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DNA damage-induced by sodium flouride (NaF) and the effect of cholicalciferol

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Abstract: It is known that the high electronegativity of fluorine affects various soft tissues, especially the bone structure in organisms. Of these tissues are the kidneys, which play an important role in the excretion of fluoride from the body. Fluoride affects many cellular mechanisms. One of these effects is DNA damage. Our study aimed to investigate the likely protective effect of cholecalciferol (vitamin D3) on genomic DNA damage-induced NaF depending on concentration and time. The IC25 and IC50 values of NaF for 3, 12 and 24 h and optimum dose of increase in proliferation to vitamin D_3 through MTT assay in NRK-52E kidney cells were determined. DNA damage was significantly increased ($p < 0.05$) compared to the control group in all groups except for vitamin D_3. It was determined that treatment with NaF together with vitamin D_3 decreased the DNA damage compared to NaF treated groups for 3 and 12 h. NaF combined with vitamin D3 was determined statistically to decrease ($p < 0.05$) DNA damage compared to NaF treated groups for 24 h. As a result, it was determined that the treatment with cytotoxic concentration NaF depending on the time significantly increased ($p < 0.05$) the genomic DNA damage, but NaF treatment together with vitamin D_3 decreased the DNA damage in renal cells depending on the time. It was concluded that vitamin D_3 may be useful in preventing DNA damage caused by NaF.

Introduction

Living organisms are exposed to fluoride from many environmental sources, namely fluoride compounds composed of various sources such as water and soil, industrial zones producing fluoride waste (Kanduti *et al.*, 2016) and fluoride-containing pesticides (Li *et al.*, 2015). While 50-60% of the daily fluoride intake is excreted by the kidneys, the remainder accumulates largely in the calcified tissues of the bones and pineal gland. There are extensive studies reporting that fluorine can affect different biological activities (Dec *et al.*, 2017). Long-time exposure to fluoride compounds causes fluorosis, which is characterized by structural disorders of the teeth and the skeleton (Krishnamachari, 1986; Yur *et al.*, 2013; Aydın *et al.*, 2014). The genotoxic effect of fluoride has been demonstrated in some studies (Zhang *et al.*, 2008; Song *et al.*, 2015). Fluoride is known to cause damage to cell structures in various tissues (Agalakova and Gusev, 2012) and oxidative DNA damage (Guney *et al.*, 2007; Yüksek *et al.*, 2017b) with apoptosis (Song *et al.*, 2015; Yüksek *et al.*, 2017b) in healthy cells.

Vitamins (such as A, C, D, E) can help protect the organism from free radical oxidation in amelioration of fluoride-induced toxicity (Chlubek, 2003; Chaiprasongsuk *et al.*, 2019; Wimalawansa, 2019). In cell and animal studies, the protective effects of vitamin D against oxidative stress or oxidative damage were reported by a multitude of research groups (Chaverri *et al.*, 2016). The role of vitamin D in fluorosis has been known for many years. Calcium intake and the vitamin D status may modify the fluorine absorption. The relatively adequate calcium intake and 25 dihydroxy vitamin D [$25(OH)_2D$] may have prevented severe skeletal fluorosis (Lidemann, 1965).

It is known that the mean serum 25- OHD3 (25 Hydroxy Vitamin D) levels were significantly lower in the fluorosis condition (Khandare *et al.*, 2005). There are some studies about the use of vitamin D in an attempt to inhibit or reverse the adverse effects of fluorosis. Vitamin D3 has been proven to be effective when in combination in reversal of dental fluorosis (Gupta *et al.*, 1994).

There are only a few studies available regarding the prevention or reducing DNA damage due to fluoride exposure. Our previous study demonstrated that the levels

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of 8-OHdG, which is an oxidative DNA damage marker, were significantly decreased in NaF + vitamin D-administered groups compared to the control and NaF-administered groups in NRK-52E cells (Yüksek *et al.*, 2017b). There have been no studies demonstrating the importance of vitamin D_3 in prevention of genomic DNA damage caused by fluoride in the literature.

For this purpose, DNA damage was induced by administering IC_{25} and IC_{50} concentrations of NaF to NRK-52E kidney cells for 3, 12 and 24 h. This study was planned to reveal the protective effect of vitamin D_3 on prevention of DNA damage caused by NaF depending on time and concentration.

Materials and Methods

Cell culture

The study material comprised rat renal epithelial NRK-52E (ATCC® CRL-1571™) cells. NRK-52E cells were cultured in vitro with cultured in a medium containing, 10% fetal bovin serum(FBS), 1% penicillin/streptomycin, 1% L-glutamine and RPMI 1640 at 37°C, 95% humidity, 5% CO_2.

