

## EXPOSURE TO SODIUM FLUORIDE VIA DRINKING WATER CAUSE CYTOTOXICITY AND OXIDATIVE DAMAGE IN LEYDIG CELLS

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**Abstract:** This study investigated the *in vitro* effects of sodium fluoride (NaF) on cytotoxicity and oxidative stress in TM3 Leydig cells. Cytotoxicity was determined with cell viability and proliferation rate, and lactate dehydrogenase leakage assay. Oxidative damage was measured by increasing lipid peroxidation product, reactive oxygen species (ROS) and decreasing cellular enzymatic and non-enzymatic antioxidants. Leydig cells were exposed to two different concentrations of NaF (2ppm and 16ppm) for 24h, 48h and 72h which are considered to be the lowest and highest fluoride concentrations based on daily intake of drinking water in Turkey. The present study showed that cell viability and proliferation decreased and oxidative stress increased in NaF-exposed Leydig cells in a concentration- and time-dependent manner. The findings suggest that fluoride-treatment can cause damage in Leydig cells, especially in antioxidant system of the cells. The results also proved that oxidative stress plays an important role in impairments on Leydig cell function.

**Key words:** Fluoride, cytotoxicity, lipid peroxidation, reactive oxygen species, antioxidant system, Leydig cell.

### Su Aracılığıyla Sodyum Florür Maruziyeti Leydig Hücrelerinde Sitotoksisite ve Oksidatif Hasara Neden Olur

**Özet:** Bu çalışma sodyum florürün (NaF) TM3 Leydig hücrelerinde *in vitro*'da sitotoksisite ve oksidatif stres üzerindeki etkilerini araştırmıştır. Sitotoksisite, hücre canlılık ve çoğalma hızı ve laktat dehidrogenaz testi ile belirlenmiştir. Oksidatif hasar, artan lipid peroksidasyon ürünü, reaktif oksijen türleri (ROS) ve azalan hücrel enzimatik ve enzimatik olmayan antioksidanlar yoluyla ölçülmüştür. Leydig hücreleri, Türkiye'de içme suyunun günlük alımına dayanan en düşük ve en yüksek florür konsantrasyonları olan iki farklı NaF (2ppm ve 16ppm) konsantrasyonuna 24 saat, 48 saat ve 72 saat maruz bırakılmıştır. Bu çalışma, NaF'ye maruz bırakılan Leydig hücrelerinde, konsantrasyon ve zamana bağlı olarak, hücre canlılığı ve proliferasyonundaki azalmanın ve oksidatif stresin arttığını göstermiştir. Bu sonuçlar florür uygulanmasının Leydig hücrelerinde ve özellikle hücrelerin antioksidan sisteminde hasara neden olabileceğini önerir. Bu aynı zamanda oksidatif stresin Leydig hücresi fonksiyonu üzerinde bozulmalarda önemli bir rol oynadığını kanıtlamaktadır.

**Anahtar kelimeler:** Florür, sitotoksisite, lipid peroksidasyonu, reaktif oksijen türleri, antioksidan sistem, Leydig hücreleri.

### Introduction

Fluorine is the lightest halogen and the most electronegative element among all other elements (Kaminsky *et al.* 1990). It is widely distributed in the environment accounting for 0.3g/kg of the earth's crust (Ayoob & Gupta 2006). It usually exists in the environment as inorganic fluorides such as sodium fluoride, calcium fluoride and hydrogen fluoride or organic fluoride compounds such as methyl fluoride, polytetrafluoroethylene and perfluorooctanesulfonic acid (Kaminsky *et al.* 1990). Fluoride is found in the environment in excessive amounts and normally enters human body through drinking water, food, industrial products, dental products, drugs, pesticides, fluoride-containing beverages, cosmetics, but the major source of daily intake is drinking water (Chinoy *et al.* 2004).

