ULTRASTRUCTURAL LOCALIZATION OF 4-HYDROXYNONENAL ADDUCTS IN FLUORIDE-EXPOSED CELLS: PROTECTIVE ROLE OF DIETARY ANTIOXIDANTS

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ABSTRACT: Fluorosis is a major health concern for people inhabiting areas with high levels of the fluoride ion (F) in the drinking water. Although the exact mechanism of F-induced pathogenesis is not clear, the role of free radicals and lipid peroxidation has been well established by various studies. The highly toxic lipid peroxidation end-product, 4-hydroxynonenal (HNE), is a nonprotein mediator of apoptosis and has been found to be involved in the pathogenesis of many diseases. HNE binds with proteins and forms HNE-protein adducts. These adducts (products resulting from the direct addition of two or more distinct molecules) accumulate in the cellular compartments, especially the endoplasmic reticulum (ER), leading to the disruption of various physiological functions. F is known to cause lipid peroxidation and ER stress, and we therefore hypothesized that F-induced lipid peroxidation end products may bind with proteins and, in turn, induce abnormalities in cellular function. We attempted to localize the HNE modified proteins in the F-exposed cells using immunogold labelling for transmission electron microscopy. Dietary antioxidants are recommended as a part of the recovery management programs for fluorosis and we therefore co-treated the cells with dietary antioxidants, namely ascorbic acid (vitamin C) and α–tocopherol (vitamin E), to investigate the protective role of these antioxidants. Our study confirmed the abundant presence of HNE conjugated proteins in the F-treated cells. Further, we found that co-treatment with the combination of the dietary antioxidants vitamins C and E was effective in protecting the cells and reducing the F-induced burden of HNE modified proteins in the cells. Our study suggests that HNE is one of the mediators of F toxicity.

Keywords: 4-hydroxynonenal (HNE); Antioxidants; Fluoride-induced lipid peroxidation; Protein modification; Ultrastructural localization; Vitamin C; Vitamin E.

INTRODUCTION

Excessive fluoride ion (F) exposure for a long-time, either through fluoridated drinking water or industrial F pollution, causes mild to severe fluorosis in man and domestic animals.[1-4] Millions of people around the world are either affected or at risk of the dreaded condition of fluorosis. F has the potential to damage both the hard issues (teeth and bones) and soft tissues (organs).[5] These F-induced adverse changes have been well studied experimentally in various species of mammals by several workers.[6-8] However, the mechanism of F toxicity is not very clear. The role of oxidative stress has been emphasized by several studies with cell cultures as well as in animal models.[9] F-induced oxidative stress leads to apoptosis via oxidative damage of mitochondria and caspase-3 activation in human leukemia cells.[10,11] It has also been suggested that there are changes in the cell cycle and in the induction of endoplasmic reticulum (ER) stress in F-exposed cells.[12,13] Based on these studies, it was hypothesized that antioxidants could help to give protection from the toxic effects of F. Many studies have shown the effectiveness of various antioxidants such as selenium, curcumin, resveratrol, gallic acid, quercetin, melatonin, and vitamin C in F-exposed cells and animals.[14-18] Nutritional supplementation with antioxidants has been recommended in the management of fluorosis to help recovery from the adverse toxic effects of F.[5,19] Dietary antioxidants, such as vitamin C and E, have been shown to be effective in ameliorating toxicity in F- exposed rats.[20]

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Lipid peroxidation end products bind with proteins and cause abnormalities in protein function, transport, and degradation systems.[21] Malondialdehyde is the most abundant lipid peroxidation end product while 4-hydroxynonenal (HNE) is believed to be the most toxic one. HNE binds with a range of cellular proteins and induces apoptosis. HNE also acts as a toxic messenger which regulates cyclins and modulates cellular function.[21] We hypothesized that F-induced lipid oxidation end products bind with proteins, cause abnormal traffic in the cell, and eventually lead to ER stress. Here, we attempted to localize the HNE adducts in F-exposed cells to prove the role of HNE-dependent pathological consequences in F toxicity. We also investigated the preventive effects of the dietary antioxidant vitamins A and E on the HNE-dependent F toxicity.

