THE EFFECTS OF SOME MINERALS ON APOPTOSIS AND DNA DAMAGE IN SODIUM FLUORIDE-ADMINISTERED RENAL AND OSTEOBLAST CELL LINES

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ABSTRACT: The present study was planned to investigate the effects of some minerals ($MgCl_2$, Na_2SeO_3, $AlCl_3$, $CaCl_2$) on the expression and translocation of certain apoptotic markers in NaF-administered (at the rate of IC_{50}) rat renal epithelial (NRK-52E) and human osteoblast (hFOB 1.19) cell lines. The NaF IC_{50} and the non-toxic mineral doses were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For the biochemical analysis, the cells were collected by trypsin treatment following a 24-hour incubation period, the cells were broken up by the freeze/thaw method, and the analysis was conducted. Caspase 9, 8, and 3 levels and gene expression, and M30 and 8-OHdG levels were determined. Target gene DNAs were propagated with the real time-PCR method. In the MTT studies, it was determined that the cell proliferation in rat renal epithelial cells (NRK-52E) treated with NaF+minerals was higher than that of all the NaF-treated groups and in the human osteoblast cells (hFOB 1.19), the cell proliferation was higher than in all the NaF-treated groups except for the $MgCl_2$ group. The reason why the NaF administration in the NRK-52E cells resulted in an average of a 2-fold decrease in caspase 3 expression compared to the control group could be attributed to the apoptotic effect of NaF based on the time we obtained the RNA. However, based on this time, when the results are assessed based on the NaF and other mineral groups, the NaF-induced cytotoxic apoptosis might have used a pathway other than the apoptotic pathway. Thus, it is considered that minerals could usually prevent NaF-induced apoptosis by a synergistic mechanism due to the ionic character of NaF. NaF+mineral administration protected the NRK-52E cells from apoptosis. In the osteoblasts, on the other hand, it was concluded that NaF+mineral administration may be useful since it inhibits increased apoptosis.

Keywords: Apoptosis; Cell culture; Minerals; NaF; Real time-PCR.

INTRODUCTION

Fluorine is the lightest and most chemically active element among the halogens.[1] It has a high electronegativity, and can easily bond with organic and inorganic substances.[1] Several studies have been conducted on fluorine and its relation to health and the environment. Fluoride ion (F) intake by humans is generally in the form of food or water but very low levels of F are also generally present in air. Besides damaging bones and teeth, excessive F intake is known to cause a wide range of adverse health effects.[2-6]

A large part of the bodily F content is stored in the skeletal system due to the high affinity of F for the calcium ion. The fluoride ion that accumulates in the skeletal system couples with the hydroxyl group and is deposited in the bone tissue in the form of hydroxyl-fluoro-apatite. Fluoride ions accumulate in the bone and disrupt the mineral structure of the bone, affecting the bonding of mineral and bone matrix proteins.[7-8] Fluoride is a toxic substance that tends to accumulate in the body. On

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average, only 50–80% of the ingested fluoride is excreted through the kidneys and the rest is accumulated in the bones, pineal gland, and other tissues.[9]

Fluoride causes various cellular effects depending on duration of exposure, the presence of other elements and compounds, and the cell type. The main toxic effect of the fluoride occurs in the cells and involves interactions with enzymes. In most cases, fluoride acts as an enzyme inhibitor, but fluoride ions can occasionally stimulate enzyme activity. The mechanisms of fluoride-induced toxicity depend on the type of enzyme involved.[10]

Apoptosis is a pathway that results in cell death and excessive apoptosis leads to different types of organ damage. In recent years, a number of studies have demonstrated that fluoride toxicity can occur due to its ability to induce protein carbonyl content as well as by altering the gene expression and inducing apoptosis.[11,12] The mechanism of fluoride-induced damage in fluorosis might be related to fluoride-induced oxidative stress damage. Cellular apoptosis could be one of the important mechanisms. The kidney is affected in fluoride toxicity and may significantly affect the level of toxicity by its role in fluoride excretion and accumulation.[13]

MATERIALS AND METHODS

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

MTT assay: 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 CYTOTOXICITY ASSAY AND REQUIRED SOLUTIONS: 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 (Sigma, P4417 catalog) was prepared using 200 mL sterile water for 1 tablet.

MTT ASSAY IMPLEMENTATION: The MTT solution, diluted with 100 µL 1/10 medium, was placed in each well after the medium in a 96-well microplate containing the cell line incubated for 24 hours at 95% humidity in 5% CO_2 at 37 ºC. It was then removed and incubated for 3 hours at 37ºC. The upper layer of the wells was then removed. 100 µL MTT lysing solution (100 mL of MTT lysing solution contained 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: To determine the adequate doses and the solution media that would be used in the study, stock solutions based on NaF,[14] $MgCl_2$,[15] Na_2SeO_3,[14,16] $AlCl_3$,[17] and $CaCl_2$[18] doses were used. The MTT viability test was performed by administering NaF at doses of 100 µM, 250 µM, 500 µM, 1,000 µM,

