BioMetals (2005) 18:207–212
DOI 10.1007/s10534-005-0336-2

Exposure to high fluoride concentration in drinking water will affect spermatogenesis and steroidogenesis in male albino rats

T. Pushpalatha, M. Srinivas & P. Sreenivasula Reddy*
*Department of Biotechnology, Sri Venkateswara University, Tirupati – 517 502, India; *Author for correspondence (Tel.: +91-877-2249320; Fax: +91-877-2249611; E-mail: reddy_1955@yahoo.co.in)*

Received 30 November 2004; accepted 10 January 2005; Published online: March 2005

Key words: sodium fluoride, sperm, male rat, testes, steroidogenic marker enzymes

Abstract

Sodium fluoride (NaF) administered orally to adult male rats at a dose level of 4.5 ppm and 9.0 ppm for 75 days caused significant decrease in the body weight, brain index and testicular index. A significant decrease in sperm count, sperm motility, sperm viability and sperm function (HOS positive) with increased sperm abnormalities was also observed in NaF-exposed male rats. The activity levels of testicular steroidogenic marker enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) were significantly decreased in NaF-treated rats indicating decreased steroidogenesis and in turn spermatogenesis in rats exposed to NaF.

Introduction

Fluoride is a wide spread natural pollutant with established toxic effects. Fluoride has a tendency to accumulate in the organisms, if the exposure persists over time, even at low concentrations (Groth 1975). Excess fluoride is reported to cause severe harmful effects on plants (Boese *et al.* 1995; Dogeroglu *et al.* 2003), insects (Davies *et al.* 1998), frogs (Agalokova *et al.* 2000; Goh & Neff 2003), livestock (Suttie 1977, 1980) and humans (Lamberg *et al.* 1997).

Most of the research on environmental toxic effects of fluoride pollution has been confined to measuring potential risks in human and other mammalian models (Chinoy & Sequeira 1989; Chinoy *et al.* 1991; Kumar & Susheela 1995; Barot 1998; Oritz *et al.* 2003). An epidemiological study on humans showed an association of decreasing total fertility rate with increasing environmental fluoride levels (Freni 1994). Infertility was found in an area of India where fluorosis is highly endemic (Neelam *et al.* 1987). Susheela & Jethanandani (1996) reported decreased testosterone concentration in skeletal fluorosis patients and suggested that fluoride toxicity may cause adverse effects in the reproductive system of males living in fluorosis endemic areas. The present study describes the responses of male rats to fluoride taking sperm analysis and marker steroidogenic enzymes as reproductive indices. The concentrations of fluoride used in the present study is equivalent the concentrations of fluoride in nature, in some parts of Andhra Pradesh, India (Ramamohan Rao & Bhaskaran 1964).

Material and methods

Maintenance of the animals

Wistar strain rats bred at Department of Biotechnology animal facility, Sri Venkateswara University were maintained under a controlled light:dark (12:12 h) schedule at 23 ± 1 °C and provided food and water *ad libitum*. The rat feed was purchased from Kamadhenu Agencies, Bangalore, India. Healthy young male rats of same

age group (40 ± 2 days) were taken from parental stock for present study. They were kept in well-cleaned sterilized cages.

Chemical

Sodium fluoride (NaF) purchased from Central Drug Private Ltd., New Delhi, was used as test chemical.

Experimental design

Rats were divided into three groups consisting of six animals in each group. The animals of group I were allowed *ad libitum* access to tap water without NaF, while the animals of group II and group III were allowed *ad libitum* access to tap water containing either 4.5 ppm or 9.0 ppm NaF for 75 days. The body weights of rats were recorded at the initiation and termination of the experiment. The rats were sacrificed by cervical dislocation on 75th day of the experiment. Testes and brain tissues were quickly excised, weighed immediately and tissue index was calculated. The testes were also used for the determination of activity levels of selected steroidogenic marker enzymes.

Methods

Sperm count

Epididymis was excised at autopsy and placed in 10-ml vial containing 1.0 ml of physiological saline at 37 °C. A suspension of spermatozoa was prepared by mincing with a sterilized razor blade on a glass slide in a few drops of physiological saline. A drop of resulting sperm suspension was used for the analysis of total sperm count. The sperms were counted using Neubauer Chamber, as described by Belsy *et al.* (1980).