MATERIALS AND METHODS

Cell culture and treatment of F and dietary antioxidants: The human brain malignant glioma (BMG)-1 cells were cultured at 37ºC with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 50 µg/mL penicillin and 50 µg/mL streptomycin. Cultures at ~80% confluence were split 1:5 in 10 cm culture dishes. Sodium fluoride (NaF) (dissolved in sterile distilled water) was added to the culture media to achieve final concentrations of 2 mM and 4 mM in the culture. The cells were observed at 12 hours and 24 hours of treatment. For a positive control, cells were treated with 100 µM etoposide. 4 mM of NaF was sufficient to induce marked morphological changes in the cultured cells and therefore this concentration was used for the further experiments. The cells were treated with a final concentration of 4 mM NaF and either 20 mM vitamin C (L-ascorbic acid), 20 mM vitamin E (α-tocopherol) (Sigma-Adrich, USA), or a combination of 10 mM vitamin C and 10 mM of vitamin E in the culture media. Ascorbic acid was dissolved in phosphate buffered saline (PBS) and α-tocopherol was dissolved in ethanol. Vehicle control groups received a corresponding volume of either ethanol, PBS, or a combination of ethanol and PBS. No gross difference was observed in the vehicle control groups as compared to the NaF treated group. Cells were harvested after 24 hours of treatment and used for lipid peroxidation assay and electron-microscopy. Light microscopic observations and images were taken using an inverted microscope (Nikon DIAPHOT 300).

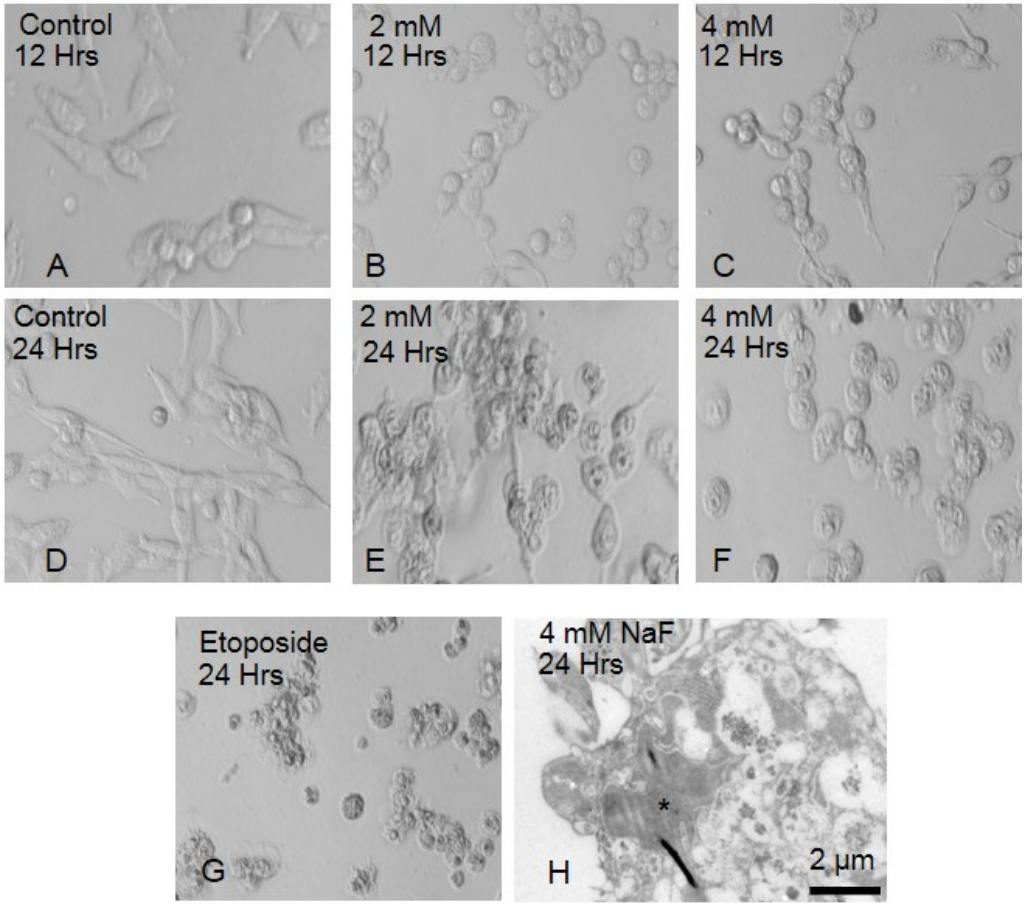
Lipid peroxidation assay: Cells were trypsinised with 0.5 mg/mL Trypsin; 0.2 mg/mL EDTA in PBS, collected, washed and lysed in radio immune precipitation assay (RIPA) buffer (Sigma-Aldrich, USA). Lipid peroxidation was measured by reaction of thiobarbiuric acid (TBA) with malondialdehyde (MDA).[22] Content of malondialdehyde was measured spectrophotometrically using a double beam spectrophotometer (Spectro UVS 2700, Labomed Inc, USA) at 532 nmwavelength. Level of thiobarbiuric acid reactive species (TBARS) was calculated from standard curve prepared from different concentrations of 1,1,3,3 tetraethoxypropane (Sigma-Aldrich, USA). Total protein contents were estimated by Bradford reagent using standards of bovine serum albumin (BSA) (Sigma-Aldrich, USA). The results were expressed as TBARS nanomoles/g (±SEM) of total protein.

