Reproductive Disorders in Pubertal and Adult Phase of the Male Rats Exposed to Vinclozolin during Puberty

Wook Joon Yu, Beom Jun Lee, Sang Yoon Nam, Byeongwoo Ahn, Jin Tae Hong, Jae Cheul Do, Young Cheul Kim, Yong Soon Lee and Young Won Yun*

(Received 26 December 2003/Accepted 12 March 2004)

ABSTRACT. Vinclozolin (VCZ) is a systemic dicarboximide fungicide with antiandrogenic activity. Reproductive toxicity of VCZ was investigated in male rats exposed to VCZ during puberty. Sprague-Dawley male rats aged with 35 days were assigned to six different groups; negative control, positive control receiving flutamide (100 mg/kg), VCZ (100, 200 and 400 mg/kg), and a combination of VCZ (200 mg/kg) + methyltestosterone (100 mg/kg). The animals were treated with test compounds by oral gavage daily during 35 to 44 days of age. In pubertal rats sacrificed on the next day after final treatment, VCZ or flutamide-treated group showed a decrease in weights of prostate, epididymis, and seminal vesicle, hypertrophy of Leydig cells in the testis, detached debris and sloughed cells in the tubules of the caput epididymis, and an increase in serum testosterone levels. On the other hand, combined treatment of VCZ + methyltestosterone decreased testicular weight, increased seminal vesicle weight, and induced degeneration of spermatocytes. In adult rats sacrificed at five weeks after final treatment, flutamide decreased testicular sperm counts, and VCZ, flutamide and VCZ + methyltestosterone also decreased epididymal sperm counts. In addition, treatment of VCZ (400 mg) or VCZ + methyltestosterone decreased some motion kinematic parameters of sperms including curvilinear velocity, mean angular displacement and lateral head displacement. Flutamide treatment also decreased lateral head displacement. These results indicate that VCZ exposure during pubertal period in male rats causes reproductive disorders in puberty and adulthood.

KEY WORDS: flutamide, serum testosterone, sperm count, sperm motility, vinclozolin.

Vincozolin (VCZ), a systemic dicarboximide fungicide, has been used in the control of diseases caused by Botrytis cinerea, Sclerotinia sclerotiorum, Rhizoctonia solani and Monilinia spp in grapes, fruits, vegetables, hops, ornamental plants, and turf grasses [10]. VCZ is also used for esculent plant cultivation in many countries including Korea, United States, Europe and etc. Fungicide effect of VCZ appears to be exerted by inhibiting ergosterol synthesis [10] and by inducing lipid peroxidation via oxygen activation in fungi [2]. VCZ is metabolized to M1 and M2 that remain commonly in the soil, the plants, and mammals. Although VCZ itself is a very weak antagonist for androgen receptor binding, both M1 and M2 are effective antagonists [4]. In addition, M1 and M2 inhibited androgen-induced transactivation mediated by a mouse mammary tumor virus promoter [12].

VCZ exposure to mother rats during periparturition period caused persistent nipples, cleft phallus with hypospadias, female-like anogenital distance, suprainguinal ectopic scrotal/testes, a vaginal pouch, epididymal granulomas, small to absent accessory sex glands in male offspring, and sexual intercourse failure with mating-proven female adult rats [3]. An exposure of VCZ from weaning to early adult stage in male rats delayed preputial separation and pubertal onset, and reduced weights of androgen-dependent tissues including the ventral prostate, seminal vesicles and epididymis [7]. Peripubertal treatment of VCZ to male rabbits decreased accessory sex organ weights but increased pooled sperm counts [8]. However, there are no available reports related to reproductive disorders in male rats treated with VCZ during puberty.

Thus, we investigated adverse effects of VCZ on morphology of male reproductive system and serum androgen level in pubertal rats, and prolonged detrimental effects of VCZ on sperm counts and sperm quality in adult rats.

MATERIALS AND METHODS

Chemicals: VCZ was purchased from Supelco Co. (Bellefonte, PA, U.S.A.). Flutamide (FLU), methyltestosterone (MT) and corn oil were purchased from Sigma Chemical Co., (ST. Louis, MO, U.S.A.).

