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THE EFFECT OF SODIUM FLUORIDE ON ALUMINUM-INDUCED OXIDATIVE STRESS AND APOPTOSIS OF PC12 *IN VITRO*

Alireza Hoorang,[a] Jamileh Saberzadeh,[b] Forough Iranpak,[b] Afshin Mohammadi-Bardbori,[a] Marjan Khorsand,[b] Mohammad Ali Takhshid[b,*]

Shiraz, Iran

ABSTRACT: The aim of the present investigation was to determine the effects of sodium fluoride (NaF) on aluminum-induced toxicity in PC12 cells, a rat pheochromocytoma cell line, including cell death and changes in apoptosis, mitochondrial membrane potential (MMP), and oxidative stress. Aluminum (Al) and the fluoride ion (F) are known as plausible pathological factors in the pathogenesis of neurodegenerative disease. Conflicting results have been reported on the interaction of fluoride and aluminum on cell toxicity. PC12 cells were treated with various concentrations of aluminum maltolate (Almal) in the presence and absence of NaF (50–2400 µM) *in vitro*. A MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was used to determine cell viability. 7AAD/annexin-V and 2',7'-dichlorofluorescin diacetate (DCFDA) flow cytometric methods were used to quantify the effects of NaF on Al-induced apoptosis and reactive oxygen species (ROS) generation, respectively. The mitochondrial membrane potential (MMP) was monitored using the retention of rhodamine 123. Catalase activity was determined by measuring the rate of H_2O_2 decomposition. Almal exposure increased cell death dose dependently and significantly (IC_{50}=1090 µM). ROS generation and apoptosis were increased while MMP and catalase activity were reduced following treatment with Almal. NaF ameliorated the Almal-induced cell toxicity in low concentrations while augmenting the Almal effects at high concentrations. The present data suggest F has antagonistic impacts on Al-induced cell toxicity in low concentrations while acting synergistically with Al to induce cell toxicity at high concentrations.

Key words: Aluminum maltolate; Apoptosis; Oxidative stress; Sodium fluoride.

INTRODUCTION

Although the fluoride ion (F) has been delivered to the teeth systemically and topically to aid in the prevention of dental caries,[1] an Iranian study found only a weak non-significant association between decay in permanent (D1) and deciduous (d1) teeth and increasing water F levels, and F has been found to have adverse effects on most of the human body systems, particularly the nervous system.[3,4] Fluoride enters to human body mainly through dental products and fluoridated water.[5-7] The metabolism of F-containing anesthetics, including isoflurane and sevoflurane, which occurs during or after surgery is another source of excess F in the body.[8] The toxicity of F for the development and functioning of the nervous system has been focus of studies in recent years.[9] The first clue was obtained from the epidemiological studies

[a]Department of Toxicology, Pharm Faculty, Shiraz University of Medical Sciences, Shiraz, Iran; [b]Diagnostic Laboratory Sciences and Technology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran (The institution where the work was done). *For correspondence: Mohammad Ali Takhshid, Diagnostic Laboratory Sciences and Technology Research Center, Faculty of Paramedical Sciences, Meshkinfam Street, Shiraz, Iran; Tel/Fax: 987132289113; E-mail: takhshidma@sums.ac.ir.

Author information: Alireza Hoorang: PharmD; Email: alirezahoorang@gmail.com, Phone: 09173919069; Forough Iranpak: Msc; E-mail: forough.iranpak@yahoo.com, Phone: 09171015707; Jamileh Saberzadeh: PhD; E-mail: saberzadej@ sums.ac.ir, Phone: 09173081069; Afshin Mohammadi-Bardbori: PhD; E-mail: toxicology@sums.ac.ir; Mohammad Ali Takhshid: PhD; E-mail: takhshidma@sums.ac.ir; Phone: 09173121699; Marjan Khorsand: PhD candidate: E-mail: ma.kh58@yahoo.com, Phone: 09173067168.

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which have found a negative association between the level of F in drinking water and the IQ of children.[10] Experimental studies in laboratory animals have confirmed the harmful effects of excess F on the anatomical structure, biochemical characteristics, and biological function of various regions of the brain including the hippocampus,[11,12] cerebrum, cerebellum, pons, medulla,[13] and prefrontal cortex.[14] Impairments in learning and memory are two major F-induced abnormalities that have been indicated by these studies.[15] On the other hand, a lower prevalence of primary degenerative dementia has been reported in geographic areas with a high content of F in the drinking water which has been attributed to the effects of F on decreasing the bioavailability of aluminum (Al),[16] an abundant neurotoxic element in the earth's crust that has been implicated as an important environmental risk factor for the neurodegenerative diseases.[17]

