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EFFECTS OF VITAMIN C ON ANTIOXIDANT SYSTEMS AND STEROIDOGENIC ENZYMES IN SODIUM FLUORIDE-EXPOSED TM4 SERTOLI CELLS

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SUMMARY: Fluoride, which is commonly found in drinking water and nutrients, threatens the environment and human life. Fluoride damages cells by causing free radical formation and affects male reproductive function by preventing spermatogenesis, changing the activity of spermatogenetic enzymes, and consequently leading to reduced reproductive capacity. A sufficient amount of antioxidants should be consumed in order to prevent oxidative stress of free radicals and minimize their effect. Vitamin C is a powerful antioxidant owing to its strong reductant activity. In this study, we treated TM4 Sertoli cells with different doses of fluoride (4 ppm and 20 ppm) for different time periods (24 hours and 48 hours) and additionally with vitamin C (50 μM), and then analyzed the activity of enzymes involved in antioxidant and steroidogenic systems. According to our results, fluoride inhibits testosterone biosynthesis by reducing the activities of 3β-HSD and 17β-HSD enzymes, which have roles in testosterone synthesis in TM4 Sertoli cells, and vitamin C has a protective role against the damage of fluoride. Fluoride was found to cause oxidative damage in TM4 Sertoli cells by reducing the amount and activities of glutathione and antioxidant enzymes (SOD, CAT, GPx, GST, γ-GT), which function in the antioxidant system. A combination of fluoride and vitamin C was found to be protective against the damage by ROS and to cause an increase in the activities of enzymes in the intracellular antioxidant system. In conclusion, fluoride may result in harmful effects in TM4 Sertoli cells and vitamin C can be protective against these effects.

Keywords: Ascorbic acid; Daily intake of fluoride; Sertoli cell; Sodium fluoride toxicity

INTRODUCTION

Male reproductive health has deteriorated significantly in the recent years. Nutrition, socioeconomic status, lifestyle, and environmental factors endanger male reproductive health. Environmental toxins are regarded as the underlying cause of reduced male fertility.[1] Many compounds present in water are toxic for living organisms, and have adverse effects on the general health.[2] Fluoride, commonly found in drinking water and nutrients, threatens the environment and human life.[3-5] Sodium fluoride is ingested into the body mostly through water, drugs, pesticides, insecticides, fertilizer, dental products, and fluoride-containing beverages and nutrient water.[6]

Despite many studies, the exact mechanism of fluoride toxicity remains unclear. Fluoride is known to be a toxic substance and its accumulation causes hematologic, hepatic, renal, and neurologic diseases.[7-9] The cellular effects of fluoride are diverse, ranging from protein damage to free radical formation. As a result, there is an increase in cellular dysfunction and apoptotic cell death.[10,11]

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Fluoride disrupts endocrine function in the male reproductive system, and reduces the reproductive capacity by decreasing testicular spermatozoa concentration and the weight of testes and accessory genital organs. Moreover, it inhibits spermatogenesis by decreasing the number of Leydig and Sertoli cells.[4,12-15]

The main toxic effects of fluoride on cells result from its relation to enzymes in a cell. Although fluoride usually acts as an enzyme inhibitor, in some cases fluoride ions can stimulate enzyme activity. The mechanism of fluoride on enzymes may vary depending on the type of enzyme.[16] Fluoride leads to reduced testosterone levels by affecting steroidogenesis. Fluoride exerts this effect by significantly reducing the enzyme activities of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD).[17-20] In addition, recent studies have shown that fluoride causes the overproduction of free oxygen radicals and a decrease in biological activities of some antioxidant enzymes (SOD, CAT, GPx, GST, γ-GT).[21]

In this study, we investigated the effect of fluoride toxicity in TM4 Sertoli cells, which are crucial for the male reproductive system, on enzymes involved in steroidogenesis, and on the antioxidant system. In addition, we examined the therapeutic role of vitamin C, which is a strong antioxidant against cellular damage.

MATERIALS AND METHODS

Experimental design: TM4 is 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-amphoterin) 50:50 DMEM/F12, in a humidified incubator containing 5% CO_2 at 37ºC.

