			
Reduced size/diffuse tubular degeneration; bilateral	0	1	0
Reduced size/no findings; bilateral	0	1	0
Macro/histopathology of epididymis			
Reduced size/oligospermia (aspermia); bilateral	0	1	0
Reduced size/no findings; bilateral	0	0	1

*$p \leq 0.05$; versus control.

Table 5b
Sperm evaluation, F2 males

	Dose group (mg/(kg bw d))		
	0	4	100
Sperm parameters, number evaluated	50	49[a]	50
Testis spermatid count (M/g)	115 ± 16	114 ± 18	114 ± 16
Cauda epididymidis sperm count (M/g)	491 ± 100	505 ± 102	514 ± 123
% abnormal sperm	1.2 ± 0.9	1.8 ± 2.2	3.1 ± 12.7[b]
Number of animals with >4% abnormal sperm	0	3	2
% motility (mean ± S.D.)	82 ± 6.1	80 ± 8.4	80 ± 8.8
Number of animals with sperm count <12 M/g testis or ≥90% abnormal morphology	0/50	1/50	1/50

[a] #489 did not have enough sperm for analysis.
[b] High variance due to #529 with 90% abnormal sperm, mainly head abnormalities and headless.

ther weight deviations of epididymides, prostate or seminal vesicle were missing for all test groups.

At necropsy, reduced size of testes only, as well as reduced size of testes and epididymides were noted in a total of 1/50 F2 offspring at 4 mg/kg each; reduced size of epididymides was noted 1/50 F2 offspring at 100 mg/kg (Table 5a). In 1/50 male at 4 mg/kg the reduced size in testes/epididymides were correlated to diffuse tubular degeneration in the testis, aspermia in the epididymis, markedly reduced homogenization-resistant spermatid/sperm counts and infertility. The 1/50 male at 4 mg/kg with apparently reduced size of testis only, had no histopathological and spermatological correlate to the macroscopical finding and was fertile. The 1/50 male at 100 mg/kg with reduced epididymis size showed a normal histopathology and sperm count, but had 90% abnormal sperm (mainly head abnormalities and headless) and was, consequentially, infertile. None of the individual incidences was statistically significantly different to control (Wilcoxon test, one-sided).

Mean testis spermatid and epididymis sperm counts did not differ between groups. Mean % abnormal sperm also did not differ statistically between groups. The variance of the 100 mg/kg group was higher than the other groups, due to the one male with 90% abnormal sperm (see above) (Table 5b).

F3 male pup development (body weight and age at preputial separation) was unaffected by treatment (Tables 3 and 6a). Mean organ weights did not differ between treated and control groups.

At necropsy, reduced size of testes (partly with cystic degeneration) and epididymides were noted in a total of 1/50 F3 offspring at 0 mg/kg, 3/50 F3 offspring at 4 mg/kg and 1/50 F3 offspring at 100 mg/kg (Table 6a). In all these animals the reported macroscopical findings were correlated to diffuse tubular degeneration in the testis and oligozoospermia/aspermia in the epididymis. Additionally, in 3/50 males at 4 mg/kg and 1/50 males at 100 mg/kg markedly reduced homogenization-resistant spermatid/sperm counts were noted, along with abnormal sperm (90–100%, mainly head abnormalities or headless) in one male in each treatment group. In the 0 mg/kg male the tubular degeneration in the left testis and oligozoospermia in the left epididymis was not accompanied by changes in spermatid/sperm counts from the right testis/epididymis, but with a slightly lower rate of motile sperm. None of the individual incidences was statistically significantly different to control (Wilcoxon test, one-sided).

Mean testis spermatid and epididymis sperm counts did not differ between groups. Mean % abnormal sperm also did not differ statistically between groups. The variance of both the 4 and 100 mg/kg groups was markedly higher than the other groups, due to one male in each treatment group with ≥90% abnormal sperm (see above) (Table 6b).

