THE EFFECTS OF VITAMINS A,D, E, AND C ON APOPTOSIS AND DNA DAMAGE IN SODIUM FLUORIDE-TREATED RENAL AND OSTEOBLAST CELL LINES

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ABSTRACT: This study was planned to investigate the effects of the antioxidant and protective vitamins A, D, E, and C, on the expression and translation of certain apoptotic markers in the NRK-52E and hFOB 1.19 cell lines treated with NaF at half the maximal inhibitory concentration (IC_{50}) for 24 hours. The IC_{50} for NaF and non-toxic vitamin doses were determined by the MTT viability test. For the biochemical assays, cells were harvested by trypsinization and lysed by the freeze/thaw method. The levels and gene expression of caspases 3, 8, and 9, and the levels of M30 and 8-OHdG were also measured with methods that included the use of ELISA and qRT-PCR. In the MTT studies, compared to the NaF-treated groups, it was found that the cell viability was higher in all the NaF+vitamin D-treated groups in the NRK-52E cell line, in some of the NaF+vitamin D-treated groups in the hFOB 1.19 cell line, and in some of the NaF+vitamin A, E, and C-treated groups for both cell lines. In the NRK-52E cell line, the NaF IC_{50} value was determined and found not to induce apoptosis sufficiently so that it was considered that mechanisms other than the apoptotic pathways were instrumental in causing cell death. In the hFOB 1.19 cell line, it was observed that the apoptotic M30 protein level was increased in the NaF+vitamin D and NaF+vitamin C groups. In addition, in the hFOB 1.19 cell line, the qRT-PCR results showed that, while the expression of caspase-3 increased with vitamin A and that of caspase-8 increased with NaF, treatment with NaF+vitamin A led to a lower levels of caspases 3 and 8. Future studies to investigate the most valid and active mechanism for NaF-induced cell death and to elucidate the inhibitory-activating effects of vitamins on this mechanism using different doses, durations of exposure, and analytic methods should be considered.

Keywords: Apoptosis; Cell culture; Sodium fluoride; Vitamins.

INTRODUCTION

Fluorine is an element with high electronegativity and the fluoride ion (F) can be found naturally in water and various nutrients.[1] The main sources of F intake by humans are usually water and food, but very low levels of F are usually present in the air. Besides damaging bones and teeth, excessive intake of F is known to cause a wide range of adverse health effects.[2-5]

F is a cumulative toxin that can alter the resorption and collapse of bone tissue.[6] The levels of various minerals with important structural or catalytic functions, including calcium, magnesium, manganese, phosphorus, and zinc, in hard tissues like bones and teeth and in soft tissues like tooth gums, may play a role in the effects of chronic fluorosis on these tissues.[7] The pro-oxidant/antioxidant status of soft tissues such as kidney, liver, and muscle are affected in different degrees by F intoxication.[8] The kidneys play a significant role in the removal of F from the body via urine.[6] Exposure to high doses of F may induce severe acute toxicity.[9]

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High concentrations of NaF administered to various human and rat cell lines have a negative effect on cell viability and proliferation, in proportion to the duration of the time of exposure.[10-11]

Apoptosis, known as programmed cell death, is a physiological cellular process that occurs in organisms during the typical development of the organism. In multicellular organisms, the increase in the number of cells via cell division is compensated for by apoptosis.[12]

The present study was planned to investigate possible cell damage mechanisms in kidney and bone cell lines involved in F metabolism and affected by F toxicity, and the role of vitamins A, D, E, and C in the prevention of these mechanisms.

MATERIALS AND METHODS

Cell culture: Rat renal epithelial (NRK-52E) cells were cultured in a medium at 37ºC temperature, 5% CO_2, and 95% humidity, and with 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, and DMEM high glucose. Human osteoblast (hFOB 1.19) cells were cultured in Ham's F12 medium at 37ºC temperature, 5% CO_2, and 95% humidity, and with 10% FBS, 2 mM L-glutamine, and 1% penicillin/streptomycin. When the cells reached 70–80% concentration, they were propagated with regular passages. When the cells reached a sufficient number, 10^4, they were planted in 96-well culture plates and at 10^6 they were planted in flasks for MTT assay.

MTT assay—required solutions and implementation: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is based on the conversion of the yellow tetrazolium salt MTT to the purple formazan crystal by metabolically active cells. MTT SOLUTION: 5 MTT mg/mL was dissolved in phosphate buffered saline (PBS) (pH = 7.0) that did not contain Ca^{2+} or Mg^{2+}. The PBS tablet (P4417, Sigma-Aldrich), was prepared using 200 mL sterile water for 1 tablet. IMPLEMENTATION: The MTT solution, diluted with 100 µL 1/10 medium, was placed in each well of a 96-well microplate containing the cell line, incubated for 24 hr at 95% humidity in 5% CO2 and at 37ºC, and then removed and incubated for 3 hr at 37ºC. The upper layer of the wells was removed. 100 µL MTT lysing (100 mL: 89 mL isopropyl alcohol 10 mL, 0.1 N HCl, and 1 mL Triton-x) was added to the wells. The intensity of the resulting color was measured at 570 nm using a spectrophotometer.

