EVALUATION OF SUBACUTE SODIUM FLUORIDE TOXICITY ON SPERMATOZOA AND TESTICULAR TISSUE OF MALE WISTAR RATS

D. K. GIRI*, R. C. GHOSH, D. K. KASHYAP AND GOVINA DEWANGAN
Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry,
Anjora, Durg, Chhattisgarh - 491 001
e-mail: giri.devesh18@gmail.com

ABSTRACT
The adverse influence of fluorides on male reproductive system has become a major concern in many countries. The present study was focussed to determine the effect of daily oral administration of sodium fluoride for 28 days on seminal characteristics of male Wistar rats at three dose levels. Intoxication of sodium fluoride to Wistar rats of group II (5mg/kg body weight, orally), group III (25 mg/kg body weight, orally) and group IV (50 mg/kg body weight, orally) caused dose dependent decrease ($p \leq 0.01$) in epididymal sperm motility, progressive sperm motility, sperm concentration and live spermatozoa along with simultaneous increase in dead spermatozoa as compared to the rats of group I (control). There was a dose dependent increase ($p \leq 0.01$) in percent morphological abnormalities (head, tail and multiple) of spermatozoa in group III and group IV as compared to rats of control group and group II. Overall, the maximum mean values for various sperm abnormalities were recorded in group IV rats. Hence, sodium fluoride exerts significant cytotoxicity to spermatozoa at higher doses adversely affecting the male reproductive system.

INTRODUCTION

Global population is increasing now a day as a result of the variations in natural and anthropogenic activities. This is leading to the contamination of various terrestrial and aquatic ecosystems with metals, nonmetals, organic and inorganic compounds. Therefore, a large part of the population in the industrialized world is exposed to variety of pollutants on daily basis, fluorides being one of them (Chouhan and Flora, 2010). Besides immunotoxic, neurotoxic and cytotoxic effects induced by fluoride, the adverse influence of fluoride on the reproductive system has become a major concern in many countries. The importance of reproductive health to offspring developments has prompted epidemiological investigations of the apparent connection between excessive fluoride exposure to male infertility and low birth rates (Sun *et al.,* 2009). Sperm quality is one of the important indices of male reproductive function. Changes in sperm quality induced by fluoride have been demonstrated *in vivo* and *in vitro* in many species, including the rat, mouse, rabbit, gerbil, guinea pig, bank vole, chicken and even people (Wan *et al.,* 2006). However, experimental results differ. Some reports indicate that sodium fluoride does not affect sperm quality in rats (Sprando *et al.,* 1997; Collins *et al.,* 2001) whereas other experimental studies suggest that fluoride can cause low sperm quality and diminished fertility (Ghosh *et al.,* 2002; Wan *et al.,* 2006). There are many conflicts regarding importance of fluoride for health and its toxic effects on vital physiological

functions of man and animals. Therefore, in view of the aforesaid controversies, the present investigation was undertaken to study the effect of sodium fluoride on seminal characteristics and histoarchitecture of testicles of male Wistar rats.

MATERIALS AND METHODS

Experimental design, animals and test chemical
The experimental investigation was planned to adjudge the toxicopathological effects of sodium fluoride on gametes and the gonads of the male Wistar rats after obtaining approval from Institutional Animal Ethics Committee. The rats were maintained under regular supervision in controlled environment with 12 hours light dark cycle and provided with standard feed and water ad libitum throughout the experimental period. The rats were acclimatized for 10 days before commencement of the experimental study. Twelve healthy male Wistar rats weighing 220 - 235g were randomly divided into four different groups having 3 male rats each. Rats of group I served as control and was given only distilled water, orally. Rats of group II, III and IV were administered NaF orally, dissolved in distilled water, at the dose rate of 5.0, 25.0 and 50.0 mg/kg body weight for 28 days in 1mL of distilled water once daily at morning hours between 7- 8 am. The test chemical sodium fluoride of 99% purity was obtained from Merck Limited, Mumbai, India- 400018.

