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Transplacental exposure to vinclozolin induces epididymal and testicular toxicity in adult male rats

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Abstract

Vinclozolin (3-(3-5-dichlorophenyl)-5-methyl-oxazolidine-2,4-dione) is extensively used in the viticulture, agriculture and commercial horticulture to control several fungal pathogens and has been shown to induce reproductive abnormalities in male rats, causing reduced fertility. The mechanism of action of vinclozolin on male reproductive system is not clear. In the present study we investigated whether prenatal administration of vinclozolin induces oxidative stress in the testes and changes in sperm quality and quantity in epididymis in adult male rats. Pregnant rats were injected with 1, 5 and 10 mg vinclozolin/Kg body weight on 1st, 7th and 14th day of pregnancy and allowed to deliver. Male pups were maintained up to postnatal day 100 and used for assessing testicular and epididymal toxicity. Embryonic exposure to vinclozolin significantly decreased in the sperm density, number of motile sperm, viable sperm and HOS tail coiled sperm in adult rats. Significant increase in the levels of lipid peroxidation with a significant reduction in the activity levels of superoxide dismutase and catalase in testis was observed in rats exposed to vinclozolin during embryonic development. It can be concluded that prenatal exposure to vinclozolin induced oxidative stress in testis of adult rats thereby decreases the sperm quantity and deteriorates sperm quality.

Key-Words: Vinclozolin, Oxidative Stress, Lipid Peroxidation, Antioxidant Enzymes, Sperm, Rat

Introduction

There is a growing distress that several chemicals released into environment disrupt reproduction of wild life and as well as humans (Colborn *et al.,* 1993). Vinclozolin (3-(3-5-dichlorophenyl)-5-methyl-oxazolidine-2,4-dione), one of the systemic fungicides extensively used in the viticulture, agriculture and commercial horticulture in several countries (Colbert *et al.,* 2005). Vinclozolin is degraded into butenoic acid (M1) and enanilide (M2) metabolites in the environment, which have been shown to be more toxic than the parent compound (Pothuluri *et al.,* 2000). Exposure to Vinclozolin is known to alter gene expression *in vivo* in an antiandrogenic manner (Kelce *et al.,* 1997). Earlier studies also suggested that *in utero* exposure to Vinclozolin causes a significant decrease in testicular and seminal vesicle weights, respectively in rodent models (Elzeinova *et al.,* 2008; Wolf *et al.,* 2004). Many studies have been reported significant reduction in sperm motility and epididymal sperm count in rodents exposed to Vinclozolin during embryonic development (Uzumcu *et al.,* 2004; Anway *et al.,* 2006b).

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Vinclozolin is also known to promote transgenerational transmission of adult onset disease through epigenetic reprogramming (i.e. DNA methylation) of male germ line (Anway *et al.,* 2005; Anway *et al.,* 2006b). Contrary reports have documented that exposure to Vinclozolin during prenatal and perinatal periods showed absence of male reproductive abnormalities (Wolf *et al.,* 2004; Schneider *et al.,* 2008). Thus, it seems apparent that Vinclozolin-induced reproductive abnormalities are highly variable and depend on various aspects, such as exposure level, length of exposure, the animal species used and the exposure during developmental stage.

One of the major factors to reproductive disorders is oxidative stress (Turner *et al.,* 2008). Oxidative stress results from increased lipid peroxidation or decreased intrinsic antioxidant defense in different tissues. In addition, Environmental contaminants have been shown to induce reactive oxygen species (ROS) generation in both intra- and extra-cellular spaces of cells or individuals leading to tissue injury and cell death (Ho *et al.,* 1998). ROS have been shown to play an important role in the defense mechanisms against pathological conditions but excessive generation of free oxygen radicals may damage tissues (Kitas *et al.,* 1991). Although earlier studies established that

Vinclozolin induces lipid peroxidation (Lioi *et al.,* 1998), the mechanism of action of Vinclozolin on the activities of antioxidant enzyme activities has not been elucidated. The present study was undertaken to evaluate the effect of embryonic exposure to Vinclozolin on the testicular lipid peroxidation and antioxidant enzymes in adult rats. We also evaluated the effect of prenatal exposure vinclozolin on epididymal sperm count and sperm motility and viability in adult male rats in order to assess the reproductive toxicity.