2,000 µM, 5,000 µM, 7,500 µM and 10,000 µM and in combination with the doses of 100 µM $MgCl_2$, 0.005 µM Na_2SeO_3, 50 µM $AlCl_3$ and 500 µM $CaCl_2$. The impacts of the minerals ($MgCl_2$, Na_2SeO_3, $AlCl_3$, $CaCl_2$) on all the NaF doses (100 µM, 250 µM, 500 µM, 1,000 µM, 2,000 µM, 5,000 µM, 7,500 µM and 10,000 µM) were determined. Using the Microsoft Excel software linear slope graph formula, NaF doses were determined as follows: NaF IC_{50} for NRK-52E cell as 6,000 µM, and for hFOB 1.19 cell NaF IC_{50} as 5,000 µM. The MTT viability test was performed for both cell lines and $MgCl_2$ 100 µM, Na_2SeO_3 0.005 µM, $AlCl_3$ 50 µM, and $CaCl_2$ 500 µM were determined as beneficial doses when compared to the control group. For the ELISA and quantitative real time PCR (qRT-PCR) studies, for each cell line, 10 groups were formed with a 10^6 cell count in each 25 cm^2 flask including the control, NaF, mineral ($MgCl_2$, Na_2SeO_3, $AlCl_3$, and $CaCl_2$), and NaF+mineral ($MgCl_2$, Na_2SeO_3, $AlCl_3$, and $CaCl_2$) groups.

Biochemical analysis: For the ELISA analysis, the cells were harvested by trypsinization following a 24-hour incubation period and 10 min at –80º. C. A 25 min freeze–thaw process was carried out 3 times to cause lysis of the cells. After the lysis phase, the suspension was transferred to Falcon tubes and centrifuged at 3,000 rpm for 20 min. Samples were prepared for analysis. The caspase 3, 8, and 9 and the M30 (Shangai LZ biotech), 8-OHdG (Elabscience, EL-0028) levels were determined using ELISA kits.

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

Quantitative real time-PCR (qRT-PCR): A Biotium brand 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. The expression primers used are listed below.

Statistical analysis: The Kruskal-Wallis test was used to determine whether there were differences between the groups based on the group properties. The Dunnet multiple comparison test was used to identify different groups. In the calculations, the statistical significance level was accepted as 5% and SPSS statistics software (v. 13) was used for the calculations.

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

RESULTS

MTT results for the NRK-52E cell line: The control group was accepted as being 100% viable in the NRK-52E cell lines. Twenty-four hours after NaF, NaF+minerals, and only minerals ($MgCl_2$, Na_2SeO_3, $AlCl_3$, $CaCl_2$) were administered, the MTT % viability results were obtained as follows (Figures 1–4).

It was determined that the highest increase in cell viability was observed in the $AlCl_3$ group after mineral administration following the apoptosis that occurred at the various NaF concentrations (100 µM, 250 µM, 500 µM, 1,000 µM, 2,000 µM, 5,000 µM, 7,500 µM, and 10,000 µM).

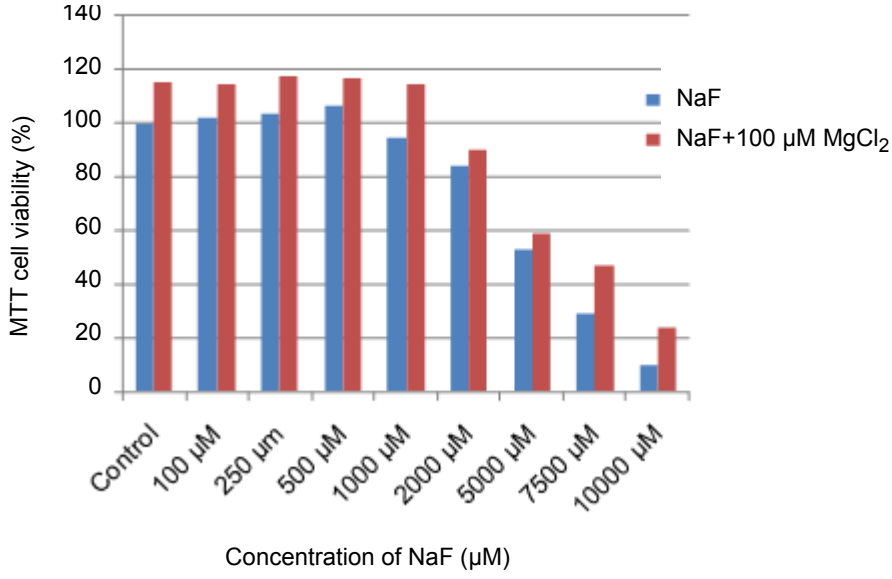

Figure 1. NRK-52E cell line NaF and NaF+100 µM $MgCl_2$ MTT chart. The MTT viability of the control group (0 µm NaF, 0 µM $MgCl_2$) was set at 100%.

The cell viability was determined as 52.9% after 24 hours of incubation in the 5,000 µM NaF-administered NRK-52E cell lines when compared to the control, while the cell viability was 58.9% in the 5,000 µM NaF and 100 µM $MgCl_2$-administered cells (Figure 1).