Preparation of analysis groups: With reference to the NaF[13], Vitamin A[14] (Retinol palmitate, Sigma-Aldrich), vitamin D[15] (cholecalciferol, Sigma-Aldrich), vitamin E[16] (alpha tocopherol, Sigma-Aldrich), and vitamin C[17] (ascorbic acid, Sigma-Aldrich) doses, stock solutions were prepared for producing the doses and solution media that would be used in the study.

MTT viability test was performed for both cell groups and the following doses were determined as beneficial compared to the control group: Vitamin A 10 µM (< 0.001% ethyl alcohol), vitamin D 10 µM(<0.05% DMSO), vitamin E 60 µM (< 0.001% ethyl alcohol), and vitamin C 100 µM. NaF in doses of 100, 250, 500, 1000, 2000, 5000, 7500, and 10000 µM, and these doses in combination with

vitamin A 10 µM, vitamin D 10 µM, vitamin E 60 µM, and vitamin C 100 µM, were administered for the MTT viability test. The effects of the vitamins (A, D, E, and C) on all the NaF doses (100, 250, 500, 1000, 2000, 5000, 7500, and 10000 µM) were determined. NaF IC_{50} doses were determined for NRK-52E cell (6000 µM), and hFOB 1.19 cell (5000 µM). For each cell series, 10 groups were formed: control (0 µM NaF+0 µM vitamin A, D, E, or C), NaF, vitamins A, D, E, and C, and NaF+vitamins A, D, E, and C, with a cell count of 10^6 in each 25 cm^2 flask.

Biochemical analysis: The cells were harvested by trypsinization following a 24-hr incubation period and the freeze–thaw process, of 10 min freezing at –80ºC followed by 25 min thawing, was repeated 3 times to lysis the cells. After the lysis phase, the suspension was transferred to falcon tubes and centrifuged at 3000 rpm for 20 min. Samples were prepared for analysis. Caspases 3, 8, 9, and M30 (Shangai LZ biotech), 8-OHDG (Elabscience, EL-0028) levels were determined using ELISA kits.

Reverse transcriptase-PCR (RT-PCR): cDNA synthesis was conducted on obtained mRNA cells for use in real time PCR expression analysis. For this purpose, the GeneAll Hyper Script First Strand Synthesis kit (Catalog: 601-005) was used for cDNA recovery. Using the protocol recommended in this kit, cDNA was obtained from 100 nanograms (ng) mRNA on average.

Quantitative real time-PCR (qRT-PCR): Biotium Fast-Plus Eva Green master kit was used for the reaction buffer, enzyme, dNTP, and $MgCl_2$ mixture. The kit contains Eva Green stain and Cheetah Taq DNA polymerase as an enzyme. Utilized expression primers are listed below.

Table 1. Primers used in expression analysis

Gene	Primer sequences
Primer caspase-3 rat	F: 5'-ATGGCCCTGAAATACGAAGTC R: 5'-GTTCCACTGTCTGTCTCAATACC
Primer caspase -8 rat	F: 5'-GAAAGCAATCTGTCCTTCCT R: 5'-ATGACCCTCTTCTCCATCTC
Primer caspase -9 rat	F: 5'- GATCCAGAAGCTGTTACACC R: 5'- CTTCACTACTTTCTGCTCCT
Control Primer rat (GAPDH)	F: 5'-AAGTCCCTCACCCTCCCAAAAG R: 5'-AAGCAATGCTGTCACCTTCCC
Primer caspase -3 human	F: 5'-AAGCGAATCAATGGACTCTG R: 5'-AAACATCACGCATCAATTCC
Primer caspase -8 human	F: 5'-GAAAGCAATCTGTCCTTCCT R: 5'-ATGACCCTCTTCTCCATCTC
Primer caspase -9 human	F: 5'-GGCTCTTCCTTTGTTCATCTCC R: 5'-ATCACCAAATCCTCCAGAACCA
Primer Control human (GAPDH)	F: 5'-GCACCACACTTTCTACAATGAG R: 5'-GAGGCATACAGGGACAACAC

Statistical analysis: Kruskal-Wallis test was used to determine whether there were differences between groups based on the group properties. Dunnet multiple comparison test was used to identify different groups. In calculations, the statistical significance level was accepted as 5% and SPSS statistics software (v. 22.0) was used for the calculations.

RESULTS

MTT results for NRK-52E cell line: The control group (0 µM NaF+0 µM vitamin) was accepted as 100% viable in the NRK-52E cell lines. After 24 hr incubation with NaF, NaF+vitamin, and only vitamin (A, D, E, and C) administration, the MTT % viability results were obtained (Figures 1–4).