Material and Methods

Animals and maintenance

Adult male Wistar rats (150 ± 10 g) were purchased from Sri Venkateswara Traders, Bengaluru, India. Animals were housed in polypropylene cages (18" x 10" x 8") under a well regulated light (12-h light/dark) schedule at 24 ± 3ºC with 50 ± 5% humidity and provided with commercial rat chow (Godrej Agrovet Ltd, Mumbai, India) and tap water *ad libitum*. This study was carried out according to guidelines for the care and use of laboratory animals (National Research Council. 1996) and approved by the Institutional Animal Ethical Committee at Sri Venkateswara University, Tirupati, India (vide No. IAEC/No-438/01/a/CPSEA).

Regularly cycling female rats (body weight 160 ± 10 g) were cohabited overnight with fertile male rats. The successful mating was confirmed by the presence of spermatozoa in vaginal smear and considered day 1 of pregnancy. Sperm positive females were randomly distributed into 4 groups of 8 rats each and housed individually.

Test chemical and dosing

Vinclozolin (CAS NO 50471-44-8, purity>99.6%) was obtained from Sigma-Aldrich Laborchemikalien, Seelze, Germany. Pregnant animals in control group received only 50 µl corn oil. Rats in experimental group II, III and IV received 50 µl Vinclozolin intra-peritoneally (vehicle as corn oil) at a dose of 1, 5 and 10 mg/Kg body weight, respectively on 1st, 7th and 14th day of pregnancy. All the rats were allowed to deliver pups. Male pups were weaned and assessed for reproductive and biochemical end points on post natal day 100. The doses selected for Vinclozolin is based on earlier report Susan *et al*. (2006), which is well below the range of no-observed-effect level (15-111 mg/Kg/day) for rats (Van Ravenzwaay, 1992).

Necropsy and spermatology

The animals were fasted overnight, weighed and killed by cervical dislocation on post natal day 100. Testes and epididymides were removed, cleared of the adhering tissue and used immediately for epididymal sperm analysis and determination of testicular oxidative status.

Sperm count

Cauda part of epididymis was removed and placed in 10 ml petridish containing 2.0 ml of physiological saline at 37ºC. Subsequently, cauda epididymis was squeezed. A drop of sperm suspension was used for the determination of total sperm count using Neubauer Chamber, as described by Belsey *et al.* (1980).

Sperm motility

Sperm sample was placed on Neubauer chamber and progressive sperm motility was evaluated by the method of Belsey *et al.* (1980). The whole process was performed within 5 minutes following their isolation from cauda epididymis. First non-motile sperm were counted followed by motile sperm. Sperm motility was expressed as a percentage of total sperm counted.

Sperm viability

The ratio of live and dead sperms was determined using 1% tryphan blue by the method of Talbot and Chacon (1981). Sperm viability was expressed as a percentage of total sperm counted.

Hypo osmotic swelling test (HOS-test)

When viable sperms are exposed to hypoosmotic medium, there will be an influx of fluid causing the tail to coil, which can be seen under phase contrast microscope and the percent of tail coiled sperm was determined by the method of Jeyendran *et al.* (1992).

Lipid peroxidation

A break down product of lipid peroxidation, malondialdehyde (MDA) was measured by the method Hiroshi *et al.* (1979). Briefly, the testes were homogenized (10% W/V) in 1.15% potassium chloride solution. 0.5 ml of saline (0.9% sodium chloride), 1.0 ml of (20% W/V) trichloroacetic acid (TCA) were added to 2.5 ml of homogenate. Samples were centrifuged for 20 minutes on a refrigerated centrifuge at 4000 x g. 0.25 ml of TBA reagent was added to 1.0 ml of supernatant, and samples were then 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 minutes at 4000 x g 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 wet weight of tissue.