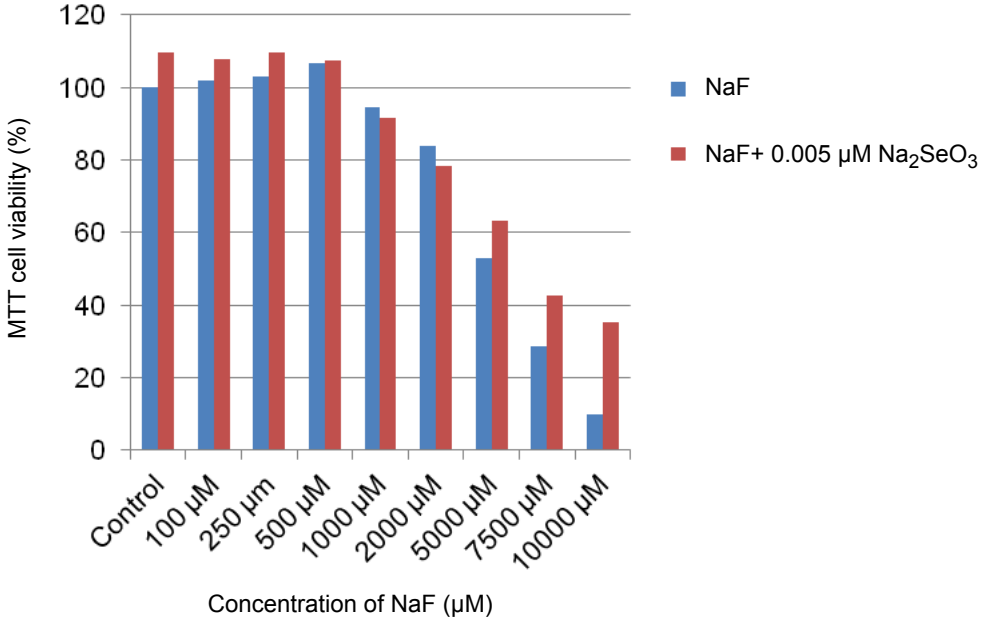

Figure 2. NRK-52E cell line NaF and NaF+0.005 µM Na_2SeO_3 MTT chart. The MTT viability of the control group (0 µm NaF, 0 µM Na_2SeO_3) was set at 100%.

Cell viability was determined as 52.9% after 24 hours of incubation in the 5,000 µM NaF-administered NRK-52E cell lines when compared to the control, while the

cell viability was 63.1% in the 5,000 µM NaF and 0.005 µM Na_2SeO_3-administered cells (Figure 2).

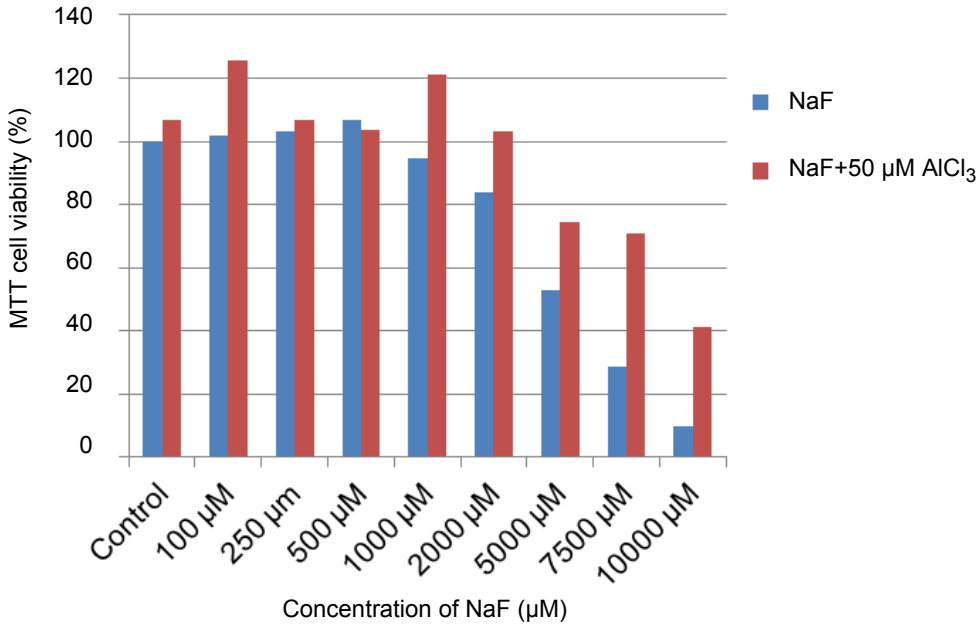

Figure 3. NRK-52E cell line NaF and NaF+50 µM $AlCl_3$ MTT chart. The MTT viability of the control group (0 µm NaF, 0 µM $AlCl_3$) was set at 100%.

The cell viability was determined as 52.9% after 24 hours of incubation in the 5,000 µM NaF-administered NRK-52E cell lines when compared to the control, while cell viability was 74.5% in 5,000 µM NaF and 50 µM $AlCl_3$-administered cells (Figure 3).

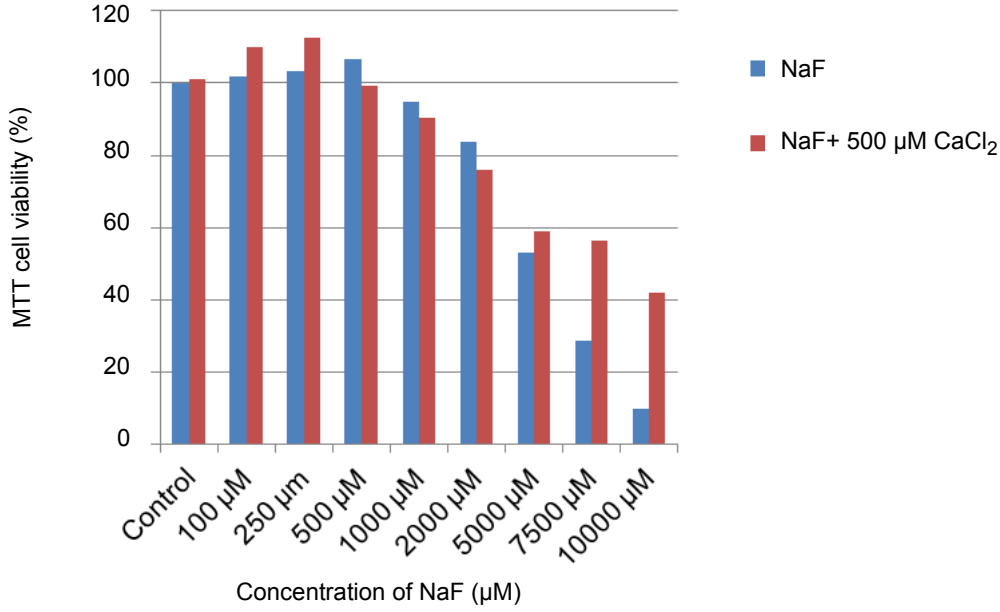

Figure 4. NRK-52E cell line NaF and NaF+500 µM $CaCl_2$ MTT chart. The MTT viability of the control group (0 µm NaF, 0 µM $CaCl_2$) was set at 100%.