Apoptotic germ cell counts were lower in the F1, F2 and F3 offspring, derived from vinclozolin-exposed dams, compared to concurrent controls. Apart from F3 males in the 4 mg/kg group, these differences were statistically significant (Table 7). Also, there appeared to be a successive decrease of apoptotic germ cell counts in all groups including controls over the F1–F3 generations (Table 8). All cross sections used for the quantitative analysis of apoptotic cells had normal testis morphology. As positively labeled apoptotic germ cells were mainly found in seminiferous tubule sec-

Table 6a
Sexual development and necropsy data, F3 males

	Dose group (mg/(kg bw d))		
	0	4	100
Number of male offspring/litters evaluated	50/49	50/49	50/47
Age at preputial separation (days, mean ± S.D.)	42.6 ± 1.8	42.3 ± 2.4	42.7 ± 1.7
Weight at preputial separation (g, mean ± S.D.)	181 ± 14	178 ± 12	182 ± 13
Relative weight: testis (% term. body weight, mean ± S.D.)	0.982 ± 0.073	0.956 ± 0.148	0.978 ± 0.129
Relative weight: epididymis (% term. body weight, mean ± S.D.)	0.289 ± 0.025	0.285 ± 0.038	0.291 ± 0.033
Relative weight: cauda epididymidis (% term. body weight, mean ± S.D.)	0.11 ± 0.011	0.108 ± 0.015	0.108 ± 0.014
Relative weight: prostate (% term. body weight, mean ± S.D.)	0.267 ± 0.039	0.267 ± 0.043	0.262 ± 0.037
Relative weight: seminal vesicles (% term. body weight, mean ± S.D.)	0.273 ± 0.039	0.277 ± 0.036	0.276 ± 0.042
Macro/histopathology of testis			
Reduced size/diffuse tubular degeneration; bilateral	0	2	1
Reduced size/diffuse tubular degeneration; unilateral	0	0	0
Cystic degeneration/diffuse tubular degeneration; bilateral	0	1	0
Cystic degeneration/diffuse tubular degeneration; unilateral	1	0	0
Macro/histopathology of epididymis			
Reduced size/oligospermia (aspermia); bilateral	0	3	1
Reduced size/oligospermia (aspermia); unilateral	1	0	0

Table 6b
Sperm evaluation, F3 males

	Dose group (mg/(kg bw d))		
	0	4	100
Sperm parameters, number evaluated	50	50	50
Testis spermatid count (M/g)	118 ± 22	113 ± 34	119 ± 26
Cauda epididymidis sperm count (M/g)	628 ± 120	629 ± 200	695 ± 184
% abnormal sperm	3.5 ± 2.2	5.2 ± 13.5[a]	5.2 ± 13.8[b]
Number of animals with >4% abnormal sperm	19	16	12
% motility (mean ± S.D.)	88 ± 7.8	88 ± 6.4	86 ± 15.2[b]
Number of animals with sperm count <12 M/g testis or ≥90% abnormal morphology	1/50	3/50	1/50

[a] $n = 48$ because #780 and #791 had too few sperm for analysis; high variance due to #763 with 96% abnormal sperm, mainly headless.
[b] High variance due to #842 with 100% abnormal sperm, all headless and immobile (spermatid/sperm counts in this animal were very low, <100/g).

Table 7
Mean values of apoptotic germ cell counts in the testes, comparison between control and test groups

	Dose group (mg/(kg bw d))		
	0	4	100
F1			
Number evaluated	50	48	48
Apoptotic germ cell counts (mean ± S.D.)	45.4 ± 10.8	30.0 ± 10.0**	27.5 ± 7.0**
Maximum	81	62	45
Minimum	28	11	15
F2			
Number evaluated	50	49	50
Apoptotic germ cell counts (mean ± S.D.)	30.9 ± 7.0	25.9 ± 4.8**	27.6 ± 5.9**
Maximum	49	44	43
Minimum	20	18	20
F3			
Number evaluated	49	48	49
Apoptotic germ cell counts (mean ± S.D.)	16.7 ± 6.5	14.7 ± 6.2	7.1 ± 4.4**
Maximum	32	31	28
Minimum	7	5	1