Assay of antioxidant enzymes

Superoxide dismutase (EC 1.15.1.1) was assayed by the method of Misra and Fridovich. (1972). 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 105,000 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 seconds 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. (1951) using bovinclozoline 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 (1955). 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 (H_2O_2). The decomposition of H_2O_2 was tracked directly by measuring the decrease in absorbance at 240 nm, at 10 seconds intervals for 1 minute in a UV-VIS spectrophotometer (Hitachi model: U-2001). The activity of the enzyme was expressed as μ moles of H_2O_2 metabolized/mg protein/min.

Statistical analysis

Statistical analyses were performed using One-way Analysis of Variance (ANOVA) followed by Dunnet's test. The differences were considered to be significant at $p < 0.05$. The data were presented as mean ± S.D.

Results and Discussion

Figure 1 shows the effect of prenatal exposure to Vinclozolin on sperm parameters in rats. A Significant decrease ($p<0.05$) was observed in total epididymal sperm count, sperm motility, sperm viability and HOS tail coiled sperms in rats exposed to vinclozolin during embryonic development when compared to controls (Fig. 1).

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 (CAT) decreased significantly in the testes of rats exposed to Vinclozolin during embryonic development when compared to control rats (Table 1).

In the present studies we have shown that prenatal exposure to vinclozolin induces oxidative stress in the testes and decreases epididymal sperm density and quality of adult male rats. Effects of vinclozolin on pubertal development and reproductive function in male rats treated during gestation and lactation have been reported (Uzumcu *et al.,* 2004). An inhibition of spermatogenesis, deterioration in sperm quality and a suppressed testicular Leydig cell functions has been reported after exposure to vinclozolin (Uzumcu *et al.,* 2004). Anway *et al* (2006b) reported a significant decrease in the sperm motility and sperm number in the vinclozolin treated rats even in F1, F2, F3, and F4 generations. Impaired sperm motility may also result in infertility due to failure of sperm to reach the site of fertilization as well as their ability to penetrate zona pellucida.

The present study also demonstrates that administration of vinclozolin during embryonic development decreased the activities of catalase and superoxide dismutase, and concomitantly increased the levels of lipid peroxidation in testes of adult rats. Superoxide dismutase is considered the first line of defense against deleterious effects of oxyradicals in the cells by catalyzing the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen (Nissen and Kreysel, 1983). Catalase allows degradation of hydrogen peroxide to water and oxygen (Jeulin *et al.,* 1989). The reduction in the activities of antioxidant enzymes and increase in lipid peroxidation could reflect the adverse effect of vinclozolin on the antioxidant system in testes.

One of the important aspects of antioxidant enzymes is their nature of synergistic functioning: a decrease in superoxide dismutase activity has been shown to increase the level of superoxide anion, which is known to inactivate catalase activity (Kono and Fridowich, 1982). Similarly, when catalase fails to eliminate hydrogen peroxide from the cell, the accumulated hydrogen peroxide has been shown to cause inactivation of superoxide dismutase (Sinet and Garber, 1981). Reactive oxygen species such as superoxide, hydroxyl radical, singlet oxygen and H_2O_2 has been shown to damage almost all macromolecules viz., proteins, lipids etc., and the changes in the sperm quantity and quality may also be due to damage induced by reactive oxygen species (Ochsendoerf, 1999).

It is well-known, that spermatozoon is highly susceptible to the damage induced by ROS because of their high content of polyunsaturated fatty acids. To counteract the effects of ROS, testes are equipped with antioxidant defense systems, which prevent cellular damage. A balance between the benefits and risks from ROS and antioxidants appears to be necessary for survival and normal functioning of spermatozoa in the testes (Aitken and Roman, 2008). Impaired antioxidant defense mechanism may induce testicular damage, low sperm counts, and infertility (Makker *et al.,* 2009). High levels of reactive oxygen species have been

correlated with reduced sperm motility (Armstrong *et al.,* 1999; Wang *et al.,* 2004).