Animals and treatments: Sprague-Dawley [Bgl-Crj:CD®(SD) IGS BR] male rats aged with 28 days were purchased from Biogenomics Co. (Gapyong, Korea) and acclimatized for 7 days prior to treatments. Animal facilities were maintained under controlled conditions with temperature of 21 ± 2°C, relative humidity of 50 ± 10% and artificial illumination (fluorescent light) with a 12-hr light/dark cycle. Animals were fed with Samyang chow (Cheonan, Korea) and filtered tap water ad libitum. One hundred twenty rats with adequate weight gain and without clinical
signs were divided by computerized and stratified randomization into six experimental groups; corn oil for negative control, flutamide (FLU, 100 mg/kg/day) for positive control, VCZ (100, 200, and 400 mg/kg/day) and a combined treatment of VCZ (200 mg/kg/day) + MT (100 mg/kg/day). Test chemicals were dissolved in corn oil and administered daily by oral gavages during 35 to 44 days of age. Dose volume was 5 ml/kg body weight. Ten rats per each group were sacrificed on the next day of final treatment and the remnants were sacrificed at 5 weeks after final treatment. Rats were anesthetized using ethyl ether and euthanized by exsanguinations. Blood was obtained from the descending vena cava and serum was stored between –65°C and –85°C until determination of circulating hormone concentrations.

Pathological evaluations: Final body weights and sex organ weights including testis, epididymis, seminal vesicle and prostate were measured, and clinical signs were observed. Sex organs were placed in formalin fixative or Bouin’s fixative. After normal tissue processing for hematoxylin & eosin staining or PAS staining, all sex organs were examined microscopically.

Determination of testosterone levels: Testosterone levels were measured with a commercial RIA kit (Orion Co., Espoo, Finland). Cross-reactivity of testosterone antiserum at 50% binding level was follows; testosterone was 100%, 5α-dihydrotestosterone was 4.5%, methyltestosterone was 0.45%, and other steroid hormones was less than 0.03%. The cross-reactivity for androgens except testosterone was therefore negligible.

Sperm counts in testis and cauda epididymis: Testicular parenchyma tissue was displaced in 12 ml distilled water at 4–6°C. The tissue was homogenized at a low speed for 1.5–2 min using a polytron homogenizer (Omni 5000 International Co., Waterburg, CT) and sonicated for 3 min at 4°C. Cauda epididymis was chopped with a sharp scissor, and then homogenized with a low speed in 10 ml distilled water for 1.5–2 min at 4–6°C. The number of homogenization-resistant spermatids was then enumerated using a hemocytometer.

Analysis of sperm kinematics: Working media for analysis of sperm kinematics was mKRB (modified Krebs-Ringer bicarbonate solution containing 94.6 mM NaCl, 4.78 mM KCl, 1.71 mM CaCl₂, 1.19 mM KH₂PO₄, 1.19 mM MgSO₄, 25.07 mM NaHCO₃, 21.58 mM sodium lactate, 0.5 mM sodium pyruvate, 5.56 mM glucose, 4.0 mg/ml bovine serum albumin, 50 µg/ml streptomycin sulfate, 75 µg/ml potassium penicillin). They were equilibrated overnight to pH 7.4 in 5% CO₂ in air at 37°C. Sperms were collected from the left vas deferens by cutting the tubule with a small scalpel blade and put into 3 ml warm medium in a 35 mm petri dish (Corning Co., U.S.A.). The sperm samples were incubated in 5% CO₂ in air for 10 min at 37°C and diluted if necessary. Sperm suspensions (40 µl) were placed on grease-free slides warmed with a slide warmer (Jisico, Korea) at 36°C, and covered with 18 × 18 mm² coverslips to achieve a chamber depth of 20–50 µm, which does not disturb sperm movements. The slides were transferred to heated plate of an inverted phase-contrast microscope (Olympus IX 70, Japan). PH2 condenser and 4X PH1 object lens were used to produce pseudo-dark-field views. Computer-assisted sperm kinematics analysis was performed using the superimposed image analysis system (SIAS 10.1, Medical supply Co., Korea). Sperm motions were captured with a color video camera (JVC, Japan). For each slide, the tracks of sperms in 10 fields were recorded for approximately 2–3 min. Frame rate was 30 frames sec⁻¹. SIAS 10.1 detects the bright image and calculates centroids or average picture element (pixel) spatial locations for each head/midpiece combination of the rat sperms in each frame. The centroids were used for estimation of motion endpoint, which includes motility (number of sperm exceeding threshold minimum velocity/total number of sperm), curvilinear velocity (VCL; mean frame-to-frame velocity), straight-line velocity (VSL; velocity between centroids in first and last frame tracked), average path velocity (VAP; velocity obtained from smoothing the original path), linearity (LIN; [VSL/VCL]X100), straightness (STR; [VSL/VAP]X100), beat cross frequency (BCF; frequency that centroid crosses average trajectory), mean angular displacement (MAD; time-average of absolute values of the instantaneous turning angle of the sperm head along its curvilinear trajectory), lateral head displacement (ALH; displacement of the centroid from a computer-calculated average trajectory).

