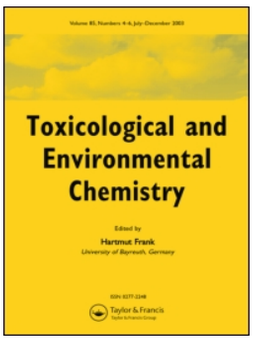

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Fluoride induces DNA damage and cytotoxicity in human hepatocellular carcinoma cells

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ABSTRACT

Humans are primarily exposed to fluoride (Fl), a widespread environmental pollutant, via contaminated drinking water and foodstuffs. The aim of this study was to examine whether sodium fluoride (NaF) exerted cytotoxic effects in human hepatocarcinoma (HepG2) cells. HepG2 cells were incubated with different concentrations of NaF and reactive oxygen species (ROS) levels, cell cycle, apoptosis, and DNA damage determined. Concentration-dependent studies showed that exposure to HepG2 cells with different concentrations of NaF for 24 hr significantly decreased cell viability and intracellular antioxidant capacity. Furthermore, NaF exposure increased lipid peroxidation levels and accumulation of intracellular ROS; and lowered antioxidant glutathione concentrations. In addition to oxidative impairments, NaF treatment enhanced HepG2 cell death via apoptotic pathway as evidenced by DNA fragmentation and cell cycle arrest. Sodium fluoride treatment unregulated p53 level, and Bax and Bcl2 expression. Diminished cell viability and changes in cell cycle accompanied a rise in p53 expression.

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KEYWORDS
HepG2 cells; DNA damage; apoptosis; cell cycle arrest; NaF

Introduction

Fluoride (Fl) occurs naturally in ground water and earth crust and presence of high concentrations is health problems in livestock (Jha et al. 2013). Sodium fluoride (NaF) is used in different types of industrial application and abundant component of dental products, food materials, and water (WHO 2002). High consumption of Fl induces fluorosis, a moderate deteriorating disorder, which affect skeletal muscle, teeth, bone, spinal cord, and brain (Shashi et al. 1994; Kaul and Susheela 1974; Franke 1976; Jha et al. 2013). In addition, accumulation of Fl in hippocampus of brain induced neuronal degeneration and changed metabolism of free radicals in heart, kidney, and liver (Mullenix et al. 1995; Patel and Chinoy 1998). Cicek et al. (2005) reported that chronic Fl treatment produced myocardial damage in rabbits and rats. Accumulation of Fl induced oxidative stress as

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evidenced by significant reduction in superoxide dismutase and glutathione-S-transferase. Fluoride induces apoptosis associated with enhanced oxidative stress-mediated lipid peroxide pathways (Karube et al. 2009). Similarly, incubation of human peripheral blood cells with NaF induced oxidative stress leading to apoptotic cell death and affected cell cycle progression (Jothiramajayam et al. 2014). Since cell cycle progression is involved in carcinogenesis, it was of interest to compare the influence of NaF on cellular processes in a hepatic carcinoma cell line HepG2 cells.

Materials and methods

Chemicals and culture wares

Fetal bovine serum (FBS), antibiotic and antimycotic solution, trypsin, Dulbecco's modified eagle's (DMEM F-12) medium, L-histidine, neutral red (NR), carbonate and phosphate buffers, 3-(4,5- dimethylthiozolyl-2)-2,5-diphenyl tetrazolium bromide (MTT), 2-7-dichlorodihydrofluorescein diacetate (DCFH-DA), NaF(NaF) were purchased from Sigma Chemical Company (St. Louis, MO).

HepG2 cell

HepG2 cells was cultured in DMEM with 10% FBS, antibiotic /antimycotic solution (1.5%) at 95% relative humidity and 5% CO_2 at 37 °C.

MTT assay

In brief, cells (2×10^3 pre-well) were seeded in 96-well plates and maintained in CO_2 incubator for 24 hr at 37 °C prior to experiment for cell adherence. The medium was changed by medium having NaF (0, 10, 20, 40, 80, 120 μg/ml) for 24 hr. Culture medium was replaced with medium containing MTT (5 μg/ml) in 100 μl. Later 4 hr incubation, media was removed and dimethyl sulfoxide (100 μl/well) was added. Absorbance was recorded at 530 nm by using multi-well microplate reader.