Fluoride has both beneficial and deleterious effects on human health (Lennon *et al.* 2004). Some studies suggested that fluoride in low concentrations is essential for mammals and humans, especially for their growth and dental and bone development (Chachra *et al.* 2010, Sun *et al.* 2016). Fluoride is also known to have disruptive effects on various tissues in the body. Studies showed that fluoride intake can cause hematological, hepatic, renal and neurological disorders (Mittal & Flora 2007, Chouhan & Flora 2010, Chouhan *et al.* 2010). Epidemiological studies showed that the relationship between excessive fluoride intake and cancer mortality is correlated in relation to concentration of fluoride in the water supply (Kaminsky *et al.* 1990). Although excessive exposure to fluorides on a daily basis might have a potential negative

impact on male fertility by affecting spermatogenesis, steroidogenesis and sperm fertilizing ability (Zakrzewska *et al.* 2002, Gupta *et al.* 2007, Long *et al.* 2009, Wang *et al.* 2009, Sun *et al.* 2010, Lu *et al.* 2014, Kim *et al.* 2015), its effects on reproductive organs are not fully understood. The present study, therefore, was performed in order to determine direct effects of sodium fluoride on Leydig cell viability, proliferation, cytotoxicity and oxidative damage *in vitro*.

## Materials and Methods

### Cell Culture and Treatment

Mouse Leydig cell, TM3, is a non-tumorigenic cell line derived from Leydig cells of 11–13d old mice and was purchased from ATCC (American Type Culture Collection, Manassas, USA). The cell was maintained in Dulbecco's Modified Eagle Medium/F12 medium (DMEM/F12, Wisent Inc., Canada) supplemented with 2.5% Fetal Bovine Serum (FBS, Wisent Inc., Canada), 5% Horse Serum (HS, Wisent Inc., Canada) and with 100U/ml of penicillin and streptomycin as antibiotics in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air in a CO<sub>2</sub> incubator (Thermo Scientific, USA).

Sodium fluoride concentrations were determined by considering fluoride concentrations humans are exposed to by drinking water (Fawell & Bailey 2006, Oruc 2008, Baba & Tayfur 2011). Leydig cells were exposed to the lowest (2 ppm) and the highest (16 ppm) fluoride concentrations based on daily intake from drinking water for 24, 48 and 72 hours. Sodium fluoride concentrations were prepared in cell culture media containing 1% HS. The control Leydig cells were treated with cell culture media containing 1% HS as a vehicle. All solutions were sterilized with 0.2µm millipore filter.

### Cytotoxicity

#### Cell Viability Assay

The MTT tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay (Roche Diagnostics GmbH, Mannheim, Germany) was performed to measure the viability of cells (Mosmann 1983). Viability ratios of cells were compared to the cell viability of the controls which was treated as 100%.

#### Cell Proliferation BrdU Incorporation Assay

The Cell Proliferation ELISA kit, BrdU (5-bromo-2'-deoxy-uridine), used is a colorimetric immunoassay for quantification of cell proliferation based on the measurement of BrdU incorporation during DNA synthesis (Roche Diagnostics GmbH, Mannheim, Germany).

#### Lactate dehydrogenase (LDH) Leakage Assay

LDH was measured using the micro plate based Cytotoxicity Detection Kit (LDH) (Roche Diagnostics GmbH, Mannheim, Germany). The results were obtained by measuring the absorbance of the red formazan product at 492nm with an ELISA reader.

### Biochemical Analysis

After sodium fluoride treatments, cells (1x10<sup>6</sup>) were harvested and transferred into ice-cold Tris-HCl buffer (pH:7.2) and were sonicated using an ultrasonicator. The resulting cell suspension was centrifuged at 1500g for 10min. at 4°C. Clear supernatants were collected for lipid peroxidation, reactive oxygen species, enzymatic and non-enzymatic antioxidants measurements as biochemical parameters.

### Determination of Lipid Peroxidation

The level of lipid peroxidation was measured using the method described by Devasagayam & Tarachand (1987). The malondialdehyde (MDA) content of the samples were expressed as nanomoles of MDA formed per milligram of protein.

### Determination of Reactive Oxygen Species

Hydroxyl radical production was evaluated following the method described by Puntarulo & Cederbaum (1988). The hydroxyl radical content of the samples was expressed as mmol/min per mg protein. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) production was quantified following the method described by Holland & Storey (1981). The H<sub>2</sub>O<sub>2</sub> content of the samples was expressed as mol/min per mg protein.