Statistics: Statistical analyses of the data were performed using the SPSS 9.0 program. The data were analyzed by one-way ANOVA followed by Dunnett’s test when the ANOVA test yielded statistical differences (p<0.05) among experimental groups.

RESULTS

Clinical signs, body weight and sex organ weight: No abnormalities in body weight and other clinical signs with noted gross findings were observed in the rats of all treatment groups. In pubertal rats sacrificed on the next day after final treatment, paired testicular weights were not affected by treatment of VCZ or FLU (Fig. 1). However, paired epididymal weights were significantly decreased by treatments of VCZ (p<0.05 at the dose of 100 mg/kg, p<0.01 at the doses of 200 and 400 mg/kg) or FLU (p<0.01), compared to the control (Fig. 1). In addition, prostate weights were significantly (p<0.01) decreased by treatments of VCZ at the doses of 200 and 400 mg/kg or FLU, compared to the control (Fig. 1). Seminal vesicle weights were also significantly (p<0.01) decreased by the treatments of VCZ or FLU, compared to the control (Fig. 1). On the other hand, the combination of VCZ + MT markedly decreased paired testicular weight (p<0.01), while it increased prostate weight (p<0.05), compared to the control (Fig. 1). However, in adult male rats sacrificed at five weeks after final treatment, any differences were not observed in overall sex organs weight of rats in all treatment groups.

Histopathological findings: VCZ treatment-related gross or histological changes in the prostate of pubertal rats were
not observed in all experimental groups. In the testis, epididymis, and seminal vesicle of rats, treatments of VCZ, FLU, or combination of VCZ + MT caused some microscopic changes (Figs. 2, 3 and 4). Leydig cell hypertrophy was observed in rats treated with VCZ or FLU (Fig. 2). Specifically, noted degeneration of pachytene spermatocytes at spermatogenic stage VII was induced by the combined treatment of VCZ+MT (Fig. 2). A slight degeneration of spermatocytes in seminiferous tubules was also observed in VCZ or FLU treatment groups. Increments of detached debris and some sloughed cells in the tubules of the caput epididymis were observed in rats of all the treatment groups (Fig. 3). The treatments of VCZ or FLU also produced some histological changes in the seminal vesicle of rats, that is, the vesicles were narrowed and occupied with epithelial cells (Fig. 4). The combined treatment of VCZ + MT did not cause any histopathological change in the seminal vesicle of rats. In adult rats sacrificed at five weeks after final treatment, there were no abnormal histopathological findings in all treatment groups.

**Serum testosterone levels**: In pubertal rats sacrificed on the next day after final treatment, the treatments of VCZ increased serum testosterone levels in a dose-dependent manner, but significantly ($p<0.05$) different at the high dose of VCZ 400 mg/kg, compared to the control (Fig. 5). FLU treatment also markedly increased serum testosterone levels above the control ($p<0.01$).

