EFFECTS OF VITAMIN C ON SODIUM FLUORIDE-INDUCED OXIDATIVE DAMAGE IN SERTOLI CELLS

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SUMMARY: The present study evaluated oxidative stress and apoptosis in Sertoli cells that had been exposed to sodium fluoride (NaF). After TM4 Sertoli cells were exposed to two doses of sodium fluoride (4 ppm and 20 ppm, equivalent to 1.81 mg F/L and 9.05 mg F/L, respectively) in the presence and absence of 50 µM vitamin C for two different exposure periods (24 hr and 48 hr), cell viability, cell proliferation, cytotoxicity, lipid peroxidation, and reactive oxygen species were measured and the apoptosis/necrosis rate was calculated. A significant decrease in the Sertoli cell viability was found with the high dose of NaF (20 ppm). The fluoride ion also caused oxidative damage by increasing cytotoxicity, reactive oxygen species, and lipid peroxidation in a dose- and time-dependent manner. The apoptosis/necrosis rate was significantly increased in both NaF groups. Vitamin C, a strong antioxidant, had a protective effect against the fluoride-induced damage in the Sertoli cells including the increased apoptosis/necrosis rate. In conclusion, the present study showed that oxidative stress plays a possible role in the apoptosis of Sertoli cells induced by NaF, and that these effects can be suppressed with vitamin C.

Keywords: Ascorbic acid; Oxidative damage; Sertoli cell apoptosis; Sodium fluoride toxicity; TM4 mouse Sertoli cells.

INTRODUCTION

In recent years, increasing concern has been felt about the hazardous effects on the male reproductive system of various environmental contaminants.[1] Amongst these contaminants is the fluoride ion (F) which is found mainly in water, drugs, pesticides, insecticides, fertilizers, dental products, and food. Studies conducted over the last three decades have provided evidence of the negative effects of F on the male reproductive system.[2] In various clinical studies and animal experiments, it has been found that F has caused structural and functional disorders in spermatozoa, a decrease in number of sperm, disturbances in reproductive hormone levels, structural changes in accessory reproductive glands, and decreased fertility.[3,4] *In vitro* F exposure at high concentrations affected certain signal pathways, such as inhibition of the cell cycle, apoptosis, and proliferation.[5-7]

F has been found to mediate the production of superoxide anion (O_2^-) and the associated production of hydrogen peroxide, peroxynitrite, and hydroxyl radicals.[8-10] In addition, F affects glutathione levels and results in toxicity that leads to an excessive mitochondrial production of reactive oxygen species (ROS), causing damage to the cellular components. The increased production of ROS causes membrane damage via lipid peroxidation, membrane depolarization, and apoptosis.[11] Free radicals attack membrane phospholipids.[11] Vitamin C is a strong

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antioxidant, with an ability to neutralize free radicals, and is widely distributed throughout the body. It also plays a protective role against oxidative stress, stimulates cell division and reproduction, protects sperm from harmful oxidative processes, and improves fertility.[12-14]Accordingly, oxidative stress is considered to play a major role in sodium fluoride-induced apoptosis of Sertoli cells

The present study examined the effects of sodium fluoride on oxidative stress and apoptosis in Sertoli cells and the protective effects of vitamin C.

MATERIALS AND METHODS

Experimental design: TM4 is a non-tumorigenic cell line derived from Sertoli cells of 11–13-day-old mice, and was purchased from ATCC (American Type Culture Collection). TM4 cells were cultured *in vitro* by regular passaging (2–3 times each week). The cells were cultured in culture medium containing 5% horse serum, 2.5% fetal bovine serum, 2.5 mM L-glutamine, 0.5 mM sodium pyruvate, 1.2 g/L sodium bicarbonate, 15 mM HEPES and PSA (penicillin-streptomycin-amphoterine) 50:50 DMEM/F12, in a humidified incubator containing 5% CO_2 at 37ºC.

The F doses applied to the TM4 Sertoli cells were chosen based on the highest and lowest average daily doses of F, from water and nutrients, that the cells of living organisms have been exposed to. The experimental groups were treated with two NaF doses, 4 ppm (NaF1) and 20 ppm (NaF2), equivalent to 1.81 mg F/L and 9.05 mg F/L, respectively, and the control groups were treated with culture medium containing 1% horse serum (HS).[15-16] In the experiments, the dose of vitamin C chosen was 50µM, which is the average antioxidant dose *in vitro*.[17] The vitamin C was dissolved in ultrapure water and was prepared freshly. The solutions of NaF and NaF+vitamin C were sterilized with a 0.2 µm millipore filter and the TM4 Sertoli cells were exposed to it for 24 hr and 48 hr.