Human beings are inevitably exposed to Al in everyday life in several ways, including with foods, pharmaceuticals, industries, vaccines, and drinking water.[18] The deleterious effects of Al on the structure and function of the brain are well known and include behavioral and cognitive deficits, enzymatic and neurotransmitter alterations, and neurofibrillary changes.[17] Due to the neurotoxicity of both Al and F, it might be expected that the simultaneous exposure to both of them would increase their neurotoxicity in a synergistic manner. In addition, Mahvi et al. showed that Al smelters result in large amounts of F being emitted into the air.[19] Varner et al. reported that Al-F caused more neurotoxic effects than NaF, both qualitatively and quantitatively.[20] Akinrinade et al. have also demonstrated that the simultaneous treatment of rats with Al and F leads to more severe adverse alterations in the histomorphology, membrane transport activities, activity of acetylcholinesterase, and expression of lysosomal and cell cycle proteins than treatment with each element used alone.[14] In contrast, it has been suggested that the formation of Al-F complexes may reduce their toxicities compared to the use of each alone. In accordance with this hypothesis, animal studies have demonstrated that the Al ion can ameliorate F-induced male reproductive toxicity.[21] The induction of oxidative stress is the most reported cause of Al- and F-induced neurotoxicity[22-24] and ameliorating effects on Al- and F-induced oxidative damage have been demonstrated with many natural antioxidants, both *in vitro* and *in vivo*.[11,22,25]

Considering the above mentioned conflicting results, there is the need for more research in order to clarify the interaction of Al and F in causing neurotoxicity. Therefore, the present study was conducted to explore the possible effects of F on Al-induced toxicity. To this end, the effects of aluminum maltolate (Almal), a lipophilic complex that allows the ready entry of Al into cells,[23] on cell death, apoptosis, and oxidative stress markers including ROS generation, mitochondrial membrane potential (MMP), and catalase (CAT) activity of PC12 cells were determined in the presence and absence of sodium fluoride.

MATERIALS AND METHODS

Cell culture and drug solution preparations: PC12 is a cell line derived from rat pheochromocytoma and is widely used as a model in research of neurotoxicity.[26] A PC12 cell line (Pasteur Institute of Iran, Tehran, Iran) was cultured and maintained in DMEM (Gibco) supplemented with 10% horse serum, 5% fetal bovine serum (Sigma-Aldrich), and 1% penicillin–streptomycin (Sigma-Aldrich) at 37ºC and 5%

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CO_2. Aluminum chloride hexahydrate ($AlCl_3$•$6H_2O$) and Maltol (3-hydroxy-2-methyl-4-pyrone) (Sigma) were used to prepare stock solution of Almal (25 mM) as described previously.[22] A stock solution of NaF (Sigma-Aldrich) was prepared in distilled water.

MTT assay: The effects of the elements aluminium and fluorine on the viability of the PC12 cells were evaluated using a MTT assay. Briefly, 5×10^3 PC12 cells were seeded per well in 96 well cell culture plates. After 24 hr, the cells were treated with various concentrations of Almal (0–1500 µM) and NaF (0–3800 µM). Forty-eight hr after the treatments, MTT assay was done after the addition of MTT solution [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] to the culture medium. After solubilizing the formed formazan using DMSO, the absorbance of the solution was measured at 570 nm.

Apoptosis assay using the Annexin V / 7-AAD flow cytometric method: An Annexin V/7-ADD apoptosis detection kit I (BD Pharmingen) was applied to investigate the possible protective effect of NaF against the Almal-induced apoptosis. In brief, the PC12 cells were treated with Almal at 1000 µM (the IC50 concentration calculated from dose response studies) in the absence and presence of two low doses of NaF (50 and 120 µM) and a high dose of NaF (2400 µM) for 48 hr. The cells were then washed in cold phosphate buffered saline (PBS) and suspended in 1 mL of ice cold binding buffer. Annexin V (5 µL) and 7-AAD (5 µL) were then added to 100 µL of the cell suspension and gently mixed and incubated for 15 min in darkness at room temperature. Apoptosis was detected using a FACS Calibur flow cytometer (BD FACS Calibur, BD Bioscience, USA). The percent of total apoptosis (percent of early and late apoptotic cells) was calculated for each sample.

Reactive oxygen species (ROS) assay: The ROS content of the PC12 cells was determined by a flow cytometric method using 2', 7'-dichlorodihydrofluorescein diacetate (DCFDA, Sigma) which is based on the oxidation of DCFDA to green fluorescent DCF by ROS.[27] Briefly, the PC12 cells were incubated with Almal (1000 µM) in the presence and absence of NaF (50, 120, and 2400 µM). Tert-Butyl hydroperoxide (TBHP, 100 µM) was used as a positive control. After treatment, the cells were washed in PBS and stained with DCFDA (20 µM in Ringer solution) at 37ºC for 30 min in the dark. After washing with PBS, the stained cells were resuspended in 200 µL of PBS and the DCF fluorescence intensity was measured at the excitation/emission wavelengths of 488 nm /520 nm on a FACS Calibur (BD).