Neutral red uptake (NRU) assay

NRU test was performed according (Ali et al. 2011).

Intracellular reactive oxygen species (ROS) production

ROS generation due to NaF (0, 10, 40, 80, 100, 120 mg/ml) exposure in HepG2 cells was quantified by Ali et al. (2015) method.

Lipid peroxidation

A total of 1×10^5 cells were seeded in 100 mm petri dishes, and treated with of NaF (0, 10, 40, 80, 100, 120 μg/ml) for 24 hr, then cells was scraped into 1 ml trichloroacetic acid (TCA) (2.5%) and homogenized. After centrifugation, supernatant was added to 0.5% (w/

v) TCA, and incubated in water bath at 95 °C for 40 min, cooled in ice bath, and centrifuged. Absorbance of supernatant was recorded at emission (532 nm) and excitation (600 nm). Concentration of malondialdehyde (MDA) is quantified using extinction coefficient $\varepsilon M = 155\ \mathrm{mM}^{-1}\ \mathrm{cm}^{-1}$ (Health and Packer 1968).

GSH levels

Glutathione (GSH) levels were determined by Elman's reagent (1959) method.

Single cell gel electrophoresis assay

Genotoxicity of NaF (0, 10, 40, 100, 120 μg/ml) was assessed by comet assay following method described by Singh et al. (1988). Cells were grown in 6 well plates and treated with different concentration of NaF. After 24 hr incubation with DMEM, cells were sandwiched between two layers of agarose. After solidification of the agarose gel, slides were immersed in lysis solution overnight at 4 °C. Electrophoretic chamber was filled with chilled alkaline electrophoresis buffer for DNA unwinding at 4 °C, 20 min. Alkaline electrophoresis was carried at 4 °C for 20 min, using 15 V (0.8 V/cm) and 300 mA. The slides were then stained with ethidium bromide. Cells were scored utilizing a fluorescent microscope (Komet 5.0).

Cell cycle analysis

Cells were treated cells with NaF (0, 100, 120 μg/ml) and incubated for 12 hr. After twice washing with PBS (phosphate buffer saline), culture medium was replaced. After 12 hr, cells were trypsinized and fixed in C_2H_5OH (70%), rinsed with cold PBS and exposed to RNase (100 μg/ml) at 37 °C for 40 min. Propidium iodide (5 μg/ml) was used to stain cells and placed in dark for 1 hr before analysis. Content of DNA in cells was measured by flow cytometry (FACS Caliber BD).

Quantitative real-time PCR analysis

Cells were incubated for 24 hr with NaF (0, 100, 120 μg/ml) and then RNA was isolated by TRIZOL reagent and quantified by a spectrophotometer (ND-1000 Thermo Scientific) at 260 nm. cDNA preparation was carried out using cDNA reverse transcription kit (Applied Biosystems). cDNA was quantified by a spectrophotometer (ND-1000 Thermo Scientific). For real-time PCR, SYBR green jumped start Taq ready mix (Sigma) was used. The sequences (Integrated DNA Technologies, USA) of forward and reverse primers were p53 (5'AGTCTAGAGCCACCGTCCAG-3'), (5'-AGTGACCCGGAAGGCAGT-3'); Bcl2 (5'-GCACCTGCACACCTGGAT-3'), (5'-AGGGCCAAACTGAGCAGA-3'); Bax (5'-AGCAAACTGGTGCTCAAGG-3', 5'-TCTTGGATCCAGCCCAAC-3'). Real-time PCR was carried out for 40 cycles on an Applied Biosystems thermal cycler.

