TOXIC EFFECTS OF SODIUM FLUORIDE ON REPRODUCTIVE FUNCTION IN MALE MICE

Chong Huang,[a,b] Ruiyan Niu,[a] Jundong Wang[a]
Shanxi, China

SUMMARY: To investigate the effects and possible mechanisms of the action of fluoride on testis cell cycle and cell apoptosis in male mice, sexually mature male Kunming mice were exposed to 50, 100, 200, and 300 mg NaF/L in their drinking water for 8 weeks. At the end of the exposure periods, sperm quality, the percentage of G1/G0 (Gap 1/Gap 0), S (synthesis), G2/M (Gap2/M, minosis), and apoptosis rate in testicle cells were measured using flow cytometry. Serum and testicular testosterone levels were determined with a radio immunoassay (RIA) kit. Effects on sperm quality and oxidative stress were also observed. Different dosages of NaF also altered the changes in the testicular cell cycle. Compared to the control, the testicular cell cycle of mice drinking 50 or 100 mg NaF/L was not significantly affected (P>0.05). However, with the higher NaF concentrations of 200 and 300 mg/L, the percentage of cells in G1/G0 phase increased significantly (P<0.01), whereas those in S phase decreased significantly (P<0.01). On the other hand, the percentage of cells in G2/M phase was similar to that of the control. In the two higher NaF concentration groups, distinct cell apoptosis of testis was observed. Likewise, sperm quality and antioxidant defenses were significantly reduced and oxidative stress occurred, whereas these effects were only slight at the lower NaF concentrations. Serum and testicular testosterone levels were also significantly lower at 100, 200, and 300 mg NaF/L (P<0.01), compared with the control group.

Keywords: Apoptosis; Cell cycle; Fluoride and male mice; Male mice; Mouse testis; Reproductive function; Testis and fluoride.

INTRODUCTION

High doses of fluoride (F) have been found to interfere with the reproductive system of animals. In addition to oxidative stress,[1-4] commonly observed effects of F in animals include damaged sperms,[5,6] reduced sperm count,[1] decreased testosterone levels,[1,9] reduced fertility,[7-9] and low birth rate.[10] As far as we are aware, laboratory studies on the effects of F on cell cycle and cell apoptosis in the testis have not yet been reported. Though testicular disorders associated with oxidative stress induced by F have been reported,[1] the detailed mechanism of these effects of F on the male reproductive system has not been elucidated.

The present study was designed to investigate the effects of F on cell cycle and cell apoptosis in testis in mice and to examine possible mechanisms of the toxic action of NaF on male reproductive function.

MATERIALS AND METHODS

Experimental animals: Six-week-old adult male Kunming mice weighing between 25 and 30 g, along with supplies of their standard diet, were obtained from the Experimental Animal Center of Shanxi Medical University. The study

[a]For Correspondence: Prof Jundong Wang, Shanxi Key Laboratory of Ecological Animal Science and Environmental Medicine, Shanxi Agricultural University, Taigu, Shanxi, 030801, PR of China; E-mail: wangjd@sxau.edu.cn [b]Peking University, Beijing, 100000, PR of China.

design was approved by the Institutional Animal Care and Use Committee of China.

Establishment of animal model: One hundred of the above mice were divided randomly into five groups of twenty animals each. The controls were given low-fluoride distilled water. Animals in the four experimental groups received 50, 100, 200, and 300 mg NaF/L, respectively, in their drinking water. After 8 weeks, all mice were weighed and injected with 20% urethane solution for fatal anaesthesia. Blood was collected from the eyeball for separation of serum. Testes and the epididymis were carefully removed, blotted free of blood, rinsed in distilled water, and used for further study.

Evaluation of sperm quality: The cauda epididymal sperm suspension was prepared in normal saline at 37ºC. Sperm count, sperm motility ratio, and the ratio of normal to abnormal sperm were measured according to the method of Cui et al.[11] Sperm survival ratio was determined by the method of Zhang et al.[12]

Separation of serum: Serum was immediately separated by centrifugation at 2000 r/min for 15 min and then stored at –20ºC for use in determining testosterone levels.