Sperm motility

Cauda epididymal tissue was teased in a 2 ml physiological saline to release the spermatozoa. Percent motility was determined using Neubauer Chamber, as described by Belsy *et al.* (1980) with in 5 min following their isolation from cauda epididymis at 37 °C.

Sperm viability

The ratio of live and dead spermatozoa was determined using 1% tryphan blue by the method of Talbot & Chacon (1981).

Morphological abnormalities

Sperms differing from normal structure can be considered as abnormal sperms. The morphological abnormalities in sperm were enumerated using light microscope and the percent of abnormality was calculated.

Hypoosmotic swelling test (HOS-test)

This test evaluates response of spermatozoa to hypoosmotic stress. The basis of this assay is, 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. The sperms were exposed to hypoosmotic medium and observed for coiled tails under the microscope and the percent of coiling was estimated by the method of Jeyendran *et al.* (1992).

Assay of testicular enzymes

The testicular tissue was homogenized in ice-cold Tris HCl buffer (pH 6.8). The microsomal fraction was separated and used as enzyme source. The activity levels of 3β-hydroxysteroid dehydrogenase (3β-HSD (EC 1.1.1.51) and 17β-hydroxysteroid dehydrogenase (17β-HSD) (EC 1.1.1.61) were measured by the method of Bergmeyer (1974). The enzyme assays were made under the conditions following zero order kinetics after preliminary standardization regarding linearity with respect to time of incubation and enzyme concentration.

The reaction mixture in a volume of 2.0 ml contained: 100 μmoles of sodium pyrophosphate buffer (pH 9.0), 0.5 μmol of cofactor NAD for 3β-HSD and NADPH for 17β-HSD, 0.08 μmol of substrate (dehydroepiandrosterone for 3β-HSD and androstenedione for 17β-HSD) and 20 mg equivalent of microsomal protein as enzyme source.

The reactions were carried out in a quartz cuvette of 1.0 cm path at 23 ± 1 °C. The absorbance at 340 nm was measured at 20 s intervals for

5 min in a UV–VIS spectrophotometer (Hitachi model U-2001) against controls. The enzyme activities were expressed in μmol of NAD converted to NADH/mg protein/min (3β-HSD) or μmol of NADPH converted to NADP/mg protein/min (17β-HSD).

Statistical analysis

The data were statistically analyzed by student's *t*-test (Pillai & Sinha 1968). $P < 0.005$ was considered significant.

Results

No mortalities were observed in control or in experimental groups. No behavioral abnormalities were observed in rats exposed to NaF.

A significant decrease in the total body weight was observed in male rats treated with NaF when compared with the control rats (Table 1). Brain index of the rats treated with 9.0 ppm NaF was significantly ($P < 0.0001$) decreased when compared with the control animals (Table 1). The decline of the testicular index in 9.0 ppm NaF-exposed rats was more (−34.50%) than the 4.5 ppm NaF-exposed animals (−10.00%).

The average sperm count in cauda epididymal plasma was found to be 42 ± 3.23 millions/gm testes in control rats. A significant ($P < 0.001$) depletion (−31.40%) in sperm count was observed in rats exposed to 4.5 ppm NaF, when compared with the control rats (Table 1). These decrease were more (−78.20%) in rats exposed to 9.0 ppm NaF (Table 1). A significant ($P < 0.001$) decrease in sperm motility was observed in NaF-treated rats, with a decrease in sperm viability (Table 1) when compared with the control rats.

All the sperms were apparently normal in control rats. Whereas in the rats exposed to NaF, many morphologically-altered sperms were observed. Two headed sperms, banana shaped head sperms, long tailed sperms and double headed sperms were the few abnormal sperms in NaF-exposed rats (Table 1). A significant ($P < 0.001$) decrease in sperm coiling percentage was observed in rats exposed to NaF (Table 1). The average sperm coiling in normal sperm is 60%, whereas the sperm coiling was decreased to 43% and 30% in rats exposed to 4.5 ppm and 9.0 ppm NaF, respectively (Table 1).

The results presented in Figures 1a and b indicate that 3β-HSD activity levels (0.012 ± 0.0018) were higher than the 17β-HSD activity levels (0.010 ± 0.0016) in rat testis. A significant decrease in the activity levels of 3β-HSD and 17β-HSD was observed in the testis of rats exposed to NaF. The decrease in the enzymes activity increased with increased dose of NaF when compared with the controls.

Table 1. Effect of sodium fluoride on body weight and sperm parameters in male rats.