Cell viability and cell proliferation assay: Cell viability was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Roche, Mannheim, Germany). Sertoli cells were seeded into each well of a 96-well plate culture, at 5,000 cells per well, with the test medium and then the plates were incubated at 37ºC with CO_2 for 24 hr and 48 hr. After 24 hr incubation, 10 µL MTT I solution were added into each well and cells were incubated for 4 hr. Immediately after incubation, 100 µL MTT II solution (SDS) was added to each well which was then stored in a CO_2 incubator for one night. The optical density of the each well was measured at 540 nm with an ELISA Reader.

The Cell Proliferation ELISA kit, BrdU (5-bromo-2'-deoxy-uridine) colorimetric, is one of the tests used to determine DNA synthesis (Roche, Mannheim, Germany). The cells seeded in 96-well plates (5×10^3 cells/well) with test medium and incubated at 37ºC with CO_2 for 24 hr and 48 hr. After incubation, the proliferating cells were marked by bromo-deoxyduridine (BrdU) at 24 hr. Briefly, the labeling medium was removed and the cells were fixed and the genomic DNA denatured by adding 200 µL FixDenat per well for 30 min at room temperature. After removing the FixDenat, anti BrdU-POD was added and the

wells were incubated at room temperature for 90 min. At the end of incubation, the cells were washed three times with 200 µL washing solution before 100 µL of substrate. Reaction products were calculated by measuring the absorbance at 450 nm wavelength with a spectrophotometer.

Lactate dehydrogenase (LDH) activity: LDH was measured using the micro plate based Cytotoxicity Detection Kit (LDH) (Roche Molecular Biochemicals, Mannheim, Germany). The cells were seeded in 96-well plates (1×10^4 cells/well) with test medium and incubated at 37ºC with CO_2 for 24 hr. After incubation, 100 µL of the kit mixture consisting of the stain and enzyme solutions was added to each well and the cells were incubated for a further 30 min. The results were obtained by measuring the absorbance of the red formazan product at 492 nm wavelength with a spectrophotometer.

Measurement of lipid peroxidation and reactive oxygen species: After the treatments, cells (5×10^5) were harvested from 6-well plates and transferred into Tris-HCl buffer (pH: 7.2). Then, the cells were sonicated using an ultrasonicator, and the resulting cell suspension was centrifuged at 14,000 g. The supernatants were collected and used for measuring lipid peroxidation and reactive oxygen species.

Lipid peroxidation was measured according to the malondialdehyde (MDA) content as in the method of Devasagayam and Tarachand.[18] This method is based on the reaction with thiobarbituric acid in an acidic pH in boiling water for 20 min. After cooling, the absorbance was measured at 532 nm wavelength with a spectrophotometer.

Hydroxyl radical production was measured according to the method of Puntarulo and Cederbaum.[19] This method is based on the formation of formaldehyde by hydroxyl radical and the reaction of formaldehyde with TCA by giving an absorbance at 570 nm wavelength.

Hydrogen Peroxide determination was measured according to the method of Holland and Storey.[20] This method is based on the increasing deviation, resulted from the oxidation of acidified ferrocytochrome c. The level of hydrogen peroxide was quantitated at 550 nm wavelength with a spectrophotometer.

Detection of apoptosis and necrosis: Hoechst 33342, a kind of blue-fluorescence dye, stains the condensed chromatin in apoptotic cells and propidium iodide (PI), a red-fluorescence dye, is permeant to dead cells. The staining pattern resulting from the simultaneous use of these dyes makes it possible to distinguish normal, apoptotic, and dead cell populations by fluorescence microscopy.

Sertoli cells were cultured in 24-well plates with 10^4 cells per well and treated with NaF and NaF+vitamin C as indicated. After the experimental periods, the cells were washed with phosphate-buffered saline (PBS, 1M) solution. Then, 20 µL PI (1 mg/mL propidium iodide) and 20 µL Ho342 (1 mg/mL Hoechst 33342) solution was prepared by dissolving in 3960 µL PBS. From the prepared solution, 0.2 mL was added to each well and incubated for 15–30 minutes at 37ºC. After the incubation, the cells were washed with PBS, once or twice, and examined under

the fluorescent microscope. Cells were examined with a UV filter via an Olympus IX71 fluorescent attachment microscope and photographed in series with equal intervals by an Olympus DP72 video camera. The ratio of viable, early apoptotic, apoptotic, and necrotic cells was calculated.

Statistical analysis: Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA) and analysis of variance was used for comparisons among all groups. The data was calculated by a one way ANOVA with Tukey's multiple comparisons test. The results were expressed as mean±standard error and values of $p<0.001$, $p<0.01$ and $p<0.05$ were considered statistically significant.