**$p \leq 0.01$ versus control.

Table 8
Mean values of apoptotic germ cell counts in the testes, comparison of the groups over 3 generations

	Generation		
	F1	F2	F3
0 mg/(kg bw d)			
Number evaluated	50	50	49
Apoptotic germ cell counts (mean ± S.D.)	45.4 ± 10.8	30.9 ± 7.0**	16.7 ± 6.5**
Maximum	81	49	32
Minimum	28	20	7
4 mg/(kg bw d)			
Number evaluated	48	49	48
Apoptotic germ cell counts (mean ± S.D.)	30.0 ± 10.0	25.9 ± 4.8**	14.7 ± 6.2**
Maximum	62	44	31
Minimum	11	18	5
100 mg/(kg bw d)			
Number evaluated	48	50	49
Apoptotic germ cell counts (mean ± S.D.)	27.5 ± 7.0	27.6 ± 5.9	7.1 ± 4.4**
Maximum	45	43	28
Minimum	15	20	1

**$p \leq 0.01$ versus F1.

tions of spermatogenesis stages VI–XIV, a further differentiation for counting was not carried out.

4. Discussion

The aim of the study reported here was to investigate a possible transgenerational effect of vinclozolin following oral exposure as this is the relevant route for risk assessment. The selection of oral administration limits in some aspects the comparability of the results of this study with those reported by Anway et al. [13] in which vinclozolin was administered following intraperitoneal administration.

In the present study, vinclozolin administered to pregnant dams from days 6–15 of gestation via oral gavage at dose levels of 4 and 100 mg/(kg bw d) did neither affect spermatogenesis nor development of the male reproductive tract in the offspring of exposed animals over 3 generations. In addition to being very low, the incidence of reproductive organ pathology was virtually the same despite a 25-fold difference in dose.

Spermatogenesis was largely defective (affected pathology, spermatology and fertility) in 4/100 F1, 1/100 F2 and 4/100 F3 vinclozolin-treated male offspring, and in 1/50 F3 untreated male offspring. None of the individual incidences in any of the 3 generations was statistically significantly different to control (Wilcoxon test, one-sided). We consider it unlikely that these individual findings represent a consistent transgenerational effect of vinclozolin.

It is of interest to note that a dose of 100 mg/(kg bw d) (comparable to a dietary administration of approximately 1000 ppm) when administered throughout gestation (days 6–20) resulted in pronounced effects in the offspring [2,4,5]. The lack of these findings in the present study very clearly demonstrated that the window of sensitivity for anti-androgenic effects on male reproductive tract in the offspring of vinclozolin-treated dams is from days 16–20 of gestation. These gestational days generally coincide with the onset of androgen receptor expression during sexual differentiation, which takes place at gestational day 15 or later in rat fetal reproductive tissue [26,27].

The difference between [13] and the present study might be related to the route of administration. Anway et al. [13] dosed 100 mg/(kg bw d) by the intraperitoneal (i.p.) route, which probably produced a different plasma peak pattern (most likely with a higher plasma peak concentration and possibly also with an altered pattern of metabolite formation) than the same dose by gavage in the present study. As the anti-androgenic effects of vinclozolin will depend both on the duration of the peak concentration (the time in which testosterone cannot bind to the androgen receptor) and metabolite formation (two metabolites of vinclozolin bind to the androgen receptor, rather than the unchanged parent compound [10]), the difference between oral and i.p. administration may have played a significant role.