Conclusion

In summary, the results of the present study reveal that prenatal exposure to vinclozolin decreased sperm quantity and deteriorated sperm quality. Further, lipid peroxidation levels increased with a decrease in activity levels of antioxidant enzymes such as superoxide dismutase and catalase in the testis of treated rats. In conclusion, decrease in sperm quantity and deterioration in sperm quality in prenatal vinclozolin exposed rats might be due to induced oxidative stress in testis of rats.

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References

1. Aitken R. J. and Roman S. D. (2008). Antioxidant systems and oxidative stress in the testes. *Adv Exp Med Biol,* 636: 154-71.
2. Anway M. D., Cupp A. S., Uzumcu M. and Skinner M. K, (2005). Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Scien,* 308: 1466-1469.
3. Anway M. D., Memon M. A., Uzumcu M. and Skinner M. K. (2006b). Transgenerational effect of the endocrine disruptor vinclozolin on male spermatogenesis. *J Androl,* 27: 868-879.
4. Armstrong J. S., Rajaskaran M., Chamulitrat W., Gatti P., Hellstrom W. J. and Sikka S.C. (1999). Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. *Free Radic Biol Med,* 26: 869-880.
5. Belsey M. A., Moghissi K. S., Eliasson R., Paulsen C. A., Callegos A. J. and Prasad M. R. N. (1980). Laboratory manual for the examination of human semen and semen-cervical mucus interaction. Singapore: Press concern. 10.
6. Chance B. and Machly A. C. (1955). Assay of catalase and peroxidase. *Methods Enzymol,* 2: 764-775.
7. Colborn T., vom Saal F. S. and Soto A. M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ Health Perspect,* 101: 378-384.
8. Colbert N.K., Pelletier N. C., Cote J. M., Concannon J. B., Jurdak N. A., Minott S. B. and Markowski V. P. (2005). Perinatal exposure to low levels of the environmental antiandrogen vinclozolin alters sex-differentiated social play and sexual behaviors in the rat. *Environ Health Perspect,* 113: 700-707.
9. Elzeinova F., Novakova V., Buckova D., Kubotava A. and Peknocova J. (2008). Effect of low dose of vinclozolin on reproductive tract development and sperm parameters in CD1 outbred mice. *Reprod Toxicol*, 26: 231-238.
10. Hiroshi O., Habuko D. and Yagi K. (1979). Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction. *Anal Biochem,* 95: 351 – 358.
11. Jeyendran R. S., Van Der Ven H. H. and Zaneveld L. J. D. (1992). The hypo-osmotic swelling test: An update. *Arch Androl,* 29: 105-116.
12. Jeulin C., Soufir J. C., Weber P., Laval Martin D. and Calvayrac R. (1989). Catalase activity in human spermatozoa and seminal plasma. *Gamete Res*. 24: 185-196.
13. Kelce W. R., Stone C. R., Gray L. E Jr. and Roberts K. (1997). ‘‘Vinclozolin and p, p' - DDE alter androgen-dependent gene expression: in ĺiĺo confirmation of an androgen receptor mediated mechanism’’. *Toxicol Appl Pharmacol,* 142: 192-200.
14. Kitas G. D., Salmon M., Young S. P. and Bacon P. A. (1991). Effects of hydrogen peroxide on lymphocyte receptor functions. *Mol Aspects Med,* 12: 149-159.
15. Kono Y. and Fridovich I. (1982). Superoxide radical inhibits catalase. *J Boil Chem,* 257: 5751-5754.
16. Lioi M. B., Scarfi M. R., Santoro A., Barbieri R., Zeni O., Di Berardino D. and Ursini M. V. (1998). Genotoxicity and oxidative stress induced by pesticide exposure in bovine lymphocyte cul-tures in vitro. *Mutat Res,* 403: 13-20.
17. Makker K., Agarwal A. and Sharma R. (2009). Oxidative stress and male infertility. *Ind J Med Res,* 129: 357-367.
18. Misra H. P. and Fridovich I. (1972). The role of superoxide anion in the autooxidation of

epinephrine and a simple assay for superoxide dismutase. J Biol Chem, 247: 3170 – 3175.