Evaluation of Spermatozoa for Male Reproductive Toxicity

At the end of the experimental period, the rats were humanely sacrificed under diethyl ether anaesthesia and the testicles were withdrawn so as to visualise cauda epididymis.

Epididymal sperm count

Epididymal sperm collection and sperm counting were carried out as per the procedure suggested by Khan and Sinha (1996). Firstly, both distal cauda epididymis were collected from rat in petridish and after several stabs on cauda with 18 G needle, it was placed in a pre-labelled and pre-warmed (37ºC) test tube containing 4mL normal saline. Then the sperms were allowed to disperse for 5 minutes and sperm counts were made by transferring small amount of the diluted suspension in the Neubauer's chamber using a Pasteur pipette by touching the edge of the cover slip allowing each chamber to be filled by capillary action. Sperms were observed at high power in five large squares.

Sperm motility

Epididymal sperm motility was carried out as per the procedure outlined by Saalu *et al.* (2010). Briefly, 10 - 20μL of cauda epididymal sperm suspension in normal saline was taken on a prewarmed slide. After applying a cover slip, the sperm motility and progressive sperm motility were recorded in percentage (%) at low power followed by high power objective lens.

Percentage viability assay/live and dead sperm count

The microscopic identification of live and dead spermatozoa was carried out following the method suggested by Roberts (1971). A drop of the epididymal sperm suspension was placed near the frosted edge of a clean prewarmed glass slide and mixed gently with small drop of eosin (3%) - nigrosin (10%) stain in 3% sodium citrate dihydrate as buffer. After a few seconds smears were made to study the stained (dead, eosinophilic) and unstained (live) spermatozoa in entire slide over a dark background produced by nigrosin. Two hundred spermatozoa of each rat were counted at high power and live and dead spermatozoa counts were recorded in percentage (%).
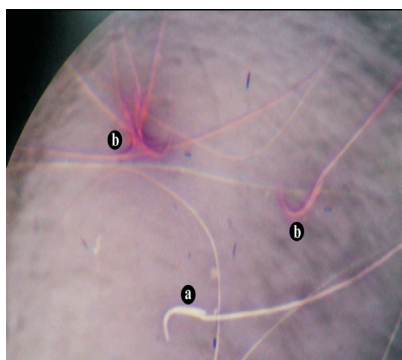
Sperm morphology

Evaluation of sperm morphology was carried out as per the method suggested by Linder *et al.* (1990). Firstly, approximately 1mL of sperm suspension collected previously was transferred to a pre-labeled test tube. Then 2 drops of 1% eosin Y was added to the test tube and mixed by gentle agitation by tapping at the bottom with a finger tip. The test tube with its contents was incubated at room temperature for 10 minutes to facilitate the staining. The slides were prepared by usual manner like preparation of thin blood smear on clean glass slides. About 200 sperms/ rat from each experimental group were examined at high power and the percentages of spermatozoa abnormalities (head, tail and/or multiple) were recorded.

Organosomatic index of testis

Following humane sacrifice the testes were carefully removed, blotted free of blood and weighed (in grams) over electronic digital balance. The organo-somatic index (OSI) was calculated by using the following formula as per Chattopadhyay *et al.* (2011).

$$\text{Organo-somatic Index (OSI)} = \frac{\text{Organ weight (g)}}{\text{Live body weight (g)}} \times 100$$

Histopathology of testis

The testicular tissue samples were collected in modified Davidson's fluid as outlined by Latendresse *et al.* (2002) in pre-labeled tissue sampling containers for histopathological examination. The tissues were thoroughly washed in running water; dehydrated in ascending grades of alcohol; cleared in benzene and embedded in paraffin at 58ºC. The paraffin embedded tissue sections of 4 - 5μm were obtained as described by Luna (1972) and stained with haematoxylin and eosin (H and E) as per the method described by Bancroft and Stevens (1990) with slight modifications. The stained sections were examined under light microscope and the lesions were recorded, if any.