The cell viability was determined as 52.9% after 24 hours of incubation of 5,000 µM NaF-administered NRK-52E cell lines when compared to the control, while the cell viability was 58.5% in the 5,000 µM NaF and 500 µM $CaCl_2$-administered cells.

MTT results for the hFOB 1.19 cell line: The control group was accepted as being 100% viable in the hFOB 1.19 cell lines. Twenty-four hours after NaF, NaF + mineral, and only minerals ($MgCl_2$, Na_2SeO_3, $AlCl_3$, and $CaCl_2$) were administered, and the MTT % viability results were obtained as follows (Figures 5–8).

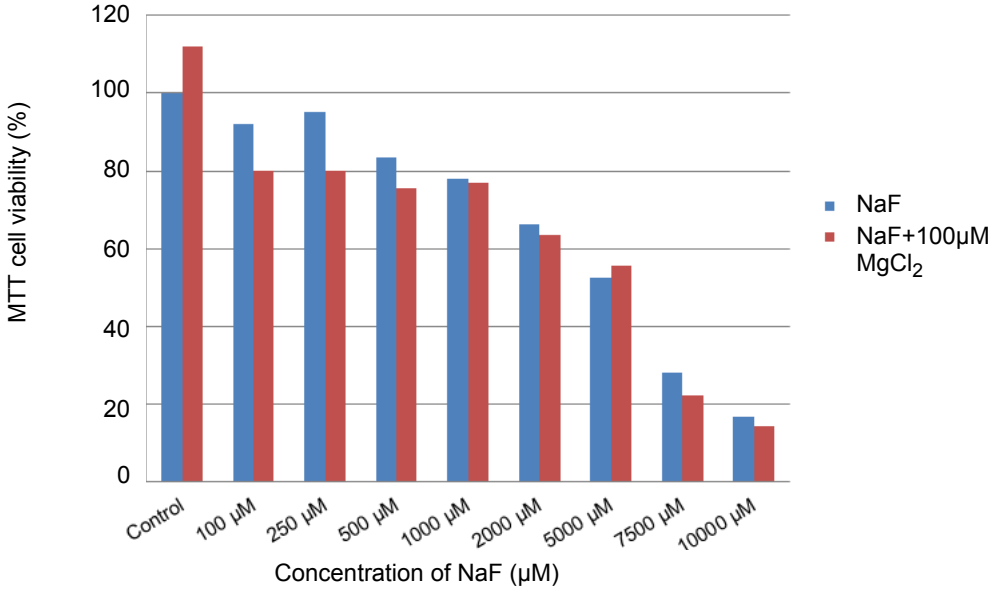

Figure 5. hFOB 1.19 cell line NaF and NaF+100 µM $MgCl_2$ MTT chart. The MTT viability of the control group (0 µm NaF, 0 µM $MgCl_2$) was set at 100%.

The cell viability was determined as 52.6% after 24 hours incubation in the 5,000 µM NaF-administered hFOB 1.19 cell lines when compared to the control, while the cell viability was 55.8% in the 5,000 µM NaF and 100 µM $MgCl_2$-administered cells (Figure 5).

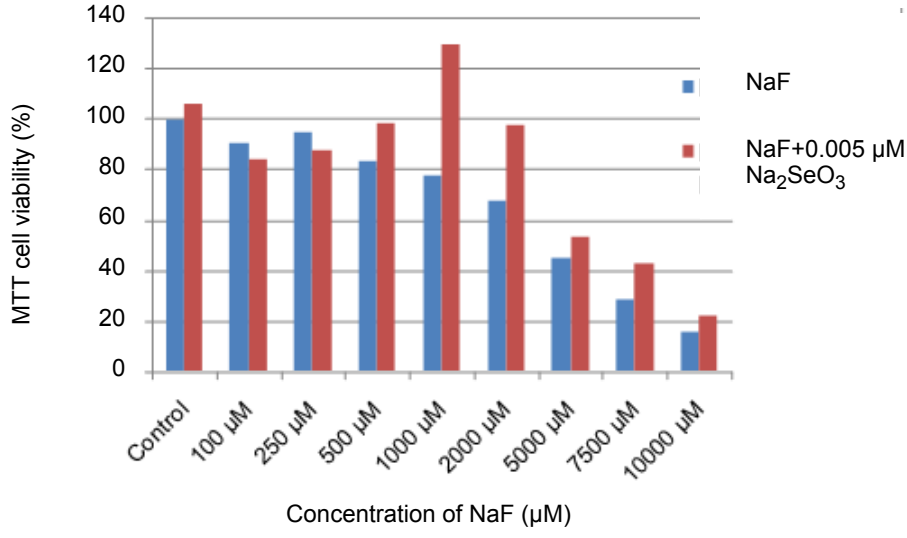

Figure 6. hFOB 1.19 cell line NaF and NaF+500 µM Na_2SeO_3 MTT chart. The MTT viability of the control group (0 µm NaF, 0 µM Na_2SeO_3 was set at 100%.