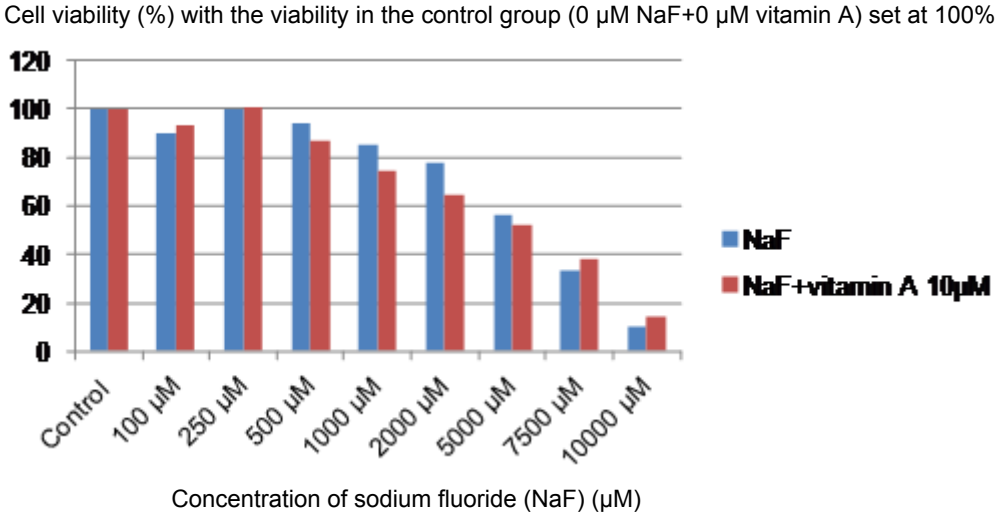

Figure 1. MTT cell viability (%) in the NRK-52E cell line treated with NaF and NaF+vitamin A 10 µM with the viability in the control group (0 µM NaF+0 µM vitamin A) set at 100%.

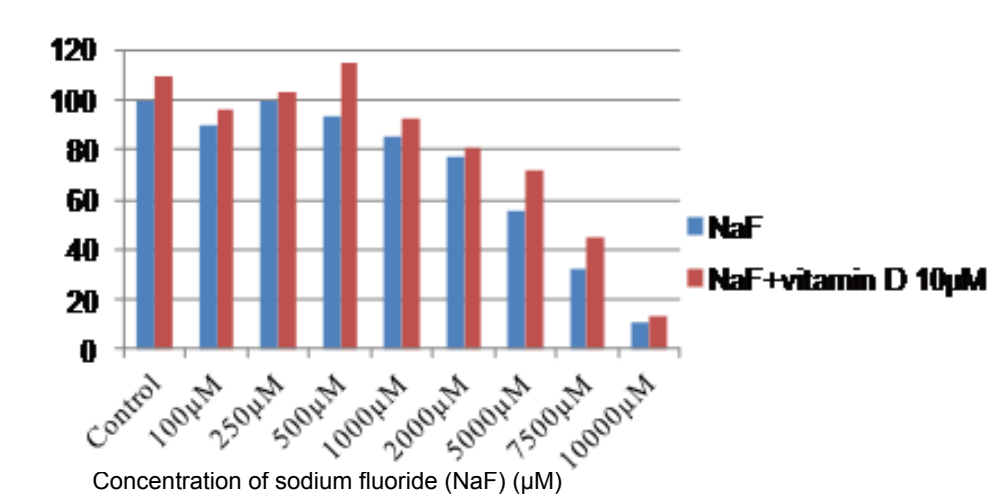

Figure 2. MTT cell viability (%) in the NRK-52E cell line treated with NaF and NaF+vitamin D 10 µM with the viability in the control group (0 µmM NaF+0 µM vitamin D) set at 100%.

In the NaF+10 µM vitamin A-treated groups, compared to the NaF groups, the cell viability increased 3% at 100 µM NaF, 8% at 7500 µM NaF, and 3% at 10000 µM NaF, while the cell viability was lower in the other groups (Figure 1).

In all the NaF+10 µM vitamin D-treated groups, cell viability was found to be 3–11% higher on average when compared to the NaF groups (Figure 2).

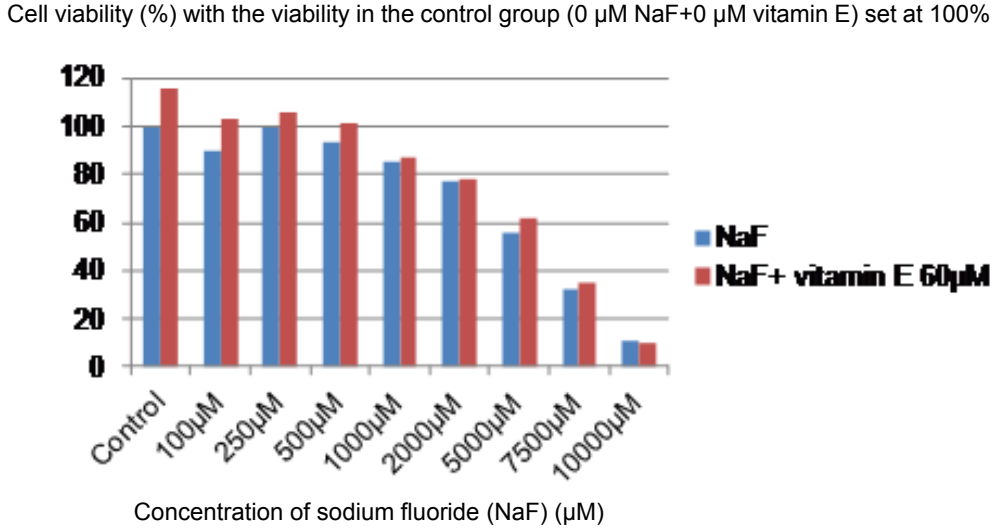

Figure 3. MTT cell viability (%) in the NRK-52E cell line treated with NaF and NaF+vitamin E 60 µM with the viability in the control group (0 µM NaF+0 µM vitamin E) set at 100%.

It was determined that cell viability was 3–10% higher in the NaF+60 µM vitamin E-treated groups when compared to 100–7500 µM NaF groups (Figure 3).