### Determination of Enzymatic and Non-Enzymatic Antioxidants

Superoxide dismutase activity (EC: 1.15.1.1, SOD) was measured as the inhibition of autoxidation of pyrogallol by using the method described by Marklund & Marklund (1974). Activity was monitored at 440nm for 180s. Data were expressed as U of SOD/mg protein. Catalase (EC 1.11.1.6, CAT) activity was determined by the method of Sinha (1972) by following the decomposition of H<sub>2</sub>O<sub>2</sub> in absorbance at 570nm. The enzyme activity was calculated using an extinction coefficient of 1.88x10<sup>4</sup> M<sup>-1</sup> cm<sup>-1</sup> and was expressed in international units (I.U.) i.e., in U of CAT/mg protein. Glutathione Peroxidase (EC 1.11.1.9, GPx) activity was measured following Hafeman *et al.* (1974) by using H<sub>2</sub>O<sub>2</sub> as a substrate and as the rate constant of glutathione decomposition. Activity was monitored at 412nm. Data were expressed as µM of glutathione consumed per mg of protein. The method by Habig *et al.* (1974) was used to determine glutathione-S-transferase (EC 2.5.1.1.8, GST). GST activity was expressed as µmol GS-DNB min<sup>-1</sup> per mg protein using an extinction coefficient of 9.6mM cm<sup>-1</sup>. Gamma-Glutamyl Transpeptidase (EC 2.3.2.2, γ-GT) activity was estimated by the method of Orłowski & Meister (1965). The level of reduced glutathione (GSH) was determined based on the reaction with Ellman's reagent (0.4% DTNB in 0.1M phosphate buffer, pH 7.4) and reduced glutathione (Ellman 1959). The absorbance was recorded at 412nm using a spectrophotometer. The GSH content was expressed as mmol of reduced glutathione/mg protein.

### Statistical analysis

All statistical analyses were carried out using the software program GraphPad Prism Version 5.0 (GraphPad Software, San Diego, CA, USA). Experimental data grouped by one variable were analyzed by one-way ANOVA followed by Tukey posttest. The results were expressed as mean  $\pm$  standard error mean. Data distribution was tested for normality using the Shapiro-Wilk test and then data were evaluated with one-way ANOVA using Tukey's multiple comparisons tests; values of  $p < 0.001$ ,  $p < 0.01$  and  $p < 0.05$  were considered as statistically significant.

## Results

### Cytotoxicity Effects of Sodium Fluoride

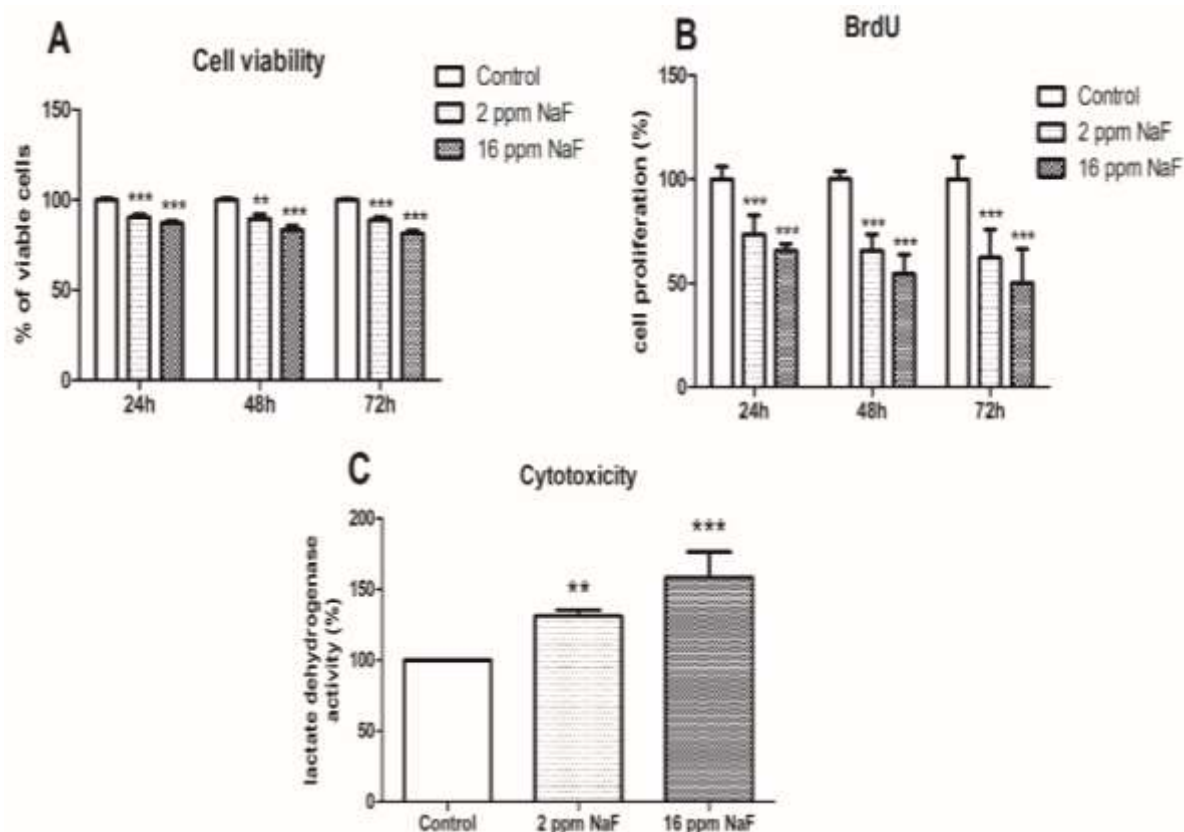
Cytotoxic effects of sodium fluoride (NaF) on TM3 Leydig cells after 24h, 48h and 72h incubation was measured by MTT and BrdU assays and the data are shown in Figure 1. According to results of MTT reduction assay, both 2ppm and 16ppm NaF concentrations reduced cell viability rate at 24h, 48h and 72h (Fig. 1A). NaF inhibited cell proliferation in a dose and time-dependent manner (Fig. 1B). The addition of 2ppm and 16ppm NaF for 24h, 48h and 72h reduced cellular 5-bromo-2'-deoxyuridine (BrdU)-uptake significantly to 73.5% (2ppm NaF,