The cell viability was determined as 52.6% after 24 hours incubation in the 5,000 µM NaF-administered hFOB 1.19 cell lines when compared to the control, while the cell viability was 53.3% in 5,000 µM NaF and 0.005 µM Na_2SeO_3-admiinistered cells (Figure 6).

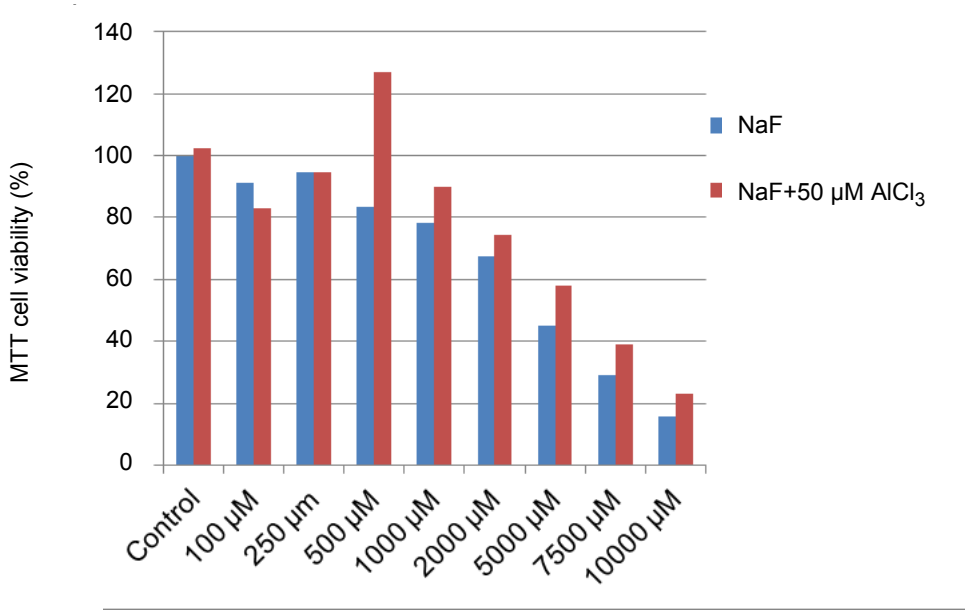

Figure 7. hFOB 1.19 cell line NaF and NaF+50 µM $AlCl_3$ MTT chart. The MTT viability of the control group (0 µm NaF, 0 µM $AlCl_3$ was set at 100%.

The cell viability was determined as 52.6% after 24 hours incubation in the 5,000 µM NaF-administered hFOB 1.19 cell lines when compared to the control, while the cell viability was 58.3% in the 5,000 µM NaF and 50 µM $AlCl_3$-administered cells (Figure 7).

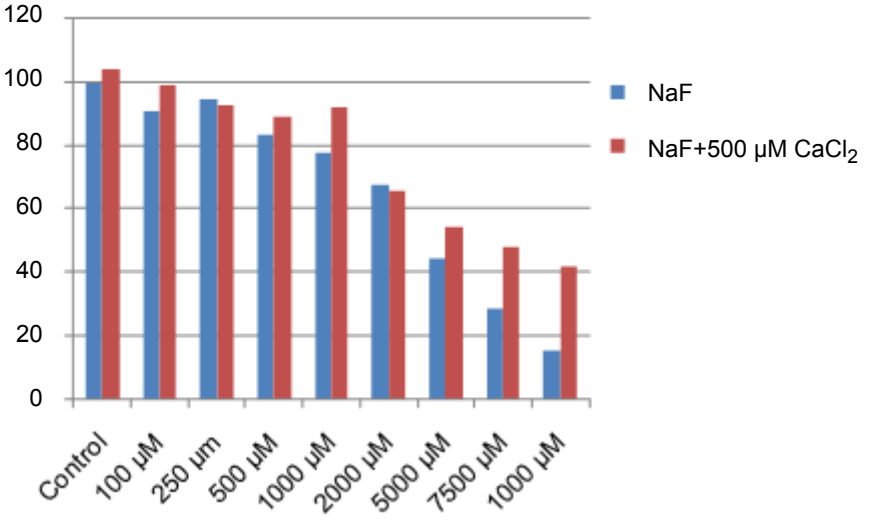

Figure 8. hFOB 1.19 cell line NaF and NaF+500 µM $CaCl_2$ MTT chart. The MTT viability of the control group (0 µm NaF, 0 µM $CaCl_2$ was set at 100%.

The cell viability was determined as 52.6% after 24 hours incubation in the 5,000 µM NaF-administered hFOB 1.19 cell lines when compared to the control, while the cell viability was 58.5% in 5,000 µM NaF and 500 µM $CaCl_2$-administered cells (Figure 8).