RESULTS

Effects of NaF and NaF+vitamin C on cell viability and cell proliferation: When the control and experimental groups were compared, it was found that Sertoli cell viability was significantly decreased in the NaF2 group, with 20 mg NaF/L, at both 24 hr and 48 hr ($p<0.05$, Figure 1). No significant difference was observed in cell proliferation.

Effects of NaF and NaF+vitamin C on lactate dehydrogenase activity: There was a significant increase in LDH levels in the all the NaF-treated groups compared to the control group ($p<0.001$, Figure 2). In addition, Vitamin C pre-treatment caused a significant decrease in LDH activity in the TM4 Sertoli cells compared to the NaF-treated groups ($p<0.001$).

Effect of NaF and NaF+vitamin C on MDA, $OH^{\bullet}$, and H_2O_2 levels: Exposure to NaF induced lipid peroxidation of the TM4 Sertoli cells with a significant increase in MDA levels at all dose levels ($p<0.001$, Figure 3). Treatment with vitamin C prevented the lipid peroxidation of the membrane induced by exposure to NaF.

Similarly, significant dose-dependent increases were observed in the hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2) levels in the NaF groups ($p<0.01$ and $p<0.001$) and a significant dose-dependent decrease was observed in the groups to which vitamin C was added ($p<0.001$, Figure 4).

Apoptosis and necrosis rate in the Sertoli cells exposed to NaF and NaF+vitamin C: Compared to the control group, the percentage of viable, early apoptotic, apoptotic, and necrotic cells was significantly altered in a dose- and time-dependent manner compared with the control group ($p<0.01$, Table).

DISCUSSION

There are only a limited number of studies evaluating the effects of F on cell viability.[8,21-24] These studies have recorded a significant decrease only with high doses of exposure. The present study found that NaF decreased cell viability in TM4 Sertoli cells *in vitro* at the dose of 20 ppm but not at 4 ppm. On the other hand, no significant difference in cell viability was observed between the two NaF groups (4 ppm and 20 ppm) using vitamin C as an antioxidant.