Western blotting

A total amount of 60 mg protein was resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membrane. Membranes were blocked in blocking buffer (1× Sigma), incubated with human monoclonal antibodies (primary) at the dilution factor of 1:1500 to β-actin, Bax Bcl2, and p53 (Abcam) which were subsequently incubated for 4 hr at room temperature, membrane washed with Tris-buffered saline (TBST) (10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween-20) three times at 5 min intervals, incubated with anti-rabbit IgG peroxidase antibody (secondary) at 1:50,000 dilutions for 2 hr at room temperature, washed in TBST four times at 10 min intervals. Protein bands were visualized after addition of immobilon western chemiluminescent (Millipore) HRP substrate on membrane. The densitometry was done with the help of image J software.

Statistical analysis

Results are expressed as mean standard error (±SE) of means (SE) for separate groups. Group means SE were compared by one-way analysis of variance followed by Tukey's *post hoc* test. A value of $p < 0.05, 0.01$ was considered statistically significant.

Results

Cell morphology

Figure 1 illustrates the comparative morphology of control and NaF-treated cells. Morphological changes in cells became visible after 24 hr as evidenced by globular shape and separated from surface.

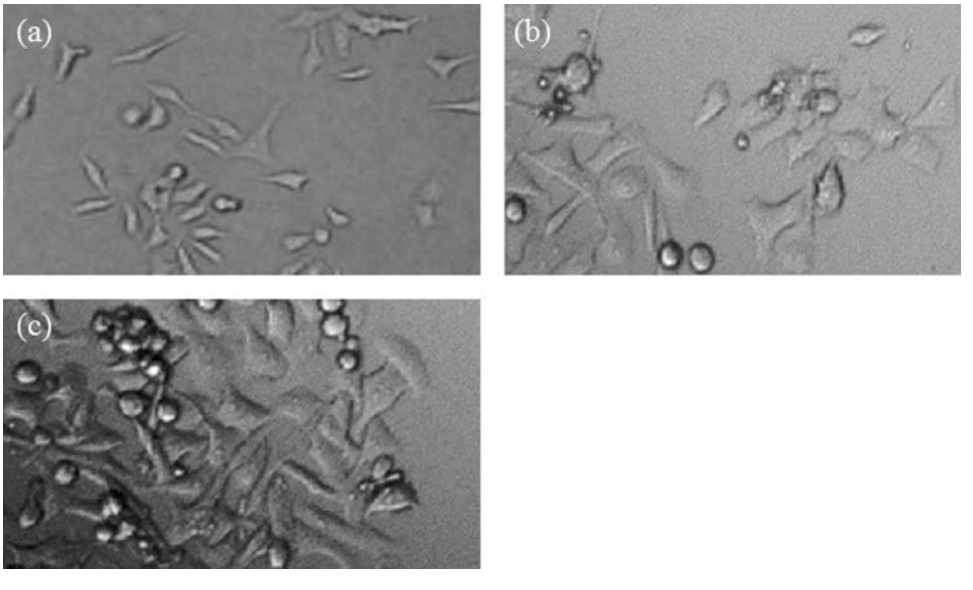

Figure 1. Morphology of HepG2 cells. (**a**) Control, (**b**) exposed with 100, and (**c**) 120 μg/ml NaF magnification 200×.