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

Table 2. NRK-52E cell line ELISA results

Group	M30 (IU/L)	Caspase-3 (ng/mL)	Caspase-8 (ng/mL)	Caspase-9 (ng/mL)	8-OHdG (ng/mL)
Control	107.52 ±12.35a	1.73 ±0.091ab	5.19 ±0.09a	4.83 ±0.12ab	17.27 ±2.43bc
NaF	94.53 ±7.31a	1.81 ±0.14ab	5.42 ±0.39ab	5.05 ±0.13ab	15.05 ±2.42bc
$MgCl_2$	89.95 ±21.30a	1.82 ±0.06ab	6.11 ±0.32de	5.09 ±1.13ab	21.10 ±1.02c
Na_2SeO_3	106.17 ±14.10a	1.60 ±0.07a	5.55 ±0.17abc	5.01 ±0.34ab	29.71 ±5.99d
$AlCl_3$	114.50 ±13.95a	2.37 ±0.52b	6.59 ±0.30e	5.82 ±0.80b	1.20 ±0.07a
$CaCl_2$	115.77 ±7.59a	2.11 ±0.92ab	5.86 ±0.16bcd	5.29 ±0.48ab	14.66 ±2.09bc
NaF+$MgCl_2$	106.32 ±6.23a	1.64 ±0.18a	5.26 ±0.21a	4.72 ±0.56a	13.99 ±2.21b
NaF+Na_2SeO_3	117.85 ±14.49a	1.89 ±0.20ab	5.47 ±0.41abc	5.04 ±0.51ab	14.76 ±8.43bc
NaF+$AlCl_3$	111.12 ±1.47a	1.90 ±0.24ab	5.97 ±0.26cd	5.69 ±0.27ab	0.64 ±0.09a
NaF+$CaCl_2$	90.03 ±25.57a	1.75 ±0.15ab	6.13 ±0.28de	5.39 ±0.26ab	11.45 ±0.42b

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

In the NRK-52E cell line, a statistically significant difference was found between the apoptosis parameters and the oxidative DNA damage ($p \leq 0.05$) in the comparison of parameters between all groups. Furthermore, a statistically significant difference was found between caspase-8 and oxidative DNA damage ($p \leq 0.05$) (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	207.49 ±29.51b	2.10 ±0.49a	7.70 ±0.89a	9.98 ±0.12b	8.19 ±1.23ab
NaF	184.90 ±6.27ab	2.61 ±0.07ab	7.28 ±0.36a	9.10 ±1.05ab	7.92 ±0.77ab
$MgCl_2$	198.06 ±13.03b	2.23 ±0.47ab	8.15 ±0.39a	10.13 ±1.75 b	7.35 ±0.38ab
Na_2SeO_3	198.15 ±20.56b	2.45 ±0.42ab	8.05 ±0.83a	9.89 ±1.30ab	9.65 ±2.82b
$AlCl_3$	182.54 ±19.28ab	2.40 ±0.36ab	8.38 ±0.87a	8.67 ±0.14 ab	7.81 ±0.51ab
$CaCl_2$	154.93 ±16.62a	2.44 ±0.10ab	8.27 ±0.30a	7.77 ±1.71a	6.45 ±0.55a
NaF+$MgCl_2$	155.88 ±26.29a	2.73 ±0.43ab	8.04 ±1.57a	9.91 ±1.50ab	6.04 ±2.34a
NaF+Na_2SeO_3	186.35 ±35.80ab	2.28 ±0.09ab	8.35 ±0.59a	10.33 ±0.44b	7.99 ±2.04ab
NaF+$AlCl_3$	196.60 ±8.23b	2.69 ±0.22ab	7.54 ±0.57a	8.68 ±0.88ab	5.98 ±1.05a
NaF+$CaCl_2$	194.34 ±12.68ab	2.78 ±0.40b	7.30 ±0.92a	9.59 ±0.79ab	7.55 ±1.44ab

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

In the hFOB 1.19 cell line, no statistically significant difference was found between apoptosis parameters and oxidative DNA damage ($p \leq 0.05$) in the comparison of parameters between all groups (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 in Tables 4A, 4B, 5A, and 5B.

Table 4A. NRK-52E cell line expressions based on real time-PCR results

Group	NaF	$MgCl_2$	Na_2SeO_3	$AlCl_3$	$CaCl_2$
GAPDH	1	1	1	1	1
Caspase-3	0.52	0.95	0.93	0.93	0.90
Caspase-8	0.18	1.67	1.44	1.17	1.21
Caspase-9	0.32	0.46	0.18	0.12	0.04

Table 4B. NRK-52E cell line expressions based on real time-PCR results

Group	NaF+$MgCl_2$	NaF+Na_2SeO_3	NaF+$AlCl_3$	NaF+$CaCl_2$
GAPDH	1	1	1	1
Caspase-3	0.59	0.67	0.63	0.68
Caspase-8	1.31	0.55	0.64	0.55
Caspase-9	0.37	0.23	0.88	0.52

Table 5A. hFOB 1.19 cell line expressions based on real time-PCR results

Group	NaF	$MgCl_2$	Na_2SeO_3	$AlCl_3$	$CaCl_2$
GAPDH	1	1	1	1	1
Caspase-3	2.25	3.43	2.51	5.16	3.86
Caspase-8	7.31	1.35	0.74	2.31	0.35
Caspase-9	1.19	1.75	0.69	0.89	3.73

Table 5B. hFOB 1.19 cell line expressions based on real time-PCR results

Group	$NaF+MgCl_2$	$NaF+Na_2SeO_3$	$NaF+AlCl_3$	$NaF+CaCl_2$
GAPDH	1	1	1	1
Caspase-3	1.61	0.70	0.76	0.60
Caspase-8	0.53	1.04	1.23	6.23
Caspase-9	2.04	2.36	0.99	3.55

Based on the real time-PCR results, in the NRK-52E cell line, caspase-9 decreased 0.7-fold in the NaF group compared to the control group. Caspase-9 expression decreased 0.6-fold in the $MgCl_2$ group and 0.7-fold in the $NaF+MgCl_2$ group. It decreased 0.8-fold in the Na_2SeO_3group, 0.8-fold decrease in the $NaF+Na_2SeO_3$ group, 0.9-fold in the $AlCl_3$ group, 0.2-fold in the $NaF+AlCl_3$ group, 9.5-fold in the $CaCl_2$ group, and 0.5-fold in the $NaF+CaCl_2$ group (Tables 4A and 4B).