$p < 0.001$ ), 65.6% (2ppm NaF,  $p < 0.001$ ), and to 62.3% (2ppm NaF,  $p < 0.001$ ) and 65.7% (16ppm NaF,  $p < 0.001$ ), 54.3% (16ppm NaF,  $p < 0.001$ ), 50.1% (16ppm NaF,  $p < 0.001$ ) respectively, compared to non-treated control cultures (100%).

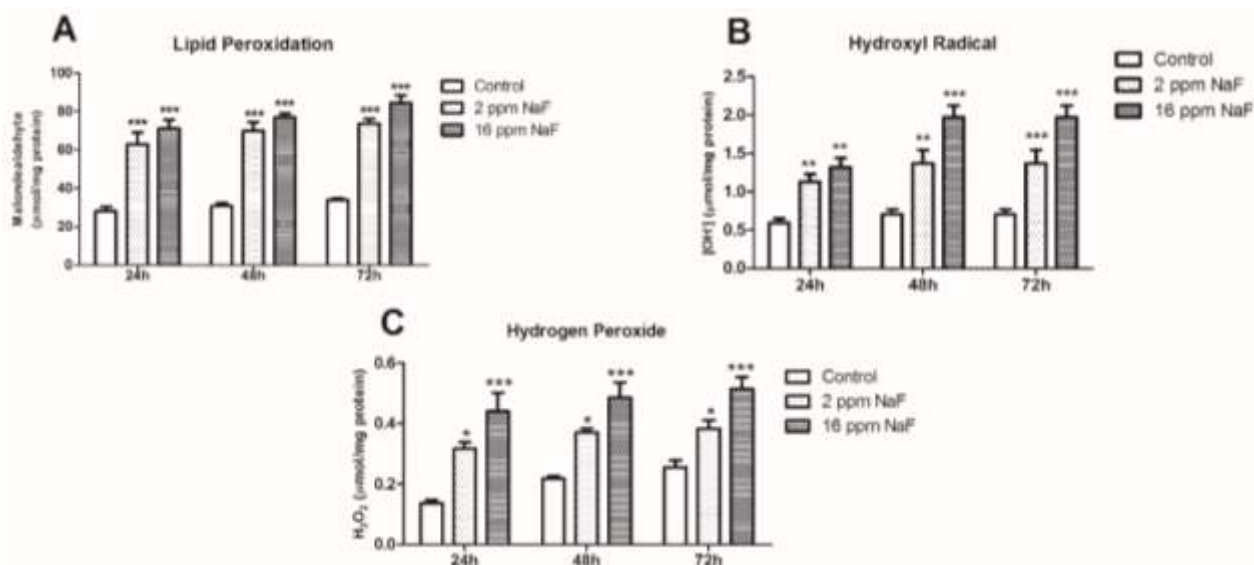
The cell membrane damage induced by NaF in TM3 Leydig cells was investigated by the release of LDH, a biomarker of membrane integrity. LDH release was significantly increased after treatment with 2ppm ( $p < 0.01$ ) and 16ppm ( $p < 0.001$ ) NaF as compared with an untreated control (Fig. 1C).