Measurement of the mitochondrial membrane potential: The MMP of the PC12 cells was measured using rhodamine 123 (Rh123) fluorescent dye. Rh123 is accumulated in the mitochondria quantitatively and in direct proportion to the MMP. Any dissipation in the MMP caused a decrease in the mitochondrial accumulation and a decrease in the fluorescence intensity of the Rh123 in the PC12 cells.[28] When cells are incubated with Rh123, some of Rh123 is absorbed by the cells and is then accumulated in the mitochondria while some remains in the incubation solution. In this study, we measured the amount of Rh123 fluorescence intensity in both the incubation solution and the cells. Because Almal causes MMP dissipation, it increases the Rh123 fluorescence intensity in the incubation solution compared to control group. Briefly, 4×10^5 PC12 cells were plated in 24 well plates for 24 hr. Then, the cells were exposed to Almal (1000 µM) for 48 hr in the presence and absence of

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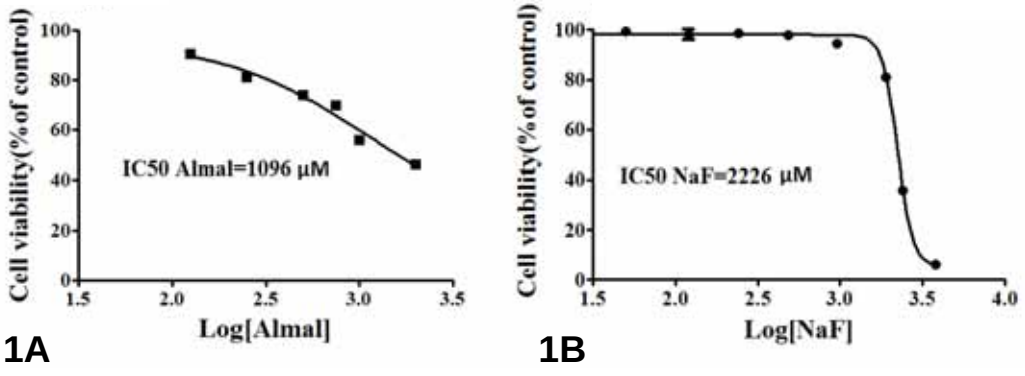
NaF (50, 120, and 2400 µM). After treatment, the cells were washed twice with PBS and were then incubated with Rh 123 (1 µM) for 30 min at 37ºC in the dark. After the incubation period, the cells were washed in PBS and the Rh-123 fluorescence was measured, in both the cells and the incubation solution, at the excitation/emission wavelengths of 490 nm/520 nm using a fluorimeter.

CAT activity assay: The close relationship between the CAT activity and the Al-induced oxidative stress and the presence of a simple and sensitive procedure for the assay of CAT activity provides a suitable tool for evaluating the cellular oxidative stress.[23] Thus, we used CAT activity as a marker of the cell response to oxidative stress in our study. In brief, PC12 cells were treated with Almal (1000 µM) in the absence or presence of NaF (50, 120, and 2400 µM) for 48 hr. The cells were then lysed using sonication on ice. The cell lysates were then centrifuged (13000 g for 20 min at 4ºC) and the supernatants were used for the determination of the protein concentration and CAT activity. The Bradford method was used for the determination of the protein concentration in the supernatant. The activity of CAT was measured based on the rate of decomposition of H_2O_2 at 25ºC, followed by a decrease in absorbance at 240 nm.[23] The CAT activity was calculated as units/µg protein and expressed as a percentage of the control cells.

Statistical analyses: The SPSS statistical program (version 21) was used to analyze the data. The data were represented as mean ± standard deviation (SD) of at least three independent experiments. Due to the absence of a normal distribution ($p>0.05$) the data were analyzed using the non-parametric Kruskal-Wallis test. $p<0.05$ was considered to be statistically significant. The IC50 values for Almal and NaF in the MTT assay experiments was calculated using the GraphPad PrismÆ 5 (Version 5.01, GraphPad Software, Inc., USA).

RESULTS

The effects of Almal and NaF on the viability of PC12 cells: The effects of various concentrations of Almal (0–1500 µM) and NaF (0–3800 µM) on the viability of the PC12 cells were investigated using the MTT assay (Figures 1A and 1B).



Figures 1A and 1B. Effects of Almal and NaF on the viability of PC12 cells. 1A: The PC12 cells were treated with various concentrations of Almal (0–1500 µM), 1B: The PC12 cells were treated with various concentrations of NaF (0–3800 µM). The cell viability was determined using the MTT assay. The represented data are mean ± SD of at least three independent experiments.

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Almal decreased the viability of the PC12 cells in a significant ($p<0.001$) and dose dependent manner (IC50 =1096 µM) compared to the control group (Figure 1A). The dose response studies revealed that NaF had no significant effect on the viability of the PC12 cells at concentrations from 10 to 950 µM. However, higher concentrations of NaF, ranging from 1900 to 3800 µM, decreased the viability of the PC12 cells dose dependently (IC50=2226 µM) and significantly ($p<0.001$) compared to the control group (Figure 1B). Based on the calculated IC50s, the concentrations of 1000 µM of Almal and 50, 120 (low doses; 0.02 and 0.05 of the calculated IC50, respectively), and 2400 µM (a concentration above the calculated IC50) of NaF were used for the further experiments.

The effects of NaF on Almal-induced PC12 cell death: The effects of various concentrations of NaF on PC12 cell death induced by 1000 µM of Almal are shown in Figure 2.