Statistical analysis

Statistical analysis was done using complete randomized design (CRD) -single factor analysis of variance by Snedecor

Figure 1: Spermatozoa of rat in subacute NaF toxicity study - Live (a, white in colour) and dead (b, pink in colour). Eosin- Nigrosin stain, 400x
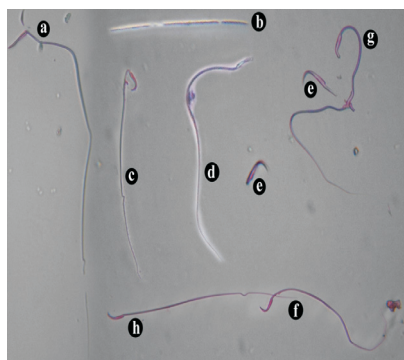

Figure 2: Various spermatozoa abnormalities in rats of group II and III. Eosin/Eosin – Nigrosin, 400x; a. Flattened head; b. Free tail (Headless sperm); c. Bent head and kinked tail; d. Banana head; e. Free head; f. Curved tail with a protoplasmic droplet; g. Banana head with curved tail; h. Flattened head with kinked tail
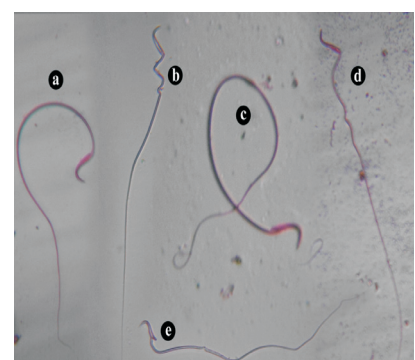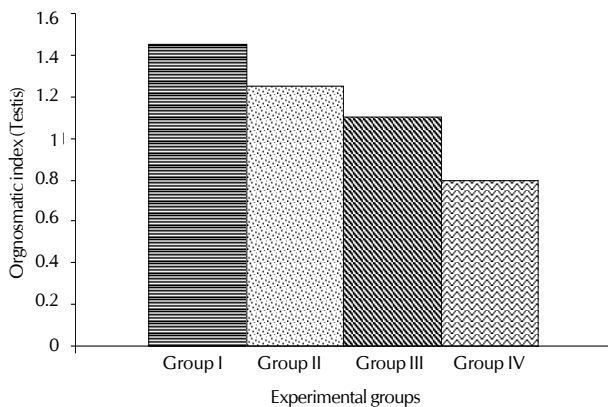

Figure 3: Multiple abnormalities of spermatozoa of rats of group IV. Eosin/ Eosin – Nigrosin, 400x; a. Bent neck and curved tail; b."Corkscrew" middle piece; c. Coiled middle piece and tail end; d. Kinked neck and tail; e. Abaxial attachment of the neck and middle piece to the head

Figure 4: Organosomatic index of testicles in experimental groups following subacute sodium fluoride toxicity

and Cochran (1968). The mean values between treatment and control groups were tested for critical difference (CD), if any. The results are expressed as Mean ± S.E.

RESULTS

Epididymal sperm evaluation

Intoxication of NaF to Wistar rats of group II, III and IV caused substantial (p ≤ 0.01) decrease in epididymal sperm motility, progressive sperm motility, sperm concentration and live spermatozoa (%) along with simultaneous increase in dead spermatozoa (%) as compared to the rats of control group (group I). The dose dependent increase in percent morphological abnormalities (head, tail and multiple) of spermatozoa were highly significant (p ≤ 0.01) in group III and group IV as compared to rats of group I and group II. Further, the spermatozoa of group II rats showed non significant increase in percent tail and multiple sperm abnormalities but significantly (p ≤ 0.05) increased head abnormality as compared to the rats of control group (group I). Overall, the maximum mean values for various sperm abnormalities (%), dead spermatozoa (%) and lowest mean values for sperm motility, epididymal sperm concentration and live spermatozoa (%) were recorded in group IV rats exposed to the repeated dose subacute oral toxicity of NaF (250mg/kg body weight). The consequences of daily oral administration of NaF on seminal characteristics of male Wistar rats is summarised in Table 1. The spermatozoa vitality and various

morphological abnormalities recorded in the present study have been shown in Fig. 1, 2 and 3 respectively.