Fluoride doses applied to TM4 Sertoli cell were confirmed by living organisms exposed to fluoride. Fluoride was prepared in the indicated doses using culture medium containing 1% Horse Serum (HS). The average daily highest and lowest doses of fluoride taken from water and nutrients were applied to the cells. The experimental groups were treated with two NaF doses, 4 ppm (NaF1) and 20 ppm (NaF2), and the control groups were treated with culture medium containing 1% HS.[22,23] In the experiments, the dose of vitamin C was determined to be 50 µM, which is the average antioxidant dose *in vitro*.[24] Vitamin C was dissolved in ultrapure water and was prepared fresh every day. The solutions of NaF and NaF+vitamin C were sterilized with a 0.2 µm millipore filter and applied to TM4 Sertoli cells for 24 and 48 hours.

Biochemical analysis: After 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 a ultrasonicator, and the resulting cell suspension was centrifuged at 14,000 g. Supernatants were collected and used for glutathione (GSH),

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superoxide dismutase (SOD), catalase (CAT), γ-glutamyl transpeptidase (γ-GT), glutathione peroxidase (GPx), glutathione S-transferase (GST) (which are involved in the antioxidant system), and 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) (which are involved in steroidogenesis) enzyme activities.

MEASUREMENT OF STEROIDOGENIC ENZYME ACTIVITY

3β-HSD enzyme activity: The method by Bergmeyer was used to assay 3β-HSD enzyme activity. 3β-HSD converts androsterone and NAD^+ into 5α-androstane-3,17-dione and NADH, and the resulting NADH gives absorbance at 340 nm, which is then detected spectrophotometrically.[25]

17β-HSD enzyme activity: The method by Bergmeyer was used to assay 17β-HSD enzyme activity. 17β-HSD converts 1,4-androstero-3,17-dione and NADPH into testosterone and NADP, and the resulting NADP gives absorbance at 340 nm, which is then detected spectrophotometrically.[25]

MEASUREMENT OF ANTIOXIDANT ENZYME ACTIVITY

Determination of superoxide dismutase: The method by Marklund and Marklund was used to determine superoxide dismutase. SOD inhibits auto-oxidation of pyrogallol, which leads to the formation of a color giving absorbance at 420 nm.[26]

Determination of catalase: The method by Sinha was used to determine catalase. Catalase converts hydrogen peroxide (H_2O_2) into water and molecular oxygen. H_2O_2 and dichromate/acetic acid indicator forms a dark blue-purple precipitate, which in turn is boiled. The resulting light green color gives absorbance at 570 nm, which is then measured spectrophotometrically.[27]

Determination of glutathione peroxidase: The method by Hafeman et al. was used to determine glutathione peroxidase levels. GPx consumes glutathione, and the resulting product forms a compound with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) indicator, which gives absorbance at 412 nm.[28]

Determination of glutathione-S-transferase: The method by Habig et al. was used to determine glutathione-S-transferase (GST). GST enzyme forms glutathione-DNB conjugate in presence of glutathione and 1-chloro-2,4-dinitrobenzene (CDNB). The resulting glutathione-DNB conjugate gives an absorbance at 340 nm, and the assay is based on measuring the absorbance values at this wavelength. The increase in the absorption rate is not directly related to GST enzyme activity.[29]

Determination of γ-glutamyl transpeptidase: The method of Orlowski and Meister was used to determine γ-glutamyl transpeptidase activity. The conversion of L-γ-glutamyl-p-nitroanilide to free p-nitro-aniline gives absorbance at 410 nm, which is then measured spectrophotometrically.[30]

Measurement of glutathione levels: The method of Ellman was used to determine glutathione levels. 5,5'-DTNB oxidizes glutathione molecules and produces oxidized glutathione and 5-thio-2-nitrobenzoic acid (TNB). The

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resulting TNB gives absorbance at 414 nm, which is then measured spectrophotometrically.[31]

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

Effect of NaF and NaF+Vitamin C on steroidogenic enzyme activities: The 3β-HSD and 17β-HSD levels at different time points (24 hr and 48 hr) in the experimental and control groups are presented in Figure 1. At both doses and both time points, all groups had significantly lower 3β-HSD and 17β-HSD levels compared to the control group ($p < 0.05$). There was a significant increase in 3β-HSD and 17β-HSD levels in all groups treated additionally with vitamin C, compared to the control and fluoride-treated groups ($p < 0.05$).

Effect of NaF and NaF+vitamin C on antioxidant enzyme activities: At both time points, SOD and CAT activities were lower in the NaF-treated groups compared to the control group. In addition, vitamin C pre-treatment caused a significant increase in the SOD activity in TM4 Sertoli cells compared to the NaF-treated groups ($p < 0.05$) (Figure 2).