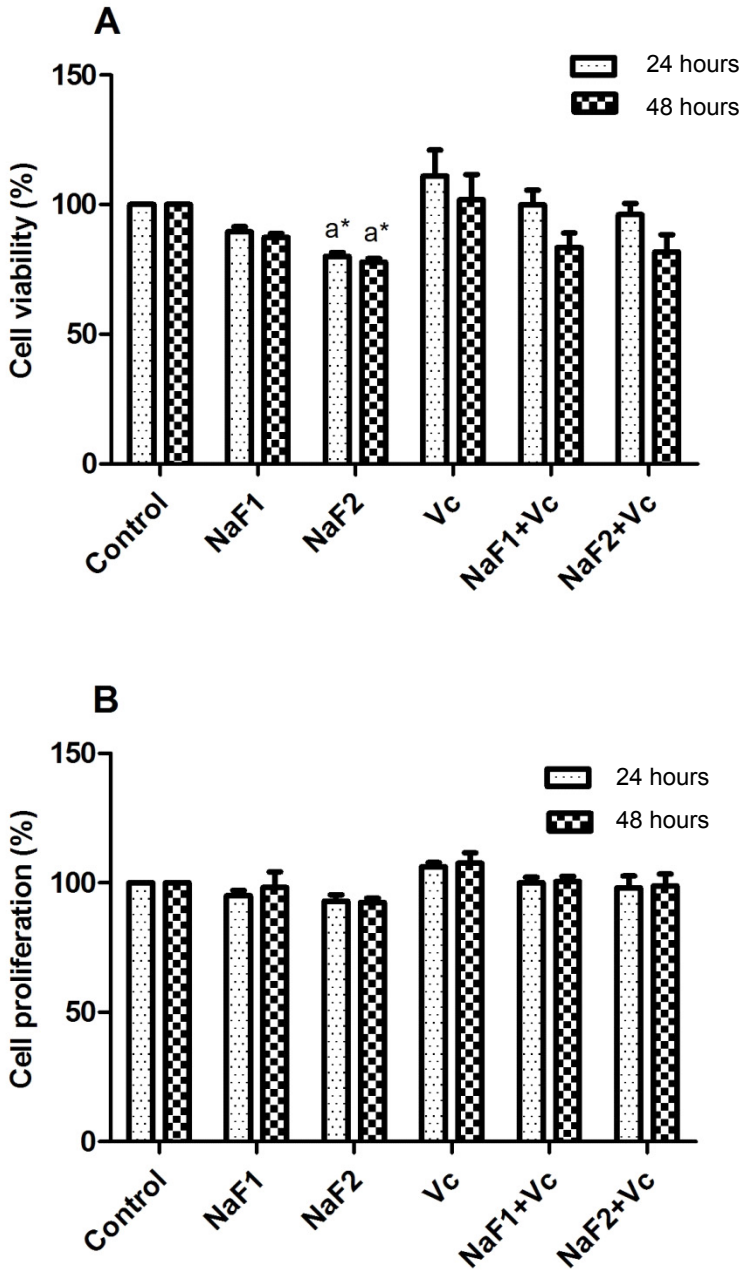

Figure 1. Dose- and time-dependent effects of NaF on cell viability (A) and cell proliferation (B) in Sertoli cells *in vitro*. Each bar denotes mean±SEM of five independent experiments carried out in triplicate. NaF1: 4 ppm NaF, NaF2: 20 ppm NaF, Vc: 50 μM Vitamin C. [a]compared with control group: *p<0.05; [b]compared with vitamin C group: *p<0.05.

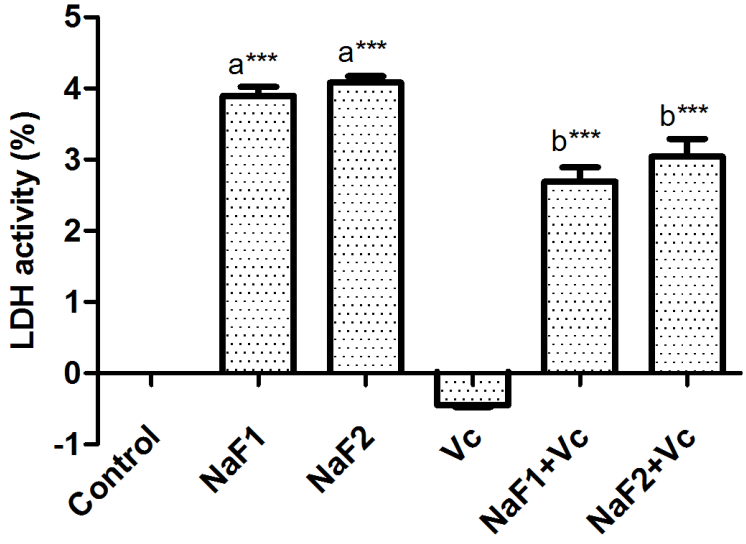

Figure 2. Dose- and time-dependent effects of NaF on LDH activity in Sertoli cells *in vitro.* Each bar denotes mean±SEM of five independent experiments carried out in duplicate. NaF1: 4 ppm NaF, NaF2: 20 ppm NaF, Vc: 50 µM Vitamin C. The value for LDH activity for the control group was 0%.
[a]compared with control group: ***p<0.001; [b]compared with vitamin C group: ***p<0.001.

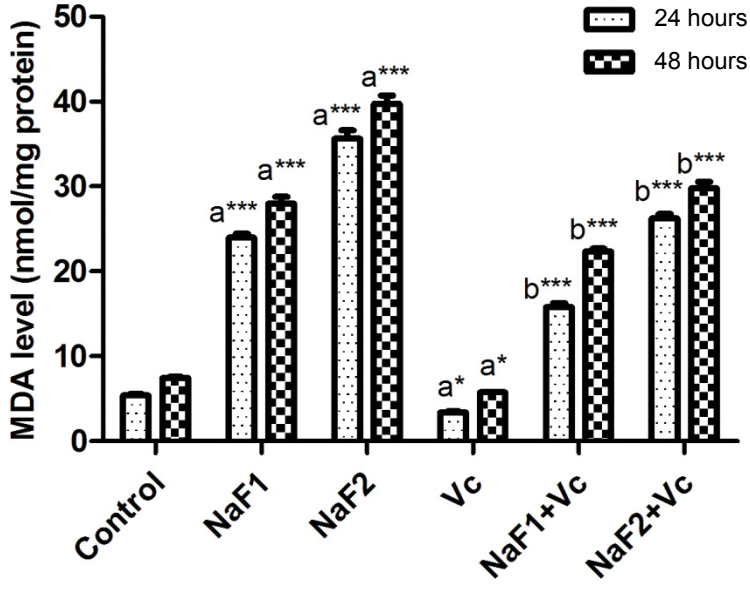

Figure 3. Dose- and time-dependent effects of NaF on MDA levels in Sertoli cells *in vitro.* Each bar denotes mean±SEM of five independent experiments carried out in duplicate. NaF1: 4 ppm NaF, NaF2: 20 ppm NaF, Vc: 50 µM Vitamin C.
[a]compared with control group: *p<0.05, ***p<0.001; [b]compared with vitamin C group: *p<0.05, ***p<0.001.