Studies with radiolabelled (^{14}C uniformly ring labeled) vinclozolin on the biokinetics of this compound following single dietary and gavage administration showed that the systemic availability were substantially similar following both routes of administration [1]. At the high dose level used in the present study (100 mg/(kg bw d)), studies on the ^{14}C kinetics of this compound at the same dose level show the following results: 5 days following a single oral dose of vinclozolin (100 mg/(kg bw d)) urinary excretion accounted for 48–54% (males and females, respectively) of the administered radioactivity. Fecal elimination accounted for 49–40% of the administered radioactivity. Approximately 1% of the dose was retained in the body. However, a major part of the radioactivity in the feces is derived from the bile as biliary excretion studies demonstrated that 64–73% of the administered radioactivity was found in the bile. Combining the fecal excretion (ca. 45% of the dose) with the biliary excretion (ca. 70% of the dose) it can be calculated that approximately 30% of the total dose was eliminated through the bile. The values are in-line with a study in which ^{14}C-vinclozolin (1 mg/(kg bw d)) was administered intravenously. In this study 23% of the dose was eliminated through the bile (http://www.inchem.org/documents/jmpr/jmpmono/v95pr18.htm). Consequently, it can be concluded that the systemic availability of vinclozolin following oral administration is quite high (approximately 80%). Assuming close to 100% bioavailability in the Anway study (following i.p. administration), the differences between both studies could be explained by several other factors affecting the concentration at the site of action (i.e. in the uterus). The i.p. administration could have induced high plasma peak levels (1), the proximity of the uterus to the peritoneal cavity could have induced high levels at the site of action (2), or reduced first pass effect due to changes in the metabolic profile following i.p. administration (3). In conclusion, the levels of active anti-androgens at the site of action may have been different despite the fact that the dose level given in both studies was 100 mg/(kg bw d).

It should also be noted that the time of administration (gestational days 6–15) is not considered to be a phase in which the fetal rat is yet particularly sensitive towards anti-androgenic effects. Thus, the dose level of 100 mg/(kg bw d) may be close to the limit of inducing effects when administered during this particular period and changes in the route of administration could have a profound effect on the outcome of a study.

Anway et al. [13] reported that intraperitoneal 100 mg/(kg bw d) was the lowest dose to produce reliable effects, intraperitoneal 50 mg/(kg bw d) produced "more variable" effects. In the present study, an oral dose of 100 mg/(kg bw d) was not sufficiently high to produce effects. In comparison with other studies using vinclozolin, the effect/no-observed adverse effect levels (NOAEL) of both our and the Anway studies (100 mg/(kg bw d)) are much higher than those seen with longer administration periods. The lowest NOAEL for reproduction toxicity was seen in a 2-generation reproduction toxicity study at 4.9 mg/(kg bw d). For subchronic and chronic systemic toxicity in rats (the most sensitive species) the NOAELs are approximately 4 mg/(kg bw d) and 1.4 mg/(kg bw d), respectively. The acceptable daily intake value, as established by WHO for vinclozolin, was set at 0–0.01 mg/(kg bw d). Other agencies have used the same study to derive reference values (EU, US-EPA) but have used different assessment factors. The most conservative reference value was set by the US-EPA for chronic dietary exposure at a value of 0.0012 mg/(kg bw d). The chronic dietary exposure estimates for the USA expressed as a percentage of the chronic reference dose were 4% for the general population and 7% for the most highly exposed population subgroup.