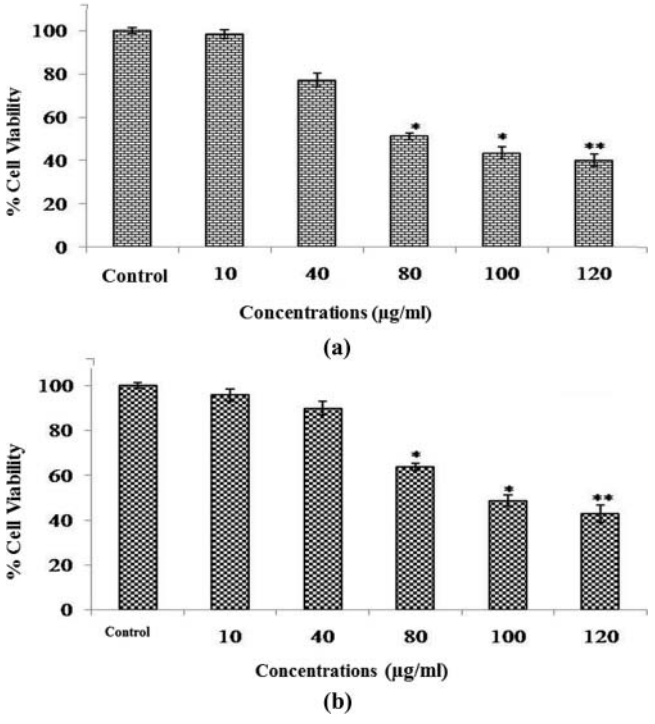

Figure 2. Percent cell viability at various concentrations of NaF (**a**) MTT assay and (**b**) NRU assay. Each value represents the mean ± SE of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ versus control.

Cell viability assay

After NaF administration, reduced cell viability was observed utilizing MTT uptake assay. Growth inhibition of HepG2 cells was concentration dependent and reduction of cell viability up to 60% versus control occurred with NaF (120 µg/ml, Figure 2(a)). Similar significant results were obtained when using NRU, 57% viability versus control with NaF (120 µg/ml, Figure 2(b)).

Intracellular ROS generation

Data in Figure 3 demonstrate the ROS levels in the NaF-treated groups were enhanced significantly as NaF concentration rose indicating a concentration-dependent response (Figure 3).

Lipid peroxidation (LPO) and glutathione

LPO was measured as MDA levels. Treatment with NaF markedly elevated (concentration dependent) levels of MDA (LPO) compared with control (Figure 4(a)) accompanied by decrease in GSH levels (Figure 4(b)).

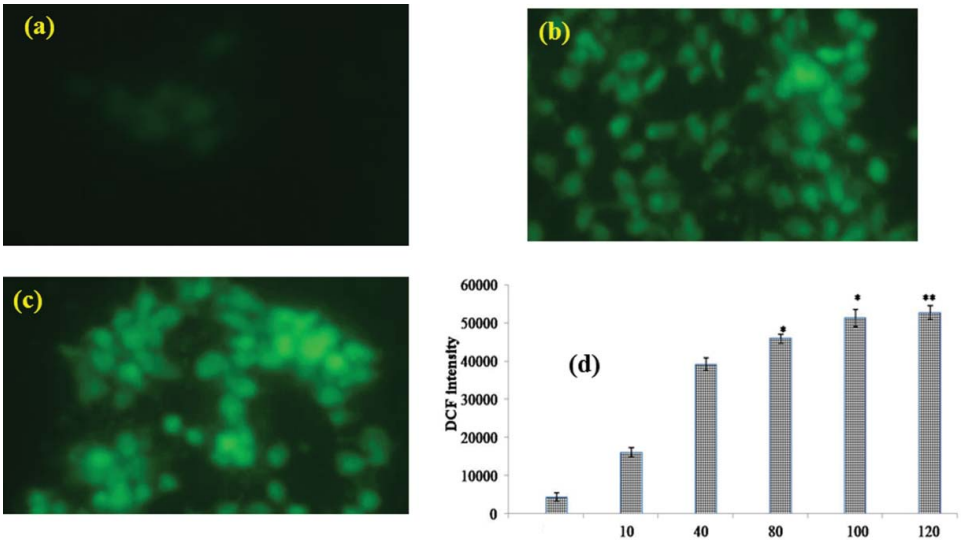

Figure 3. DCF-fluorescence intensity after exposure for 24 hr to different concentration of NaF. (**a**) Control, (**b**) at 100 μg/ml, (**c**) at 120 μg/ml, and (**d**) percent ROS generation. Each value represents the mean ± SE of three experiments, $^{*}p < 0.05$, $^{**}p < 0.01$ versus control.