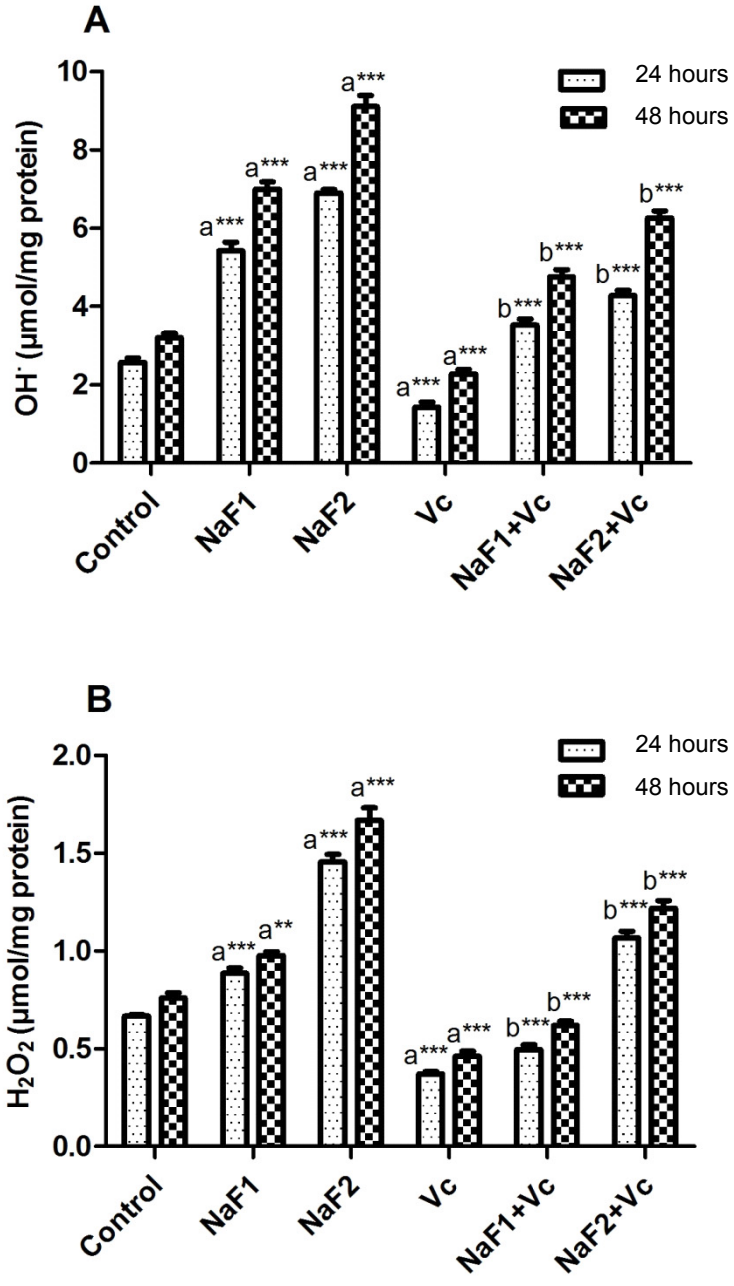

Figure 4. Dose- and time-dependent effects of NaF on the reactive oxygen species, hydroxyl radical ($OH^{\bullet}$) and hydrogen peroxide (H_2O_2), levels in Sertoli cells *in vitro.* Each bar denotes mean±SEM of five independent experiments carried out in duplicate. NaF1: 4 ppm NaF, NaF2: 20 ppm NaF, Vc: 50 µM Vitamin C.
[a]compared with control group: **p<0.01, ***p<0.001; [b]compared with vitamin C group: **p<0.01, ***p<0.001.

Table. Dose- and time-dependent effects of NaF and vitamin C on apoptosis and necrosis in Sertoli cells *in vitro* (Each value for the percentage of cells in a group denotes the mean±SEM of five independent experiments carried out in duplicate)