Anway et al. [13] also reported that vinclozolin *increased* the number of apoptotic germ cells in testis in adult F1 and subsequent (untreated) F2–F4 generations of offspring. In contrast, in the present study, testicular apoptosis was numerically *reduced* in exposed F1 males and subsequent generations of untreated offspring. However, taking the fluctuations of apoptotic cells in control animals over time into account, we consider it unlikely that this finding is test substance induced. Great care was taken in the evaluation procedure for testicular apoptosis, all histotechnical procedures and countings follow strict standard operating procedures, positive labeling is compared with the morphologic features of apoptotic cell death, and all animals of all generations were assessed within a narrow age frame (maximum difference 8 days), to reduce technical and age-related bias. Thus it is unlikely that technical or procedural problems were causative for varying control values in the different generations. The only caveat that we have to make is that the TUNEL assay was used for a tissue for which we have no previous experience. This adds to the uncertainty of this apparent effect. In the Anway study, apoptosis rates in controls were constant or marginally increased from F1 to F4 [13], whereas there appeared to be a successive decrease in all groups over the F1–F3 generations in the present study. A possible explanation for the difference in direction of effect between Anway et al. [13] and the present study is that testicular apoptosis rates are age-related. Jeyaraj et al. [28] reported that testicular apoptosis is age-dependent in the mouse, and also reported an age-dependent effect in genetically androgen-deficient mice, which initially (up to 60 days of age) have a higher apoptosis rate than wild-type mice, but which subsequently (day 90–360) have a lower rate than wild-type, whose rates after a peak at 30 days, steadily increase (approximately double) from days 60–360 of age. Age at sacrifice may therefore have a significant effect on both apoptosis rates and treatment effects. It is not clear whether age at sacrifice was the same in Anway et al. [13] as in the present study; Anway et al. [13] reported age at sacrifice as "60–180 days". In our study, age at sacrifice was 127–134 days.

The different outcome of Anway et al. [13] and the present study might be related to different susceptibilities of the rat strains used. Accurate comparisons between the Sprague–Dawley strain used by Anway et al. [13] and the Wistar strain used for our study have not been performed with regard to transgenerational effects. However, the sensitivity of different rat strains with respect to the anti-androgenic effects of Vinclozolin was demonstrated by Hellwig et al. [6].

In conclusion, no transgenerational effect on the male reproductive system was found. Vinclozolin exposure in utero had no statistically significant effect on reproduction in terms of sexual maturity (age at preputial separation), time to mate, or mating and fertility indices, either in in utero-exposed F1 offspring or in subsequent generations (F2 and F3). Spermatogenesis was not affected by vinclozolin exposure in utero, as it was reported by Anway et al. [13]. Neither histopathology nor quantification of apoptotic cells, examined in the testes of F1–F3 generation males at an age of approximately 130 days, revealed adverse effects of the test compound. The average number of apoptotic germ cells was somewhat lower in the offspring derived from vinclozolin-exposed dams, and there appeared to be a successive decrease in all groups over the F1–F3 generations. Spermatid/sperm count, sperm morphology and sperm motility were not affected in F1–F3 generation male offspring.

The NOAEL of vinclozolin in this study was >100 mg/(kg bw d).