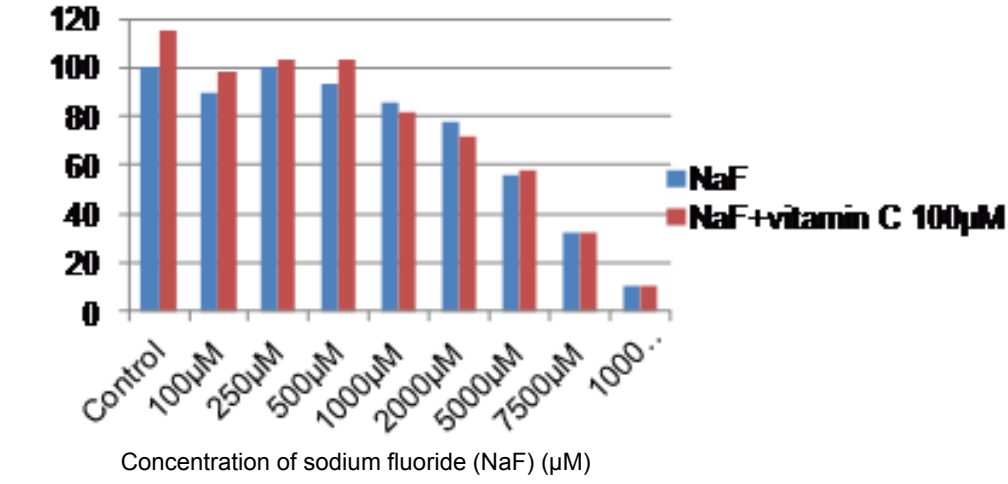

Figure 4. MTT cell viability (%) in the NRK-52E cell line treated with NaF and NaF+vitamin C 100 µM with the viability in the control group (0 µM NaF+0 µM vitamin C) set at 100%.

Cell viability was higher in the NaF+100 µM vitamin C-treated groups, from 100–500 and at 5000 µM NaF, when compared to the NaF groups (Figure 4).

MTT results for hFOB 1.19 cell line: The control group (0 µM NaF+ 0 µM vitamin) was accepted as 100% viable in the hFOB 1.19 cell lines. After 24 hr incubation with NaF, NaF+vitamin, and only vitamin (A, D, E, and C) administration, the MTT % viability results were obtained (Figures 5–8).

Cell viability (%) with the viability in the control group (0 µM NaF+0 µM vitamin A) set at 100%

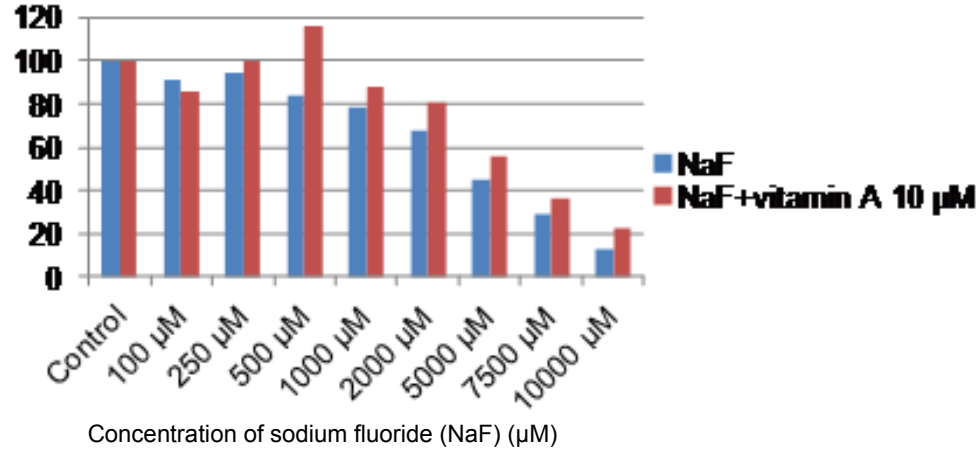

Figure 5. MTT cell viability (%) in the hFOB 1.19 cell line treated with NaF and NaF+vitamin A 10 µM with the viability in the control group (0 µM NaF+0 µM vitamin A) set at 100%.

In NaF+10 µM vitamin A-treated groups, it was determined that cell viability decreased at 100 µM NaF dose, while in all other doses cell viability increased by 2–5% when compared to the NaF group (Figure 5).

Cell viability (%) with the viability in the control group (0 µM NaF+0 µM vitamin D) set at 100%

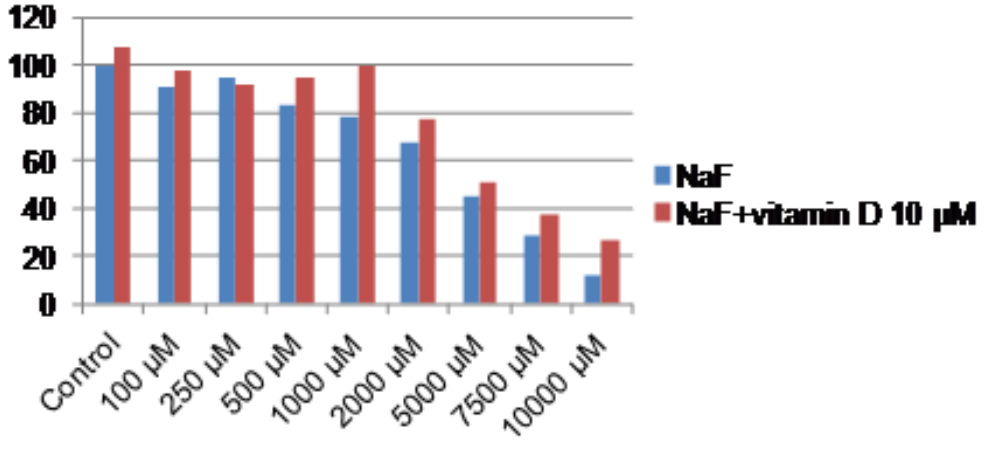

Figure 6. MTT cell viability (%) in the hFOB 1.19 cell line treated with NaF and NaF+vitamin D 10 µM with the viability in the control group (0 µM NaF+0 µM vitamin D) set at 100%.