Considering GPx and GST activities for 24–48 hours, there was a significant reduction in GPx and GST activities in the 20 ppm NaF-treated groups at both time points. There was a significant increase in GPx activities in NaF+vitamin C-treated groups at 24 hr (both doses) compared to the control group, whereas there was a significant increase in GPx activity in the 4 ppm NaF-treated groups at 48 hr ($p < 0.05$) (Figure 3).

There was a significant reduction in γ-GT activity in all NaF-treated groups compared to the control group ($p < 0.05$). On the other hand, there was a significant reduction in all NaF+vitamin C-treated groups compared to the control group ($p < 0.05$). There was a significant increase in both doses in NaF+vitamin C-treated groups only at 24 hours ($p < 0.05$) (Figure 4).

Effect of NaF and NaF+Vitamin C on glutathione levels: The intracellular glutathione levels, which are measured spectrophotometrically, in the TM4 Sertoli cells in the control and experimental groups at 24 hr and 48 hr are presented in Figure 5. There was a significant reduction in glutathione levels in the 20 ppm NaF-treated group at both time points compared to the control group ($p < 0.05$). There was no significant difference in glutathione levels between vitamin C-treated groups ($p < 0.05$).
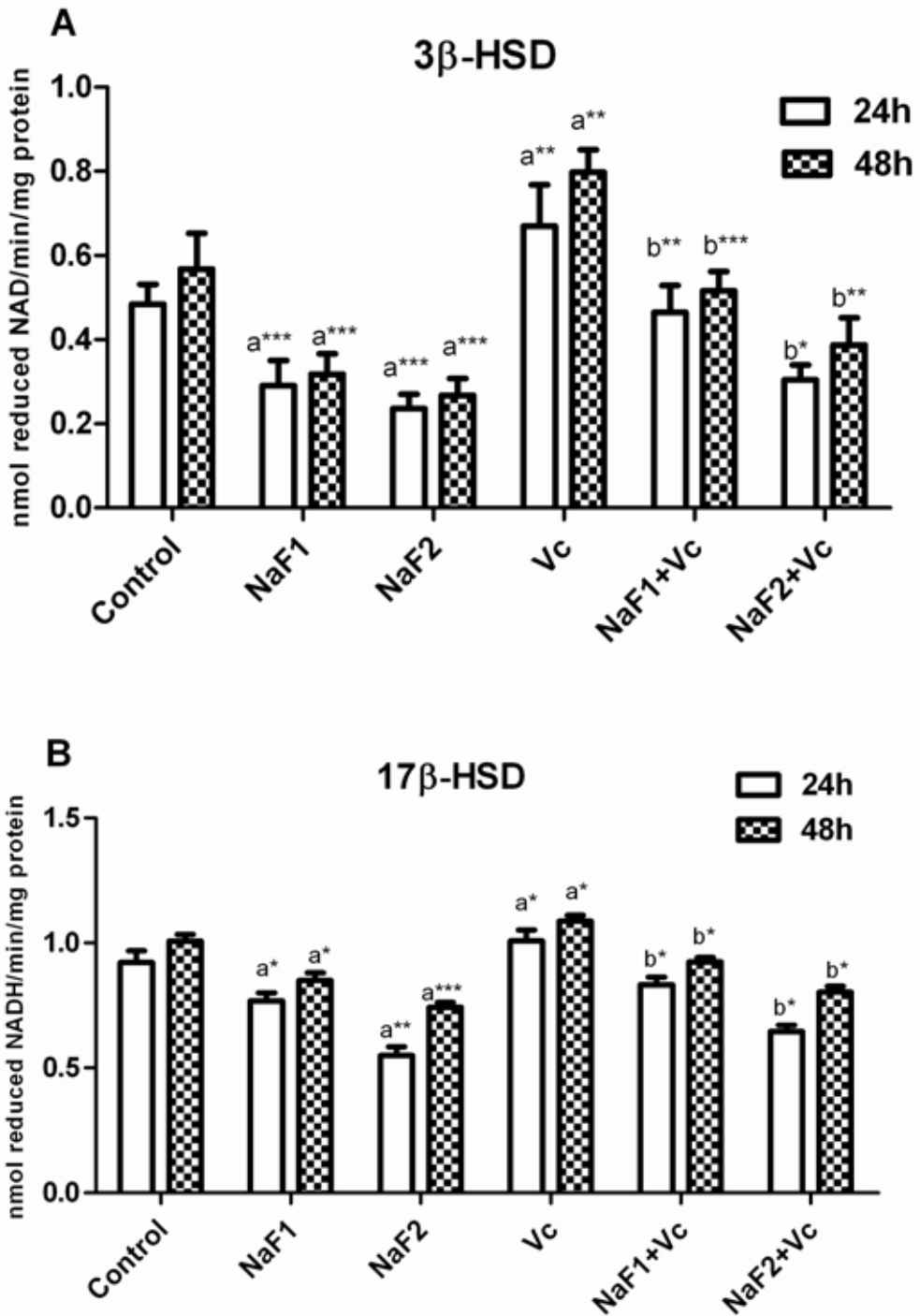
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Figure 1. Dose- and time-dependent effects of NaF on 3β-HSD (A) and 17β-HSD (B) activities of Sertoli cell *in vitro*. Each bar denotes mean (±SEM) of five independent experiments carried out in duplicates. Significance at *p<0.05, **p<0.01, ***p<0.001. (a) compared with control and (b) compared with vitamin C. Control = 1% horse serum; NaF1 = 4 ppm F; NaF2 = 20 ppm F; Vc = 50 µg vitamin C.
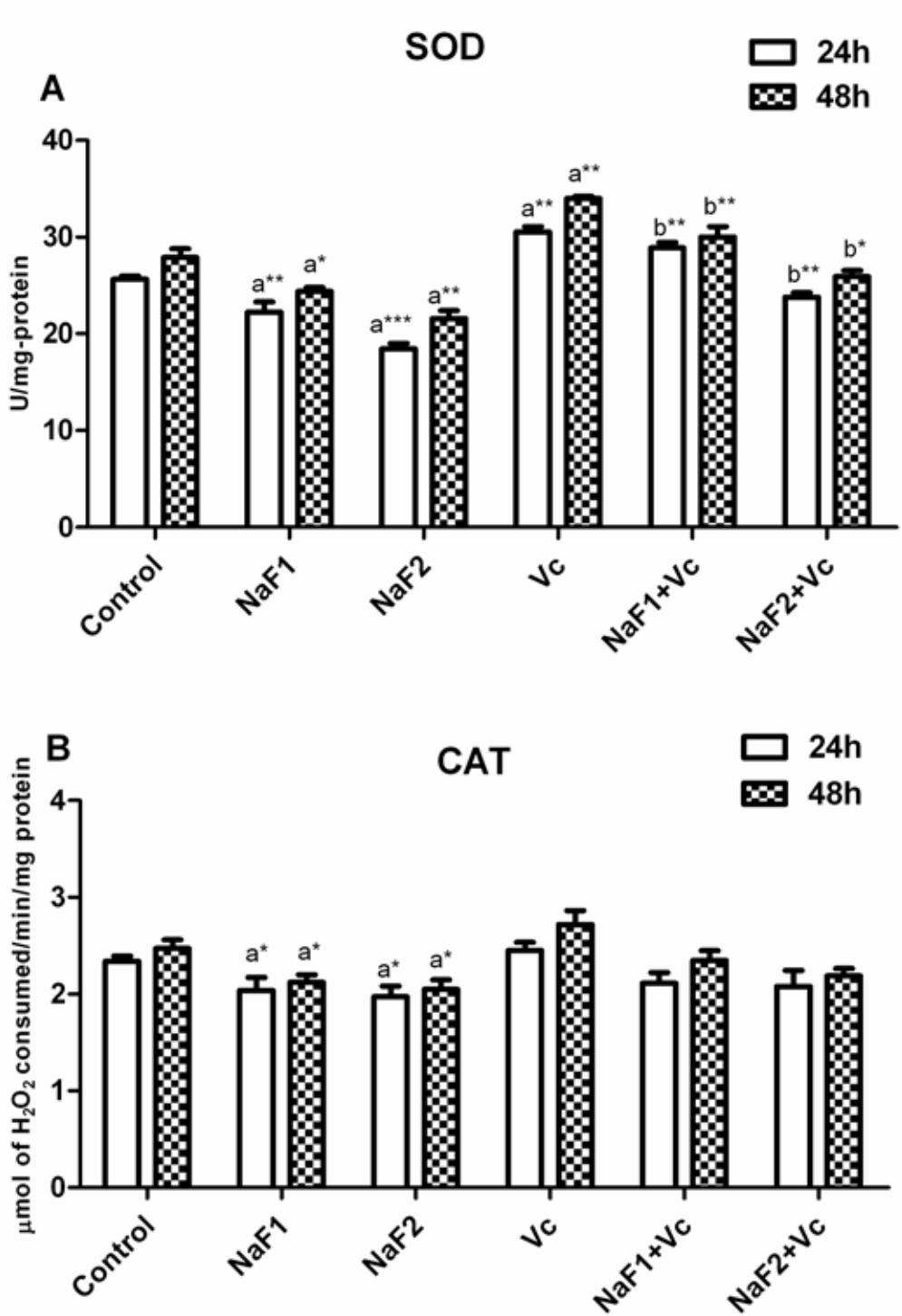
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Figure 2. Dose- and time-dependent effects of NaF on SOD (A) and CAT (B) activities of Sertoli cell *in vitro*. Each bar denotes mean (±SEM) of five independent experiments carried out in duplicates. Significance at *p<0.05, **p<0.01, ***p<0.001. (a) compared with control and (b) compared with vitamin C. Control = 1% horse serum; NaF1 = 4 ppm F; NaF2 = 20 ppm F; Vc = 50 µg vitamin C.
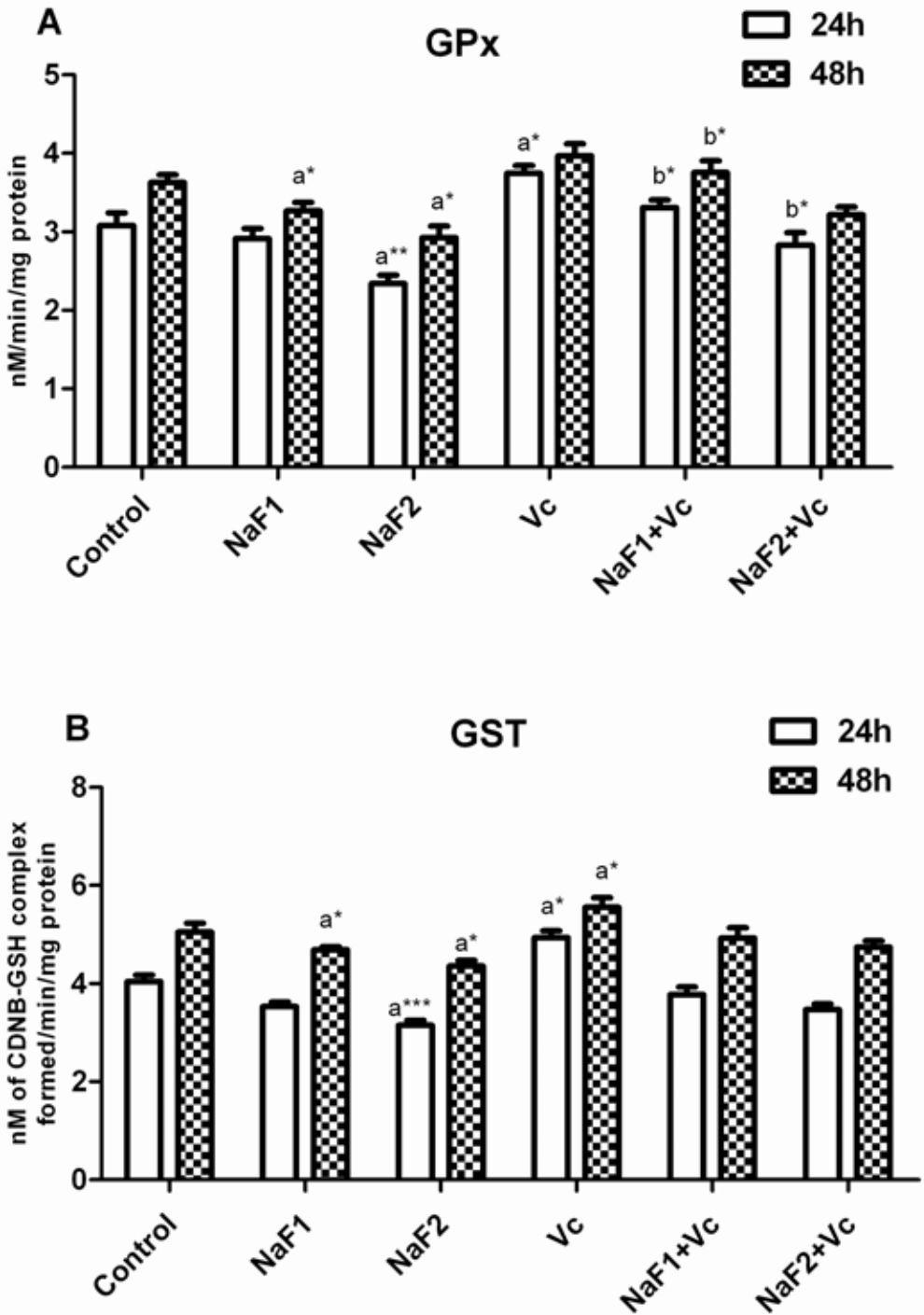
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Figure 3. Dose- and time-dependent effects of NaF on GPx (A) and GST (B) activities of Sertoli cell *in vitro*. Each bar denotes mean (±SEM) of five independent experiments carried out in duplicates. Significance at *p<0.05, **p<0.01, ***p<0.001. (a) compared with control and (b) compared with vitamin C. Control = 1% horse serum; NaF1 = 4 ppm F; NaF2 = 20 ppm F; Vc = 50 µg vitamin C.
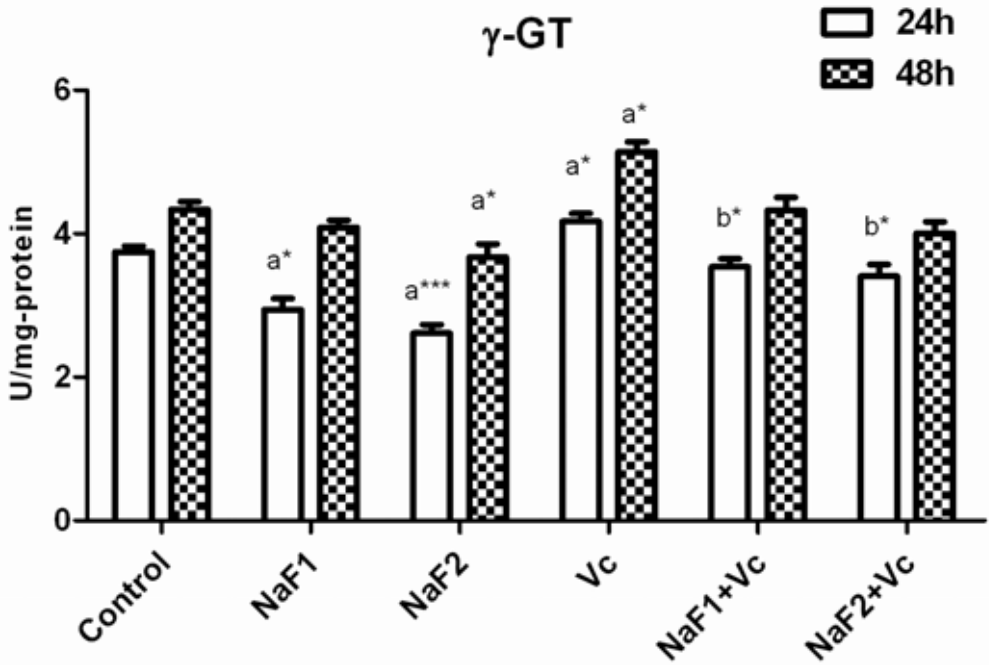
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Figure 4. Dose- and time-dependent effects of NaF on γ-GT activities of Sertoli cell *in vitro*. Each bar denotes mean (±SEM) of five independent experiments carried out in duplicates. Significance at *p<0.05, ***p<0.001. (a) compared with control and (b) compared with vitamin C. Control = 1% horse serum; NaF1 = 4 ppm F; NaF2 = 20 ppm F; Vc = 50 μg vitamin C.
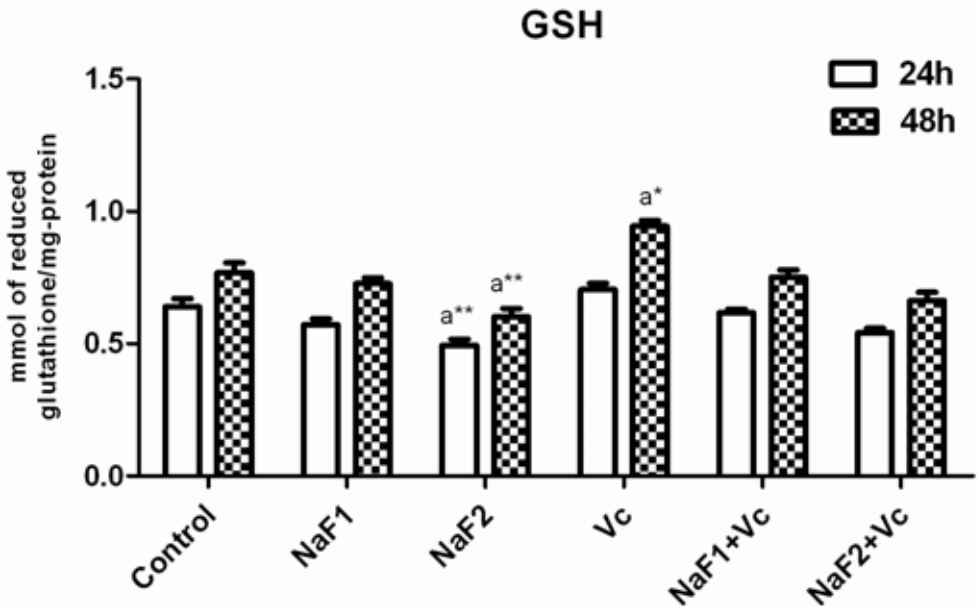