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References

[1] WHO-JMPR. Vinclozolin; 1995. p. 375–404.
[2] van Ravenzwaay B. Discussion of prenatal and reproductive toxicity of reg. no. 83-258 (vinclozolin). Data submission to U.S. EPA from BASF Corporation, MRID 425813-02; 1992.
[3] Matsuura I, Saitoh T, Ashina M, Wako Y, Iwata H, Toyota N, et al. Evaluation of a two-generation reproduction toxicity study adding endpoints to detect endocrine disrupting activity using vinclozolin. J Toxicol Sci 2005;30:163–88 [special issue].
[4] Gray Jr LE, Ostby JS, Kelce WR. Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rats. Toxicol Appl Pharmacol 1994;129:46–52.
[5] Wolf CJ, LeBlanc G, Ostby JS, Gray Jr LE. Characterization of the period of sensitivity of fetal male sexual development to vinclozolin. Toxicol Sci 2000;55:152–61.
[6] Hellwig J, van Ravenzwaay B, Mayer M, Gembardt C. Pre- and postnatal oral toxicity of vinclozolin in Wistar and Long–Evans rats. Regul Toxicol Pharmacol 2000;32(1):42–50.
[7] Ostby JS, Gray Jr LE, Kelce WR, Wolf CJ, Huey OP. Sexual differentiation in male rats exposed to low doses of the antiandrogen vinclozolin. Biol Reprod 1997;56(Suppl. 1):99.
[8] Kelce WR, Monosson E, Gamcsik MP, Laws SC, Gray Jr LE. Environmental hormone disruptors: evidence that vinclozolin developmental toxicity is mediated by anti-androgenic metabolites. Toxicol Appl Pharmacol 1994;126:276–85.
[9] Wong C, Kelce WR, Sar M, Wilson EM. Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. J Biol Chem 1995;270:19998–20003.
[10] Kelce WR, Lambright C, Gray Jr LE, Roberts K. Vinclozolin and pp′-DDE alter androgen-dependent gene expression: in vivo confirmation of an androgen receptor mediated mechanism. Toxicol Appl Pharmacol 1997;142:192–200.
[11] Molina-Molina JM, Hillenweck A, Jouanin I, Zalko D, Cravedi JP, Fernández MF, et al. Steroid receptor profiling of vinclozolin and its primary metabolites. Toxicol Appl Pharmacol 2006;216:44–54.
[12] Uzumcu M, Suzuki H, Skinner MK. Effect of the anti-androgenic endocrine disruptor vinclozolin on embryonic testis cord formation and postnatal testis development and function. Reprod Toxicol 2004;18:765–74.
[13] Anway MD, Cupp AS, Uzumcu M, Skinner MK. Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 2005;308(5727 (June 3)):1466–9 [+Science Supporting Online Material, http://www.sciencemag.org/cgi/data/308/5727/1466/DC1/1, dated 31 May, 2005].
[14] OECD. Guideline for testing of chemicals, no. 416, two-generation reproduction toxicity study, adopted 22 January 2001. Paris: Organisation for Economic Cooperation and Development; 2001.
[15] OECD. Principles of good laboratory practice. Paris: Organisation for Economic Cooperation and Development; 1981.
[16] German Chemicals Act. Chemikaliengesetz; Bundesgesetzblatt Teil I, 22 March, 1990 and 29 July, 1994, Germany; 1994.
[17] Slott VL, Suarez JD, Perreault SD. Rat sperm motility analysis: methodologic considerations. Reprod Toxicol 1991;5:449–58.
[18] Feuston MH, Bodnar KR, Kerstetter SL, Grink CP, Belcak MJ, Singer EJ. Reproductive toxicity of 2-methoxyethanol applied dermally to occluded and nonoccluded sites in male rats. Toxicol Appl Pharmacol 1989;100(1):145–61.
[19] Salewski E. Färbemethode zum makroskopischen Nachweis von Implantationsstellen am Uterus der Ratte. Naunyn Schmiedebergs Arch Pharmakol Exp Pathol 1964;247:367.
[20] Dunnett CW. A multiple comparison procedure for comparing several treatments with a control. J Am Stat Assoc 1955;50:1096–121.
[21] Dunnett CW. New tables for multiple comparisons with a control. Biometrics 1964;20:482–91.
[22] Hettmansperger TP. Statistical inference based on ranks. New York: John Wiley & Sons; 1984. p. 132–42.
[23] Nijenhuis A, Wilf HS. Combinatorial algorithms. New York: Academic Press; 1978. p. 26–38.
[24] Siegel S. Non-parametric statistics for the behavioral sciences. New York: McGraw-Hill; 1956.
[25] Holm S. A simple sequentially rejective multiple test procedure. Scand J Stat 1979;6:65–70.
[26] Majdic G, Millar MR, Saunders PT. Immunolocalisation of androgen receptor to interstitial cells in fetal rat testes and to mesenchymal and epithelial cells of associated ducts. J Endocrinol 1995;147:285–93.
[27] You L, Casanova M, Archibeque-Engle S, Sar M, Fan L, Heck H. Androgen receptor expression in the testes and epididymides of prenatal and postnatal Sprague–Dawley rats. Endocrine 1998;9:253–61.
[28] Jeyaraj DA, Grossman G, Petrusz P. Dynamics of testicular germ cell apoptosis in normal mice and transgenic mice overexpressing rat androgen-binding protein. Reprod Biol Endocrinol 2003;1:48.