In the NaF-treated hFOB 1.19 cell line, cell viability tended to gradually decrease with increasing doses. In NaF+10 µM vitamin D-treated groups, cell

viability decreased at the 250 µM dose while at all the other doses, the cell viability increased 1–11% when compared to the NaF group (Figure 6).

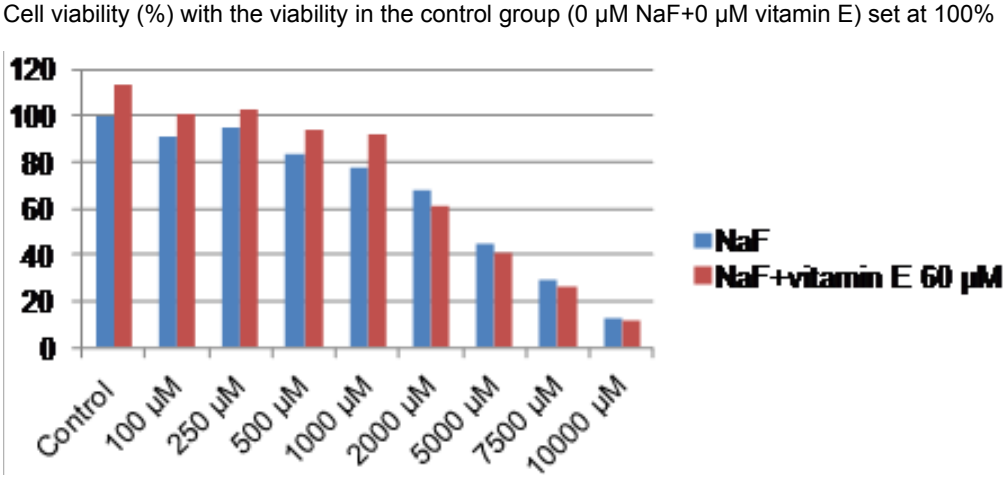

Figure 7. MTT cell viability (%) in the hFOB 1.19 cell line treated with NaF and NaF+vitamin E 60 µM with the viability in the control group (0 µM NaF+0 µM vitamin E) set at 100%.

In the NaF-treated hFOB 1.19 cell line, cell viability tended to decrease gradually with increasing doses. In the NaF+60 µM vitamin E-treated groups, compared to the NaF groups, cell viability increased at 100, 250, 500, and 1000 µM and decreased in the 2000, 5000, and 7500 µM dose groups (Figure 7).

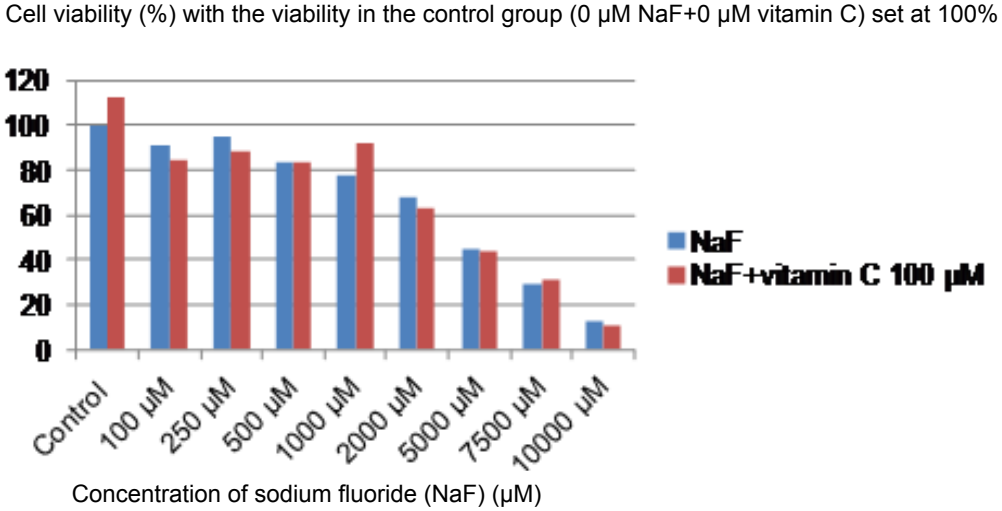

Figure 8. MTT cell viability (%) in the hFOB 1.19 cell line treated with NaF and NaF+vitamin C 100 µM with the viability in the control group (0 µM NaF+0 µM vitamin C) set at 100%.

In the NaF-treated hFOB 1.19 cell line, cell viability gradually decreased as the dose increased. In NaF+100 µM vitamin C-treated groups, cell viability generally decreased when compared to NaF groups, while cell viability increased 11% in NaF+vitamin C group at 1000 µM (Figure 8).