Organosomatic index of testis

When compared with the control rats of group I, the OSI of testis was significantly decreased in (p < 0.05) in group II. The decrease (p < 0.01) was more pronounced in group III and IV. The results have been summarised and presented in Fig. 4.

Pathomorphology of testis

No appreciable gross lesions were seen in testes of all the experimental groups. However, there were significant pathomorphological changes in histoarchitecture of testicles of intoxicated rats. The microscopic lesions in testis of group IV rat showed moderate necrotic changes in different layers of seminiferous tubules along with their defoliation. Abnormal seminifeous tubules having increased size of lumen with clumped spermatids were also noticed. Congestion in testicular vessels was observed (Fig. 5 and 6). Similar but, moderate to milder changes were also observed in the histologic sections of group III rats (Fig. 7 and 8).The histological sections of testis in group II (Fig. 9) and group I rats was apparently normal.

DISCUSSION

Epididymal sperm evaluation

The results of our study are in accordance with Huang *et al.* (2007) who reported that exposure to high concentrations of NaF (200ppm and 300ppm) in drinking water lead to decrease sperm count, sperm motility, sperm survival and an increased sperm abnormality in male mice. Wan *et al.* (2006) also reported a decline in sperm viability, sperm density and a significant increase of sperm abnormalities by NaF at 150mg/L in the drinking water when given to male Wistar rats for 50, 80, 100 and 120 days. Akin to our findings, Kour and Singh (1980) also reported that, the testicular spermatogenic process was affected in mice administered with fluoride at a dose of 500 and 1000ppm in drinking water. On the other hand, Li *et al.* (1987) claimed that fluoride did not have adverse effects on spermatogenesis or sperm morphology. Similarly, Collins *et al.* (2001) also indicated that fluoride does not adversely affect spermatogenesis or endocrine function at 25, 100, 175 and 250ppm in the drinking water of male rats. However, Cui *et al.* (2003) found that NaF at the dose rate of 150ppm in drinking water for 10 weeks caused a significant decrease in sperm count and sperm motility of male rats.

Table 1: Effect of daily oral administration of sodium fluoride on seminal characteristics of male Wistar rats (n = 3)

| Various Sperm Parameters	Experimental Groups			
	Group I	Group II	Group III	Group IV
Sperm Motility (%)	81.0 ± 0.58 [a]	76.0 ± 1.15 [b**]	61.0 ± 0.58 [c**]	53.0 ± 0.58 [d**]
Sperm Progressive motility (%)	67.67 ± 0.33 [a]	54.33 ± 0.33 [b**]	41.67 ± 0.88 [c**]	36.67 ± 0.33 [d**]
Spermatozoa Concentration (millions/ml)	84.33 ± 0.88 [a]	75.67 ± 0.88 [b**]	60.33 ± 0.88 [c**]	36.0 ± 0.58 [d**]
Live Spermatozoa (%)	80.0 ± 0.58 [a]	77.33 ± 0.33 [b**]	71.33 ± 0.33 [c**]	57.33 ± 0.33 [d**]
Dead Spermatozoa (%)	20.0 ± 0.58 [d]	22.67 ± 0.33 [c**]	28.67 ± 0.33 [b**]	42.67 ± 0.33 [a**]
Sperm Head abnormalities (%)	6.0 ± 0.00 [c]	6.67 ± 0.33 [c*]	10.67 ± 0.33 [b**]	13.0 ± 0.00 [a**]
Sperm Tail abnormalities (%)	6.67 ± 0.88 [b]	10.0 ± 0.58 [a]	12.00 ± 1.00 [a**]	12.33 ± 0.33 [a**]
Multiple Sperm abnormalities (%)	7.00 ± 0.58 [c]	7.33 ± 0.67 [c]	11.67 ± 1.45 [b**]	23.0 ± 0.58 [a**]

