

Elevation of oxidative stress in the testes of rat by vinclozolin

B. Bhavanarayana and P. Sreenivasula Reddy*

Department of Zoology, S.V. University, Tirupati-517 502, India.

*Corres.author: reddy_1955@yahoo.co.in
Tel: +91-877-2249320; Fax: +91-877-2249611.

Abstract: Vinclozolin is one of the environmental contaminants, that has been shown to induce reproductive abnormalities in male rats. The aim of this study was to explore the adverse effects of vinclozolin on testicular oxidative stress in rats. Adult (100 day old) Wistar rats were injected inperitoneally with 1, 5 and 10 mg/kg bw vinclozolin on days 1, 7, 14 and 21 and maintained up to 50 days. The animals were killed by cervical dislocation at the end of maintainance period. Testes were isolated, cleared from adhering tissue/fluid and used to analyze oxidative status. A significant increase in the levels of lipid peroxidation was observed in the testis of vinclozolin exposed rats when compared with control rats. Furthermore, a significant decrease in the activity levels of antioxidant enzymes such as, superoxide dismutase, catalase was observed in the testis of vinclozolin treated rats when compared to control rats. The results were indicated that exposure to vinclozolin induces oxidative stress in the testis by increasing lipid peroxidation and decreasing the activities of antioxidant enzymes in rats. Our findings are consistent with a major role for accumulated free radical damage in the disruption of spermatogenic process in vinclozolin exposed rats thereby affects reproduction.

Key-words: Antioxidant enzymes, Lipid peroxidation, Oxidative stress, Rat, Vinclozolin.

Introduction

Vinclozolin is an environmental contaminant, which is extensively used as a fungicide in several countries (1). Vinclozolin (3-(3-5-dichlorophenyl)-5-methyl-oxazolidine-2,4-dione) is widely used to control fungi in fruits, vegetables, viticulture and turf grass (2,3). Vinclozolin has been characterized as an androgen receptor antagonist *in vitro* and *in vivo* (4,5). Vinclozolin has two primary metabolites namely, M1 and M2 (6). Exposure to vinclozolin during puberty and adulthood induces wide spectrum of reproductive abnormalities including altered secondary sexual characters(7-9), reduction of reproductive organ weights (10,11) and androgen levels (12). Moreover, several studies suggested that vinclozolin alters the central endocrine activity and the regulation of the hypothalamo-pituitary-gonadal (HPG) axis in male rats (13-15). Previous studies from our laboratory have shown that environmental contaminants alter epididymal spem quality and quantity, circulatory testosterone levels, testicular steroidogenesis and oxidative stress in rats (16-18).

It is well established that reactive oxygen species (ROS) has been implicated in tissue dysfunction leading to reproductive disorders (19). A few toxicological studies have been addressed the possible relationship between reproductive toxicity and oxidative stress by exposure to toxic chemicals that generate

ROS (20). ROS includes superoxide anion radical, hydroxyl radical, hydrogen peroxide, nitric oxide and peroxy nitrite. These ROS can cause damage to membranes (lipid peroxidation) and molecular modifications of proteins including protein carbonyl formation, nitration, and covalent modification by lipid aldehydes (21). Although, free radicals are generated in the body through various mechanisms, the cells respond to the oxidative stress by increasing the production of antioxidant enzymes and other defense mechanisms. Such adaptive responses to the cellular stress may contribute to the beneficial effects on health (22, 23). Several studies have also been documented that male reproductive organs suffer functional decline characterized by reduced serum levels of testosterone and production of sperm after exposure to xenobiotics (24-26). The causes for dysfunction of testes during exposure to xenobiotics are unknown, but oxidative stress is implicated (27, 28). Here, we report that exposure to vinclozolin induces oxidative stress in testes which might be one of the causative factors for suppression of reproduction in rats exposed to vinclozolin.

Materials and Methods

Procurement and Animal Maintenance

Adult Wistar strain male rats (190 ± 10 g, 110 days) were obtained from Sri Venkateswara Traders, Bengaluru, India. The animals were housed in identical polypropylene cages (18" x 10" x 8") lined with sterilized paddy husk as bedding in the same room within the animal facility, and were provided with commercial pellet diet (Godrej Agrovet Ltd, Mumbai, India) and tap water *ad libitum*. The animals were maintained under a well regulated light (12:12-h light/dark) schedule at $24 \pm 2^\circ\text{C}$ with $50 \pm 5\%$ relative humidity. The experiments were carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (29), Government of India. The protocol was approved by the Institutional Animal Ethical Committee of Sri Venkateswara university, Tirupati, India (vide resolution No. IAEC/No- 438/01/a/CPSEA).