Preparation of solutions

Stock solutions of NaF and vitamin D_3 used in the study were prepared by referring to the concentrations in our previous study (Yüksek *et al.*, 2017b). NaF was dissolved in the medium. Vitamin D in DMSO was kept at the non-toxic (≤0.05%) level. Cell viability was measured by MMT assay to measure the cytotoxic effect of NaF at different times and concentrations. NRK-52E cells were treated with various concentrations of NaF (Fig. 2) for 3, 12 and 24 h.

MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide) cell viability tests were performed and proliferative doses of vitamin D_3, and IC_{25} and IC_{50} concentrations of NaF (Tab. 1) for 3, 12 and 24 h were determined that according to the control group. The cells were cultured in 96-well plates at 7×10^3 cells/well density in 100 µl medium and incubated overnight for attachment to the surface of the plate. The control and the study groups and the cross study groups were designed

Comet assay

The cells were cultured in 25 cm^2 flasks at 2×10^6 cells density in medium, and incubated overnight for attachment to the surface of the flask. The cells were treated only with IC_{25} and IC_{50} concentrations of NaF and in combination with vitamin D_3 for 3, 12 and 24 h. Cells, the incubation times of which had expired, were collected by the trypsinization-EDTA method. The single cell neutral comet assay phase was passed. The level of DNA damage was determined by calculating the genetic damage index (GDI). GDI, Arbitrary Units (AU) = $(0 \times N_0 + 1 \times N_1 + 2 \times N_2 + 3 \times N_3 + 4 \times N_4/N_0 + N_1 + N_2 + N_3 + N_4) \times 100$. 'N' shows the number of cells according to the degree of damage (García *et al.*, 2011; Taspinar *et al.*, 2013).

Statistical analysis

The *p* values of the study groups were determined according to the Z-ratio test comparison (Minitab Statistical Software, 18 version).

Results

As a result of the MTT test, different NaF IC_{25} and IC_{50} concentrations at different times were obtained, which have been presented in Tab. 1, and it was determined that the best concentration of vitamin D_3 in Fig. 1 that improved cellular proliferation was 10 µM.

Cellular proliferation increased at concentrations up to 250 µM in the planned three test durations when compared to the control group. During all the incubation periods, it was observed that NaF cytotoxicity began after 500 µM. After 500 µM, it was found that cell viability decreased based on time and concentration in Fig. 2.

Genomic DNA damage has been presented in Tab. 2 and the statistical results associated with the data presented in Tab. 3.

It was determined that DNA damage was significantly higher ($p < 0.05$) in all groups except for the vitamin D_3 group when compared to the control group. A separate analysis of the incubation periods demonstrated that at the 3 h, NaF (IC_{25} and IC_{50}) significantly increased ($p < 0.05$) the DNA damage when compared to the control group. Concomitant administration of vitamin D_3 with the above mentioned concentrations did not reduce the damage ($p > 0.05$).

At the 12 h, it was determined that the NaF IC_{25} concentration significantly increased the DNA damage compared to control group ($p < 0.05$). At this concentration, it was demonstrated that vitamin D_3 treatment decreased the damage by 20% ($p > 0.05$). During the same incubation period, NaF IC_{50} administration increased the DNA damage ($p < 0.05$); however, vitamin D_3 administration decreased the damage by 15% ($p > 0.05$).

At the 24 h, NaF IC_{25} concentration significantly increased the DNA damage when compared to the control group ($p < 0.05$). In this concentration, concomitant vitamin

TABLE 1

Time dependent cytotoxic NaF concentrations as determined in the MTT assay

Incubation times	NaF IC_{25} (µM)	NaF IC_{50} (µM)
3 h	7750	9600
12 h	2750	5500
24 h	1600	3200

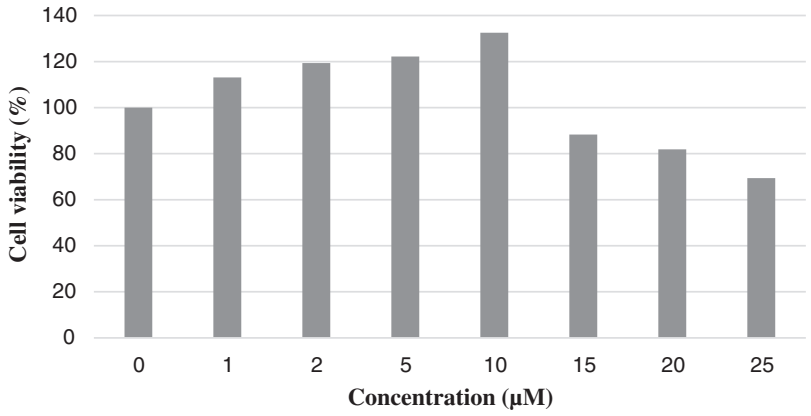

FIGURE 1. MTT cell viability (%) in the NRK-52E cell line treated with vitamin D_3 in the control group (0 µM vitamin D_3) set at 100%.