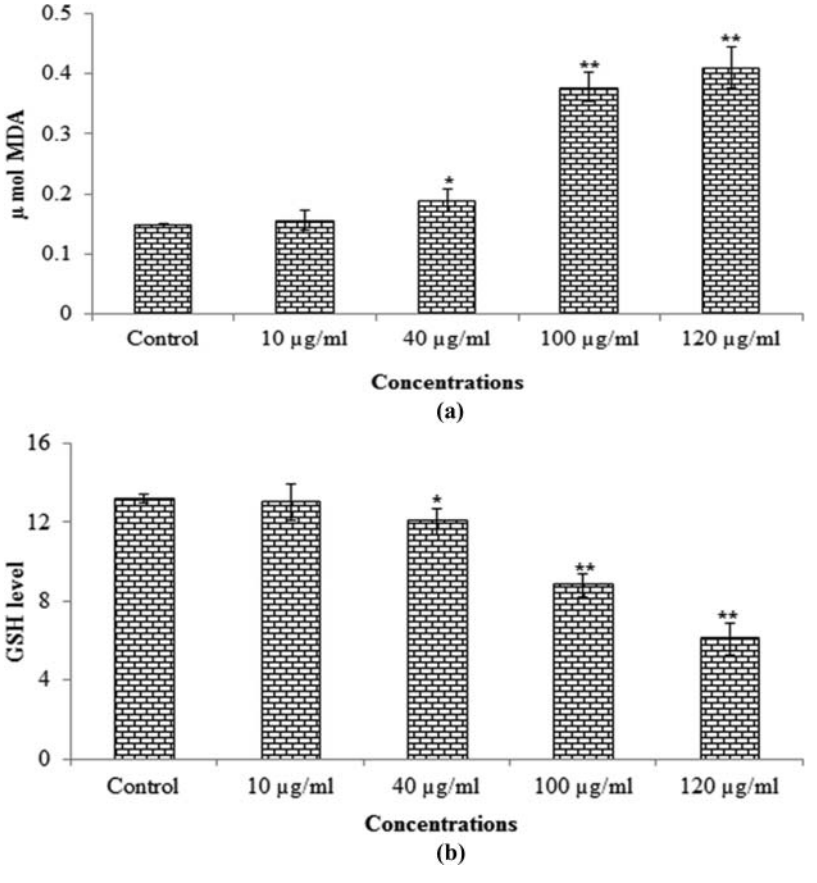

Figure 4. Effect of NaF on the levels of (**a**) LPO (MDA) and (**b**) GSH in HepG2 cells. Values are given as means ± SE of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$.

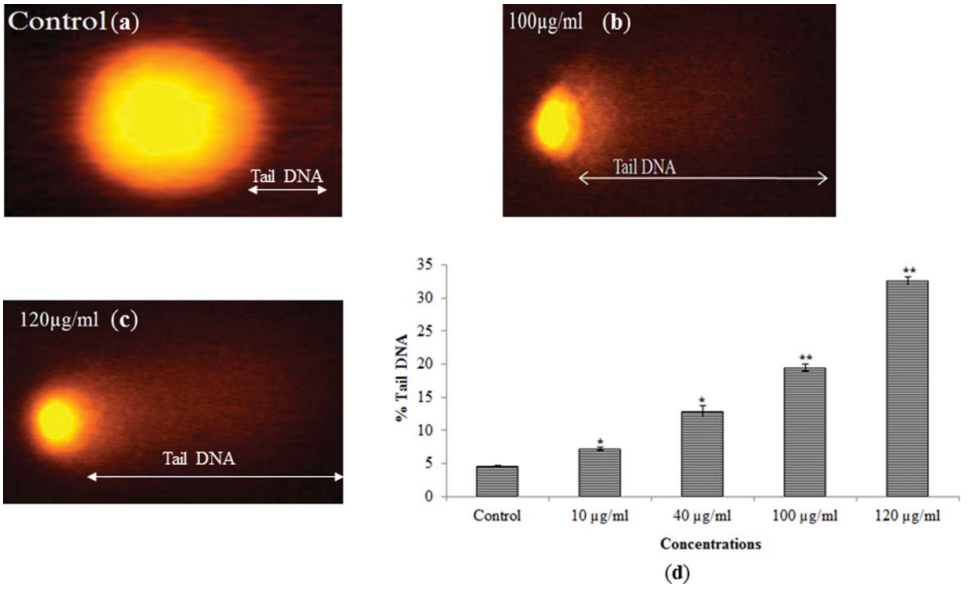

Figure 5. (**a**)–(**c**) Microphotograph of tail DNA formation and (**d**) percent tail DNA induced by NaF.

