Sodium Fluoride Induces Apoptosis in H9c2 Cardiomyocytes by Altering Mitochondrial Membrane Potential and Intracellular ROS Level

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Abstract Chronic excessive fluoride intake is known to be toxic, and effects of long-term fluorosis on different organ systems have been examined. However, there are few studies about the effects of fluorosis on cardiovascular systems. Here, we studied the fluoride-induced apoptosis in H9c2 cells and determined the underlying molecular mechanisms including the cell viability, intracellular reactive oxygen species (ROS) level, the changes of mitochondrial membrane potential ($\Delta\Psi$m), and the cell apoptosis. Sodium fluoride (NaF) at concentrations of 0, 2, 4, 8, and 16 mg/L was administered to cultured H9c2 cells for up to 48 h. After the treatment, H9c2 cells were collected and the associated parameters were measured by flow cytometry. Our study found that fluoride not only inhibited H9c2 cell proliferation but also induced cell apoptosis. With the increment of NaF concentration, the apoptotic rates and ROS generation were increased, while the $\Delta\Psi$m was decreased. In summary, these data suggested that NaF-induced H9c2 cell apoptosis is mediated by direct increased intracellular ROS and downregulated $\Delta\Psi$m.

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Introduction

The natural background fluoride levels of surface water is continuously increasing due to rapid industrialization, use of fluoride-containing pesticides, and discharges of fluoridated municipal water [1]. Fluoride is reported to prevent caries [2]. But long-term excessive intake of fluoride can cause chronic fluorosis [3], characterized by tooth discoloration and skeletal manifestations such as crippling, osteoporosis, and osteosclerosis. The latest report showed that around 200 million people from 25 countries around the world are affected by fluoride toxicity [4], and China is one of the most populous countries in the world that threatened by the dreadful fate of fluorosis [5].

It is well known that fluoride can cause the different effects on different organ systems, and the cardiovascular system is susceptible to be disrupted by a high concentration of fluoride. Fluorine is an active nonmetallic element, which easily interacts with calcium and magnesium in the blood, thus causing blood calcium metabolism disorder, leading to the functional and organic damage to the cardiovascular and other systems [6]. Studies showed that fluoride has severe detrimental effects on cardiac function in atherosclerosis-related myocardial injury and pathophysiological remodeling [7–11]. However, very few studies have ever addressed the mechanism of action of fluorosis in cardiovascular system. Our investigations have previously demonstrated that fluoride is a cytotoxic agent inducing damage in myocardial tissues by oxidative stress through the Bcl-2/Bax signaling pathway [12]. Fluoride-induced oxidative stress plays an important role in the progression of a variety of cardiac disorders such as cardiac failure and ischemia [13]. Knowing that reactive oxygen species (ROS) plays a crucial role in fluoride-induced cardiotoxicity and oxidative stress, studies have been carried out on the cardioprotective action

of antioxidants against fluoride-induced toxicity in cardiac tissues [13].

As "power plants" of the cell, mitochondria provide with more than 90 % energy of the body. Not only does it generate energy, but also it moderates cell aging and death. Mitochondrial dysfunction has been shown to participate in the induction of apoptosis and has even been suggested to be central to the apoptotic pathway [14]. In some apoptotic systems, loss of mitochondrial membrane potential ($\Delta\psi$m) may be an early event in the apoptotic process. However, emerging data suggest that, depending on the model of apoptosis, the loss of $\Delta\psi$m may not be an early requirement for apoptosis, but on the contrary, may be a consequence of the apoptotic-signaling pathway [14]. Mitochondrial permeability transition (MPT), which involves a sudden increase of the mitochondrial membrane permeability, is a central coordination event of apoptosis [15–17]. It has been postulated that MPT could be caused by exogenous and endogenous ROS [15]. Some studies suggest that mitochondria are the main source of ROS, and that MPT contributes the O_2^- generation and subsequent H_2O_2 generation [18–20].

The toxicity of fluoride on cardiac system can be evaluated directly on in vitro cultured cardiomyocytes. The H9c2 cell line derived from embryonic rat hearts maintains the features of cardiomyocytes and has been used as an in vitro model to study the effects of NaF on the heart [21]. Therefore, we used H9c2 cells as an in vitro model in the present study to determine the underlying molecular mechanisms including the cell viability, intracellular ROS level, mitochondrial membrane potential, and the cell apoptosis, which provides information for further elucidating the cardiac toxicity of fluoride.