ELISA results: The levels of M30, caspase 3, 8, and 9 (apoptosis markers) and the 8-OHdG (oxidative DNA damage product) of both cell lines are presented in Tables 2 and 3.

Table 2. NRK-52E cell line ELISA results

Groups	M 30 (IU/L)	Caspase-3 (ng/mL)	Caspase-8 (ng/mL)	Caspase-9 (ng/mL)	8-OhdG (ng/mL)
Control	94.69±10.22^{a}	1.69±0.17^{ab}	5.49±0.49^{a}	5.70±0.36^{a}	17.27±.2.43^{cd}
NaF	111.34±7.45^{ab}	1.94±0.15^{ab}	6.12±0.41^{a}	5.82±0.46^{ab}	15.05±2.42^{c}
Vitamin A	111.67±4.58^{ab}	1.56±.43^{a}	5.50±0.34^{a}	5.85±0.19^{ab}	24.80 ±10.27^{e}
Vitamin D	106.86±8.11^{ab}	1.95±0.18^{ab}	5.24±0.34^{a}	5.68±0.45^{a}	30.19±2.95^{e}
Vitamin E	106.39±5.36^{ab}	1.76±017^{ab}	6.01±0.23^{a}	6.50±0.18^{b}	3.2740.57^{a}
Vitamin C	97.84±6.53^{ab}	1.76±0.19^{ab}	5.56±037^{a}	6.00±0.35^{ab}	23.28±5.17^{de}
NaF+vit. A	112.79±6.56^{ab}	2.10±0.20^{b}	5.50±0.37^{a}	5.89±0.45^{ab}	13.55±2.58^{bc}
NaF+vit. D	94.10±20.73^{a}	1.56±0.29^{a}	5.73±0.50^{a}	6.02±0.47^{ab}	5.129±0.75^{a}
NaF+vit. E	135.19±37.25^{b}	1.70±0.29^{ab}	5.60±0.34^{a}	5.65 ±0.29^{a}	7.12±3.68^{ab}
NaF+vit. C	130.39±41.80^{ab}	1.56±.350^{a}	6.12±0.35^{a}	6.09±0.30^{ab}	5.35±0.17^{a}

*Columns depicted with different letters are statistically significant ($p \leq 0.05$).

In the NRK-52E cell line study, there was no correlation between the apoptosis parameters, M30 and caspases 3, 8, and 9, and the marker for oxidative DNA damage, 8-OHdG, for all the groups. Compared to the respective control groups, the caspase-3 and caspase-8 levels were not significantly different in any of the other groups while the caspase-9 level was significantly increased in the vitamin E group ($p \leq 0.05$). Compared to the respective NaF groups, the caspase-3, caspase-8, and caspase-9 levels were not significantly different in any of the other groups. Compared to the control group, the M30 levels were increased in the NaF+vitamin E group ($p \leq 0.05$). Compared to the NaF group, the M30 levels were not significantly different in any of the other groups (Table 2).

Compared to the control group in the NRK-52E cell line study, the 8-OHdG levels were increased in the vitamin A and D groups and reduced in the vitamin E

and NaF+vitamins D, E, and C groups ($p \leq 0.05$). Compared to the NaF group, the 8-OHdG levels were increased in the vitamin A, D, and C groups and reduced in the vitamin E and NaF+vitamins D, E, and C groups ($p \leq 0.05$) (Table 2).

Table 3. hFOB 1.19 cell line ELISA results

Groups	M30 (IU/L)	Caspase-3 (ng/mL)	Caspase-8 (ng/mL)	Caspase-9 (ng/mL)	8-OhdG (ng/mL)
Control	157.69±21.04^{ab}	2.80±0.37^{b}	7.86±0.25^{a}	8.55±0.26^{abc}	7.31±1.74^{a}
NaF	126.94±25.32^{a}	2.78±0.18^{a}	7.15±0.50^{a}	9.33±0.82^{abc}	7.01±0.86^{a}
Vitamin A	168.94±28.54^{bc}	2.52±0.047^{ab}	7.13±0.24^{a}	8.095±0.43^{ab}	7.54±1.04^{a}
Vitamin D	178.37±11.68^{bc}	2.62±0.27^{ab}	7.47±0.37^{a}	7.84±2.03^{a}	14.15±4.03^{b}
Vitamin E	170.938±12.07^{bc}	2.50±0.21^{ab}	7.31±1.05^{a}	9.71±0.38^{abc}	7.80±0.84^{a}
Vitamin C	199.05±2.32^{cd}	2.45±0.16^{ab}	7.92±0.59^{a}	9.72±1.14^{abc}	13.34±3.37^{b}
NaF+vit. A	181.73±13.35^{bc}	2.41±0.25^{ab}	7.99±1.16^{a}	9.23±0.81^{abc}	7.23±1.12^{a}
NaF+vit. D	200.40±4.164^{d}	2.27±0.11^{a}	7.40±1.27^{a}	10.02±1.60^{bc}	6.65±0.57^{a}
NaF+vit. E	156.78±34.25^{ab}	2.27±0.20^{a}	8.93±2.40^{a}	10.02±2.14^{abc}	9.75±2.10^{a}
NaF+vit. C	220.64±14.76^{d}	2.54±0.20^{ab}	7.79±0.32^{a}	10.57±1.20^{c}	9.04±0.89^{a}

*Columns depicted with different letters are statistically significant ($p \leq 0.05$).