Values indicate Mean ± S.E. Superscripts may read row wise for comparison of means. Mean values with similar superscripts do not differ significantly from each other. *Indicates significant at p ≤ 0.05 and **indicate significant at p ≤ 0.01 from control.
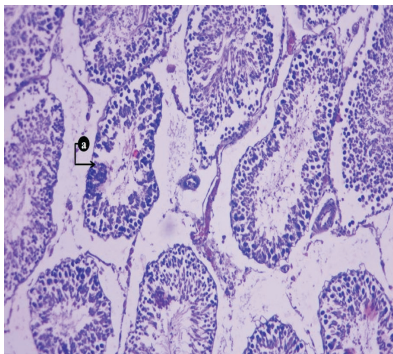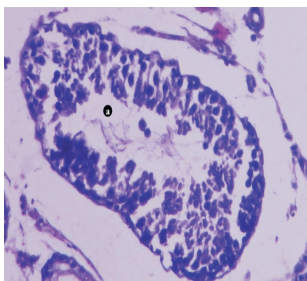
Figure 5: Testis of group IV rat (50 mgNaF/ kg body weight, orally) showing moderate necrotic changes in different layers of seminiferous tubules (a) together with clumping of spermatids in distorted seminiferous tubules. H and E. 100x
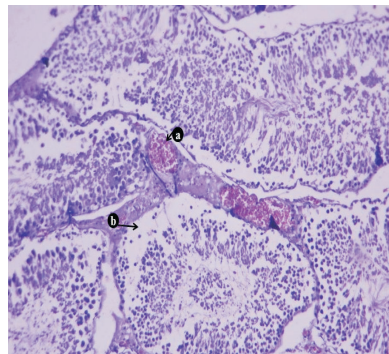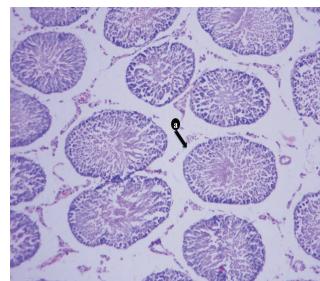

Figure 6: Testis of group IV rat (50 mgNaF/ kg body weight, orally) showing congestion in testicular vessel (a) and defoliation of spermatogonial cells in distorted seminiferous tubules (b). H and E. 100x
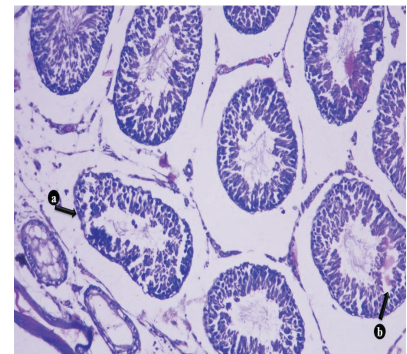
Figure 7: Testis of group III rat (25 mgNaF/ kg body weight, orally) showing mild degenerative changes of spermatogonial cells (a) and serous exudation in seminiferous tubules (b). H&E. 100x.

Figure 8: Higher magnification of Fig. 7 showing defoliated cells and scanty spermatids in the lumen of seminiferous tubules (a). H&E. 400x

Figure 9: Testis of group II rat (5 mgNaF/kg body weight, orally) showing apparently normal seminiferous tubules (a). H&E. 100x

The effect of fluoride toxicity on spermatogenesis might be because fluoride reduces the testosterone levels and by reducing the testicular zinc levels, it impairs angiotensin-converting enzyme activity and hence causes inhibition of spermatogenesis (Ghosh *et al.,* 2002). However, Wan *et al.* (2006) reported that epidermal growth factor and its receptor, which plays an important role in male reproductive functions of rat, were significantly decreased in Leydig cells, spermatogonia and spermatocytes on exposure to 68ppm fluoride for 10 days.

According to Pati and Bhunya (1987), NaF also induces cytogenetic damage in bone marrow and sperm cells viz. chromosomal aberrations, micronuclei and alterations in sperm morphology respectively. Dominok and Miller (1990) also reported that NaF induces recessive lethal mutations in the germ cells of male fruitfly (*Drosophila melanogaster*).