Cell type	Time (hr)	Proportions (%) of viable cells, cells in early apoptosis, cells in apoptosis, and necrotic cells under control conditions and with the addition, for 24 and 48 hours, of sodium fluoride (NaF1: 4 ppm, NaF2: 20 ppm) and vitamin C (50 µM)					
		Control (% of cells)	NaF1 (% of cells)	NaF2 (% of cells)	Vitamin C (% of cells)	NaF1 + vitamin C (% of cells)	NaF2 + vitamin C (% of cells)
Viable cells	24	92.9±0.26	88.3±0.57[a**]	80.8±0.68[a**]	94.8±0.12[a**]	91.5±0.36[b**]	88.0±0.53[b**]
	48	89.9±0.56	85.2±0.65[a**]	76.8 ±0.76[a**]	91.5±0.19[a**]	87.8±0.58[b**]	84.6±0.40[b**]
Cells in early apoptosis	24	5.7±0.26	8.5±0.56[a**]	11.1±0.73[a**]	4.6±0.14[a**]	6.3±0.25[b**]	8.5±0.48[b**]
	48	7.7±0.38	9.6±0.49[a**]	13.9±0.78[a**]	7.0±0.17[a**]	8.5±0.36[b**]	10.9±0.46[b**]
Cells in apoptosis	24	0.7±0.07	2.6±0.18[a**]	5.9±0.19[a**]	0.4±0.07[a**]	1.3±0.07[b**]	2.4±0.17[b**]
	48	2.1±0.13	4.3±0.22[a**]	7.3±0.17[a**]	1.2±0.10[a**]	2.7±0.36[b**]	3.1±0.19[b**]
Necrotic cells	24	0.6±0.07	0.6±0.10[a**]	2.2±0.26[a**]	0.3±0.04[a**]	0.9±0.07[b**]	1.1±0.11[b**]
	48	0.4±0.09	0.8±0.07[a**]	2.1±0.16[a**]	0.3±0.03[a**]	0.9±0.10[b**]	1.4±0.08[b**]

[a]compared with control group: **p<0.01; [b]compared with vitamin C group: **p<0.01.

Although Chinoy and Sarma[21] found increased cell viability with the addition of vitamin D and vitamin E to NaF exposure, vitamin C is thought to have no effect on cell viability in TM4 Sertoli cells at the administered doses. It is well known that F inhibits cellular reproduction by arresting the cell cycle before the synthesis phase.[25,26] Although F decreased significantly DNA synthesis in human dental pulp cells at doses of 5×10^{-4}, 10×10^{-4}, and 20×10^{-4} mM, DNA synthesis in TM4 Sertoli cells was not significantly affected by F exposure at the doses of 4 ppm NaF (9.5×10^{-2} mM) and 20 ppm NaF (47.6×10^{-2} mM) used in the present study.[27] The lack of a significant difference with the addition of vitamin C to the F doses suggests that vitamin C has no effect on the reproduction of TM4 Sertoli cells.

In *in vitro* studies, cell death or damage to the plasma membrane causes an increase in activity of the lactate dehydrogenase (LDH) enzyme.[9] The production of superoxide anion and the increase in hydroxyl levels caused by F exposure results in increased LDH production, as a consequence of oxidative damage to the cell membrane.[28] Increased lactate dehydrogenase activity in TM4 Sertoli cells after exposure to NaF suggests that F has an cytotoxic effect on Sertoli cells, as in

other cell types.[11,29] The significant decrease in activity of lactate dehydrogenase observed after the addition of vitamin C to NaF exposure suggests that vitamin C decreases the activity of lactate dehydrogenase, as shown in the study of Takhshid et al.[30]

Oxidative stress can cause damage to many biological molecules particularly, DNA, proteins, and lipids.[31] This effect is mediated by the reactive oxygen species found within cells.[32] There are many studies evaluating the effects of F on lipid peroxidation.[8,33-36] Similar to studies evaluating only NaF exposure, the present study also found a significant increase in lipid peroxidation. The decreased lipid peroxidation in Sertoli cells of groups that received simultaneously NaF and vitamin C is consistent with the findings of Das et al.[34] and Sarkar et al.[35] who recorded decreased oxidative stress with the addition of testosterone, vitamin E, and $CaCl_2$. In the present study, F, at both the 4 ppm and the 20 ppm NaF doses of exposure, equivalent to 1.81 mg F/L and 9.05 mg F/L, respectively, caused lipid peroxidation in cells, while vitamin C played a protective role against this.

High levels of ROS play a role in the pathogenesis of many disorders and mediate the toxicity of various compounds.[9] F can attack oxygen species and cause the production of ROS.[11] In addition, F can affect glutathione levels and cause toxicity that leads to an excessive mitochondrial production of ROS, which in turn can cause damage to cellular components.[7] In the present study, the increased production of hydroxyl radicals and levels of hydrogen peroxide, occurring in a time- and dose-dependent manner with exposure to NaF, provides support for the findings in similar studies on the effect of F on reactive oxygen species.[11,37] A significant decrease in hydroxyl radicals and intracellular hydrogen peroxide levels in the groups that received NaF and vitamin C suggests that vitamin C protected the Sertoli cells from the hydroxyl radicals and hydrogen peroxide, concurring with the findings of Ghosh et al.[33] in their study of arjunolic acid and vitamin C.