Preparation of testicular homogenates: For each mouse one of the two testes was weighed and washed two times with phosphate buffered saline (PBS: NaCl 8.0 g, KCl 0.2 g, $Na_2HPO_4 \cdot 12H_2O$ 2.8 g, KH_2PO_4 0.2 g/L). The tissue was then chopped into small pieces and homogenized in 1.0 mL of PBS. The homogenate was centrifuged at 3500 rpm for 15 min at 4ºC, and the supernatant was suitably diluted with PBS (usually 1:10) before use for radioimmunoassay of testicular testosterone levels.

Determination of testosterone levels: The serum level of testosterone was assayed by a radioimmunoassay kit provided by the Chinese Institute of Atomic Energy, Beijing, China. The inter-assay variation was 6.5% for testosterone. All the samples were run at the same time to minimize such variation.

Flow cytometry analysis of cell cycle and cell apoptosis: The procedure used to obtain testis monocellular suspensions is described in detail elsewhere.[13] Briefly, after removal of the tunica albuginea, parts of the left testis were first minced with surgical scissors and were filtered through nylon mesh. The resulting monocellular suspensions were centrifuged at 1000 r/min for 10 min. The supernatant layers were decanted and washed two times with PBS. Next they were resuspended in PBS, immediately fixed in 95% ethanol, and stored at –20ºC for 18 hr. The cell suspension was then adjusted to approximately 10^6 cells per mL in PBS. For each test, 1 mL of cell suspension was removed, washed three times with PBS, and then stained with 50 μg/mL of propidium iodide (PI) for 30 min in the dark at 37ºC. Flow cytometric analysis of the DNA content was performed using a Coulter Epics XL flow cytometer (Coulter Electronics, USA) equipped with a 488 nm argon ion laser.[14,23]

Testis protein and oxidative lesion indexes: After collection, testes were immediately weighed and then homogenized with 1:9 (w/v) 0.9% saline solution

at –4ºC. Total protein content, glutathione (GSH) activity, glutathione peroxidase (GSH-Px) activity, superoxide dismutase (SOD) activity, and the malondialdehyde (MDA) content in the testis tissue were determined with the reagent kit provided by the Nanjing Jianchen Biological Institute.

Statistical analysis: Data are expressed as mean ±SD. Differences between control and NaF-exposed groups were analyzed using SPSS 11.5 software. $P<0.05$ was considered significant.

RESULTS

Effects of NaF on sperm quality: As seen in Table 1, compared to the controls, a significant progressive decrease in epididymal sperm count ($p<0.01$) was observed at 200–300 mg NaF/L in the drinking water of the mice after 8 weeks. Moreover, even at 100–300 mg NaF/L, sperm motility, sperm survival, and sperm abnormality were also significantly affected with increasing severity ($p<0.05$ to $p<0.01$).

Table 1. Sperm quality in male mice after 8 weeks of exposure to different levels of NaF in their drinking water (n=8; mean±SD)

Groups (mg NaF/L)	Sperm count (10^6/ml)	Sperm motility (%)	Sperm survival (%)	Sperm abnormality (%)
Control	17.50±2.26	90.38±1.70	92.30±3.44	6.213±0.57
50	16.83±2.18	85.93±1.40	89.22±4.70	6.618±0.11
100	15.13±2.73	80.93±4.26*	80.18±3.99†	13.11±1.24†
200	14.30±1.04†	62.20±2.75†	69.70±9.50†	20.34±3.10†
300	11.10±2.03†	40.99±12.70†	67.07±3.27†	19.15±2.35†

*P<0.05; †P<0.01 (compared with the control group).

Effects of NaF on serum and testicular testosterone levels: As seen in Table 2, administration of NaF at 100, 200, and 300 mg/L significantly lowered serum and testicular testosterone levels but the decrease was not significant at 50 mg/L ($p<0.01$).

Table 2. Testosterone levels in serum and testis of male mice after 8 weeks of exposure to different levels of NaF in their drinking water (n=8; mean±SD)

Group (mg NaF/L)	Serum testosterone (ng/dL)	Tissue testosterone (ng/dL)
Control	238.03±12.02	204.40±21.34
50	230.23±4.44	181.33±5.13
100	205.59±8.08†	174.35±4.74†
200	151.54±5.73†	127.17±15.05†
300	139.93±2.73†	111.84±22.69†

*P<0.05; †P<0.01 (compared with the control group).