Epididymis is the site for maturation of spermatozoa. Spermatozoa acquire motility and fertilizability in epididymis. Therefore, normal internal milieu of the epididymis is necessary for maintenance of structural and functional integrity of sperms. NaF treatment disturbs the homeostasis in the epididymis by hampering sialomucoproteins (essential for maturation of spermatozoa and maintenance of structural integrity of sperm) leading to alteration in sperm motility, viability and morphology along with a resultant decrease in fertilizability of spermatozoa. By and large, epididymis is very prone to NaF induced alteration

causing lowered semen quality (Chinoy *et al.* 1995; Chinoy *et al.* 1997).

Dose dependent reduction in values of cauda epididymal spermatozoa concentration by NaF might be due to the adverse effect of NaF on epididymis and resulting malformations. The decreased vitality of spermatozoa with an increase occurrence of dead (necrosed) spermatozoa and abnormal spermatozoa in our study might be due to increased lipid peroxidation, mutagenicity and cell membrane toxicity to gonadal cells by fluoride. The increased abnormalities of spermatozoa observed in our study suggest that NaF might lead to genotoxic damage in germ cells. A more severe level of NaF produced higher levels of abnormal sperm, showing a dose dependent response in the present study.

Pathomorphology of testis

The findings on the altered histoarchitecture of testis corroborates well with the earlier reports. Chinoy and Sequeira (1989) reported that 30 days of treatment with NaF (10 mg/kg body weight) to mice resulted in defoliation of the spermatogenic cells in the luminal region of seminiferous tubules of the testis leading to disorganisation of their epithelium. This caused a complete absence of spermatogenesis in the testis. Wan *et al.* (2006) also found that NaF (150mg/L of drinking water) caused disorganization, denudation and reduction in germinal epithelial cells of the seminiferous tubules and an accompanying absence of sperm in the lumina in histological sections of rats.

Sekhar *et al.* (2011) found that cypermethrin and NaF - treated mice testes exhibited clumped spermatozoa, vacuolation, severe necrosis and degenerative changes with increased lumen of seminiferous tubules. Beside these degenerative changes in spermatids, atrophied seminiferous tubules, necrotic changes in theca albuginea and scattered spermatids were also reported. Sakr and Azab (2001) reported abnormal seminiferous tubules with many vacuoles, marked reduction in spermatogenic cells and degenerated Leydig cells in albino rats inhaling pyrethroids. In addition, Manna *et al.* (2004) found edema between seminiferous tubules, vacuolization and hyalinization in the tubules of the testes of rats exposed to a-cypermethrin. In another study Ghosh *et al.* (2002) reported

a decrease in luminal sperm and apparent dilation of tubules and opined that oxidative stress might be associated with testicular damage from NaF in rats.

The histopathologic findings in the present study, justifies the findings noted during cauda epididymal spermatozoa evaluation. Therefore, it might be concluded that NaF in higher doses acts as a potential reproductive toxicant leading to significant alteration in internal milieu of testicular tissue, altered semen characteristics and various morphological abnormalities in spermatozoa. The cytotoxicity of spermatogonial cells and spermatozoa by fluoride; is an important finding in the present study in view of the increasing endemicity of fluorosis in Indian as well as the global scenario.

ACKNOWLEDGEMENT

REFERENCES

Bancroft, J. D. and Stevens, A. 1990. Theory and practice of histological techniques. *Churchill Livingstone, Edinburgh*, pp- 113-305.

Chattopadhyay, A., Podder, S., Agarwal, S. and Bhattacharya, S. 2011. Fluoride - induced histopathology and synthesis of stress protein in liver and kidney of mice. *Arch. Toxicol.* **85:** 327-335.

Chinoy, N. J. and Sequeira, E. 1989. Effects of fluoride on the histoarchitecture of reproductive organs of the male mouse. *Reprod. Toxicol.* **3:** 261-267.

Chinoy, N. J., Shukla, S., Walimbe, A. S. and Bhattacharya, S. 1997. Fluoride toxicity on rat testis and cauda epididymal tissue components and its reversal. *Fluoride.* **30:** 41-50.

Chinoy, N. J., Narayana, M. V., Dalal, V., Rawat, M. and Patel, D. 1995. Amelioration of fluoride toxicity in some accessory reproductive glands and spermatozoa of rat. *Fluoride.* **28:** 75-86.