Materials and Methods

Chemicals and Reagents

Dulbecco's modified Eagle's medium (DMEM) and methyl thiazolyl tetrazolium (MTT) kit were purchased from BOSTER (Wuhan, China). Fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Biological Engineering Material Company (Hangzhou, China). The apoptosis detection kit containing Annexin V-FITC and propidium iodide (PI) was from Nanjing KeyGen Biotechnology Company (Nanjing, China). Penicillin-streptomycin solution was purchased from Solarbio Biological Technology Company (Shanghai, China). Mitochondrial membrane potential assay kit with JC-1 and reactive oxygen species assay kit were obtained from Beyotime Institute of Biotechnology (Shanghai, China). NaF was purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade.

Cell Culture

H9c2 cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The H9c2 cells were cultured with DMEM supplemented with 10 % FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in 5 % CO_2 incubator. Medium was changed every 2 days. Cells were subcultured at a ratio of 1:3 every 3 days using 0.2 % trypsin plus 0.02 % EDTA. For experiments, when the cells had again grown to 70–80 % confluence, they were exposed to different NaF concentrations (0, 2, 4, 8, and 16 mg/L) incubated in 5 % FCS-containing medium for 48 h. At the end of 48 h, cells were subjected to ROS, mitochondrial membrane potential, and cell apoptosis assays with FACS calibur flow cytometry.

Cell Survival Assay

To study the effects of fluoride on the survival of H9c2 cells, the viability of cells was evaluated at 24 and 48 h by utilizing the MTT assay. The cells were treated with different NaF concentrations (0, 2, 4, 8, and 16 mg/L). Then, 10 μL MTT solution was added to the media for another 4 h at 37 °C. Later, 100 μL formazan-dissolved solution was added to dissolve the formazan crystals. Finally, the optical density in each well was determined by spectrophotometry at a wavelength of 570 nm.

HE staining

Hematoxylin-eosin staining (HE staining) was used for morphological examination. The H9c2 cells were subcultured on the cover-slips at a concentration of 1×10^4 cells/mL in 24-well plates. DMEM complete medium containing 0, 2, 4, 8, and 16 mg/L NaF were added in each well for 48 h. After treatment, the cover-slips were took out of 24-well plates and then washed three times with PBS solution. The H9c2 cells were fixed in 95 % ethanol for 20 min followed by a rinse with PBS solution (1 min every time). After washing in PBS, the cells were stained with hematoxylin for 15 min and washed with tap water. Then the H9c2 cells were stained with eosin for 1 min followed by a rinse with tap water. After drying open-air, the cover-slips were mounted on glass slides with neutral gum for microscopic examination.

Detection of Intracellular ROS Level

To determine ROS generation within fluoride-treated cells, fluorescence-activated cell sorting (FACS) analysis was performed. After trypsinized to collect, H9c2 cells were centrifugated and then suspended again in 10 μmol/L diluted 2′,7′-dichlorofluorescin diacetate (DCFH-DA). The

suspension cells were incubated for 20 min in 5 % CO$_2$ incubator, blending them every 3–5 min. After treatment, cells were washed three times with DMEM without FBS, and after centrifugation, 250 µL binding buffer was added to each sample. The labeled cells were detected by FACS calibur flow cytometry.

Mitochondrial Membrane Potential Assay

Mitochondrial membrane potential assay kit with JC-1 was used to detect the $\Delta\Psi$m within fluoride-treated cells. H9c2 cells were cultured in DMEM complete medium containing 0, 2, and 16 mg/L NaF for 48 h. After treatment, cells were trypsinized to collect, after centrifugated and then suspended again in DMEM complete medium at a concentration of $1{\sim}6{\times}10^5$ cells/mL. The cells were incubated for 20 min in 5 % CO$_2$ incubator after blending with the 1ml JC-1 stain liquid. Then cells were washed two times with JC-1 stain buffer solution (1×), after centrifugation, cells were suspended again in 500 µL JC-1 stain buffer solution (1×) and then detected by FACS calibur flow cytometry.

Flow Cytometry Analysis of Cell Apoptosis

For apoptosis analysis, Annexin V-FITC/propidium iodide (PI) staining was performed using flow cytometry according to the manufacturer's guidelines. H9c2 cells were cultured in DMEM complete medium containing 0, 2, and 16 mg/L NaF for 48 h. After treatment, cells were trypsinized and washed two times with PBS (pH 7.4) and then suspended again in binding buffer at a concentration of $1{\times}10^6$ cells/mL. The suspension cells were incubated with 5 µL Annexin V-FITC and 5 µL PI for 15 min in the dark at 37 °C, then, 250 µL binding buffer was added to each sample. The labeled cells were detected directly by FACS calibur flow cytometry.