### Effects of Sodium Fluoride on Lipid Peroxidation and ROS

Malondialdehyde (MDA) content as a marker of lipid peroxidation after exposure to NaF is presented in Figure 2A. The results indicated that MDA levels significantly increased in NaF treated groups in a dose and time-dependent manner compared with the control (Fig. 2A). To determine NaF-induced ROS generation, we measured hydroxyl radical and hydrogen peroxide in Leydig cells under different NaF concentrations. ROS generation in the NaF-treated cells resulted in a significant and concentration and time-dependent increase ( $p < 0.05$ , 0.01 or 0.001) (Fig. 2B and 2C).



**Fig. 1.** Concentration- and time-dependent effects of NaF on Leydig cell viability (A), proliferation (B) and cytotoxicity (C). Data are expressed as mean  $\pm$  S.E.M from three repeated experiments. \*indicates significantly different from control (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ).

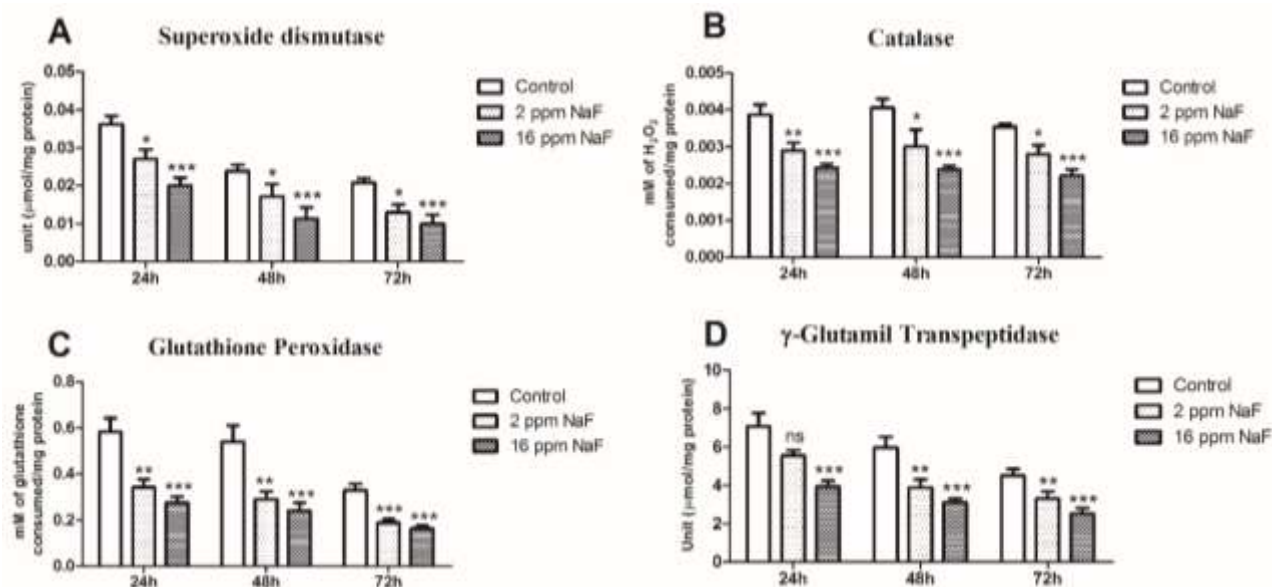


**Fig. 2.** Effects of NaF concentrations on MDA levels (A), hydroxyl radical (B) and H<sub>2</sub>O<sub>2</sub> (C) in Leydig cells. Data are expressed as mean  $\pm$  S.E.M from three repeated experiments. \* indicates significantly different from control (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ).