Immunogold-electronmicroscopy: The treated cells and the control cells were fixed with 2.5% glutaraldehyle solution in 0.1M phosphate buffer (pH 7.4) and processed for immunogold labeling as described in 2000 by Dasgupta et al.[23] Briefly, fixed cells were scraped from the culture plates, pelleted, washed with 0.1 M sodium phosphate

buffer (pH 7.2), and dehydrated with serial ascending ethanol. Blocks were prepared in LR white resin (TAAB laboratories, UK). Ultrathin sections were cut by using UCT ultramicrotome (Leica, Austria). Structural changes in cellular structure were observed under 80 kV transmission electron microscope (CM10, Philips, Germany). Polyclonal rabbit anti-HNE-Michael adduct antibody (Calbiochem, CA, USA) (1:1000 dilution) and goat anti-rabbit secondary antibody conjugated with 10 nm nanogold particles (Electron Microscopy Sciences, USA) (1:100 dilution) were used to localize the proteins modified by HNE. For quantification of immunogold labeling, five cells from three samples of each group were randomly selected and total number of gold particles was manually counted. The results were expressed as number of gold particles per cell (±SEM).

RESULTS

Cell morphology: The morphology of the F-exposed cells changed with time (Figures 1A–1F).

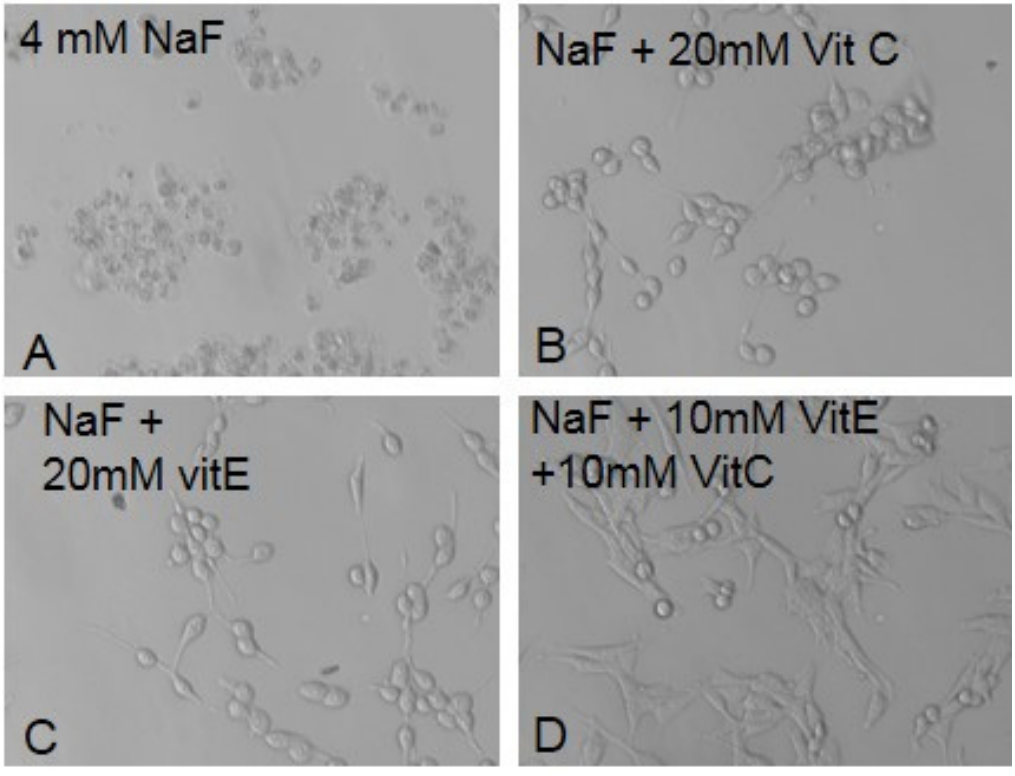