19. Nissen H. P. and Kreysel H. W. (1983). Superoxide dismutase in human sperm. *Klinische Wochenschrift*, 61: 63-65.
20. Ochsendoerf F. R. (1999). Infection in the genital tract and reactive oxygen spercies. *Hum reprod Update,* 5: 399-420.
21. Pothuluri J. V., Freeman J. P., Heinze T. M., Beger M. D. and Cernigalia C. E. (2000). Biotransformation of vinclozolin by fungus Cunnighamella elegans. *J Agri Food Chem,* 48: 6138-6148.
22. Schneider S., Kaufmann W., Buesen R. and van Ravenzwaay B. (2008). Vinclozolin-The lack of a transgenerational effect after oral maternal exposure during organogenesis *Reprod Toxicol,* 25: 352 – 360.
23. Sinet, P. M. and Garber P. (1981). Inactivation of human copper-zinc superoxide dismutase during exposure to oxygen and H_2O_2. *Arch Biochem Biophys,* 212: 411-416.
24. Susan M. A. and Vincent P. M. (2006). Learinig deficits expressed as delayed contiction of a conditione running response following perinatal exposure to vinclozolin. *Neurotoxicol Teratol,* 28: 482-488.
25. Talbot P. and Chacon R.S. (1981). A triple stain technique for evaluating normal acrosome reaction of human sperm. *J Exp Zool,* 215: 201-208.
26. Turner T. and Lysiak J. J. (2008). Oxidative Stress: A Common Factor in Testicular Dysfunction. *Androl,* 29: 488-498.
27. Uzumcu M., Suzuki H. and Skinner M. K (2004). Effect of the anti-androgenic endocrine disruptor vinclozolin on embryonic testis cord formation and postnatal testis development and function. *Reprod Toxicol,* 18: 765-774.
28. Van Ravenzwaay B. (1992). Discussion of Prenatal and Reproductive Toxicity of Reg. No. 83-258 Vinclozolin. Data submission to US Environmental Protection Agency from BASF, MRID 42: 5813-02.
29. Wang Y., Song L., Chen J., He J., Liu R., Zhu Z. and Wang X. (2004). Effects of di-butyl phthalate on sperm motility and oxidative stress in rats. *Zhonghua Nan Ke Xue,* 10: 253-260.
30. Wolf C. J., LeBlanc G. A. and Jr LEG. (2004). Interactive effects of vinclozolin and testosterone propionate on pregnancy and sexual differentiation of the male and female SD rat. *Toxicol Scien,* 78: 135-143.

Table 1: Effect of prenatal exposure to vinclozolin on lipid peroxidation levels and catalase and superoxide dismutase activity levels in testis of adult rats

Parameter	Control	1 mg/kg BW	5 mg/kg BW	10 mg/kg BW
Lipid peroxidation (μ moles of malondialdehyde formed/g wet wt.)	$6.18^{a} \pm 2.43$	$7.38^{a} \pm 0.68$ (19.41)	$11.87^{b} \pm 3.64$ (92.07)	$14.27^{b} \pm 0.75$ (130.9)
Superoxide dismutase (Units/mg protein/min.)	$0.84^{a} \pm 0.11$	$0.61^{a,b} \pm 0.52$ (-27.38)	$0.32^{b} \pm 0.09$ (--58.33)	$0.31^{b} \pm 0.16$ (-63.09)
Catalase (μ moles of H_2O_2 metabolised/mg protein/min)	$0.027^{a} \pm 0.007$	$0.02^{a,b} \pm 0.01$ (-25.92)	$0.016^{b} \pm 0.007$ (-40.74)	$0.012^{b} \pm 0.009$ (-55.55)

Values are mean ± S.D. of 6 individuals.
Values in parentheses are percent change from control.
Mean values in a row that do not share the same superscript differ significantly at $p<0.05$.