#### Effects of Sodium Fluoride on Cellular Antioxidant System

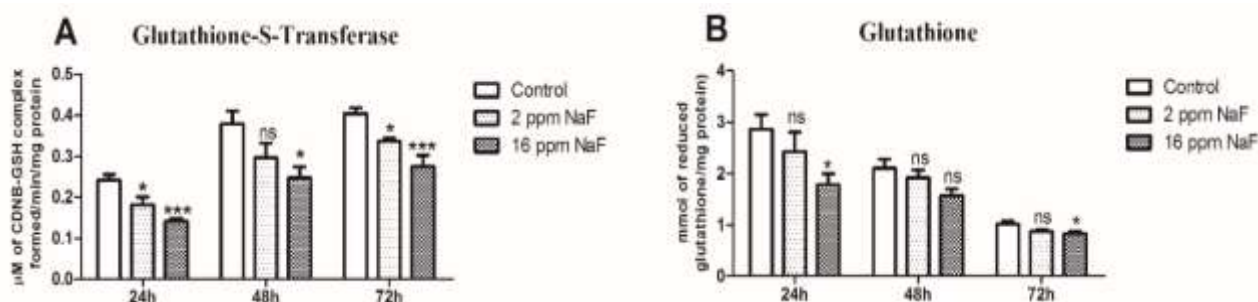
Figures 3 and 4 provide data on cellular levels of antioxidant enzymes such as SOD, CAT, GPx, GST,  $\gamma$ -GT, and non-enzymatic antioxidant such as glutathione in control and NaF-exposed Leydig cells for 24, 48 and 72h. The activity of this group of enzymes was significantly diminished in a dose and time-dependent manner when compared with the respective control. SOD, CAT and GPx were significantly lower at 2ppm ( $p < 0.05$ ,  $p < 0.01$ ) and 16ppm ( $p < 0.001$ ) concentrations for all time points

(Fig. 3A, B and C). Gamma-Glutamyl Transpeptidase ( $\gamma$ -GT) activity was significantly reduced except for 2ppm NaF-treated group for 24h (Fig. 3D). GST enzyme activity was significantly diminished for 2ppm ( $p < 0.05$ ) and 16ppm ( $p < 0.001$ ) NaF-treated group at 24 and 72h, however there is no significant difference for 2ppm NaF-treated group at 48h (Fig. 4A). Glutathione depletion was regarded as the marker of oxidative stress in cells, glutathione level in NaF-treated groups decreased significantly in 16ppm NaF group for 24 and 72h ( $p < 0.05$ ) (Fig. 4B).



**Fig. 3.** Effects of NaF concentrations on SOD (A), catalase (B), GPx (C) and  $\gamma$ -GT (D) in Leydig cells. Data are expressed as mean  $\pm$  S.E.M from three repeated experiments. \* indicates significantly different from control (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ).





**Fig. 4.** Effects of NaF concentrations on GST (A), GSH (B) in Leydig cells. Data are expressed as mean  $\pm$  S.E.M from three repeated experiments. \* indicates significantly different from control (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ).

## Discussion

Various environmental toxicants are capable of causing oxidative stress, developmental and reproductive abnormalities and infertility. Fluoride is known as an environmental contaminant that causes various hazard effects on organisms (Kaminsky *et al.* 1990, Ayoob & Gupta 2006, Long *et al.* 2009, Dey & Giri 2016). Fluoride is found in all natural waters. It can be extremely high in groundwater, depending on a number of factors, such as volcanic rocks and minerals. Drinking water is the largest fluoride source, adding to your exposure from dental products (Fawell *et al.* 2006). Previous studies demonstrated that fluoride has toxic effects on male reproductive system, including reproductive organ damage (Chinoy *et al.* 2004, Gupta *et al.* 2007), endocrine disruption of reproductive system (Long *et al.* 2009) and the expression of steroidogenesis genes (Dong *et al.* 2016).

Several studies suggested that fluoride exposure decrease cell viability in different cells including Sertoli (Yang *et al.* 2015, Yilmaz & Erkan 2015), Leydig (Song *et al.* 2014), human lung BEAS-2B (Ying *et al.* 2017), lung epithelial cells (A549) (Ameeramja *et al.* 2016), primary rat ameloblast (Wang *et al.* 2016) and primary hippocampal neurons (Zhang *et al.* 2007) dose- and time-dependent manner. In addition, some studies showed that germinal and interstitial cell count (Bataneh & Nusier 2006), epididymal sperm count and viability (Wan *et al.* 2006) were significantly reduced after NaF treatment. According to findings in this study, 2ppm NaF exposure decreased cell viability by 90% at 24h, 89% at 48h, 88% at 72 h and 16ppm NaF decreased cell viability by 86% at 24h, 83% at 48h, 81% at 72 h in Leydig cells meaning that NaF decrease cell viability in a dose- and time-dependent manner. It is well known that BrdU incorporation assay measures the amount of DNA synthesized by the dividing cells and can be a good measure of cell proliferation. Previous studies reported that NaF had a biphasic effect on cell proliferation depending on concentration in ameloblast lineage cells (Yan *et al.* 2007, Riksen *et al.* 2011). According to Yan *et al.* (2007), low fluoride concentrations increased cell proliferation, while high fluoride concentrations significantly decreased cell proliferation in ameloblast-derived cell lines. Besides, fluoride inhibited cell growth, proliferation,