Parameter	Control	4.5 ppm NaF-exposed	9.0 ppm NaF-exposed
Body weight (g)	329 ± 1.0	301.66 ± 7.63^{ns} (−8.31)	272.33 ± 3.05* (−17.22)
Brain index	1.18 ± 0.02	0.57 ± 0.35* (−51.27)	0.55 ± 0.08* (−53.30)
Testicular index	1.10 ± 0.01	0.90 ± 0.014* (−10.00)	0.72 ± 0.073* (−34.50)
Sperm count (millions/ml)	39 ± 1.44	26.73 ± 0.90* (−31.40)	8.5 ± 0.20* (−78.20)
Sperm viability (%)	81 ± 1.44	55.7 ± 1.83* (−31.20)	46.6 ± 2.16* (−43.20)
Sperm motility (%)	75.5 ± 6.36	48.8 ± 1.13* (−35.30)	38.5 ± 3.10* (−49.00)
Sperm physiological response (HOS coiling) (%)	60 ± 0.0	43 ± 2.8* (−28.30)	30.3 ± 2.16* (−49.50)
Abnormalities (%)	–	43.5 ± 0.7	46.6 ± 3.78
a. Banana shape	–	40%	–
b. Rod shaped sperms	–	–	40%
c. Two headed sperms	–	1%	3%
d. Long tailed sperms	–	1%	1%
e. Two tailed sperms	–	1%	2%

Values are mean ± S.D. of six individuals. Values in parentheses are percent change from control. *Significant at $P < 0.001$ when compared with control; ns = Not significant.

Discussion

Semen analysis including sperm morphology assessment has been suggested to be a useful indication of the factors in man's macro-environment, which can modulate or damage spermatogenesis (Mac Leod & Gold 1953). The present study was aimed to determine the reproductive toxic effects of male rat after ingestion of NaF through drinking water. The route chosen in this study for exposure was via drinking water to mimic human exposure and to reflect the impact on fertility, after chronic ingestion.

The decreased sperm number and motility observed in experimental rats might be responsible for decreasing male fertility. Decrease in male reproductive potential was observed in rats and rabbits after exposure to fluoride (Kumar & Susheela 1994, 1995; Narayana & Chinoy 1994; Zhang *et al*. 2000; Collins *et al*. 2001). Besides decreased sperm count, sperm motility, the sperm viability and HOS sperm coiling percentages were also adversely affected in NaF-exposed rats. These changes were greater in rats exposed to higher dose of NaF.

The decreased testicular steroidogenic enzyme activity levels may lead to decreased steroidogenesis in experimental rats, which in turn may suppress the reproductive activities in the male rats. Oral administration of NaF caused decrease in 3β-HSD and 17β-HSD along with significant diminution in plasma levels of testosterone (Ghosh *et al*. 2002). The decrease in testicular 17β-HSD activity levels with a decrease in male reproductive potential have been reported following exposure to several xenobiotics in rats (Pant *et al*. 1995, 1997; Pant & Srivastava 2003).

The activity level of 3β-HSD which is involved in the formation of pregnenolone from cholesterol