Figure 5. Dose- and time-dependent effects of NaF on GSH activities of Sertoli cell *in vitro*. Each bar denotes mean (±SEM) of five independent experiments carried out in duplicates. Significance at *p<0.05, **p<0.01. (a) compared with control and (b) compared with vitamin C. Control = 1% horse serum; NaF1 = 4 ppm F; NaF2 = 20 ppm F; Vc = 50 μg vitamin C.

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DISCUSSION

Several studies have demonstrated that fluoride causes metabolic, functional, and structural tissue damage.[32-34] Our understanding of the intracellular pathways of cell-fluoride interaction is limited due to the complexity and diversity of the underlying molecular events. On the other hand, several lines of evidence have significantly demonstrated that fluoride stimulates the rate of intracellular oxidation, gene expression, and protein accumulation. In addition, fluoride affects steroidogenesis and leads to reduced levels of testosterone.[35] Different *in vivo* studies have demonstrated that fluoride reduces testosterone levels by affecting steroidogenesis, and exerts this effect by reducing activity of 3β-HSD and 17β-HSD, which are important enzymes for steroidogenesis.[17-20] In this study, there was a significant reduction in 3β-HSD and 17β-HSD levels in both doses and at both time points. When we considered 3β-HSD and 17β-HSD levels, we observed a protective effect of vitamin C in groups treated with NaF+vitamin C. This result is similar to the study by Das Sarkar et al., where they used vitamin E and $CaCl_2$ in addition to NaF.[20]

Fluoride may attack oxygen species, may alter the normal oxygen metabolism, and additionally cause ROS production.[33] Fluoride increases the formation of superoxide anion (O_2^-) and mediates the formation of hydrogen peroxide, and peroxinitric and hydroxyl radicals.[11,36] Moreover, fluoride may alter glutathione levels, and cause excessive ROS production in mitochondria, leading to toxicity, which damages cellular components.[35] There are several cellular defense mechanisms that prevent the formation of ROS. Antioxidant enzymes represent such a defense mechanism, and are responsible for detoxification of ROS. Elevated levels of antioxidant enzymes indirectly indicate elevated ROS production.[37] Superoxide dismutase (SOD) is also a part of this defense mechanism, and catalyzes the reaction between superoxide anion and hydrogen peroxide to form molecular oxygen and water.[33] On the other hand, catalase (CAT), reduces hydrogen peroxide and protects the tissues from hydroxyl radicals. Catalase contains iron (Fe), and inactivates hydrogen peroxide by converting it into water and oxygen.[38] In addition, catalase activity is inhibited by superoxide radicals.[39] The significant reduction in SOD activity in previous studies is similar to our results in NaF-treated Sertoli cells.[11,20,33] In addition, the increase in SOD levels in vitamin C-treated groups is similar to the study by Das Sarkar et al., where they combined NaF with vitamin E or CaC_2.[20] These results suggest that vitamin C has a protective role in SOD-related NaF toxicity in Sertoli cells. The significant reduction in catalase activity is similar to the findings by Ghosh et al. and Das Sarkar et al.[18,40] We did not observe a significant effect of vitamin C on CAT levels, which suggests that vitamin C does not have a protective effect *in vitro* on CAT-related NaF toxicity at the given concentration.