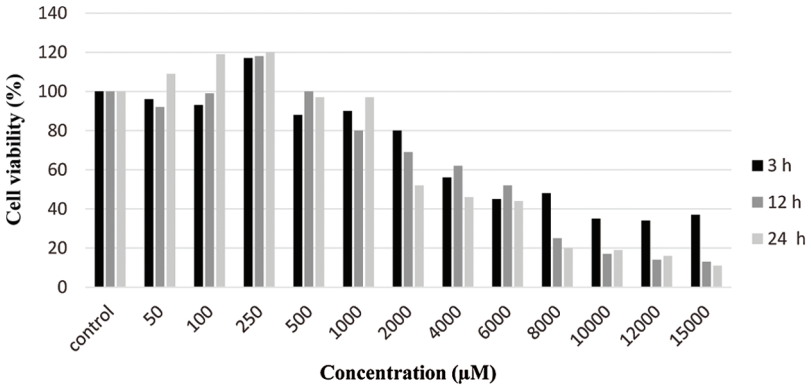

FIGURE 2. MTT cell viability (%) in the NRK-52E cell line treated with NaF in the control groups (0 µM NaF) set at 100%.

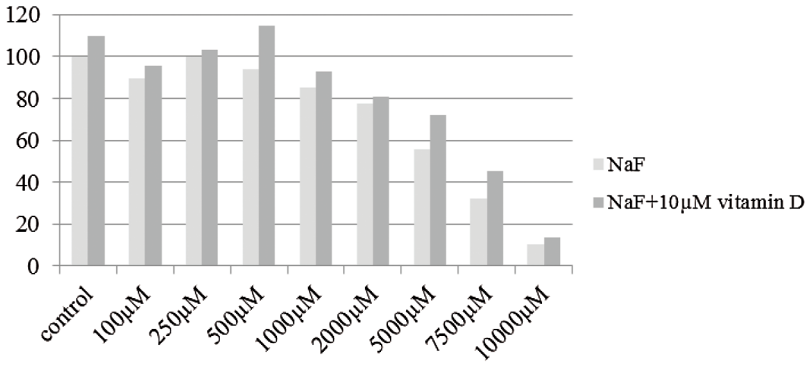

FIGURE 3. MTT cell viability (%) in the NRK-52E cell line treated with NaF and NaF + vitamin D_3 the viability in the control group (0 µM NaF + 0 µM vitamin D_3) set at 100% for 24 h (Yüksek *et al.*, 2017a).