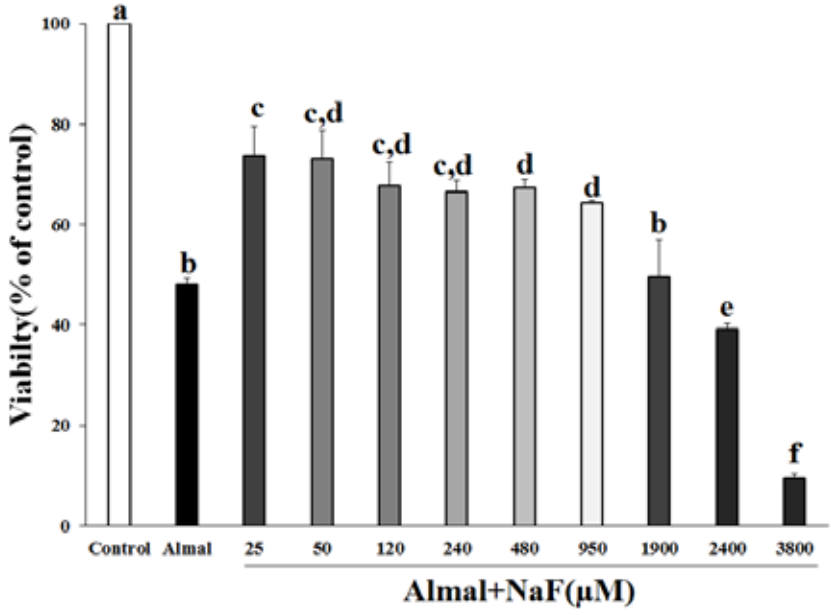

Figure 2. The effect of various concentrations of NaF on Almal-induced cell death. The PC12 cells were treated with Almal (1000 µM) in the absence or presence of NaF (0–3800 µM). The cell viability was then evaluated using a MTT assay. The data are mean ± SD of at least three independent experiments. The non-parametric Kruskal-Wallis test was used to determine the presence of a significant difference between the groups. The rectangles in the histogram denoted by common letters are not significantly different in the group comparisons while the groups denoted by dissimilar letters are significantly different.

As can be seen in Figure 2, NaF in concentrations from 25 up to 950 µM significantly protected the PC12 cells from Almal-induced cell death. The maximum protective effect was obtained at the 25 µM concentration of NaF. At the 1900 µM concentration, NaF had no protective effects while at higher concentrations of NaF (2400–3800 µM) PC12 cell viability was dramatically decreased following the concomitant treatment with NaF and Almal.

The effect of NaF on Almal-induced apoptosis: The AnnexinV/7ADD flow cytometric assay was used to explore the effects of Almal and NaF on the apoptosis of the PC12 cells. The findings revealed that the treatment of the PC12 cells with Almal (1000 µM) increased the percentage of total apoptotic cells [early (Q2) + late

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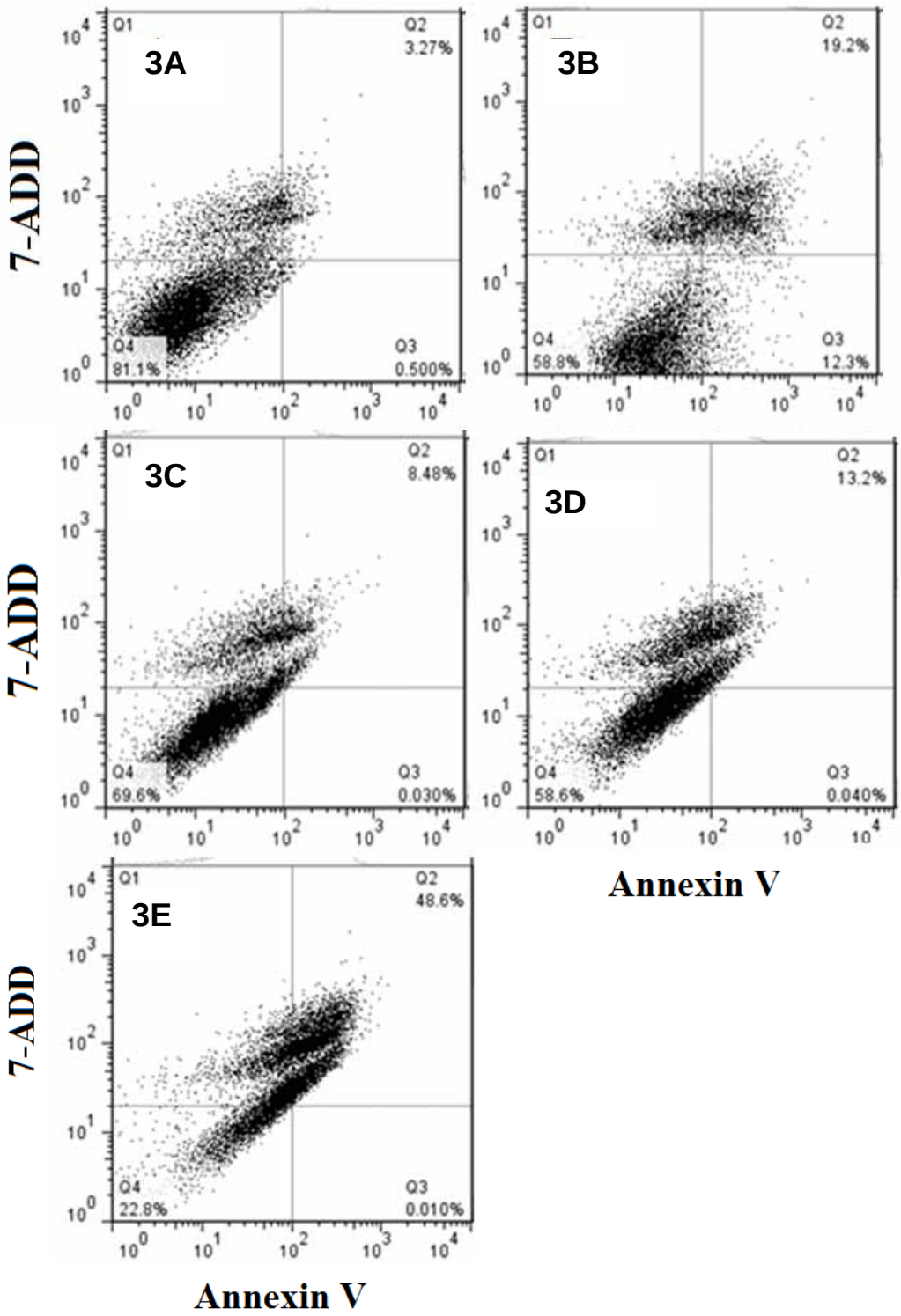
apoptotic (Q3) cells] and reduced the live cells (Q4) compared to the control group ($p<0.001$) (Figures 3A–3F).