mitochondrial activity and protein synthesis in cultured human pulp cells in a concentration-dependent manner (Chang & Chou 2001). Song *et al.* (2013) investigated cell cycle arrest, apoptosis and DNA damage of fluoride in TM3 Leydig cells *in vitro*. Fluoride reduced proliferating index in Leydig cells to 81% at 16.79ppm, 70% at 25.19ppm and 59% at 33.59ppm at 24h, however proliferating index did not alter at 8.39ppm concentrations. Nevertheless, fluoride did not significantly affect Sertoli cells proliferation in 4ppm and 20ppm concentrations (Yilmaz & Erkan 2015). In our present study, 2ppm concentration fluoride treatment significantly decreased cell proliferation in TM3 Leydig cells to 73.5% at 24h, 65.6% at 48h, 62.3% at 72h and 16ppm concentration to 65.7% at 24h, 54.3% at 48h, 50.1% at 72h. These results revealed that NaF decreased amount of DNA synthesized related to decrease in cell proliferation in Leydig cells as in other cell types (Chang & Chou 2001, Riksen *et al.* 2011, Song *et al.* 2013, Yan *et al.* 2007).

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme stored in various cell types. It has been widely used to evaluate damage of plasma membrane and toxicity of cells (Weyermann *et al.* 2005). Earlier studies showed that increased LDH release of Sertoli cells (Yilmaz & Erkan 2015), primary human hepatocytes (Wang *et al.* 2004) and primary hippocampal neurons (Zhang *et al.* 2007) after treatment of NaF were notably enhanced, while fluoride had no major cytotoxic effect on the LS-8 ameloblast derived cell line (Riksen *et al.* 2011). In the present study, 2ppm (131.2%) and 16ppm (158.4%) NaF concentrations significantly increased LDH release in Leydig cells, meaning that fluoride has cytotoxic effects on TM3 Leydig cells.

Reactive oxygen species (ROS) are used for oxygen-derived free radicals (superoxide, hydroxyl radical, nitric oxide) and non-radical oxygen derivatives of high reactivity (singlet oxygen, hydrogen peroxide, peroxynitrite, hypochlorite), which can cause damage to biological targets such as lipid membranes, DNA and cellular proteins. In return, cells have their own defense system which are composed of antioxidant enzymes and other molecules (SOD, catalase, GPx, GST and GSH) (Kohen & Nyska 2002). Fluoride can disrupt this prooxidant/antioxidant balance with increasing ROS and

lipid peroxidation product and with decreasing cellular antioxidant enzymes such as SOD, catalase and GPx in various tissues and cells (Akdoğan *et al.* 2002, Zhang *et al.* 2007, Rao & Bhatt 2012, Yang *et al.* 2013, Orta & Erkan 2014, Feng *et al.* 2015). Various *in vivo* studies showed that NaF in high doses can induce membrane lipid peroxidation in liver, kidney (Mittal & Flora 2006, Mittal & Flora 2007), testis (Rao & Bhatt 2012, Zhang *et al.* 2013) and spermatozoa (Izquierdo-Vega *et al.* 2008). In addition, several *in vitro* studies demonstrated that the effect of NaF on lipid peroxidation depends on dose and time dependent manner. Lipid peroxidation increased significantly in Sertoli cells after 4ppm and 20ppm NaF treatment for both 24h and 48h (Yilmaz & Erkan 2015), however there was no significant change in lipid peroxidation level in primary Sertoli cells after 6ppm NaF treatment (Yang *et al.* 2015). Moreover, lipid peroxidation product significantly increased in 80ppm NaF dose for 24h in human hepatocyte (Wang *et al.* 2004) and in 20ppm, 40ppm and 80ppm doses for 24h in primary hippocampal neurons (Zhang *et al.* 2007). Our present study showed that both 2ppm and 16ppm NaF treatment increased lipid peroxidation in Leydig cells. Several recent studies demonstrated that NaF increased ROS levels in Sertoli cells (Yang *et al.* 2015, Yilmaz & Erkan 2015), primary hippocampal neurons (Zhang *et al.* 2007), liver, kidney and brain tissues (Chouhan & Flora 2008). Our present results revealed that NaF increased hydrogen peroxide and hydroxyl radical both at 2ppm and 16ppm exposures for 24h, 48h and 72h, suggesting that free radicals and non-radical oxygen derivatives were involved in oxidative damage.