Apoptosis is a complex process that involves gene expression and/or protein activity, as well as a delicate balance in the signal proteins.[7] In general, studies have shown that apoptosis is triggered by oxidative stress, particularly by reactive oxygen species, and this effect can be mediated by an increase in lipid peroxidation and mitochondrial dysfunction.[38,39] The present study has shown that NaF induced apoptosis in Sertoli cells at both the 4 ppm and 20 ppm doses, at both 24 hr and 48 hr, consistent with the results of previous *in vitro* studies.[11,40] In addition, the finding of an increased necrosis rate at both doses as a result of NaF-induced oxidative damage in Sertoli cells confirms the findings of Ghosh et al.[33] who identified an increased rate of necrosis. Güney et al.[12] found that administering vitamin C and vitamin E prevented F-induced apoptosis, and the observation of similar effects on Sertoli cells suggests that vitamin C may have reduced F-induced damage.

CONCLUSION

In conclusion, NaF induced oxidative damage in TM4 Sertoli cells, which in turn triggered apoptosis. Vitamin C may have protective effects against apoptosis by inhibiting the production of the reactive oxygen species and free radicals caused by F exposure. Consequently, NaF may act as an endocrine disrupter and, with daily consumption, cause impairments in the male reproductive system.

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REFERENCES

1 Mathur PP, D'Cruz SC. The effect of environmental contaminants on testicular function. Asian J Androl 2011;13(4):585-91.
2 Zhang J, Liang C, Ma J, Zhou B, Wang J. Changes in testis protein and metabolic enzyme activities in rats induced by sodium fluoride and sulfur dioxide. Fluoride 2006;39(3):179-84.
3 Long H, Jin Y, Lin M, Sun Y, Zhang L, Clinch C. Fluoride toxicity in the male reproductive system. Fluoride 2009;42(4):260-76.
4 Inkielewicz-Stepniak I, Czarnowski W. Oxidative stress parameters in rats exposed to fluoride and caffeine. Food Chem Toxicol 2010;48(6):1607-11.
5 Wang AG, Xia T, Chu QL, Zhang M, Liu F, Chen XM, et al. Effects of fluoride on lipid peroxidation, DNA damage and apoptosis in human embryo hepatocytes. Biomed Environ Sci 2004;17(2):217-22.
6 Mendoza-Schulz A, Solano-Agama C, Arreola-Mendoza L, Reyes-Mórquez B, Barbier O, Del Razo LM, et al. The effects of fluoride on cell migration, cell proliferation, and cell metabolism in GH_4C_1 pituitary tumour cells. Toxicol Lett 2009;190(2):179-86.
7 Barbier O, Arreola-Mendoza L, Del Razo LM. Molecular mechanisms of fluoride toxicity. Chem Biol Interact 2010;188(2):319-33.
8 Izquierdo-Vega JA, Sánchez-Gutiérrez M, Del Razo LM. Decreased *in vitro* fertility in male rats exposed to fluoride-induced oxidative stress damage and mitochondrial transmembrane potential loss. Toxicol Appl Pharmacol 2008;230(3):352-7.
9 Hassan HA, Yousef MI. Mitigating effects of antioxidant properties of black berry juice on sodium fluoride induced hepatotoxicity and oxidative stress in rats. Food Chem Toxicol 2009;47(9):2332-7.
10 García-Montalvo EA, Reyes-Pérez H, Del Razo LM. Fluoride exposure impairs glucose tolerance via decreased insulin expression and oxidative stress. Toxicology 2009;263(2-3):75-83.
11 Zhang M, Wang A, He W, He P, Xu B, Xia T, et al. Effects of fluoride on the expression of NCAM, oxidative stress, and apoptosis in primary cultured hippocampal neurons. Toxicology 2007;236(3):208-16.
12 Guney M, Oral B, Demirin H, Karahan N, Mungan T, Delibas N. Protective effects of vitamins C and E against endometrial damage and oxidative stress in fluoride intoxication. Clin Exp Pharmacol Physiol 2007;34(5-6):467-74.
13 Ghosh J, Das J, Manna P, Sil PC. Cytoprotective effect of arjunalic acid in response to sodium fluoride mediated oxidative stress and cell death via necrotic pathway. Toxicol In Vitro 2008;22(8):1918-26.
14 Baba NA, Raina R, Verma PK, Sultana M, Prawez S, Nisar NA. Toxic effects of fluoride and chlorpyrifos on antioxidant parameters in rats: protective effects of vitamin C and E. Fluoride 2013;46(2):73-9.
15 Fawell J, Bailey K, Chilton J, Dahi E, Fewtrell L, Magara Y. Fluoride in drinking-water. London: WHO; 2006.
16 Kucukesmen C, Sonmez H. Diş hekimliğinde florun, insan vücudu ve dişler üzerindeki etkilerinin değerlendirilmesi. SDÜ Tıp Fakül Der 2008;15(3):43-53.[in Turkish].
17 Jialal I, Grundy SM. Preservation of the endogenous antioxidants in low density lipoprotein by ascorbate but not probucol during oxidative modification. J Clin Inves 1991;87(2):597-601.