Effects of NaF on sperm protein and oxidative lesion indexes: As seen in Table 3, the testis MDA level decreased with increasing F concentration in an obvious dose-effect relationship. In comparison with the control group, the values of MDA in mice exposed to 200 and 300 mg NaF/L were significantly higher ($P<0.01$). However, even at 50 and 100 mg NaF/L, the increase was still significant ($P<0.05$). Table 3 also shows that the testis SOD, GSH-Px, and GSH activity levels decreased with increasing F concentration, showing a clear

dose-effect relationship. As compared with that of the control group, the activities of SOD and GSH-Px decreased markedly at 200 and 300 mg NaF/L ($P<0.01$). The testis GSH level decreased significantly at 50 and 200mg NaF/L ($p<0.05$), and 300 mg NaF/L ($p<0.01$), but not at 100 mg NaF/L.

Table 3. Protein, MDA, GSH levels and SOD and GSH-Px activity in testis of mice (n=8; mean±SD)

Groups (mg NaF/L)	Protein (mg/100mg tissue wt)	MDA (nmol/mg Pro)	SOD (U/mg Pro)	GSH-Px (U/mg Pro)	GSH (mg/mg Pro)
Control	10.78±2.46	0.67±0.29	204.65±1.29	10.08±0.56	27.83±3.05
50	9.21±0.84	1.03±0.39	205.68±26.39	9.15±0.89	23.77±1.79*
100	9.09±1.12	0.9±0.13	190.48±2.53	8.33±0.81†	26.80±0.88
200	8.83±0.57*	1.2±0.53†	164.73±15.70†	8.06±0.36†	23.70±2.36*
300	8.47±0.30*	2.0±0.56†	122.70±8.79†	7.11±0.71†	21.33±4.60†

*P<0.05; †P<0.01 (compared with the control group).

Effects of NaF on testicular cell cycle and cell apoptosis: When the mice were exposed to 50 and 100 mg NaF/L, no effect on cell cycle of testis was observed, but when they were exposed to 200 and 300 mg NaF/L, significant effects were observed, which included a increase in percentage of G1/G0 stage cell and a decrease in the percentage of S stage cells. The percentage of G2 stage cells showed no effect. A significant increase in cell apoptosis ratio was observed after exposure to 200 and 300 mg NaF/L. The cell apoptosis ratio in testes of the F-treated groups was higher than in the control group, and the apoptosis ratio increased with increasing doses of NaF.

Table 4. Effects of different concentrations of NaF on cell cycle in testis of mice (n=8; mean±SD)

Groups (mg/L)	G0/G1 (%)	S (%)	G2/M (%)	Apoptosis ratio (%)
Control	70.96±3.06	18.50±5.32	10.53±2.91	17.90±6.82
50	74.73±7.91	13.87±5.59	8.93±2.15	18.77±3.67
100	68.83±5.66	18.27±6.33	9.23±5.18	22.75±3.35
200	81.63±3.92*	5.70±1.78†	12.63±3.06	26.13±1.80*
300	81.57±6.86*	8.17±3.49*	10.27±3.63	33.80±4.98†

*P<0.05; †P<0.01 (compared with the control group).

DISCUSSION

Effects of NaF on sperm quality and serum and testicular testosterone levels: In previous investigations, we have demonstrated adverse effects of NaF at a single dose on the sperm quality in male rats.[15,16] The present study also shows similar effects in mice. Exposure to high concentrations of NaF (200 mg/L and 300 mg/L) was found to decrease sperm count, sperm motility, sperm survival, and to increase sperm abnormality, whereas lower concentrations of NaF (100 mg/L or even to 50 mg/L) resulted in only a slight influence on these indexes.

Male reproductive function is evaluated by the above indexes,[17] and they are directly related to the structure and function of the testis[18] which is regulated by the hypothalamic-pituitary-testicular axis (HPTA). Testosterone, which is produced by Leydig cells in the testis, plays an important role in this regulation process.[19] Recently, Reddy et al. reported that when the sperm-positive female rats were exposed to NaF during gestation and lactation, the serum testosterone of their

male offspring was significantly decreased.[20] Meanwhile, the sperm count, sperm motility, sperm coiling, and sperm viability in those male offspring were also decreased. In the present study, we found that with increasing concentrations of NaF the drinking water, serum and testicular testosterone levels in male mice show a distinct descending trend in a dose-dependent relationship and thus affect the sperm quality in mice.