**Sperm counts**: The counts of homogenization-resistant sperm heads retrieved from the testis and cauda epididymis of adult rats sacrificed at five weeks after final treatment were shown in Fig. 6. FLU treatment significantly ($p<0.01$) decreased testis sperm counts in rats, compared to the control (Fig. 6A). VCZ did not affect the testis sperm counts in rats. VCZ treatments at the doses of 200 mg/kg ($p<0.01$) and 400 mg/kg ($p<0.05$) significantly decreased sperm counts in the cauda epididymis (Fig. 6B). The treatment of FLU or the combination of VCZ + MT also significantly decreased sperm counts in the cauda epididymis of rats ($p<0.01$).

**Sperm kinematics**: Motion kinematics of sperms retrieved from vas deferens of adult male rats at five weeks after final treatments were present in Fig. 7. The treatment of VCZ at the dose of 400 mg/kg significantly ($p<0.05$) decreased VCL and ALH below the control value. MAD was also markedly ($p<0.01$) decreased in rats treated with VCZ at the doses of 200 mg/kg and 400 mg/kg, compared to the control. In combined treatment group of VCZ + MT, VCL ($p<0.05$), MAD ($p<0.01$), and ALH ($p<0.01$) were also decreased below the control value. VCZ treatments caused a slight decrease in motility, VSL, VAP, LIN, BCF and STR, but the values were not significantly different from those of the control.

**DISCUSSION**

The present study was carried to evaluate reproductive disorders in male rats exposed to fungicide VCZ during pubertal period (35 to 44 days of age) and recovery effects by methyltestosterone on antiandrogenicity of VCZ were also investigated. The pubertal period from 35 to 44 days of age in rats is highly sensitive to exposures of various chemicals, thereby causing dramatic histopathological and functional changes in reproductive systems. We selected two different sacrifice days; the first sacrifice was made on the next day following final treatment for examining reproductive disorders inducible immediately following VCZ treatments during puberty in rats and the second sacrifice was executed at 5 weeks after final treatment for investigating how VCZ treatments during pubertal period affects reproductive functions in adulthood. The treatments of VCZ or FLU during pubertal period of rats caused reproductive disorders in adulthood as well as puberty.

Decreases in accessory sex organ weights including epididymis, prostate, and seminal vesicle by VCZ or FLU treatment were in agreement with those in other studies reported previously [1, 7–9]. In general, treatments of an antiandrogen induce a microscopic alteration in the testis of rats, commonly characterized by hypertrophy of Leydig cells [1, 7]. In this study, the treatments of VCZ or FLU actually caused the hypertrophy of Leydig cells in the testis. The combined treatment of VCZ + MT caused degenerations of pachytene spermatocytes at the stage VII of spermatogenesis, which is common change pattern of testicular histology induced by hormonally active compounds [11]. Although microscopic alterations of seminiferous tubules were not observed in
VCZ or FLU treatment group, several evidences for spermatogenesis disorders were present in the epididymis. In VCZ or FLU treatment group, detached debris and some sloughed cells, which are considered as germ cells originated from seminiferous tubules, were found in the caput epididymis, implying that VCZ or FLU caused spermatogenic disorders in the testis. In this study, VCZ or FLU treatment increased serum testosterone levels most likely due to suppression on feedback inhibition of androgens on the anterior pituitary gland [1, 5, 7, 9]. The suppression by VCZ or flutamide may induce secondarily LH secretion in pituitary gland and a blockade of Leydig cell androgen receptors, resulting in stimulating the secretion of testosterone [1, 5, 7, 9].

In this study, the counts of homogenization-resistant sperms retrieved from the testis were decreased only in FLU treatment group. VCZ treatment groups did not affect testicular sperm counts. The discrepancy on the sperm counts between VCZ and FLU treatment groups may be due to an actual difference in antiandrogenic potency between FLU and VCZ. In accessory sex organs weight and serum testosterone level, flutamide treatment displayed stronger antiandrogenic effects than those of VCZ. Although VCZ treatments did not affect sperm counts in the testis, they significantly decreased sperm counts in the cauda epididymis. Sperm counts in the cauda epididymis were also decreased by FLU treatment. These results in the present study indicate that the treatment of antiandrogen for ten consecutive days during puberty in male rats could affect sperm counts of cauda epididymis in adulthood. Moorman et al. [8] reported a controversial result that showed an increased count of pooled sperms in the rabbit. The reason for the discrepancy is unclear. However, it is likely that VCZ displays differently its antiandrogenicity on different species. Monsson et al. [7] reported that sperm counts in the cauda epididymis were markedly decreased in male rats treated with VCZ for 22 to 54 days of age. In addition, FLU treatment from 6 to 10 weeks of ages in male rats also decreased
sperm counts in the cauda epididymis [1]. These reports strongly support our present results on sperm counts in cauda epididymis, even if treatment conditions were different from those in this study.