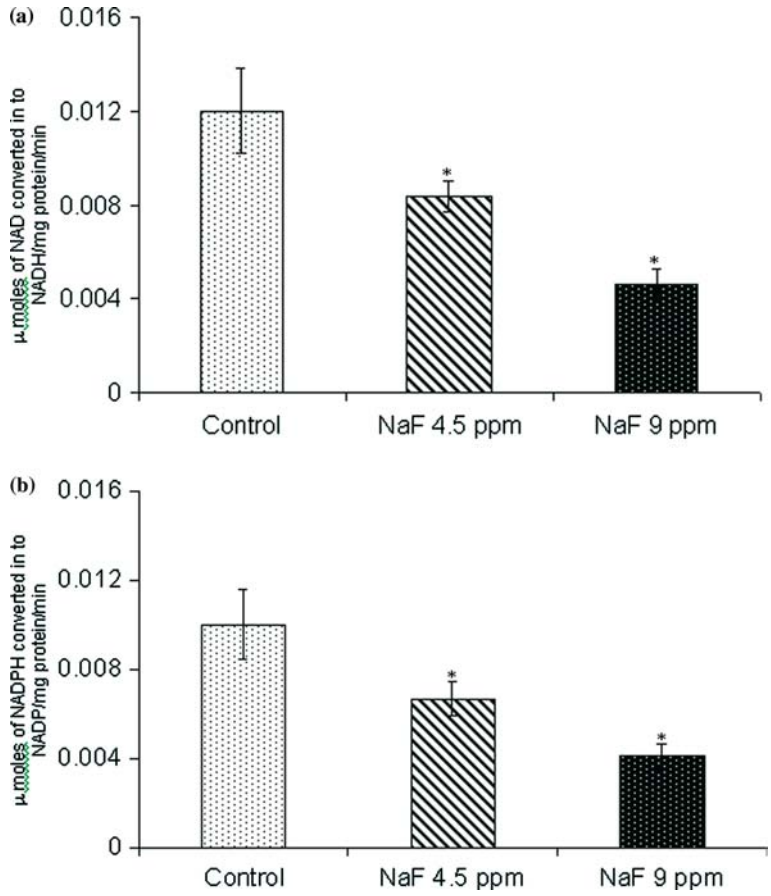

Figure 1. Effect of NaF on testicular 3β-HSD (a) and 17β-HSD (b) activity. Vertical bars represent mean $\pm$ SD of six rats. Values are significant at $^*P < 0.001$ from controls.

was significantly decreased and the decrease in enzyme activity affects steroid synthesis in three ways; (1) by reducing the conversion of pregnenolone to progesterone, (2) by reducing the conversion of 17β-pregnenolone to 17α-hydroxyprogesterone and (3) also by reducing the conversion of dehydroepiandrostenone (DHEA) to androstenodione. Similarly the decreased activity level of 17β-HSD, which denotes the degree of formation of androgen, has been found significantly inhibited in NaF-exposed rat testis.

In the present study, exposure to NaF resulted in alterations in the activity of testicular steroidogenic enzyme (3β-HSD and 17β-HSD) activities, along with a decrease in sperm count, sperm motility, viability and sperm function. In line with these results, significant reduction in reproduction was observed in screech owls collected from fluoride-polluted areas (Hoffman *et al*. 1985). Though extrapolation of rat data to human is not relevant but the sodium fluoride level in some parts of Andhra Pradesh is equivalent to the dose exposed to the rats in the present study.

It can therefore be concluded that the fluoride-exposed rats though apparently normal but exhibits poor reproductive efficiency as indicated by decreased sperm quality and quantity and that these parameters can be used as a good indicators of fluoride- induced reproductive toxicity in humans.

Acknowledgements

The authors thank Dr. K.V.S. Sharma, Professor of Statistics, Sri Venkateswara University for statistical analysis of data. We also thank S. Umasankar for maintaining the rat colony in the Department. The experiments were conducted following the rules and regulations of Department of Biotechnology Ethical Committee and comply with the laws of the country. Part of the data was submitted to Sri Venkateswara University in partial fulfillment for the award of the degree of Master of Science in Biotechnology by MS.

References

Agalokova WI, Lapin AV, Gusev GP. 2000 Effects of fluoride and vanedate on K^+ transport across the erythrocyte membrane of *Rana temporaria*. *Membr Cell Biol* **13**, 527–536.

Barot VV. 1998 Occurrence of endemic fluorosis in human population of North Gujurat, India: human health risk. *Bull Environ Contam Toxicol* **61**, 303–310.

Belsy MA, Moghissi KS, Eliasson R, Paulsen CA, Callegos AJ, Prasad MRN. 1980 *Laboratory Manual for the Examination of Human Semen and Semen Cervical Mucus Interaction*. Singapore: Press Concern.

Bergmeyer HU. 1974 β-hydroxysteroid dehydrogenase. In: Bergmeyer ed. *Methods of Enzymatic Analysis*, Vol. I. New York: Academic Press; 447–489.

Boese SR, Mac Lean DC, El-Mogazi D. 1995 Effects of fluoride on chlorophyll and fluorescence in spinach. *Environ Pollut* **89**, 203–208.

Chinoy NJ, Rao MV, Narayana MV, Neelakanta E. 1991 Microdose vasal injection of sodium fluoride in the rat. *Reprod Toxicol* **5**, 505–512.

Chinoy NJ, Sequeira E. 1989 Effects of fluoride on the histoarchitecture of reproductive organs of the male mouse. *Reprod Toxicol* **3**, 261–267.