Statistical Analysis

All data were expressed as means±standard error (SE). Differences between groups were determined by the one-way ANOVA with $P<0.05$ considered as statistically significant.

Results

Effect of NaF Treatment on H9c2 Cell Viability

H9c2 cell viability was measured after NaF treatments for 24 and 48 h by cell viability assays. Compared with the control group, numbers of viable cells were moderately increased at low concentrations but decreased with high NaF concentration. For 48-h incubation, 16 mg/L NaF treatment dramatically depressed H9c2 cell proliferation. These results indicate that long time exposure to NaF inhibits H9c2 cell proliferation (Fig. 1).

HE Staining of H9c2 Cell after NaF Treatment

After incubation with serial concentrations NaF for 48 h, we evaluated the cellular morphology by using an inverted microscope. In the control group without fluoride, cells were presented to fusiform, lined up in order, and the intercellular space were tight. With the increase of NaF concentrations, the density of H9c2 cells was decreased. The majority of H9c2 cells became curled, flaked, and contracted with a dosage of 16 mg/L of NaF.

NaF-Induced Intracellular ROS Production in H9c2 Cells

The effects of NaF on the production of intracellular ROS were examined by DCFH-DA staining. Following exposure to 0, 2, and 16 mg/L of NaF for 48 h, the number of cells with positive fluorescence indicating ROS generation was higher than that of the control group, and it shows a dose-dependent fashion. These results demonstrate that NaF upregulates the intracellular ROS level in H9c2 cells (Fig. 2).

Effect of NaF on Mitochondrial Membrane Potential

The $\Delta\Psi$m of H9c2 cells was analyzed by a fluorescent dye JC-1. JC-1 is capable of selectively entering mitochondria, where it forms monomers and emits green fluorescence when $\Delta\Psi$m is relatively low. At high $\Delta\Psi$m, JC-1 aggregates and gives a red fluorescence. When H9c2 cells were treated with different concentrations of NaF for 48 h, we found that the percentage of cells with green fluorescence increased with the increment of NaF concentration. This suggests that $\Delta\Psi$m in NaF-induced H9c2 cells was down-regulated (Fig. 3).
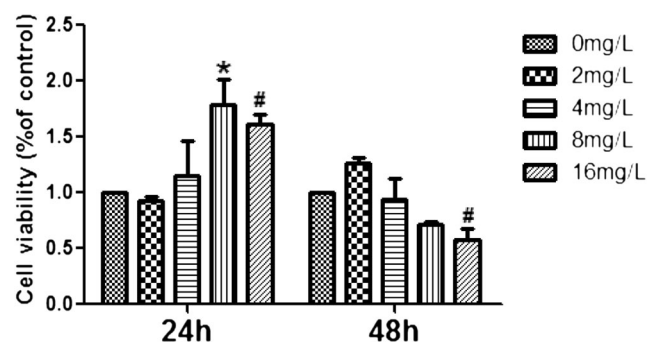

Fig. 1 The histogram revealed H9c2 cell viability in control, 2, 4, 8, and 16 mg/L NaF group for 24 and 48 h. Values represent mean±SEM of three independent experiments performed. (*$P<0.05$ vs. control; #$P<0.05$ vs. control)