Chouhan, S. and Flora, S. J. S. 2010. Arsenic and fluoride: two major ground water pollutanats. *Indian J. Exp. Biol.* **48:** 666-678.

Collins, T. F. X., Sprando, R. L., Black, T. N., Shackelford, M. E., Bryant, M. A., Olejnik, N., Ames, M. J., Rorie, J. I. and Ruggles, D. I. 2001. Multigenerational evaluation of sodium fluoride in rats. *Food. Chem. Toxicol.* **39:** 601-613.

Cui, L. X., Jiang, C. X., Wang, X. L. and Chen, X. M. 2003. Experimental study on effect of fluoride on reproductive system of male rats. *Chin. J. Endemiol.* **22:** 195-197.

Dominok, B. and Miller, G. 1990. Effects of fluoride on *Drosophila melanogaster* in relation to survival and mutagenicity. *Fluoride.* **23:** 83-91.

Ghosh, D., Das, S. S., Maiti, R., Jana, D. and Das, U. B. 2002. Testicular toxicity in sodium fluoride treated rats: association with oxidative stress. *Reprod. Toxicol.* **16:** 385-390.

Huang, C., Niu, R. and Wang, J. 2007. Toxic effects of sodium fluoride on reproductive function in male mice**.** *Fluoride.* **40:** 162-168.

Khan, P. K. and Sinha, S. P. 1996. Ameliorative effects of vitamin C on murine sperm toxicity induced by three pesticides (endosulphan, phosphamidon, and mancozep). *Mutagen.* **11:** 33-36.

Kour, K. and Singh, J. 1980. Histological findings in mice testes following fluoride ingestion. *Fluoride.* **13:** 160-162.

Latendresse, J. R., Warbrittion, A. R., Jonassen, H. and Creasy, D. M. 2002. Fixation of testes and eyes using a modified Davidson's fluid: comparison with Bouin's fluid and conventional Davidson's fluid. *Toxicol. Pathol.* **30:** 524-533.

Li, Y., Dunipace, A. T. and Stookey, G. K. 1987. Effects of fluoride on the mouse sperm morphology test. *J. Dent. Res.* **66:** 1509-1511.

Linder, R. E., Strader, L. F., Barbee, R. R., Rehnberg, G. L. and Perreault, S.D. 1990. Reproductive toxicity of a single dose of 1, 3 - dinitrobenzene in two ages of young adult male rats. *Fundam. Appl. Toxicol.* **14:** 284-298.

Luna, L. G. 1972. Manual of histological staining methods of the Armed Forces Institue of Pathology. *W. B. Saunders, Philadelphia*.

Manna, S., Bhattacharya, D., Basak, D. K. and Mandal, T. K. 2004. Single oral dose toxicity study of a-cypermethrin in rats. *Indian J. Pharmacol.* **36:** 25-28.

Pati, P. and Bhunya, S. 1987. Genotoxic effect of an environmental pollutant, sodium fluoride in mammalian *in vivo* test system. *Caryologia.* **40:** 79-87.

Roberts, S.J. 1971. Infertility in male animals in Veterinary obstetrics and genital diseases (Theriogenology). *CBS pub. and distri.* New Delhi. pp- 712-719.

Saalu, L. C., Kpela, T., Benebo, A. S., Oyewopo, A. O., Anifowope, E. O. and Oguntola, J. A. 2010. The dose-dependent testiculoprotective and testiculotoxic potentials of *telfairia occidentalis hook f.* leaves extract in rat. *Int. J. Appl. Res. Nat. Prod.* **3:** 27-38.

Sakr, S. A. and Azab, A. E. 2001. Effect of pyrethroid inhalation on the testis of albino rat. *Pak. J. Biol. Sci.* **4:** 498-500.

Sekhar, P. R., Savithri, Y., Kishore, S., Jayasankar, A. and Rao, K. J. 2011. Synergistic effect of sodium fluoride and cypermethrin on the somatic index and histopathology of albino mice testes**.** *Fluoride.* **44:** 103-111.