Collins TF, Sprando RL, Black TN *et al*. 2001 Multigenerational evaluation of sodium fluoride in rats. *Food Chem Toxicol* **39**, 601–613.

Davies MT, Port GR, Davison AW. 1998 Effects of dietary and gaseous fluoride on the aphid *Aphis fabae*. *Environ Pollut* **99**, 405–409.

Dogeroglu T, Cicek A, Kara S. 2003 Short-term effects of hydrogen fluoride on *Nicotiana tobaccum* L. *J Environ Sci Health B* **38**, 561–570.

Freni SC. 1994 Exposure to high fluoride concentrations in drinking water is associated with decreased birth rates. *J Toxicol Environ Health* **42**, 109–121.

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

Goh EH, Neff AW. 2003 Effects of fluoride on *Xenopus* embryo development. *Food Chem Toxicol* **41**, 1501–1508.

Groth E. 1975 An evaluation of the potential for ecological damage by chronic low level environmental pollution by fluoride. *Fluoride* **8**, 224–240.

Hoffman DJ, Pattee OH, Wiemeyer SN. 1985 Effects of fluoride on screech owl reproduction: teratological evaluation, growth, and blood chemistry in hatchlings. *Toxicol Lett* **26**, 19–24.

Jeyendran RS, Vander Ven HH, Zaneveld LID. 1992 The hypoosmatic swelling test: an update. *Arch Androl* **29**, 105–116.

Kumar A, Susheela AK. 1994 Ultrastructural studies of spermatogenesis in rabbit exposed to chronic fluoride toxicity. *Int J Fert Men Std* **39**, 164–171.

Kumar A, Susheela AK. 1995 Effects of chronic fluoride toxicity on the morphology of ductus epididymis and the maturation of spermatozoa of rabbit. *Int J Exp Pathol* **76**, 1–11.

Lamberg M, Hausen H, Vartiamen T. 1997 Symptoms experienced during periods of actual and supposed water fluoridation. *Community Dent Oral Epidemol* **25**, 291–295.

Mac Leod J, Gold RZ 1953 The male factor in fertility and infertility, VII: semen quality in relation to age and sexual activity. *Fertil Steril* **4**, 194–199.

Narayana MV, Chinoy NJ. 1994 Effect of fluoride on rat testicular steroidogenesis. *Fluoride* **27**, 7–12.

Neelam K, Suhasini RV, Sudhakar RY. 1987 Incidence of prevalence of infertility among married male members of

endemic fluorosis district of Andhra Pradesh. In: *Abstract Proceedings of Conference of the International Society for Fluoride Research*. Nyon, Switzerland.

Ortiz-Perez D, Rodriguez-Martinez M, Martinez F *et al*. 2003 Fluoride-induced disruption of reproductive hormones in men. *Environ Res* **93**, 20–30.

Pant N, Shankar R, Srivastava SP. 1997 *In utero* and lactational exposure of carbofuran to rats: effect on testes and sperm. *Hum Exp Toxicol* **16**, 267–272.

Pant N, Srivastava SP. 2003 Testicular and spermatotoxic effects of quinalphos in rats. *J App Toxicol* **23**, 271–274.

Pant N, Srivastava SC, Prasad AK, Shankar S, Srivastava SP. 1995 Toxic effect of carbaryl on male reproductive system of rat. *Vet Hum Toxicol* **47**, 421–425.

Pillai SK, Sinha HC. 1968 *Statistical Methods for Biological Workers*. Agra, India: Ram Prasad and sons.

Ramamohan Rao NV, Bhaskaran CS. 1964 Endemic fluorosis; study of distribution of fluorine in water sources in the Kurnool district of Andhra Pradesh. *Indian J Med Res* **52**, 180–186.

Susheela AK, Jethanandani P. 1996 Circulating testosterone levels in skeletal fluorosis patients. *J Toxicol Clin Toxicol* **34**, 183–189.

Suttie JW. 1977 Effects of fluoride on livestock. *J Occup Med* **19**, 40–48.

Suttie JW. 1980 Nutritional aspects of fluoride toxicosis. *J Anim Sci* **51**, 759–766.

Talbot P, Chacon RS. 1981 A triple stain technique for evaluating normal acrosome reaction of human sperm. *J Exp Zool* **215**, 201–208.

Zhang C, Liang B, Liu J, Wang G. 2000 Toxic effect of fluoride–arsenic on the reproduction and development of rats. *Wei Sheng Yan* **29**, 138–140.