Figures 3A–3E. The effect of NaF on Almal-induced apoptosis of PC12 cells. The flow cytometric graphs illustrate percentage of live cells (Q4), early apoptotic (Q3), late apoptotic (Q2), and death cells (Q1). 3A: Control group, 3B: Almal alone, 3C: Almal (1000 µM) + NaF (50 µM), 3D: Almal (1000 µM) + NaF (120 µM), and 3E: Almal (1000 µM) + NaF (2400 µM).

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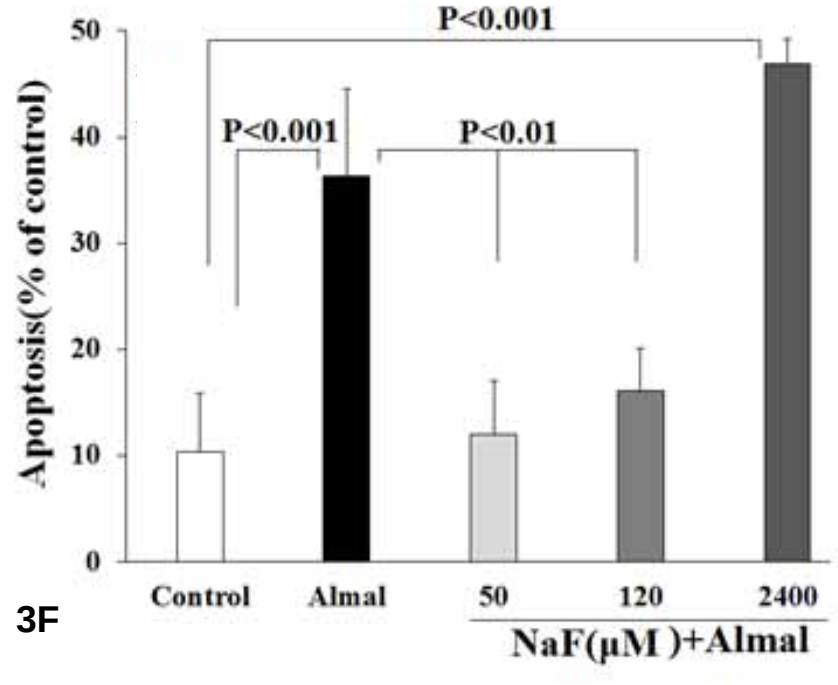

Figure 3F. The effect of NaF on Almal-induced apoptosis of PC12 cells. The histogram reveals the effects of NaF on Almal-induced apoptosis. The data are mean ± SD of at least three independent experiments which were analyzed using the non-parametric Kruskal-Wallis test.

Co-treatment with NaF (50–120 μM) and Almal (1000 μM) reduced the percentage of apoptotic cells and increased the live cells compared to the Almal-treated group ($p<0.001$). In contrast, concomitant treatment of PC12 cells with 1000 μM of Almal and 2400 μM of NaF increased apoptosis of the PC12 cells compared to the co-treatment with NaF (50–120 μM) and Almal (1000 μM).

Protective effects of NaF against Almal-induced ROS generation: The generation of ROS in the PC12 cells following the treatment with Almal alone or in combination with NaF was determined using the DCF flow cytometric assay. As revealed in Figures 4A and 4B, a significantly higher amount of ROS ($p<0.001$) was detected in PC12 cells treated with Almal (1000 μM) compared to the control group. A decrease in the ROS content was observed in the cells that were treated concomitantly with Almal (1000 μM) and NaF (50 and 120 μM) as compared to the Almal (1000 μM) group ($p<0.001$). However, there was no significant difference between the groups with NaF concentrations of 50 and the 120 μM.