Based on the real time PCR results, in the NRK-52E cell line, caspase-8 expression decreased 0.8-fold in the NaF group compared to the control group. It was determined that it increased 1.7-fold in the $MgCl_2$ group and 1.3-fold in the $NaF+MgCl_2$ group. While it increased 1.4-fold in the Na_2SeO_3 group, it was determined that it decreased 0.5-fold in the $NaF+Na_2SeO_3$ group. It increased 1.1-fold in the $AlCl_3$ group, while a 0.4-fold decrease was observed in the $NaF+AlCl_3$ group. While a 1.2-fold increase was observed in the $CaCl_2$ group, it decreased 0.5-fold in the $NaF+CaCl_2$ group (Tables 4A and 4B).

Based on the real time-PCR results, in the NRK-52E cell line, caspase-3 expression decreased 0.5-fold in the NaF group when compared to the control group. A 0.4-fold decrease was observed in the $NaF+MgCl_2$ group, while no change was observed in the Na_2SeO_3 group. It decreased 0.4-fold in the $NaF+Na_2SeO_3$ group and in the $NaF+AlCl_3$group, 0.3-fold in the $NaF+CaCl_2$ group, and it remained unchanged in the $CaCl_2$ group. The caspase-3, caspase-8, and caspase-9 expression graphs based on the real time PCR results conducted on the hFOB 1.19 cell line study groups are presented below (Tables 4A and 4B).

Based on the real time-PCR results, in the hFOB 1.19 cell line, caspase-9 expression increased 1.2-fold in the NaF group and 1.7-fold in the $MgCl_2$ group when compared to the control group. In the $NaF+MgCl_2$ group, a 2-fold increase was observed. A 0.3-fold decrease in the Na_2SeO_3 group and a 2.3-fold increase in the $NaF+Na_2SeO_3$ group were determined. The expression decreased 0.1-fold in the $AlCl_3$ group and there was no change in the $NaF+AlCl_3$ group. The increase in the $CaCl_2$ group was 3.7-fold and the increase in the $NaF+CaCl_2$ group was 3.5-fold (Tables 5A and 5B).

In the real time PCR results, in the hFOB 1.19 cell line, the caspase-8 expression increase was 7.3-fold in the NaF group, in the NaF group the increase was7.3-fold, in the NaF+$MgCl_2$ group there was a 0.4-fold decrease, there was no change in the Na_2SeO_3 group, the decrease was 0.4-fold in the NaF+Na_2SeO_3 group, the expression was not changed in the $AlCl_3$ group, it decreased 0.4-fold in the NaF+$AlCl_3$group, there was no change in the $CaCl_2$ group, and the decrease was 0.3-fold in the NaF+$CaCl_2$ group (Tables 5A and 5B).

Real time PCR results, in the hFOB 1.19 cell line, demonstrated that caspase-3 expression increased 2.25-fold in the NaF group, 3.4-fold in the $MgCl_2$ group, and 1.6-fold in the NaF+$MgCl_2$ group when compared to the control group. The increase was 2.5-fold in Na_2SeO_3 group and there was a 0.3-fold decrease in the NaF+Na_2SeO_3 group. The expression increase in the $AlCl_3$ group was 5-fold, while it decreased 0.3-fold in the NaF+$AlCl_3$ group. The increase in the $CaCl_2$ group was 3.8-fold, while the expression decreased 0.4-fold in the NaF+$CaCl_2$ group (Tables 5A and 5B).

DISCUSSION

Endemic fluorosis, which can affect human health systemically, is observed in several parts of the world. Fluorosis cannot only damage skeletal tissues and teeth, but can also harm soft tissues such as the brain, liver, kidneys, and the spinal cord. In addition to the studies that demonstrated fluoride (F) induced DNA damage and chromosomal instability,[19,20] there are also studies that reported no genotoxic effects.[21,22]

Fluoride has a high penetrative ability and can easily penetrate the cell membrane. It may enter deeper soft tissues such as the liver, brain, and kidney, and therefore, nephrotoxicity could occur due to the accumulation and retention of inorganic fluoride in the renal tubules.[23]

Fluoride has a cytotoxic effect based on the dose of exposure.[24] In the present study, it was determined that NaF exhibited a dose-dependent cytotoxic effect. The findings demonstrated that although low NaF dose (0–500 μM) administration triggered cell proliferation independent of the cell type, high doses (>1,000 μM) had toxic effects on the cell.

Çetin and Yur[25] investigated mineral substance levels in muscle and kidney tissues obtained from sheep with endemic fluorosis. They reported that fluorosis significantly altered the mineral metabolism in the muscle and kidney and resulted in high levels of mineral accumulation and excretion.

Song et al.[26] investigated whether sodium fluoride induced apoptosis in rat kidneys. To find the answer, they investigated the mechanisms underlying NaF-induced apoptosis. Their findings demonstrated that NaF-induced renal cell apoptosis was mediated by caspase-mediated and death receptor pathways, and that DNA damage might also play a role in this process. Concurrently, an increase in systolic cytochrome c and caspase-3, 8, and 9 protein levels was reported.