In the hFOB 1.19 cell line study, the comparison of the parameters in all groups showed negative correlations were present between the apoptosis parameters, M30 and caspase-3, and the marker for oxidative DNA damage, 8-OHdG. A positive correlation was determined between caspase-8 and caspase-9. Compared to the control and NaF groups, caspase-3 was significantly lower in the NaF+vitamin D and NaF+vitamin E groups ($p \leq 0.05$). Compared to the control and NaF groups, there were no statistically significant differences in the caspase-8 and caspase-9 levels for the cell line study groups. Compared to the control and NaF groups, the M30 levels were significantly higher in the vitamin C, the NaF+vitamin D, and the NaF+vitamin C groups ($p \leq 0.05$). Compared to the control and NaF groups, the 8-

OHdG levels were significantly higher in the vitamin C and vitamin D groups ($p \leq 0.05$) (Table 3).

Gene expression results: Various expressions in the NRK-52E and hFOB 1.19 cell line study groups based on the qRT-PCR results are presented tables 4 and 5.

Table 4. NRK-52E cell line expressions based on qRT-PCR results

Groups	NaF	Vit. A	Vit. D	Vit. E	Vit. C	NaF + vit. A	NaF+ vit. D	NaF+ vit. E	NaF+ vit. C
GAPDH	1	1	1	1	1	1	1	1	1
Caspase-3	0.52	1.21	0.87	1.04	0.85	0.59	0.35	0.77	0.65
Caspase-8	0.18	1.36	0.90	1.28	0.86	0.83	0.51	1.09	1.91
Caspase-9	0.32	0.47	4.82	0.18	0.97	2.04	0.34	0.49	2.75

In the NRK-52E cell line study, based on the qRT-PCR results, there was no change in caspase-3 levels in the vitamin E group when compared to the control group (GADPH). Caspase-8 was reduced 0.8-fold in the NaF group compared to the control. Caspase-9 decreased 0.7-fold in the NaF-treated group (Table 4).

Table 5. hFOB 1.19 cell line expressions based on qRT-PCR results

Groups	NaF	Vit. A	Vit. D	Vit. E	Vit. C	NaF + vit. A	NaF+ vit. D	NaF+ vit. E	NaF+ vit. C
GAPDH	1	1	1	1	1	1	1	1	1
Caspase-3	2.25	3.89	3.53	0.28	2.49	0.96	3.07	6.40	4.92
Caspase-8	7.31	0.90	1.15	0.82	1.49	0.47	1.90	6.19	1.15
Caspase-9	1.19	1.14	1.31	0.49	0.60	1.85	2.51	23.26	1.49

In the hFOB 1.19 cell line study, it was determined that caspase-3 expression increased 2.2-fold in the NaF group, and, apart from the vitamin E and NaF+vitamin A groups, all the groups demonstrated higher expressions compared to NaF group. Caspase-8 expression increased 7.3-fold in NaF group and decreased in the vitamin A, vitamin E, and NaF+vitamin A-treated NaF groups.

Caspase-9 expression increased 1.2-fold in the NaF group, and 1.9- and 1.5-fold in the NaF+vitamin A and the NaF+vitamin C groups, respectively. Interestingly, the largest increase in the caspase-9 gene expression, 23.3-fold, occurred when NaF and vitamin E were used together (Table 5).

DISCUSSION

As demonstrated in previous studies, NaF has a significant impact on cell proliferation, based on dose and time. *In vitro* studies have demonstrated that, although the effects of NaF vary between the studies conducted with different organisms and/or different cell lines in the same organism, the NaF-induced inhibition of cell proliferation increases as the NaF dose and the duration of the exposure increase.

Apoptosis is an activity of regulated cell death and includes a series of biochemical and morphological changes such as caspase family activation, nucleosome DNA breaks, increased cell death counts, and chromatin condensation.[18] NaF induces apoptosis in HGF (human gingival fibroblast) cell lines along with chromatin condensation and DNA fragmentation, increases the transition of cytochrome C from mitochondria to cytosol and increases caspase-3, -8 and -9 activities, and BcL-2 family mediated mitochondrial and death receptor pathways in HGF cells.[10] NaF induces various parameters related to apoptosis in HL-60 cells, including affecting cell viability.[20]

In the present study conducted on the kidney epithelial cell NRK-52E cell line, M30, caspase-3, -8 and -9 levels were investigated as apoptosis markers. It was determined that the M30 value did not change in the NaF group when compared to the control. In groups where vitamins and NaF were administered together, although cell viability increased relative to the NaF group in the vitamin D, E, and C supplemented groups, this finding was not consistent with the results found with the apoptotic M30 protein. However, when the inhibition of apoptosis was considered, the lowest M30 level was detected in the NaF+vitamin D-treated group. This suggested that vitamin D, a natural antioxidant, might inhibit M30, an apoptosis marker, by reducing oxidative stress, as noted in previous studies. While, compared to the control and NaF groups there was no significant change in the caspase-3 level in the NaF+vitamin A-treated group in the NRK-52E cell line, the level in the NaF+vitamin A-treated group, it was significantly higher than in the vitamin A-treated group and consistent with the MTT results. Compared to the control and the NaF groups, there was a decrease in the caspase-3 levels in the NaF+vitamin D and NaF+vitamin C groups.