The potential harmful effects of ROS are controlled by cellular antioxidant defense system. Cellular antioxidant defense system is classified into two major groups, enzymatic antioxidants such as SOD and non-enzymatic antioxidants such as glutathione (Bhattacharya 2015). SOD is an antioxidant enzyme known to eliminate superoxide radical. On the other hand, catalase and glutathione peroxidase (GPx) remove hydrogen peroxides by converting them into water and oxygen. Glutathione-S-transferase (GST) catalyzes intracellular detoxification reactions, including inactivation of many toxic substances by catalyzing their conjugation with glutathione. Another antioxidant enzyme is  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT) that is functional in glutathione metabolism (Kohen & Nyska 2002). Some previous *in vitro* studies showed that SOD activity decreased in a concentration-dependent manner in primary hippocampal neurons, primary Sertoli cells, TM4 Sertoli cells and TM3 Leydig cells (Zhang *et al.* 2007, Yang *et al.* 2013, Orta & Erkan 2014, Yang *et al.* 2015). The result of present study demonstrated that both 2ppm and 16ppm concentrations for all exposure durations decreased SOD activity in TM3 Leydig cells when compare to control. Recent studies indicated that exposure to NaF decreased catalase and GPx activity in a concentration and time-dependent manner in Sertoli and Leydig cells (Yang *et al.* 2013, Orta & Erkan 2014).

Another study revealed that 20, 40 and 80ppm NaF treatment declined GPx activity in primary hippocampal neurons (Zhang *et al.* 2007). The results in this study showed that catalase and GPx enzyme activities reduced significantly after NaF treatment in TM3 Leydig cells. Several studies indicated that GST activity was decreased after NaF treatment in testis and TM4 Sertoli cells (Rao & Bhatt 2012, Orta & Erkan 2014). The activity of  $\gamma$ -GT also decreased significantly after exposure to NaF in kidney, semen and Sertoli cells (Akdoğan *et al.* 2002, Zakrzewska *et al.* 2002, Orta & Erkan 2014). According to the result of the present study, 2ppm and 16ppm NaF treatment decreased  $\gamma$ -GT activity at 48h and 72h, while only 16ppm NaF decreased  $\gamma$ -GT activity at 24h in Leydig cells.

Glutathione (GSH) acts as a cofactor for enzyme peroxidase, thus serving as an indirect antioxidant for hydrogen peroxide (Kohen & Nyska 2002). Previous *in vitro* studies demonstrated that fluoride affects GSH level in Sertoli cells (Orta & Erkan 2014), primary hippocampal neurons (Zhang *et al.* 2007) and primary human hepatocytes. According to findings of a recent study, GSH level decreased only after a treatment with 20ppm concentration for 24 and 48h in Sertoli cells (Orta & Erkan 2014). Moreover, GSH levels significantly decreased after 20, 40 and 80ppm treatments in primary hippocampal neurons and 80ppm treatment in primary human hepatocyte (Wang *et al.* 2004, Zhang *et al.* 2007). The current study showed that there was no significant difference in GSH levels after 2ppm NaF treatment at 24, 48 and 72h, however there was a significant decline in 16ppm concentration at 24 and 72h. These findings suggest that low doses of NaF do not affect GSH levels.

## Conclusion

To maintain the function of Leydig cells, which are responsible for testosterone biosynthesis and are an important cell type in the male reproductive system, are the key factor for improvement of male infertility. When daily intake of minimum and maximum fluoride amount from drinking water supply is taken into consideration, it is very important to determine possible effects of fluoride on Leydig cells. The results of our present study revealed that fluoride has toxic effects on Leydig cells even with low doses. Fluoride builds up these effects by increasing the lipid peroxidation, level of ROS and cell membrane damage and by decreasing the level of cell viability, cell proliferation and antioxidant level. Although these results provide information about the cytotoxicity of fluoride on Leydig cells, further studies are necessary to investigate molecular interaction of oxidative stress markers and cell dynamics.

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