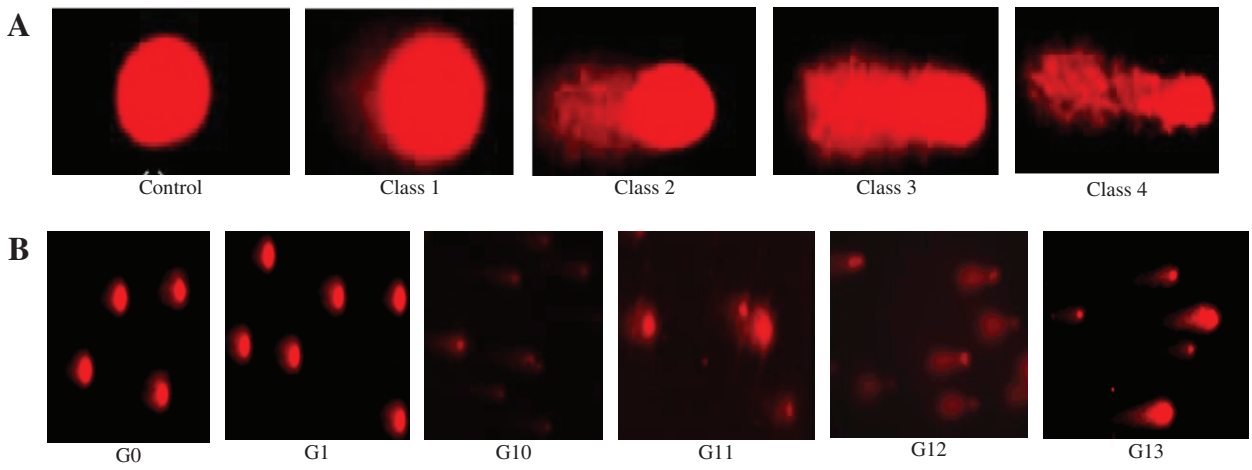

FIGURE 4. (A) Assessed by DNA damage using the comet assay. The cells were evaluated image and received class 0 (undamaged) to 4 (maximally damaged), according to the size and shape of the tail. (B) Showed the representative images of comet assay to NRK-52 cells in control (G0), vitamin D_3 (G1), NaF IC_{25} (G10), NaF IC_{25} + vitD_3 (G11), NaF IC_{50} (G12) and NaF IC_{50} + vitD_3 (G13) groups for 24 h.

TABLE 2

Genetic Damage Index (GDI) obtained from different levels of NaF and vitamin D_3 treated groups

	Groups	Group no.	GDI × 100 (AU)
	Control	G0	16
	Vitamin D_3	G1	29
3 h	NaF IC_{25}	G2	246
	NaF IC_{25} + vitD_3	G3	250
	NaF IC_{50}	G4	254
	NaF IC_{50} + vitD_3	G5	254
12 h	NaF IC_{25}	G6	101
	NaF IC_{25} + vitD_3	G7	80
	NaF IC_{50}	G8	196
	NaF IC_{50} + vitD_3	G9	165
24 h	NaF IC_{25}	G10	194
	NaF IC_{25} + vitD_3	G11	161
	NaF IC_{50}	G12	234
	NaF IC_{50} + vitD_3	G13	160

TABLE 3

***p* values for group DNA damage rates based on Minitab Statistical Software and Z-ratio comparison**

	G0	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12	G13
G0	0	0,045	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,001
G1			0,050	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,001	0,001
G2				0,836	0,680	0,680	0,001	0,001	0,001	0,007	0,005	0,001	0,530	0,001
G3					0,837	0,837	0,001	0,001	0,001	0,004	0,003	0,001	0,403	0,001
G4						1,00	0,001	0,001	0,001	0,002	0,001	0,001	0,298	0,001
G5							0,001	0,001	0,001	0,002	0,001	0,001	0,298	0,001
G6								0,101	0,001	0,001	0,001	0,001	0,001	0,001
G7									0,001	0,001	0,001	0,001	0,001	0,001
G8										0,071	0,091	0,809	0,001	0,762
G9											0,910	0,041	0,038	0,035
G10												0,053	0,029	0,046
G11													0,001	0,951
G12														0,001
G13														

D_3 and NaF administration reduced the damage by about 15%; however, the decrease was not statistically significant ($p > 0.05$). It was determined that treatment with IC_{50} concentration in the same period significantly increased the DNA damage ($p < 0.05$) and vitamin D_3 treatment decreased ($p < 0.05$) DNA damage by 35%.

Discussion

The fluoride molecule exists in nature and in several vital resources. Fluoride accumulates in several living organism tissues that have been exposed to fluoride for extended periods of time. Accumulated fluoride molecules lead to structural damages in organisms. In humans, the kidney is the tissue where these damages are observed most. The underlying reasons for fluoride-induced tissue damage include oxidative stress and toxicity pathways.

The correlation between fluoride-induced oxidative stress and fluoride toxicity has been demonstrated in in vivo (Yüksek *et al.*, 2017a; Lu *et al.*, 2017) and in vitro (He and Chen, 2006) studies. In studies performed with in vitro approaches, it was demonstrated that fluoride could lead to dose-dependent genomic DNA damage (Zhang *et al.*, 2008; He and Chen, 2006), apoptosis (Yüksek *et al.*, 2017b;

Lu *et al*., 2017) and cell cycle variations (He and Chen, 2006; Otsuki *et al*., 2005). The present study findings also demonstrated that NaF could lead to dose-dependent genomic DNA damage. Thus, the present study findings were consistent with those reported in other studies.

In molecular studies investigating fluoride-induced cellular damage and necrosis, NaF has been used as the fluoride source similar to the present study. He *et al*. (2015) reported that NaF inhibited cell proliferation after 320 µM and led to increases in necrosis in human RPMI8226 cell lines in a study where different concentrations of NaF were administered for 48 h. In another study carried out on HL-60 cells, it was reported that the cell count decreased rapidly in the 2.5 mM NaF administered group, and only 11% of the cells remained alive in the 7.5 mM administered group (Otsuki *et al*., 2005). In anonther study performed on H9c2 cells, it was reported that a 16 mg/L (380 µM) concentration of NaF increased the cellular proliferation in the first 24 h and led to cytotoxicity after 48 h (Yan *et al*., 2015). In two previous studies, we reported that cellular proliferation decreased after the 500 µM concentration in HfOB 1.19 and NRK-52E cell lines when NaF was administered for 24 h (Yüksek *et al*., 2017b; Çetin *et al*., 2019).