Glutathione peroxidase (GPx) is a selenium-dependent enzyme that catalyzes the reduction of hydrogen peroxide or lipid hydroperoxide (LOOH) by glutathione. When clearing hydrogen peroxide, GPx uses glutathione as the hydrogen donor, and produces GSSG (oxidized from of glutathione) and water.[41-43] Fluoride is

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believed to inhibit GPx activity.[11,36] According to our results, there was no significant difference in GPx levels, compared to the control, at 24 hr in the 4 ppm NaF-treated group, while there were significant differences in the GPx levels, compared to the control, in the 4 ppm NaF-treated group at 48 hr and in the 20 ppm NaF-treated groups at both 24 hr and 48 hr. Similar to the forementioned studies, our results also suggest that NaF reduces GPx activity. We observed a protective effect of vitamin C in the 4 ppm NaF+vitamin C-treated group, similar to the findings by Das Sarkar et al., where they combined fluoride with testosterone or vitamin E and observed a protective effect on GPx levels.[40] On the other hand, the vitamin C dose did not have a protective effect on NaF toxicity at 20 ppm. This outcome differs from the previous findings and the use of higher vitamin E and testosterone doses may explain the different outcome.

Glutathione-S-transferase (GST) is responsible for the conjugation of glutathione (GSH).[44] GST is an antioxidant enzyme that catalyzes the conversion of xenosubstances, which are converted into intermediate products in the liver by the function of cytochrome P450 system, into less reactive species.[45] According to our results, 4 ppm NaF treatment did not have a significant effect on GST activity at 24 hr but it reduced GST activity at 48 hr. In addition, 20 ppm NaF treatment reduced GST activity at both 24 hr and 48 hr. The reduction in GST activity at 100 mM NaF,[46] which is significantly higher than 4 and 20 ppm, suggests that NaF does not affect GST at lower doses, but significantly reduces its activity at higher doses. The vitamin C dose was not sufficient to increase GST activity significantly in the NaF+vitamin C-treated groups.

γ-glutamyl transpeptidase (γ-GT) is one of the key enzymes in glutathione metabolism. γ-GT cleaves extracellular glutathione into glutamic acid and cystenyl glycine for cellular uptake, and then catalyzes its reformation within the cell.[47] In addition, γ-GT is one of the key enzymes determining the intracellular toxicity in Sertoli cells.[47,48] The number of studies investigating the effect of fluoride on γ-GT activity is limited.[49-52] Our study aimed to identify the toxicity of fluoride on TM4 Sertoli cells, and we observed a significant reduction in γ-GT activity in NaF-treated groups in both doses. Our findings are parallel to the reduction in γ-GT levels in Sertoli cells after the administration of toxic substances. γ-glutamyl transpeptidase activity was higher in the 4 ppm NaF+vitamin C-treated group compared to the NaF-treated groups, whereas vitamin C treatment did not affect fluoride toxicity at the 20 ppm dose. These results suggest that vitamin C does not create a protective effect at a high fluoride concentration (e.g., 20 ppm).

Glutathione (GSH) is synthesized as an intracellular tripeptide, and is a natural antioxidant, which protects the cell against toxicity by using NADPH to reduce oxidized molecules.[53] Glutathione plays an important role in the cellular defense system by scavenging free radicals and other oxidants. In addition, it maintains the reduced sulfhydryl groups (-SH) in proteins and protects them against oxidation.[54] Fluoride is known to reduce intracellular GSH levels and inhibit various enzymes that require GSH as a cofactor. A fluoride-dependent reduction in GSH levels is mediated by the reduction in other antioxidant enzymes and the excessive increase

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in ROS production.[5,33,46,55,56] According to our results, there was no significant difference in glutathione levels in the 4 ppm NaF-treated groups at 24 hr and 48 hr, whereas there was a significant reduction in glutathione level in the 20 ppm dose groups. These findings suggest that lower doses of NaF are not sufficient to significantly reduce glutathione levels, and that glutathione levels are affected by high doses of NaF (e.g., 20 ppm), similar to the results found by Ghosh et al.,[46] Chinoy et al., and Ghosh et al. They also demonstrated that vitamin C increased glutathione levels *in vivo*. These findings suggest that the administration of low vitamin C doses *in vitro* is not sufficient to increase glutathione levels to the comparable levels *in vivo,* where higher vitamin C doses are administered.[46,57]

In conclusion, our results suggest that NaF has a toxic effect on TM4 Sertoli cells, and this toxic effect reduces the activities of the enzymes involved in testosterone biosynthesis, and vitamin C treatment (in addition to NaF) may reduce this toxic effect. In addition, we found that NaF caused cellular damage by reducing glutathione levels and the activities of the enzymes involved in the antioxidant system, and that vitamin C might have a protective role against this damage.

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