Figures 1A–1H. Effect of 2 mM and 4 mM of NaF on BMG-1cells at 12 hours and 24 hours after treatment. A-G: Representative images showing morphological changes in 2 mM and 4 mM treatment groups in comparison with control at 12 hours (A-C) and 24 hours (D-F). G: 100 µM etoposide treatment for 24 hours as a positive control. Images in 1A–1G taken at 200×. H: representative electron micrograph showing nuclear fragmentation (asterisk marked) in a cell treated with 4 mM sodium fluoride (scale bar in 1H=2 µm).

Many cells left the plate surface and came into the medium after 24 hours of 4 mM NaF treatment (Figure 1F). The morphology was very similar to the morphology of

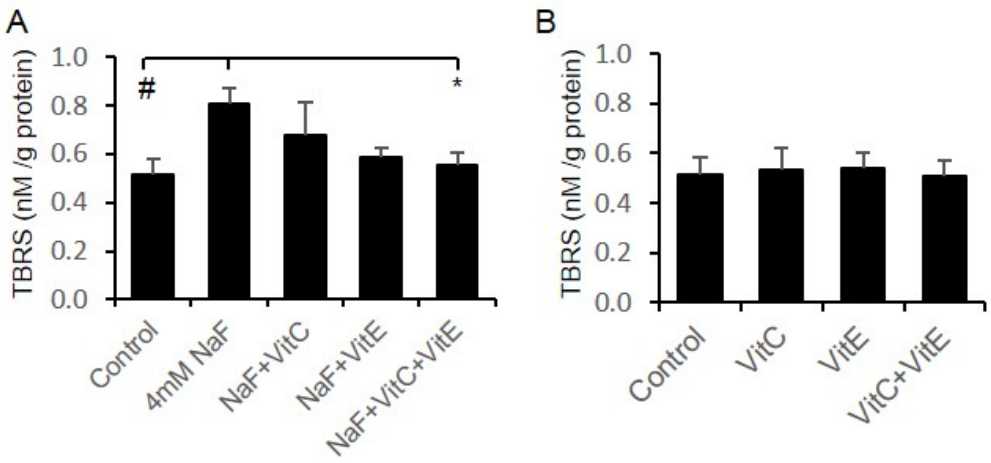
the positive control cells which was induced by treatment of 100 μM of etoposide (Figure 1G). Electron microscopic observation of 4 mM NaF-treated cells showed characteristic nuclear fragmentation in many cells confirming apoptotic cell death (Figure 1H). 2 mM concentration was also able to cause defects in morphology but most of the cells remained adhered with the surface (Figure 1B and 1E).

We used 4 mM concentration for further studies to investigate the protective role of dietary antioxidants. We found that treatment with either 20 mM of vitamin C (Figure 2B) or 20mM of vitamin E (Figure 2C) protected cells from NaF induced toxicity (Figure 2A). Furthermore, the combination of 10 mM vitamin C and 10 mM vitamin E (Figure 2D) was found to be most effective for protecting the cellular morphology from NaF-induced toxicity.