Effects of NaF on sperm protein and oxidative lesion indexes: In the present study, there was evidence of oxidative stress in the testes of male mice exposed to different doses of F (as NaF), in agreement with other reports.[21-23] Because of its dense negative charge, F has a very strong hydrogen bonding capacity and is prone to bind various antioxidants and anti-oxidation enzymes. It thus interferes with many metabolic processes, including promotion of reactive free radical oxygen species that attack cell membranes and even lead to cell apoptosis.[24] It has also been confirmed that germ cells are more susceptible to oxidative stress than somatic cells.[21] Therefore it is plausible that F in the testis of mice causes an increase in MDA and a decrease in the activity of SOD and GSH-Px.

Effects of NaF on testicular cell cycle and cell apoptosis: In regard to the effects of F on testicular cell cycle and apoptosis in relation to reproductive function in mice, our findings clearly indicate that NaF can disrupt normal cell cycle and apoptosis. Compared with the control group, lower doses of NaF (50 and 100mg/L in the drinking water) had no significant effects on testicular cell cycle, but high doses of NaF (200 and 300 mg/L) administered for 8 weeks, caused a significant increase in the percentage of testicular cells in the G0/G1 phase of cell cycle, whereas the percentage in the S phase decreased significantly. These results indicate that F-induced inhibition of testicular cell proliferation may be attributed to blockage of the G1 phase. This dose-effect relation also appeared in the testis cell apoptosis ratios.

In apoptosis there is a balance between the withdrawal of positive signals and the receipt of negative signals.[25] Testosterone, which is secreted by Leydig cells in the testes, plays an important role in testicular cell proliferation and differentiation.[26] However, F can adversely affect the production of testosterone. Decreased serum levels of testosterone in both humans and experimental animals with skeletal fluorosis has been reported,[27,28] as found here. We therefore postulate that F diminishes positive signals for testosterone formation needed for continued growth of germ cells. On the other hand, one mechanism of cell apoptosis is the increased levels of oxidants within the cell that damage DNA.[28-30] At the same time, F can also affect oxygen metabolism and increase of reactive oxygen species.[16] Our results show that high doses of F in the mice caused an increase in MDA and a decrease in the activity of SOD and GSH-Px in their testes. Hence oxidative stress is considered a negative signal for adverse effects on testicular function.

In conclusion, one of the mechanisms of reproductive toxicity induced by F appears to be testicular cell metabolism dysfunction and cell cycle disruption characterized by blockage of phase G1/G0 and induction of cell apoptosis. Further

elucidation of details of the molecular mechanism of the effects of F on the male reproductive system awaits future experiments.

ACKNOWLEDGEMENTS

This research was sponsored by the China National Natural Science Foundation (Grant No. 30671545), the Shanxi Province Returnee's Science Foundation (Grant No. 2004043), and the Shanxi Province Science and Technology Bureau Program (Grant No. 2006026).