Some sperm motion parameters including VCL, MAD, and ALH were markedly decreased by VCZ treatments and other parameters also showed a trend of dose-dependent decreases. Andrews et al. [1] reported that sperm motility and viability were decreased in male rats treated with FLU for 6 to 10 weeks of ages. In the present study, VCZ or FLU treatment, decreased sperm motion parameters in adult rats, implying that pubertal exposure to antiandrogenic chemicals in rats may induce some deterioration of sperm quality in their adulthood.

Additional application of methyltestosterone recovered, in part, reproductive disorders by VCZ in rats. Combined treatment of VCZ + MT recovered the decrease in the weights of paired epididymis and seminal vesicle by VCZ treatments. In addition, serum testosterone level in the combined treatment group of VCZ + MT was comparable, compared to controls. However, the MT treatment did not recover the decrease in the sperm counts in the cauda epididymis, sperm motion kinematics by VCZ. In weight and histopathology of the testis, co-treatment of VCZ + MT caused a considerable deterioration more than VCZ or FLU treatment did. In humans, co-treatment of cyproterone acetate, having dual progestin/antiandrogenic effects, and testosterone enathate induced male contraception by impairing spermatogenesis [6]. Although the detrimental effects of co-treatment of pure androgen and anti-androgen on testicular function have been first illustrated in this study, the toxic mechanism needs to be further studied in future.

In conclusion, the oral application of VCZ to pubertal male rats impairs normal differentiation of reproductive organs during puberty and it causes significant decreases in both sperm count and sperm motions in their adulthood, possibly by disturbing the endogenous androgen profile essential for normal development and function in reproductive system.

ACKNOWLEDGEMENTS. This work was supported by Chungbuk National University Grant.
Fig. 4. Histopathology of the seminal vesicle in the pubertal male rat treated daily with vinclozolin (200 mg/kg), flutamide (100 mg/kg), and a combination of vinclozolin (200 mg/kg) + methyltestosterone (100 mg/kg) during 35 to 45 days of age. A, The view of control regimen. B, The view of flutamide treatment regimen. C, The view of vinclozolin treatment regimen. D, The view of vinclozolin + methyltestosterone treatment regimen. The treatments of flutamide, vinclozolin, and vinclozolin + methyltestosterone caused narrow vesicles occupied with epithelial cells. Magnifications: × 40.

Fig. 5. Changes in serum testosterone levels in the pubertal male rat treated daily with vinclozolin (V100: 100 mg/kg, V200: 200 mg/kg, V400: 400 mg/kg), flutamide (100 mg/kg, FLU), and a combination (V+M) of vinclozolin (200 mg/kg) + methyltestosterone (100 mg/kg) during 35 to 44 days of age. Data were expressed as the mean ± S.D. (n=10). Asterisks on the bars indicate a significant difference, compared to the controls (* p<0.05, ** p<0.01).

Fig. 6. The counts of homogenization-resistant sperm heads recovered from the testis (A) and the cauda epididymis (B) in the adult male rats at five weeks after daily treatments with vinclozolin (V100: 100 mg/kg, V200: 200 mg/kg, V400: 400 mg/kg), flutamide (100 mg/kg, FLU), and a combination (V+M) of vinclozolin (200 mg/kg) + methyltestosterone (100 mg/kg) during 35 to 44 days of age. Data were expressed as the mean ± S.D. (n=10). Asterisks on the bars indicate a significant difference, compared to the controls (* p<0.05, ** p<0.01).
REFERENCES