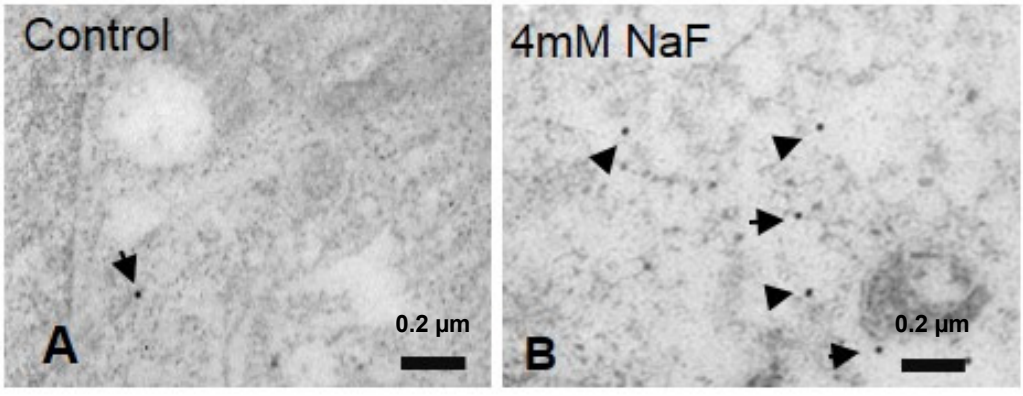
Figures 2A–2D. Effect of dietary antioxidants on NaF treated BMG-1cells. Cells grown for 24 hours in culture media containing either 4 mM NaF alone (A) or 4 mM NaF supplemented with 20 mM vitamin-C (B), 20 mM vitamin-E (C), or combination of 10 mM vitamin-C and 10 mM vitamin-E (D). Images in 2A–2D taken at 100×.

Lipid peroxidation (TBARS measurement): We found that 4 mM NaF treatment to the cells for 24 hours induced an increase, by about 1.5 times, of the TBARS level in comparison with that of the control cells ($p=0.023$) (Figure 3A). Co-treatment with the dietary antioxidants, 20 mM Vitamin E and 20 mM Vitamin C, alone gave some protection to the cells against lipid peroxidation but not however at a statistically significant level (vitamin C, $p=0.29$ and vitamin E, $p=0.08$). Treatment with both the vitamins in combination, at a concentration for each of 10 mM, significantly protected the cells against lipid peroxidation and the TBARS level came near the TBARS level of the cells of control group ($p=0.048$). No significant difference was observed in TBARS levels of cells treated with antioxidants alone (Figure 3B).