REFERENCES

1 Ghosh D, Das Sarkar S, Maiti R, Jana D, Das UB. Testicular toxicity in sodium fluoride treated rats: association with oxidative stress. Reprod Toxicol 2002;16(4):385-90.
2 Das SS, Maiti R, Ghosh D. Induction of oxidative stress on reproductive and metabolic organs in sodium fluoride treated male albino rats: protective effect of testosterone and Vitamin E co-administration. J Toxicol Mech Method 2005;15(4):271-7.
3 Krechniak J, Inkielewicz I. Correlations between fluoride concentrations and free radical parameters in soft tissues of rats. Fluoride 2005;38(4):293-6.
4 Chlubek D. Fluoride and oxidative stress. Fluoride 2003;36(4):217-28.
5 Chinoy NJ, Sequeira E. Effects of fluoride on the histoarchitecture of reproductive organs of the male mouse. Reprod Toxicol 1989;3(4):261-8.
6 Zakrzewska H, Udała J, Błaszczyk B. *In vitro* influence of sodium fluoride on ram semen quality and enzyme activities. Fluoride 2002;35(3):153-60.
7 Messer HH, Armstrong WD, Singer L. Influence of fluoride intake on reproduction in mice. J Nutr 1973;103(9):1319-26.
8 Chinoy NJ, Momin R, Sorathia HP, Jhala DD. Recovery from fluoride+aluminium toxicity in vas deferens, seminal vesicle, and ventral prostate of mice by vitamin C. Fluoride 2005;38(2):122-6.
9 Bataineh NH, Nusier MK. Impact of 12-week ingestion of sodium fluoride on aggression, sexual behavior, and fertility in adult male rats. Fluoride 2006;39(4):293-301.
10 Freni SC. Exposure to high fluoride concentration in drinking water is associated with decreased birth rates. J Toxicol Environ Health 1994;42(1):109-12.
11 Cui LX, Jiang CX, Wang XL, Chen XM. Experimental study on effect of fluoride on reproductive system of male rats. Chin J Endemiol 2003;22(3):195-7. [in Chinese].
12 Zhang XS, Zhu JL, Zhou YF, Jiang XZ, Wang YL. Study on the effects of lead acetate on spermatogenesis in male mice. Acta Acad Med Shanghai 1993;20(1):5-10. [in Chinese].
13 Spanň M, Pacchierotti F, Mauro F, Quaggia S, Uccelli R. Flow cytometric analysis of the effects of 0.4 MeV fission neutrons on mouse spermatogenesis. Int J Radiat Biol Relat Stud Phys Chem Med 1987;51(3):401-19.
14 Bonanni G, Calcagno A, Mammana G, Chemello E, Pennelli N, Mastrogiacomo I. DNA flow cytometry of left and right testes in normospermic patients affected by left varicocele. Hum Reprod 1997;12(1):64-7.
15 Wan SX, Zhang JH, Wang JD. Effects of high fluoride on sperm quality and testicular histology in male rats. Fluoride 2006;39(1):17-21.
16 Zhang JH, Liang C, Ma JJ, Niu RY, Wang JD. Effects of sodium fluoride and sulfur dioxide on sperm motility and serum testosterone in male rats. Fluoride 2006;39(2):126-31.
17 Zhao ZL, Wu NP, Gao WH. Influence of fluoride on contents of testosterone and cholesterol in rat. Fluoride 1995;28(3):128-30.
18 Wan SX, Zhang JH, Wang JD. Fluoride-induced changes in the expression of epidermal growth factor and its receptor in testicular tissues of young male rats. Fluoride 2006;39(2):121- 5.
19 Holstein AF, Schulze W, Davidoff MS. Understanding spermatogenesis is a prerequisite for treatment. Reprod Biol Endocrinol 2003;1:107. Available from: http://www.rbej.com/content/1/1/107

20 Reddy PS, Pushpalatha T, Reddy PS. Suppression of male reproduction in rats after exposure to sodium fluoride during early stages of development. Naturwissenschaften 2007; 94(7):607-11.
21 Zhang JH, Liang C, Ma JJ, Zhou BH, Wang JD. Effects of sodium fluoride and sulfur dioxide on oxidative stress and antioxidant defenses in rat testes. Fluoride 2006;39(3):185-90.
22 Zhang JH, Liang C, Ma JJ, Zhou BH, Wang JD. Changes in testis protein and metabolic enzyme activities in rats induced by sodium fluoride and sulfur dioxide. Fluoride 2006; 39(3):179-84.
23 Chinoy NJ, Mehta D, Jhala DD. Effects of different protein diets on fluoride induced oxidative stress in mice testis. Fluoride 2005; 38(4):269-75
24 Ge YM, Ning HM, Feng CP, Wang HW, Yan XY, Wang SL, Wang JD. Apoptosis in brain cells of offspring rats exposed to high fluoride and low iodine. Fluoride 2006;39(3):173-8.
25 RCN [homepage on the Internet]. Herndon: Corporation; c2007 [updated 2007 Apr 25; cited 2007 May 4]. Apoptosis; [about 8 screens]. Available from: http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/A/Apoptosis.html
26 Chinoy NJ. Studies on effects of fluoride in 36 villages of Mehsana district, North Gujarat. Fluoride 1992;25(3):101-10.
27 Aardema MJ, Tsutsui T. Sodium fluoride-induced chromosome aberrations in different cell cycle stages. Mutat Res 1995;331(1):171-2.
28 Beck MA. Selenium and host defence towards viruses. Proc Nutr Soc1999;58(3):707-11.
29 Ozaki M, Deshpande SS, Angkeow P, Bellan J, Lowenstein CJ, Dinauer MC, et al. Inhibition of the Rac1 GTPase protects against nonlethal ischemia/reperfusion-induced necrosis and apoptosis in vivo. FASEB J 2000;14(2):418-29.
30 Cho KS, Lee EH, Choi JS, Joo CK. Reactive oxygen species-induced apoptosis and necrosis in bovine corneal endothelial cell. Invest Ophthalmol Vis Sci 1999;40(5):911-9.