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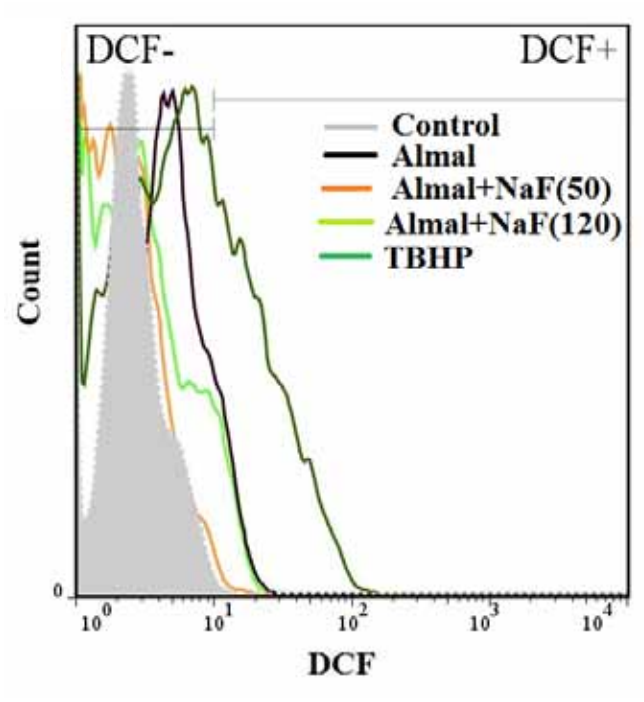
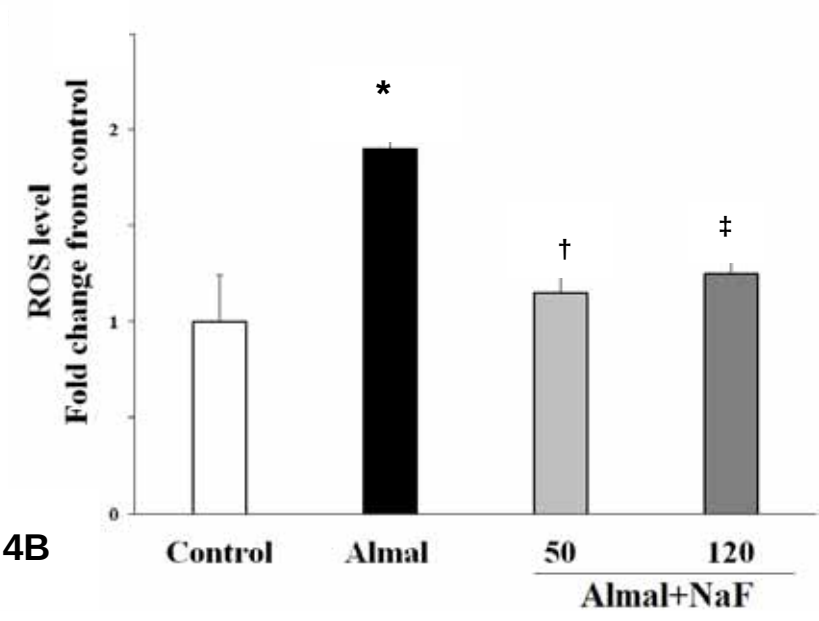


4A



4B

Figures 4A and 4B. Measurement of intracellular ROS content using DCF flow cytometery in PC12 cells treated with Almal in the presence and absence of NaF. 1A: The representative spectra of fluorescent DCF in cells treated with Almal and Almal+NaF with cells treated with TBHP as a positive control. 1B: The comparative analysis of the DCF fluorescence in Almal treated cells in the absence (Almal) and presence of NaF (50 and 120 µM). The data are mean ± SD of at least three independent experiments which were analyzed using the non-parametric Kruskal-Wallis test. Compared to control group: *p=0.007; compared to the Almal-treated group: †p=0.029 and ‡p=0.09.

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Protective effect of NaF against Almal-induced MMP dissipation: The Rh123 fluorescence assay was used to evaluate the effects of Almal and NaF on the MMP of the PC12 cells. Figure 5 illustrates the changes in the MMP level of the PC12 cells following the treatment with Almal alone or in combination with NaF. The Almal (1000 µM) treatment caused a 40% decrease in MMP compared to the control cells ($p<0.001$), indicating a dissipation of MMP by Almal. Co-treatment with NaF at 50 and 120 µM concentrations restored the Almal-induced MMP dissipation and increased MMP by 27% and 28%, respectively, compared to the Almal treated group ($p<0.001$), suggesting a protective role of NaF against the Almal-induced MMP dissipation (Figure 5).