18 Devasagayam TP, Tarachand U. Decreased lipid peroxidation in the rat kidney during gestation. Biochem Biophys Res Commun 1987;145(1):134-8.
19 Puntarulo S, Cederbaum AI. Effects of oxygen concentration on microsomal oxidation of ethanol and generation of oxygen radicals. Biochem J 1988;251(3):787-94.
20 Holland MK, Storey BT. Oxygen metabolism of mammalian spermatozoa. Generation of hydrogen peroxide by rabbit epididymal spermatozoa. Biochem J 1981;198(2):273-80.
21 Chinoy NJ, Sharma A. Amelioration of fluoride toxicity by vitamins E and D in reproductive functions of male mice. Fluoride 1998;31(4):203-16.
22.Pushpalatha T, Srinivas M, Sreenivasula Reddy P. Exposure to high fluoride concentration in drinking water will affect spermatogenesis and steroidogenesis in male albino rats. BioMetals 2005;18(3):207-12.
23 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.
24 Gupta RS, Khan TI, Agrawal D, Kachhawa JB. The toxic effects of sodium fluoride on the reproductive system of male rats. Toxicol Ind Health 2007;23(9):507-13.
25 Yan Q, Zhang Y, Li W, Denbesten PK. Micromolar fluoride alters ameloblast lineage cells *in vitro*. J Dent Res 2007;86(4):336-40.
26 Riksen EA, Kalvik A, Brookes S, Hynne A, Snead ML, Lyngstadaas SP, et al. Fluoride reduces the expression of enamel proteins and cytokines in an ameloblast-derived cell line. Arch Oral Biol 2011;56(4):324-30.
27 Chang YC, Chou MY. Cytotoxicity of fluoride on human pulp cells cultures *in vitro*. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001;91(2):230-4.
28 Kaczor JJ, Ziolkowski W, Popinigis J, Tarnopolsky MA. Anaerobic and aerobic enzyme activities in human skeletal muscle from children and adults. Pediatr Res 2005;57(3):331-5.
29 Zakrzewska H, Udala J, Blaszczyk B. *In vitro* influence of sodium fluoride on ram semen quality and enzyme activities. Fluoride 2002;35(3):153-60.
30 Takhshid MA, Tavasuli AR, Heidary Y, Keshavarz M, Kargar H. Protective effect of vitamins E and C on endosulfan-induced reproductive toxicity in male rats. Iran J Med Sci 2012;37(3):173-80.
31 Cai Q, Rahn RO, Zhang R. Dietary flavonoids, quercetin, luteolin and genistein, reduce oxidative DNA damage and lipid peroxidation and quench free radicals. Cancer Lett 1997;28;119(1):99-107.
32 Halliwell B, Gutteridge JM. Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol 1990;186:1-85.
33 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.
34 Das S, Maiti R, Ghosh D. Induction of oxidative stress on reproductive and metabolic organs in sodium fluoride-treated male albino rats: protective effects of testosterone and vitamin E coadministration. Toxicol Mech Methods 2005;15(4):271-7.
35 Sarkar SD, Maiti R, Ghosh D. Management of fluoride induced testicular disorders by calcium and Vitamin-E co-administration in the albino rat. Reprod Toxicol 2006;22(4):606-12.
36 Oncu M, Kocak A, Karaoz E, Darici H, Savik E, Gultekin F. Effect of long-term fluoride exposure on lipid peroxidation and histology of testes in first- and second-generation rats. Biol Trace Elem Res 2007;118(3):260-8.
37 Chouhan S, Lomash V, Flora SJ. Fluoride-induced changes in haem biosynthesis pathway, neurological variables and tissue histopathology of rats. J Appl Toxicol 2010;30(1):63-73.
38 Matsui H, Morimoto M, Horimoto K, Nishimura Y. Some characteristics of fluoride-induced cell death in rat thymocytes: cytotoxicity of sodium fluoride. Toxicol In Vitro 2007;21(6):1113-20.
39 Karube H, Nishitai G, Inageda K, Kurosu H, Matsuoka M. NaF activates MAPKs and induces apoptosis in odontoblast-like cells. J Dent Res 2009;88(5):461-5.
40 Xu B, Xu Z, Xia T, He P, Gao P, He W, et. al. Effects of the Fas/Fas-L pathway on fluoride-induced apoptosis in SH-SY5Y cells. Environ Toxicol 2011;26(1):86-92.