DNA damage

To assess the genotoxicity effect of NaF in HepG2 cells, the comet assay was employed. Percent tail DNA noted was 12.83%, 19.74%, and 32.61% at 40, 100, and 120 µg/ml NaF, respectively, relative to control. Microphotograph of tail DNA formation is presented in Figure 5.

Cell cycle detection

Cell cycle phase distribution was performed, using flow cytometry, to determine if NaF also affected the cell cycle kinetics. A significant progressive increase in number of cells in sub-G1 phase was apparent over time. This effect was accompanied by a progressive fall in percent cells in the G0/G1 phase. The expression of p53 reflects inhibition of proliferation by a cell cycle arrest (sub-G1). NaF exposure, at 120 µg/ml, induced a significant increase p53 expression (Figure 6).

RT-PCR analysis of Bax /Bcl-2 and p53 mRNA and protein expression in HepG2 cells

The expressions of p53, anti-apoptotic gene Bcl-2 versus pro-apoptotic gene Bax were analyzed by quantitative RT-PCR (Figure 7). Data showed significant down regulation of Bcl-2 with treatment versus control. Alteration in Expression of Bax was non significant but a reduction was found in the ratio of Bax/Bcl-2 expression. Furthermore, up regulation of p53 gene was observed following treatment with NaF. Qualitative measurement (expression of related protein) through western blot images of the corresponding proteins of analyzed genes displayed a similar pattern (Figure 8).

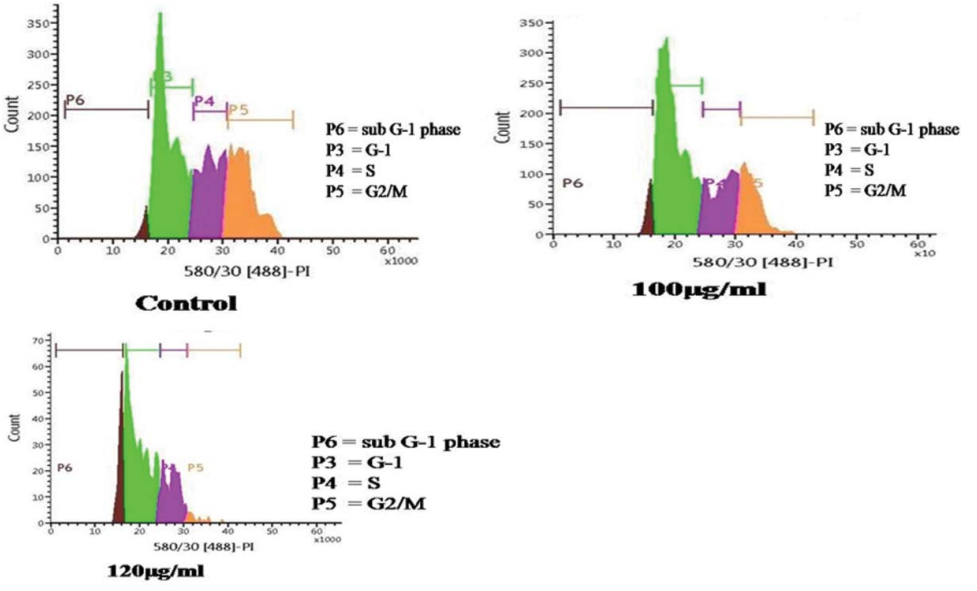

Figure 6. Effect of NaF on cell cycle in HepG2 cells by flow cytometry.