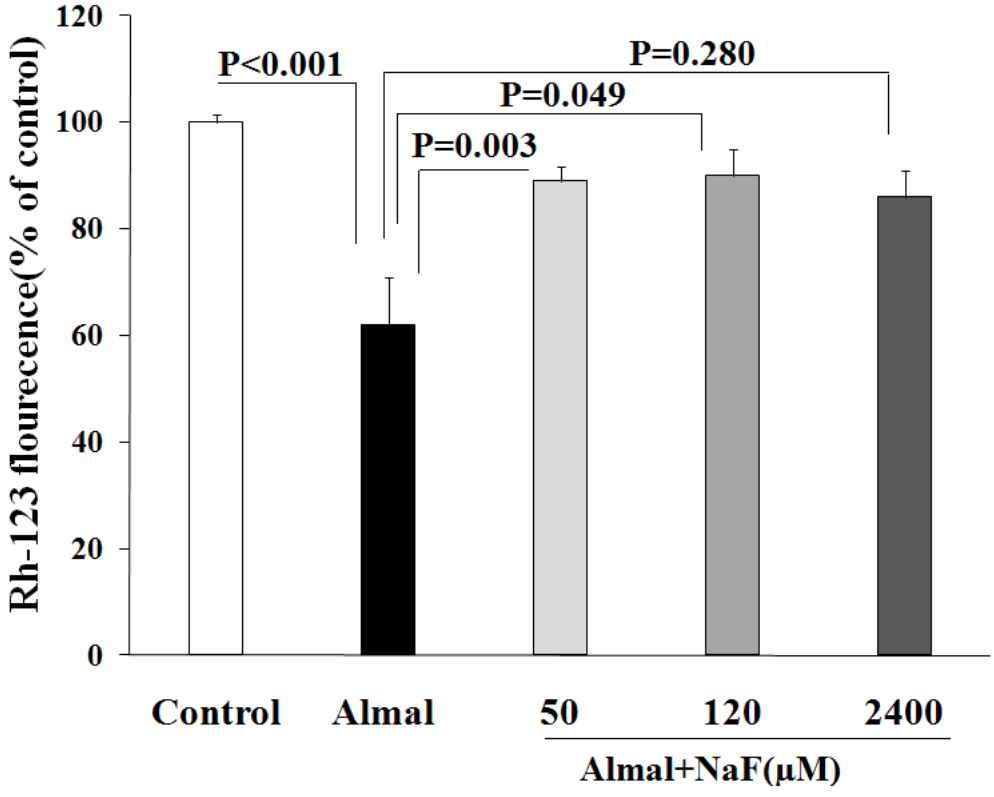

Figure 5. The mitochondrial membrane potential of PC12 cells following treatment with Almal and NaF. The Rh123 fluorescence obtained from the PC12 cells treated with Almal (1000 µM) in the absence and presence of varying concentrations of NaF and expressed as a percentage of the control untreated cells. The data are mean ± SD of at least three independent experiments which were analyzed using the non-parametric Kruskal-Wallis test.

Effect of Almal and NaF on catalase activity: The activity of catalase was measured as an enzymatic marker of oxidative stress. The Kruskal-Wallis test results revealed that the CAT activity is significantly different among the experimental groups ($p=0.015$). Pair wise comparisons revealed that Almal reduced the activity of CAT to about 33% of the control cells ($p=0.007$). Co-treatment with Almal and NaF (50, 120, and 2400 µM) increased the CAT activity in a dose dependent manner; However, no

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significant effects were observed with the co-treatment groups compared to those of Almal-treated group (Figure 6).

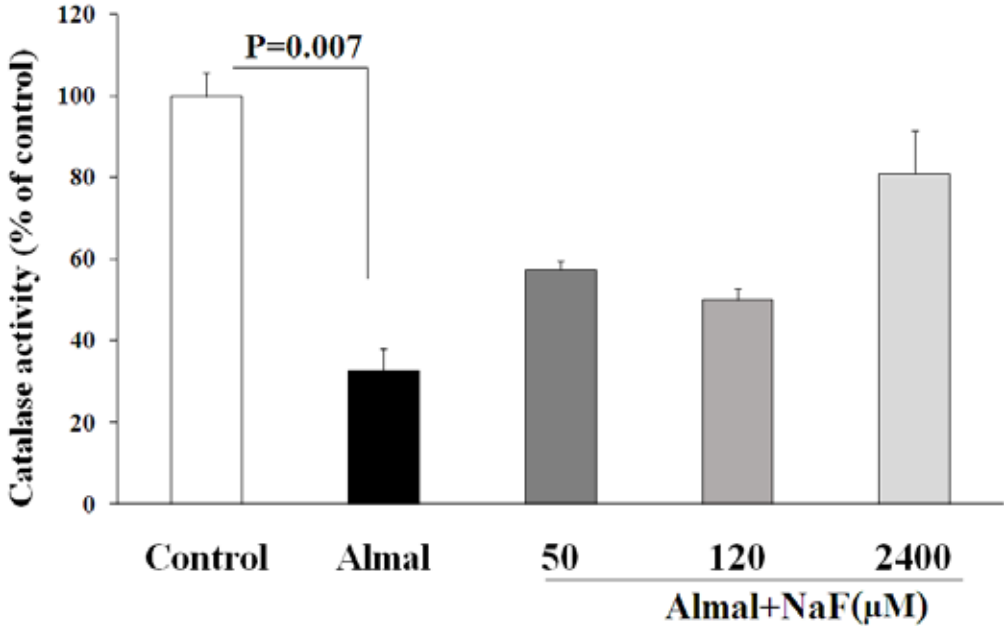

Figure 6. Effects of Almal and NaF on catalase activity. The PC12 cells were treated with Almal (1000 μM) in the absence or presence of various concentrations of NaF (50, 120, and 2400 μM) and the catalase activity was measured in the supernatant of the cells. The data are mean ± SD of at least three independent experiments which were analyzed using the non-parametric Kruskal-Wallis test.