According to the qRT-PCR analysis results on the kidney epithelial cell NRK-52E cell line, only vitamin A increased caspase-3 expression and caspase-3 expression decreased in the NaF and NaF+vitamin D groups. Although the NaF dose administered in the present study increased cell death, it was found that there were no differences between the vitamin-administrated groups with respect to the cell death. Caspase-8 levels were not significantly affected in the NRK-52E cell line in all study groups. Caspase-8 expression decreased in the NaF-treated study

groups. Furthermore, similar to caspase-3, the administration of vitamin A increased caspase-8 expression. Interestingly, however, administration of vitamin A and D together with NaF resulted in a decrease in caspase-8 expression. However, it was determined that these variations in expression were not reflected in the enzyme concentration. For caspase-9, the results were consistent with respect to the levels and expressions.

Yang et al. demonstrated that NaF administration reduced cell viability in MC3T3-E1 osteoblast cells based on time and concentration, and also increased apoptosis even at low concentrations.[11] It was determined that NaF displayed a positive effect on cell proliferation in human osteosarcoma MG-63 cells (5×10^3 μmol), while it inhibited cell proliferation (2×10^4 μmol).[21]

Yan et al. demonstrated the effects of fluoride on osteoblast cell proliferation and caspase-3 and -9 expression and found that cell proliferation was inhibited in a dose dependent manner and even in the lowest NaF dose (0.5 mg/L), a significant increase was observed in osteoblast apoptosis (after 24 and 72 hr) and osteoblast apoptosis further increased in response to higher doses.[22]

In the present study in the hFOB 1.19 osteoblast cell line, apoptotic parameters were evaluated to understand the mechanisms of NaF-induced cell death. With respect to the apoptosis mechanism and the effect of antioxidant vitamins on the resulting apoptosis mechanism, after the detection of NaF levels leading to IC_{50} level apoptosis in the osteoblast cell line, it was determined, based on the qRT-PCR results, that the solitary administration of both NaF and vitamin A increased the expression of caspase-3 while their combined administration decreased this. The solitary administration of NaF increased the expression of caspase-3 while the administration of NaF+vitamin A decreased it. The apoptotic caspase-3 and the apoptotic M 30 protein were increased in the NaF+vitamin D group compared to the NaF group. In addition, the apoptotic M 30 protein was increased in the vitamin C group compared to the NaF group.

8-OHdG is an important biomarker in the assessment of DNA damage.[23] Studies conducted with humans, experimental animals, and cell cultures on the relationship between F toxicity and oxidative stress, have shown a close correlation to exist between them at various levels. It was shown that excessive F could lead to DNA damage.[24] Oxidative stress that occurs as a response to F was reported in different types of cells, experimental tissues that were exposed to F, *in vitro* or *in vivo*, in animal tissues and in humans living in F endemic regions.[25]

The present study demonstrated that, in the kidney epithelial cell NRK-52E cell line, the levels of 8-OHdG, an oxidative DNA damage marker, were significantly decreased in the NaF+vitamin D, E, and C-treated groups when compared to the control and NaF-treated groups. Interestingly, it was observed that oxidative DNA damage significantly increased in only the vitamin A and D-treated groups. On the other hand, in the osteoblast hFOB 1.19 cells, 8-OHdG levels were increased only in the groups where only vitamin C or vitamin D were administered. No correlation was found between the apoptosis parameters (M30, caspase-3, -8, and -

9) and between the apoptosis parameters and the oxidative DNA damage parameter (8-OHdG) in the comparisons conducted between the parameters in all the groups.

CONCLUSIONS

The results of the present study for both cell lines are summarized as follows:

Based on the selected IC_{50} value for NaF, apoptotic pathways function differently based on the cell type in apoptosis. The M30 apoptotic protein did not vary significantly between either cell type. In the renal epithelial NRK-52E cell line, downregulations were detected, based on the qRT-PCR results, of the caspase-3, -8 and -9 enzymes in NaF group. Caspase-3 and 8 enzyme expression increased in the human osteoblast hFOB 1.19 cell line with NaF administration. There was no significant consistency between the caspase enzyme concentrations and their expressions, which might have occurred due to the sample collection time. There was no significant correlation between oxidative DNA damage and the apoptosis parameters. The bone tissue was more susceptible to the effect of NaF, considering that the apoptosis parameters increased only in the NaF-treated group in the osteoblast cell line. Fluoride induced apoptosis via caspase-8 using the external apoptotic pathway in osteoblast cells. The administration of NaF+vitamin D in the osteoblast cells blocked the caspase-8 apoptotic pathway. Significant changes in cytotoxicity were observed between NaF and NaF+vitamin-treated groups. Since NaF and the vitamins used different apoptotic pathways, the apoptosis effect increased in some groups when they were used in conjunction, while in others it was inhibited. The effect of NaF on apoptotic pathways was through the inhibition of the gene transcription factors in the pathway enzymes, preventing the formation of enzyme mRNA. As a result of the analysis of the data obtained in the present study, it was concluded that further studies should be conducted to reveal, especially, the most valid and active mechanism in NaF-induced apoptosis and the inhibition and activation effects of vitamins on this mechanism using different doses, times and analytic methods, based on the apoptotic pathways that NaF and vitamins utilize and parameters related to these pathways.

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