Wei et al.[27] investigated the expression of the genes of BMP-2 and BMP-3, members of bone morphogenetic protein family, and the possible effects of these genes on cell viability in human osteosarcoma MG-63 cells. In this study,[27] it was reported that sodium fluoride (NaF) increased proliferation based on dose. Cell

proliferation was at the maximum level at 5×10^3µmol/L concentration and inhibited at 2×10^4 µmol/L. In the present study, NaF increased cell proliferation at low doses in the hFOB 1.19 osteoblast cell line, while proliferation was inhibited at high doses (>1000 µM). The dose-dependent cytotoxic effect was found to be consistent with the findings of the above mentioned study,[27] while the IC_{50} value was found as 5,000 µM. This finding suggested that NaF might have different cytotoxic effects on cells despite the cells having the same origin.

There are several studies on fluorosis-induced apoptosis in tissues.[26,27,28] Zhang et al.[28] reported that NaF could induce apoptosis in the osteosarcoma Saos-2 cell line depending on the dose and time, and that the apoptotic pathway could consist of both mitochondrial and receptor pathways. In this study,[2] it was also reported that the expression and protein levels of certain genes in the mitochondrial pathway increased, while others decreased. However, the underlying molecular pathway was not clarified and the presence of a complex NaF-mediated apoptosis pathway structure was identified. In a study conducted with ameloblast cells,[29] it was reported that caspase activation increased as a result of 5,000 µM NaF administration for 6 hours and apoptosis was triggered in the cells. The present study demonstrated that apoptosis was induced in 6,000 µM NaF-administered NRK-52E kidney cells and 5,000 µM NaF-administered hFOB 1.19 osteoblast cells. Our findings were consistent with the literature.

In the present study, M30, and caspase-3, 8, and 9 levels were investigated as an apoptosis marker in an experimental study on the kidney epithelial NRK-52E cell line. ELISA results demonstrated no change in the M30 levels.

Yang et al.[30] examined Bcl-2, and Bax mRNA expression by real time-PCR after exposure to different NaF concentrations for 48 hours to investigate the cellular mechanism underlying NaF-induced apoptosis in MC3T3-E1 osteoblast cell lines. Bax-2 mRNA expression significantly decreased after exposure to NaF, while Bax mRNA expression significantly increased. Higher concentrations (5×10^{-4} and 10^{-3} M) caused an increase in Bax expression, while it was reduced at low NaF concentrations (10^{-5} and 5×10^{-5} M). They also reported that the Bcl-2/Bax ratio in cells exposed to NaF at 5×10^{-5}, 10^{-4}, 5×10^{-4}, and 10^{-3} M concentrations decreased significantly.

The findings in the present study on the osteoblast cell line demonstrated that cell viability increased with the administration of NaF in conjunction with Na_2SeO_3, $AlCl_3$, and $CaCl_2$, while $MgCl_2$ administration decreased cell viability. Apoptotic parameters were evaluated to understand the mechanisms that influenced cell viability. Among these parameters, it was found that the M30 protein remained unchanged in NaF-administered hFOB 1.19 cells and was not different, when compared to control, except for the NaF+$MgCl_2$ and $CaCl_2$ administered groups. This suggested that cell death might be caused not only by apoptotic pathways but also by other mortal pathways.

NaF increased apoptosis in the osteoblast cell line via caspase-8, in other words, the extrinsic pathway. On the other hand, $MgCl_2$ increased the apoptotic effect at the doses given both through the intrinsic and the extrinsic pathways. However, it was determined that $MgCl_2$ prevented apoptosis by blocking the extrinsic pathway, hence the solitary effect of NaF. The application of Na_2SeO_3 reduced the apoptotic effect of

NaF 7-fold. $AlCl_3$ triggers apoptosis via the extrinsic pathway similar to NaF when administered alone. However, since $AlCl_3$ forms a complex with NaF, the apoptotic effect is reduced when they are administered together. $CaCl_2$ triggers apoptosis using the mitochondrial (intrinsic) pathway. $CaCl_2$ forms a complex with NaF, inhibiting the NaF extrinsic pathway.

In the present study, the levels of 8-OHdG, an oxidative DNA damage marker, significantly decreased in the NaF+mineral groups in the kidney cell line NRK-52E cells when compared to the control group. It was determined that the oxidative DNA damage increased only in the Na_2SeO_3 and $MgCl_2$ administered groups. However, there was a decrease in the NaF+mineral groups. It was observed that the 8-OHdG levels were not affected in the control and NaF+mineral groups in the osteoblast cells.

CONCLUSION

In conclusion, in the present study, the results obtained by the investigations conducted on both cell lines and utilized analysis methods were evaluated and can be summarized as follows:

Based on the IC_{50} value selected for NaF, apoptotic pathways function differently in apoptosis based on the cell type. The M30 apoptotic protein did not vary significantly in either cell type. The fluorostatic effect is cell and tissue specific. The mode of action has changed according to the dose, duration, and cell type. Death type in fluorosis can be apoptotic, autophagic, and necrotic. Which of these three modes death occurs depends on the fluoride doses which affect the expression of the apoptotic markers. When fluoride is used in low doses, it increases short-term cellular acute proliferation.

In the present study, the possible apoptotic effects of NaF+mineral combinations on kidney and osteoblast cells were investigated for the first time and it was demonstrated that minerals inhibited fluorosis induced-intercellular signal pathways based on the cell type. Similarly, the significance of the electronegativity of minerals in inhibiting the effects of fluorosis was examined and the findings were that the higher the electronegativity of the mineral, the more effective the it was in inhibiting fluorosis-induced necrosis. Finally, it was concluded that further studies on the elucidation of the roles of minerals in NaF-induced cellular death should be planned with different doses, times, and analytic methods.

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