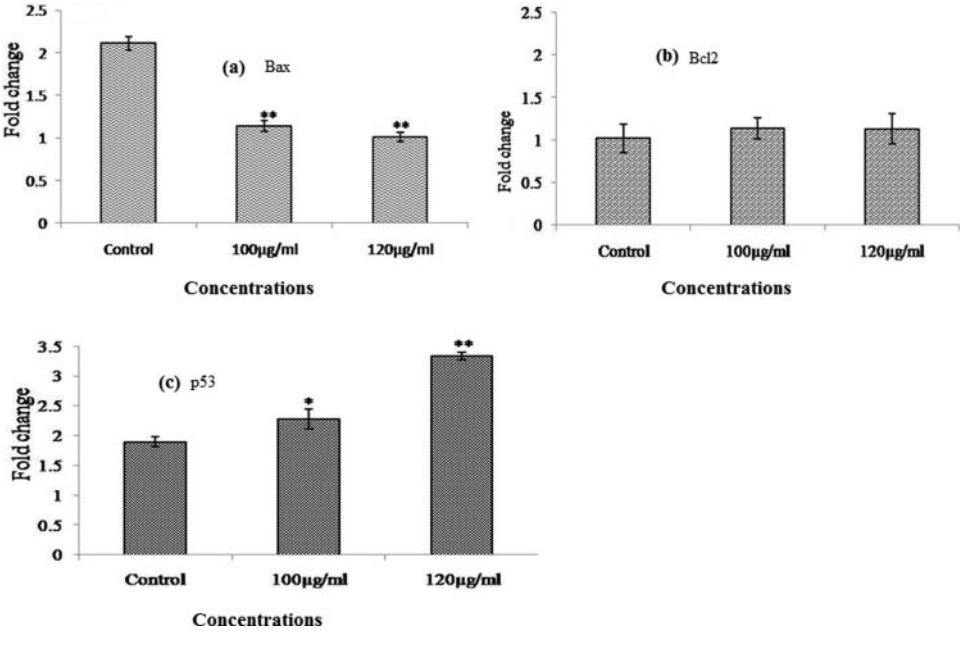

Figure 7. Effect of NaF on mRNA expression of apoptotic genes like (**a**) Bax, (**b**) Bcl2, and (**c**) p53 in HepG2 cells through real-time PCR. Genes were normalized to β-actin mRNA level and results are expressed as fold change from control.

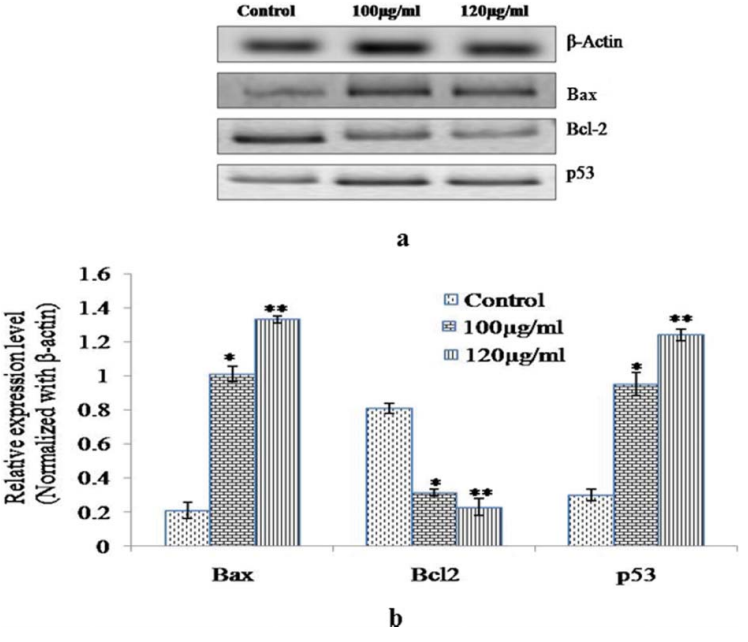

Figure 8. Effect of NaF on protein expression through (**a**) western blot and (**b**) relative densitometry analysis of Bax, Bcl2, and p53. $^{*}p < 0.05$ or $^{**}p < 0.01$.