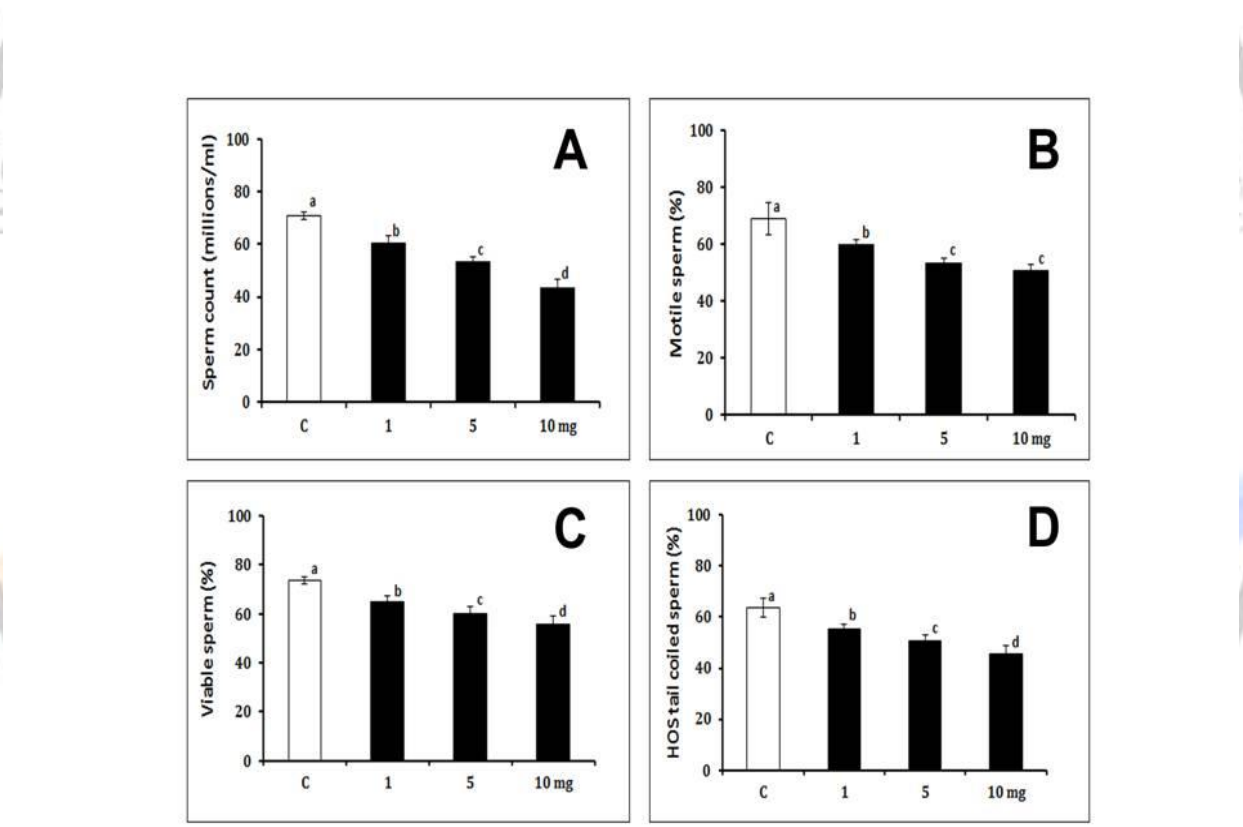

Fig. 1: Effect of prenatal exposure to vinclozolin on elididymal sperm count (A), sperm motility (B), sperm viability (C) and HOS tail coiled sperm (D) in the control rats (C) and rats exposed to 1, 5 and 10 mg/kg BW vinclozolin (10 mg).
Each bar represents the mean ± SD of 6 rats.
Bars with same superscript do not differ significantly from each other at $p<0.05$.