On the other hand, it was determined that NaF increased the cellular proliferation at low concentrations (50, 100 and 250 µM) and similarly decreased at high concentrations (500 µM and above) in the NRK-52E kidney cell line in the present study. Furthermore, it was shown that 10000 µM NaF administration reduced the cell viability to below 20% when compared to the control. The present study findings demonstrated that NaF exhibited cytotoxic effects and the finding was consistent with other studies, which reported that the cytotoxicity varied based on the cell type, concentration and the administration period (Otsuki *et al*., 2005; He *et al*., 2015; Yan *et al*., 2015; Yüksek *et al*., 2017b; Çetin *et al*., 2019).

Otsuki *et al*. (2005) reported that 5 mM and higher NaF concentrations induced DNA fragmentation in HL-60 cells. It was reported that NaF could induce oxidative stress and DNA damage in the rat oral mucosal and hepatocyte cells and lead to apoptosis and cell cycle variations (He and Chen, 2006). In a study performed by Song *et al*. (2014), it was reported that 50, 100 and 200 mg/L NaF administration led to renal tissue damages in rats and the damages increased with administered NaF concentration. In the present study, it was also determined that NaF led to DNA damage based on concentration. Thus, the present study findings were consistent with the studies in the literature.

Several studies have been performed on the use of vitamins (Yüksek *et al*., 2017a), flavonoids (Pal and Sarkar, 2014) and natural molecules (Çetin *et al*., 2017) in the prevention of fluoride-induced tissue damage (Guney *et al*., 2007), organ damage, and DNA damage (Pal and Sarkar, 2014).

It was determined that apoptosis significantly decreased in concomitant fluoride and vitamins E and C administered rats with endometrial damage, and vitamin E and C combination could prevent fluoride-induced apoptosis (Guney *et al*., 2007). In a study performed by Pal and Sarkar (2014), it was reported that resveratrol administration may be beneficial in preventing fluoride-induced DNA damages. In a previous study (Yüksek *et al*., 2017b), it was demonstrated that vitamin D_3 administration may be beneficial in preventing NaF-induced oxidative DNA damage.

The molecular basis of fluoride intoxication is interesting and highly investigated at recent years. There is a limited number of studies in the literature regarding the molecular approach of the protective role of vitamin D_3 in prevention of NaF-induced DNA damage, and the present study aimed to clarify the protective role of vitamin D_3 in prevention of NaF-induced DNA damage *in vitro*.

In the current study, the possible preventive effect of vitamin D_3 against time- and dose- dependent NaF-induced DNA damage was investigated. It was determined that NaF led to DNA damage after the 3 h and vitamin D3 was not effective in preventing the damage. Vitamin D_3 reduced the NaF-induced DNA damage at the 12 and 24 h. The reduction in DNA damage was statistically significant only at the 24 h ($p < 0.05$). The present study findings were consistent with previous study findings, which demonstrated that vitamins and similar natural molecules could prevent NaF-induced DNA damage. Several intracellular mechanisms could play a role in the protective effect of vitamin D_3 against NaF toxicity. Vitamin D_3 can induce cellular proliferation and increase viability against NaF. Furthermore, by binding directly to NaF and/or through the binding of metabolites formed in vitamin D_3-mediated metabolism with NaF, vitamin D_3 can lead to the binding of NaF to the DNA and consequently prevent DNA damage. Vitamin D_3 is known to lead to several epigenetic changes that affect the chromatin dynamics (Nurminen *et al*., 2019). These epigenetic changes may affect the NaF metabolism and reduce the impact of NaF on DNA damage. However, in order to determine these effects, further detailed studies on genetic and epigenetic factors are required.

In conclusion, it was demonstrated in the present study that NaF administration increased genomic DNA damage in renal cells at the 3, 12 and 24 h and it exhibited a genotoxic effect, and vitamin D_3 administration prevented NaF-mediated DNA damage based on time. Based on the obtained results from this study, it was decided to carry out new studies with different and detailed parameters related to vitamin D_3 metabolism, showing the effect of protective roles of vitamin D_3 on fluoride induced DNA damage.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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