Discussion

Exposure to excessive Fl disturbs the processes of individual growth and development at cell, tissue, and organism levels. The effects of Fl on cellular metabolism and physiology vary according to concentration, cell type, and time of exposure (Barbier et al. 2010). Although in teeth and bone tissues, μM concentrations of Fl elicit potentially positive effects by promoting cell proliferation and growth, whereas Mm doses suppress cell proliferation and induce apoptosis in cells of different organs and tissues, including liver (Wang et al. 2004; Zhan et al. 2006), brain (Zhang et al. 2008), pancreas (Elliott et al. 2001), kidneys (Bai et al. 2010), erythrocytes (Chouhan and Flora 2008), lymphocytes (Jothiramajayam et al. 2014) as well as carcinoma cells (Song et al. 2002). Toxicity of Fl to HepG2 cell was evidenced by reduction in cell viability in a concentration-dependent manner. In this study, HepG2 cells incubated with NaF 10−120 μg/ml for 24 hr showed decreased MTT reduction.

Sodium fluoride may inhibit metabolism of O_2, producing imbalance between oxidative stress and anti-oxidative defense mechanisms, resulting in increased production of cellular ROS (Nabavi et al. 2012). Higher ROS levels were accompanied by elevated MDA levels (Ilhan et al. 2006) and activation of Bcl-2 and Bax in Fl-induced apoptosis (Di Dong et al. 2014). Similar results were found by Wang et al. (2011) in primary mouse osteoblasts and Nguyen Ngoc et al. (2012) in mouse embryonic stem cells, which correlated to ROS-mediated signaling pathway. Jacinto-Aleman et al. (2010) reported that cell apoptosis of ameloblasts and odontoblasts induced by Fl was associated with oxidative stress. Data in our study showed that NaF exposure enhanced levels of oxidative stress via both increasing ROS level and MDA content in HepG2 cells. Furthermore, our results

demonstrated that DNA damage was induced in HepG2 cells by NaF at the concentration dependent manner of 10−120 μg/ml for 24 hr. Evidence indicated that Fl generated ROS, enhanced lipid peroxidation, DNA damage, and apoptosis. Lipid peroxidation and apoptosis may co-exist in the beginning when tissues are exposed to excess Fl and generates ROS that may be sufficient to induce apoptosis. In this study, the results oxidative stress, DNA damage, apoptosis and modifications of membrane lipids were induced in HepG2 in by excess Fl. In conclusion, excess Fl produced oxidative stress, DNA damage, apoptosis, and alteration of cell cycle in HepG2 cells.

By activating the specific transcription dependent and transcription-independent pathways in nuclei and mitochondria, p53 is able to mediate expression of various apoptosis-related proteins such as Bcl-2, Fas, and Bax, regulate the mitochondria redox balance and integrity, and contribute to regulation of oxidative phosphorylation, fatty acid oxidation, and antioxidant response. Alterations in these proteins activities might trigger apoptotic cascade involving p53, what leads to chromosomal instability DNA damage-inducible protein and cell cycle checkpoint arresting cells at G2/M phase in response to DNA damage (Zhang et al. 2008). This finding indicated that number of HepG2 in G2/M phase was significantly lower in Fl group, although there were no marked changes in cell number in G0/G1 and S phase. Cell proliferation was also decreased with increasing Fl concentration group. Anti-apoptotic members of the Bcl-2 family (among which Bcl-2 is of particular significance), inhibit the release of these apoptogenic factors, whereas pro-apoptotic members such as Bax, promote its release (Tsujimoto 2002). Both Bax and Bcl-2 play critical roles in caspase activation and regulation of apoptosis (Zhonga et al. 2011). In this study, in HepG2 cells in response to F transcription (as demonstrated by RT-PCR) and translation (as demonstrated by western blot analysis) of pro-apoptotic Bax gene was significantly enhanced while the transcription and translation of anti-apoptotic Bcl-2 gene were significantly reduced.

Disclosure statement

No potential conflict of interest was reported by the authors.

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