Fig. 2 NaF-induced intracellular ROS production in H9c2 cells after 48 h of treatment. **a–c** The intracellular ROS production was measured by a fluorescent dye DCFH-DA after 48 h of exposure to 0, 2, and 16 mg/L NaF concentrations. **d** The mean fluorescence intensity of ROS in control, 2 mg/L NaF, and 16 mg/L NaF group for 48 h. Values represent mean±SEM of three independent experiments performed. (**P<0.01 vs. control, ##P<0.01 vs. control)
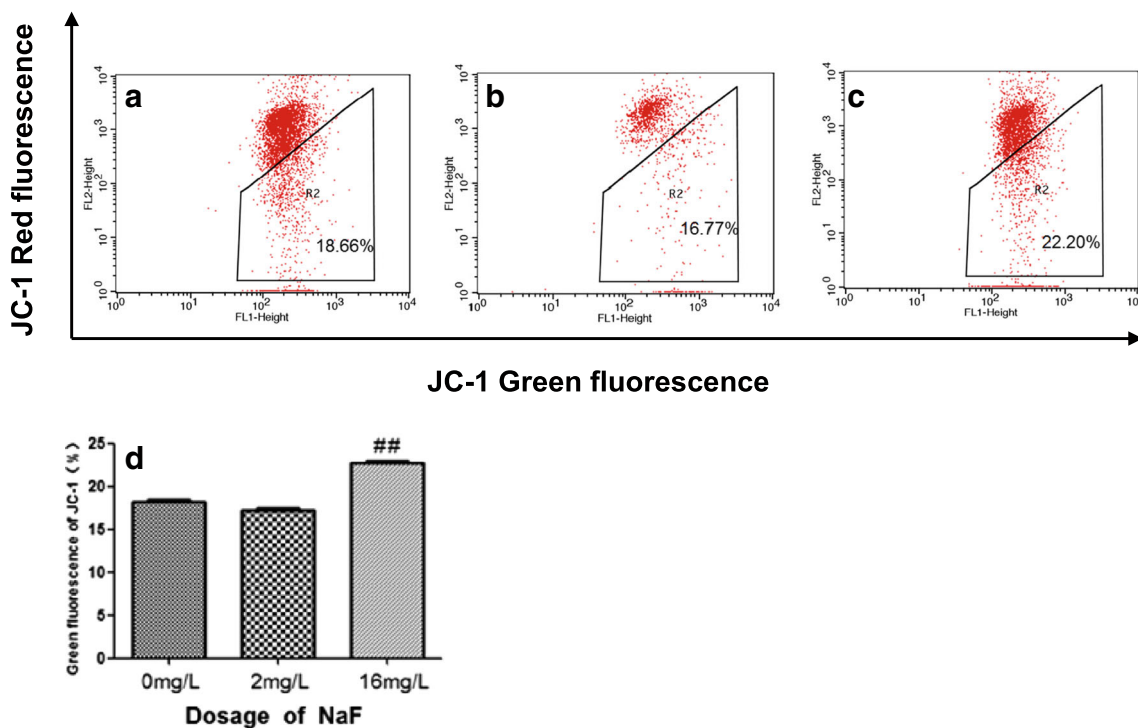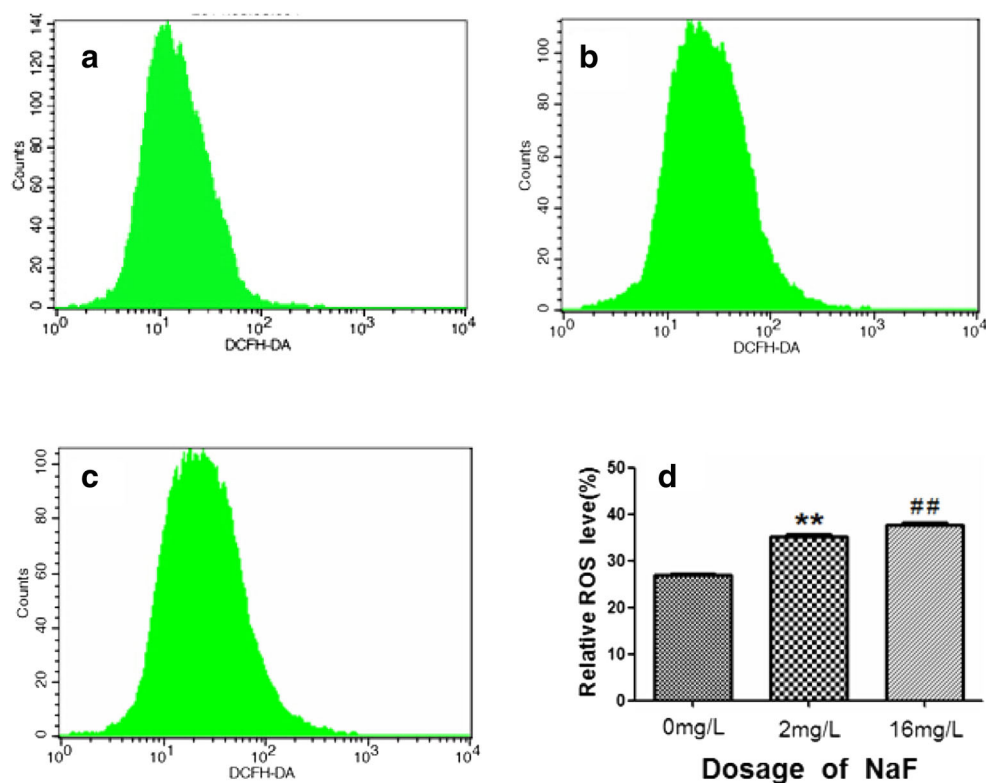
Fig. 3 Effects of NaF on ΔΨm in H9c2 cells by flow cytometry assay. **a–c** The ΔΨm was measured by a fluorescent dye JC-1 after 48 h of exposure to 0, 2, and 16 mg/L NaF concentrations. Cells in R2 area represented ratio of JC-1 from polymer to monomer. **d** The percentage of green fluorescence of JC-1 in control, 2 mg/L NaF, and 16 mg/L NaF group for 48 h. Values represent mean±SEM of three independent experiments performed. (##P<0.01 vs. control)