Chemicals

Vinclozolin (CAS NO 50471-44-8, purity >99.6%) was obtained from Sigma-Aldrich Laborchemikalien, Seelze, Germany. HSD substrates were purchased from Sigma-Aldrich and Loba chemie, India. All other chemicals used for various assays were of analytical grade and obtained from local commercial sources.

Experimental Design

Rats were randomly divided into 4 groups. Rats in group I served as control and were given 50 μl of vehicle (corn oil) alone in a 50 μl volume. Rats in experimental group II, III and IV were received vinclozolin intra-peritoneally (vehicle as corn oil) at a dose of 1, 5 and 10 mg/Kg body weight, respectively on 1, 7, 14 and 21 days in a 50 μl volume and maintained up to 50 days. Vinclozolin doses selected for the present study were based on earlier report (30), which is well below the range of no-observed-effect level (15-111 mg/Kg body weight/day) for rats (10). The weight of each rat was monitored once in 10 days until the end of the treatment period.

Necropsy

The animals were fasted overnight, weighed and killed by cervical dislocation. Testes were dissected out, cleared from adhering tissue or fluid, washed in cold saline, weighed and used immediately for biochemical analysis.

Lipid Peroxidation

The levels of lipid peroxidation in the testis was measured in terms of malondialdehyde (MDA; a breakdown product of lipid peroxidation) content and determined by using the thiobarbituric acid (TBA) reagent. The reactivity of TBA is determined with minor modifications of the method adopted by Hiroshi (31). Briefly, testes were homogenized (10% W/V) in 1.15% potassium chloride solution. To 2.5 ml of homogenate, 0.5 ml of saline (0.9% NaCl) 1.0 ml (20% W/V) trichloroacetic acid (TCA) was added. The contents were then centrifuged for 20 minutes on a refrigerated centrifuge at 4000 Xg. To 1.0 ml of supernatant, 0.25 ml of TBA

reagent was added and the contents were incubated at 95°C for 1 h. One ml of n-butanol was added to it. After thorough mixing the contents were centrifuged for 15 min at 4000 Xg in a refrigerated centrifuge. The organic layer was transferred into a clear tube and its absorbance was measured at 532 nm. The rate of lipid peroxidation was expressed as μ moles of malondialdehyde formed/gram tissue.

Assay of Superoxide Dismutase and Catalase

Superoxide dismutase (EC 1.15.1.1) was assayed by the method of Misra and Fridovich (32). Briefly, the testes were homogenized (10% W/V) in 50 mM ice-cold sodium phosphate buffer (pH 7.0) containing 0.1 mM EDTA. The homogenate was centrifuged at 1,05,000 x g for 60 min. The supernatant (cytosol) fraction was used for the assay of the enzyme activity. The reaction mixture in a final volume of 2.0 ml contained: 0.05 M carbonate buffer (pH 10.2), 30 mM epinephrine (freshly prepared) and the enzyme extract. Changes in absorbance were recorded at 480 nm, measured at 10 sec. intervals for 1 minute in a UV-VIS spectrophotometer (Hitachi model: U-2001). The protein content in the enzyme source was determined by the method of Lowry et al (33) using bovine serum albumin as standard. The enzyme activity was expressed as Units/mg protein/min.

Catalase (EC. 1.11.1.6) was assayed by the method of Chance and Machly (34). Testes were homogenized in 50 mM phosphate buffer (pH 7.0). The homogenized sample was centrifuged at 1,05,000 x g for 60 minutes. The supernatant (cytosol) fraction was used for the assay of the enzyme activity. The reaction mixture in a final volume of 2.5 ml contained: 0.05 M phosphate buffer (pH 7.0), and appropriate amount of enzyme protein. The reaction was initiated by the addition of 19 mM hydrogen peroxide (H₂O₂). The decomposition of H₂O₂ was tracked directly by measuring the decrease in absorbance at 240 nm, at 10 sec. intervals for 1 minute in a UV-VIS spectrophotometer (Hitachi model: U-2001). The activity of the enzyme was expressed as μ moles of H₂O₂ metabolized/mg protein/min.

Statistical Analysis

Statistical analyses were performed using One-way Analysis of Variance (ANOVA) followed by Dunnet's test using SPSS (student version 7.5, SPSS Inc., UK). The differences were considered to be significant at $p < 0.05$. All data were presented as mean \pm S.D.