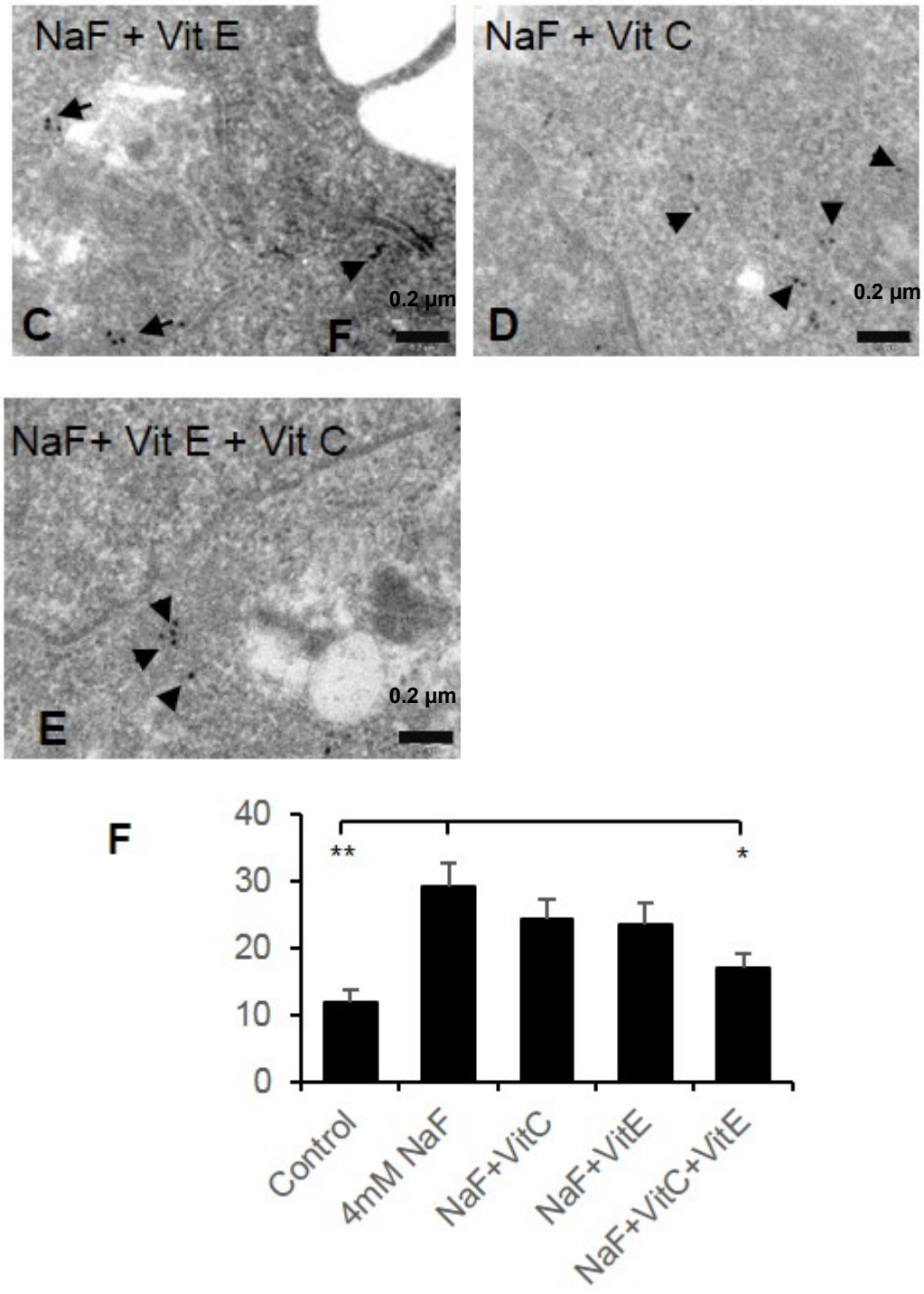
Figures 3A and 3B. Graphical representation of TBARS levels after NaF treatment with either vitamin C, vitamin E, or a combination of both (3A). #Significant increase in lipid peroxidation in the 4 mM NaF group when compared with vehicle control group ($p<0.05$). *Significant reduction in lipid peroxidation in the groups co-treated with combination of vitamin C and E ($p<0.05$) in comparison to the group treated with 4 mM NaF. Note that the treatment of antioxidant without fluoride does not have significant effect on lipid peroxidation (3B)

Immunoelectron localization of HNE-adducts: Antibody against HNE-BSA adduct was used to analyze the localization of lipid peroxidation products bound with cellular proteins (Figures 4A–4E).



Figures 4A and 4B. Representative electron micrographs showing immunogold-labeling of HNE modified proteins in BMG-1 cells (A-B). Control cells grown in normal culture medium without any treatment (A) and cells grown in medium containing 4 mM NaF (B). Scale bar in 2A and 2B=0.2 µm.

We found the number of gold-immuno labels was much higher in the 4 mM treated cells (Figure 4B) in comparison with control cells (Figure 4A) ($p=0.001$). Immuno gold particles were distributed unevenly in the all cellular compartments. In the antioxidant treated groups, nanoparticles were more localized in endoplasmic reticulum (Figures 4C–4E). The total number of nanoparticles in the antioxidant treated groups were less than the number of particles in the 4 mM NaF group but the difference was not statistically significant. Co-treatment with vitamin C and E significantly lowered immune labeling of HNE-adducts ($p=0.046$) (Figure 4F).



Figures 4C–4F. Representative electron micrographs showing immunogold-labeling of HNE modified proteins in BMG-1 cells (C-E). Cells treated with 4 mM NaF and Vitamin C (C), Vitamin E (D), or a combination of 10 mM Vitamin C and 10 mM E (E). Scale bar in 2C–2E=0.2 µm. F: Graphical representation of number of HNE labeled immunogold particles in ultrathin sections of cells with treatment with NaF and combination of antioxidant. **p<0.01; *p<0.05.