Sprando, R. L., Collins, T. F. X., Black, T. N., Rorie, J. I., Ames, M. J. and O'Donnell, M. 1997. Testing the potential of sodium fluoride to affect spermatogenesis in the rat. *Food Chem. Toxicol.* **35:** 881-890.

Snedecor, G. W. and Cochran, W. G. 1968. Statistical methods. *Iowa State University Press, Ames, Iowa.*

Sun, Z., Wang, B., Niu, R., Zhang, J. H. and Wang, J. D. 2009. Decreased sperm hyperactivation and low catsper1 expression in mice exposed to fluoride. *Fluoride.* **42:** 167-173.

Wan, S. X., Zhang, J. H. and Wang, J. D. 2006. Effects of high fluoride on sperm quality and testicular histology in male rats. *Fluoride.* **39:** 17-21.

INSTRUCTION TO AUTHORS

The Bioscan

An International Quarterly Journal of Life Science

THE JOURNAL

The Bioscan is an international quarterly journal of life sciences with international editorial board. The journal is online and details can be seen (downloaded from the site. www.thebioscan.in). For any query e-mail at m_psinha@yahoo.com & dr.mp.sinha@gmail.com can be used.

AIM & SCOPE

The journal aims to publish original peerly reviewed/ refereed research papers/reviews on all aspects of life sciences.

SUBMISSION OF MANUSCRIPT

Only original research papers are considered for publication. The authors may be asked to declare that the manuscript has not been submitted to any other journal for consideration at the same time. Two hard copies of manuscript and one soft copy, complete in all respects should be submitted. The soft copy can also be sent by e-mail as an attachment file for quick processing of the paper.

FORMAT OF MANUSCRIPT

All manuscripts must be written in English and should be typed double-spaced with wide margins on all sides of good quality A4 paper.

First page of the paper should be headed with the title page, (in capital, font size 16), the names of the authors (in capitals, font size 12) and full address of the institution where the work was carried out including e-mail address. A short running title should be given at the end of the title page and 3-5 key words or phrases for indexing.

The main portion of the paper should be divided into Abstract, Introduction, Materials and Methods, Results, Discussion (or result and discussion together), Acknowledgements (if any) References and legends.

Abstract should be limited to 200 words and convey the main points of the paper-outline, results and conclusion or the significance of the results.

Introduction should give the reasons for doing the work. Detailed review of the literature is not necessary. The introduction should preferably conclude with a final paragraph stating concisely and clearly the aims and objectives of your investigation.

Materials and Methods should include a brief technical description of the methodology adopted while a detailed description is required if the methods are new.

Results should contain observations on experiment done illustrated by tables and figures. Use well known statistical tests in preference to obscure ones.

Discussion must not recapitulate results but should relate the author's experiments to other work on the subject and give their conclusions.

All tables and figures must be cited sequentially in the text. Figures should be abbreviated to Fig., except in the beginning of a sentence when the word Figure should be written out in full.

The figures should be drawn on a good quality tracing/ white paper with black ink with the legends provided on a separate sheet. Photographs should be black and white on a glossy sheet with sufficient contrast.

References should be kept to a minimum and listed in alphabetical order. Personal communication and unpublished data should not be included in the reference list. Unpublished papers accepted for publication may be included in the list by designating the journal followed by "in press" in parentheses in the reference list. The list of reference at the end of the text should be in the following format.

1. **Witkamp, M. and Olson, J. S. 1963.** Breakdown of confined and non-confined Oak Litter.*Oikos.* **14**:138-147.

2. **Odum, E.P. 1971.** *Fundamentals of Ecology.* W. B. Sauder Co. Publ. Philadelphia.p.28.

3. **Macfadyen, A.1963.** The contribution of microfauna to total soil metabolism. In:*Soil organism*, J. Doeksen and J. Van Der Drift (Eds). North Holland Publ. Comp., pp 3-16.

References in the text should be quoted by the **author's name and year** in parenthesis and presented in year order. When there are more than two authors the reference should be quoted as: first author followed by *et al.,* throughout the text. Where more than one paper with the same senior author has appeared in on year the references should