DISCUSSION

In this study, we evaluated the effects of NaF on Almal-induced cell toxicity in PC12 cells. Our study had three main findings. Firstly, Almal decreased the viability of PC12 in a dose dependent manner (IC50 =1096 μM). The induction of apoptosis, which was probably mediated by an increase in ROS generation, mitochondria damage, and a decrease in the activity of catalase were responsible in the observed Almal toxic effects. The second finding was that at low concentrations of NaF (below 950 μM) there were no significant toxic effects on the viability of the PC12 cells while at higher concentrations (IC50 =2226 μM) it reduced cell viability. Finally, in non-toxic doses NaF ameliorated the adverse effects of Almal on cell viability, apoptosis, ROS generation, and MMP dissipation while in high doses it increased the Almal-induced adverse effects, suggesting hormetic effects of NaF against Almal-induced toxicity.

Hormesis is defined as a reagent having opposite effects on the rate of a reaction when it is used at low and high concentrations, i.e., the rate of the reaction is increased with low concentrations of the reagent and is decreased at higher concentrations, or vice versa.[29] In the current study, the results of the MTT assay and the annexin V/7-AAD apoptosis assay revealed the hormesis effects of NaF against Almal-induced adverse effects in PC12 cells. The highest protective effect of NaF against the Almal-induced toxic effects was observed at 50 μM or lower

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concentrations and then it gradually decreased until it reached a concentration of 950 µM. At higher concentrations, we observed an opposite effect of NaF in which the presence of NaF augmented the Almal-induced cell death and apoptosis. The present findings are in accordance with the existence of hormesis for NaF effects that has also been reported by other authors. Tang et al. [30] have demonstrated hormesis effects of NaF on cell death and apoptosis of rat kidney cells, in which NaF showed stimulatory effects on cell proliferation in low concentrations and anti-proliferative effects at high concentrations (above 1000 µM). Qu et al.[31] have also revealed that NaF at concentrations of 0.01 to 10 µM stimulated caprine osteoblast cell proliferation, but inhibited cell proliferation and induced apoptosis at higher concentrations (100 to 1000 µM). In a primary culture of rat chondrocytes, Meng et al.[32] showed that NaF at a concentration lower than 1.0 mM had no effects on cell viability while an inhibition of cell activities occurred with higher concentrations from 1.5–4.0 mM. Furthermore, it has been demonstrated that 1 mM NaF had no significant impact on the proliferation of human embryonic stem cells, but at a 2 mM or higher concentration it significantly decreased the viability of the cells.[33]

It has been demonstrated that the dissipation of MMP causes an excessive generation of ROS and the release of cytochrome C from the mitochondria to the cytoplasm which may ultimately trigger apoptosis.[34] In the present study, we found a 40% reduction in the level of MMP in the PC12 cells after exposure to Almal. Co-treatment of Almal with NaF at 50 and 120 µM concentration restored the MMP to nearly that of the control cells while a higher concentration of NaF (2400 µM) had no significant effects on the Almal-induced MMP dissipation. Consistent with our results, it has been reported that NaF increased MMP loss in H9c2 cardiomyocytes[35] and in mouse osteoblastic MC3T3-E1cell lines, suggesting that high concentrations of F have toxic effects on mitochondria.

The results of a ROS determination revealed that NaF diminishes Al-induced ROS generation in low concentrations while at high concentration it increases ROS generation. A CAT activity assay showed that NaF had similar effects on CAT activity. At low concentrations NaF had no significant effects on CAT activity while at higher concentrations it increased the activity of catalase. These results suggest that NaF has hermetic effects on ROS generation and CAT activity. The same findings have been reported by Mukhopadhyay et al.[16] with NaF elevating the activity of antioxidant enzymes including CAT, superoxide dismutase, and glutathione reductase in the brain of zebrafish exposed in the laboratory to NaF. Moreover, it has been demonstrated that the effect of NaF of increasing CAT activity, in zebrafish liver [37] and in the brain cells of *Drosophila melanogaster*,[38] is through the enhancement of the expression of the genes of CAT and other antioxidant enzymes.

Although the precise mechanisms are not clear, one proposed mechanism that may describe the interaction of NaF and Al toxicity is the formation Al-F complexes. It has been revealed that Al^{3+} and F can form Al-F complexes in a pH and concentration dependent manner.[39] The formation of such complexes may affect the concentration of both Fe and Al^{3+} in the solution and thereby modulate their toxic effects. Alternatively, a close structural similarity has been demonstrated between an Al-F complex and the phosphate group in the transition state. Thus Al-F can bind to

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and modulate the activity of a variety of biomolecules including the heterotrimeric and small G proteins involved in signal transduction and energy producing pathways.[40] Therefore, in addition to their individual pathological effects, the combination of Al^{3+} and F may affect the functions of tissues through the formation of Al-F complexes.

CONCLUSIONS

The findings from this study provide evidence on hormetic effects of NaF against Almal-induced toxicity in the PC12 cell line in which at low concentrations NaF protects the cells against Al toxicity and at high concentrations increases Al toxicity. Further studies are needed to clarify the molecular mechanisms involved in the hermetic effect of NaF against Al toxicity.

CONFLICT OF INTEREST

None declared.

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