Table 1: Effect of vinclozolin on levels of lipid peroxidation and activity levels of catalase and superoxide dismutase in testes of rats.

Parameter	Control	1 mg/kg BW	5 mg/kg BW	10 mg/kg BW
Lipid peroxidation (μ moles of malondialdehyde formed/g wt.)	6.09 ^a \pm 2.14	12.74 ^b \pm 2.61 (109.19)	13.42 ^b \pm 3.57 (120.36)	17.79 ^c \pm 2.47 (191.11)
Superoxide dismutase (Units/mg protein/min.)	0.91 ^a \pm 0.11	0.63 ^b \pm 0.15 (-30.76)	0.41 ^c \pm 0.14 (-56.04)	0.27 ^d \pm 0.14 (-70.32)
Catalase (μ moles of H ₂ O ₂ metabolised/mg protein/min)	0.035 ^a \pm 0.01	0.021 ^b \pm 0.007 (-36.36)	0.017 ^b \pm 0.009 (-48.48)	0.011 ^c \pm 0.008 (-66.66)

Values are mean \pm S.D. of 8 individuals.

Values in parentheses are percent change from control.

Mean values in a row that do not share the same superscript differ significantly at $p < 0.05$.

Results and Discussion

The levels of lipid peroxidation products (malondialdehyde) are presented in Table 1. A significant ($p < 0.05$) increase in malondialdehyde (MDA) content in the testes of vinclozolin treated rats was observed when compared with testis of control rats. The activity levels of superoxide dismutase (SOD) and catalase

decreased significantly ($p < 0.05$) in the testes of vinclozolin exposed rats when compared to control rats (Table 1).

The present study clearly demonstrates that exposure to vinclozolin induced testicular lipid peroxidation and decreased antioxidant enzyme activities in the testis of rats. Our studies are consistent with a major role for accumulated free radical damage in the disruption of spermatogenic process during vinclozolin intoxication (24,26,35,36). Our results are also in consonance with earlier reports (37).

Lipid peroxidation, an indicator of oxidative stress in tissues cause peroxidative damage of cellular lipid content and is assessed through estimation of thiobarbituric acid-reactive substances (TBARS) in a tissue. Malondialdehyde (MDA) being the end product remains an important molecular marker of lipid peroxidation. Generation of the reactive oxygen species within a tissue is neutralized by the antioxidant enzymes present in the tissue. Increase in the testicular MDA content detected in the testis of experimental rats indicative of the oxidative stress induced by vinclozolin. This was further affirmed by the noted decrease in the antioxidant enzyme activities viz., superoxide dismutase and catalase.

Antioxidant enzymes constitute a mutually supportive team of defence against ROS. Reactive oxygen metabolites such as superoxide, hydroxyl radical, singlet oxygen and H_2O_2 are generally considered cytotoxic agents because of their ability to induce lipid peroxidation in tissues and cell membrane. In addition, superoxide is an important source of hydroperoxides and deleterious free radicals (38). Hydrogen peroxide and superoxide reacts are catalyzed by transition metals to form the deleterious hydroxyl radical (39). In addition, most toxic effects are due to hydroxyl radical formation, which also initiates lipid peroxidation (38). SOD inhibits lipid peroxidation by catalyzing the conversion of superoxides into hydrogen peroxide and oxygen (40-43). Catalase converts hydrogen peroxide to water and molecular oxygen, thereby reducing the amount of free hydroxyl radical formation (44). In the present study the activities of antioxidant enzymes, superoxide dismutase and catalase are decreased in vinclozolin treated rats. Thus in the present study an increase in lipid peroxidation with a reduction in antioxidant enzymes indicates the oxidative stress induced by vinclozolin. Similar results were also observed earlier (45, 46). Impaired antioxidant defense mechanism may induce testicular damage, low sperm counts, and infertility (47).

In conclusion, our results indicate that exposure to vinclozolin causes impairment in the testicular antioxidant metabolism of rats as evidenced by elevation in the lipid peroxidation products and decrease in the activities of superoxide dismutase and catalase.

Conflict of Interest

The authors declare that there are no conflicts of interest with respect to the authorships or publication of this article.

Acknowledgements

The authors are grateful to the Head, Department of Zoology, S.V. University, Tirupati for providing facilities. PSR acknowledge the financial support received from the University Grants Commission, New Delhi in the form of research grant. We thank Prof. K.V.S. Sarma for the statistical analysis of data. Technical help received from S. Umasankar is gratefully acknowledged. The authors declare that the experiments conducted during these studies comply with the current laws of their country.

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