DISCUSSION

F induces oxidative burden and lipid peroxidation in experimental animals and cell cultures.[10,12-20] HNE is considered to be the most toxic lipid peroxidation end product and is highly reactive towards proteins producing thioether adducts that further undergo cyclization and act as a non-protein mediator for apoptosis.[24] HNE adducts have been found colocalized with active caspase-3 in apoptotic cells suggesting its direct role in the apoptosis mechanism.[25]HNE has a strong hydrophobic nature and can diffuse to other cellular compartments. It is known to bind to a variety of cellular proteins including cytoskeletal proteins, various enzymes, ion channels, and well receptors.[26] It also makes adducts with all four bases of DNA and is known to occur in various diseases such as Alzheimer's disease and cancer. [27-29] We found that immune labeling of HNE-adduct was distributed in most of the compartments of cells and possibly HNE-conjugated proteins are localized in most of the organelles in F-treated cells.

There are controversial reports about oxidative stress in fluorosis patients. Several researchers have observed imbalanced oxidative status in the children with prolonged F exposure.[30,31] Similarly, a 50% decrease was reported in cytoplasmic superoxide dismutase in pancreas of F-administered rats.[32] On the other hand, other workers were unable to find any significant alterations in the antioxidant system in fluorosis patients and F-administered rabbits.[33] Calcium, vitamin C, and vitamin D supplements were found to be effective in reducing plasma superoxide dismutase levels.[30] It has been suggested that dietary antioxidant levels should be considered for analyzing and reporting on the oxidative status in patients.[30] In cell culture models, it is possible to manage and monitor the levels of the F and antioxidants and therefore we performed a cell culture study to analyze F toxicity and the role of dietary antioxidants on the oxidative damage in the cells. We used BMG-1, a human brain malignant glioma cell line, which has been used previously for cytotoxicity and oxidative stress related studies.[34,35] We found that the combination of vitamin E and vitamin C was more effective in protecting the cells from F-induced lipid peroxidation. The supplement of antioxidants is considered be protective from oxidative damage in many disease conditions. The combination of vitamin E, folate, and acetyl-L-carnitine synergistically protects neuroblastoma cells against oxidative stress resulting from exposure to amyloid-beta.[36] Vitamin E enhances the levels of glutathione and reduces oxidative stress induced by immobilization in rats.[37] The nutritional supplementation of antioxidants and calcium is suggested as an approach in the management of fluorosis and in the recovery from the adverse health effects of F.[5,19] Our study also supports the recommendation for using antioxidants for the prevention of fluorosis.

Protein conjugates of lipid peroxidation end-products cause misfolding in proteins and disrupt the proteasomal scavenging system of the cell. These conjugated proteins accumulate in the ER and induce ER stress.[38]The ER stress further induces oxidative stress and dysregulation in cellular metabolism.[38] F is known to inhibit protein transportation and to induce ER stress in cultured cells.[39,40] In our study, most of the immune labeling in the antioxidant treated groups was localized near ER. This suggests that most of the HNE conjugated proteins accumulate in the ER and there is a substantial reduction in the diffused HNE-conjugated proteins after antioxidant treatment.These cells may experience ER stress and abnormalities in cellular

function, even after a significant reduction of lipid peroxidation and protein modification. In humans and animals, a subtoxic concentration of F causes abnormalities at the cellular level in different tissues after prolonged exposure.[8,41,42] We hypothesize that chronic exposure to mild levels of F may cause abnormal cellular function due to modified proteins and, in turn, these disrupt the physiological function of organ systems.

CONCLUSION

The study confirms the abundant presence of HNE conjugated proteins in F-treated cells and the effectiveness of the combination of vitamin C and E in reducing the burden of HNE modified proteins. The study suggests that protein modifications by HNE may play important role in F-induced toxicity and abnormal cellular function. Further investigations are required to explain the HNE-mediated subtoxic effects of F in presence of antioxidants on various cells and tissues.

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