NaF Induces H9c2 Cell Apoptosis

The H9c2 cell apoptosis in response to NaF treatment was assessed by flow cytometry after staining with Annexin V-FITC and PI. Cells in the lower left quadrant of each picture correspond to normal cells (Annexin V /PI). Cells in right lower quadrant correspond to early apoptotic cells (Annexin V+/PI−). Cells in the right upper quadrant correspond to late apoptotic/dead cells (Annexin V+/PI+). NaF treatment induces both early and late stage cell apoptosis in a dose-dependent fashion. At the end of 48 h, the total apoptotic cells were increased by 23.16 and 28.27 % in the presence of 2 and 16 mg/L NaF, respectively (Fig. 4).

Discussion

Taking fluoride at recommended concentration is suggested to be beneficial to health [22–24]. However, more and more evidence showed that fluoride intoxication produces oxidative stress and damages various tissues [25–28]. The cardiovascular systems are susceptible to a high concentration of fluoride, because many cardiovascular dysfunctions have been observed, such as the decreasing of cardiac output, the occurrence of arrhythmias, and heart block [7, 8, 29]. However, the mechanisms of fluorosis in the cardiovascular system need to be further illustrated.

In our study, we detected the level of intracellular ROS increased with the increasing of NaF for 48 h. It is showed that NaF causes cardiac cell oxidative metabolic abnormalities, breaks the ROS dynamic balance, and leads to the body system in oxidative stress condition [30]. This produces cytotoxic effect and eventually causing irreversible damage to cells [31, 32]. NaF treatment leads to cardiomyocyte dysfunction in which cells become curled, flaked, and contracted.

As one type of signaling molecule, ROS have participated in signal transduction of cell proliferation, differentiation, and apoptosis. Our results indicated that the $\Delta\Psi m$ in NaF-induced H9c2 cells were downregulated compared with the control group. Furthermore, the NaF induces the H9c2 cells apoptosis even at low dose (2 mg/L). ROS formation may cause the disruption of the mitochondrial membrane potential and subsequent release of cytochrome C, which leads to caspase-3 cleavage and finally cell apoptosis [33, 34].

Apoptosis is mediated by two central pathways including mitochondrial pathway and death receptor pathway [35]. Mitochondria are a target of ROS and a source for the additional ROS generation. In situ generated ROS can cause the opening of mitochondrial permeability transition pore (mPTP), with subsequent $\Delta\Psi$ loss and cell apoptosis. Excessive ROS are the trigger in the early stages of apoptosis to induce the depolarization of the $\Delta\Psi m$. This eventually results in an increase in the level of ROS along with other proapoptotic molecules in the cytosol [36–38].

In conclusion, our study suggested that NaF exerts a toxic effect on apoptosis in H9C2 cells via regulating the mitochondrial pathway, such as increasing of ROS generation and decreasing of $\Delta\Psi m$. Simultaneously, NaF inhibited the cells proliferation and induced morphological damage in H9C2 cells. This research provides insightful evidences for the

Fig. 4 Effects of NaF on cell apoptosis in H9c2 cells by flow cytometry assay. **a–c** Cell apoptosis was measured by Annexin V-FITC and PI double-staining after 48 h of exposure to 0, 2, and 16 mg/L NaF. **d** The percentage of early apoptosis, late apoptosis, and total cell death in control, 2 mg/L NaF, and 16 mg/L NaF group for 48 h. Values represent mean±SEM of three independent experiments performed. (**P<0.01 vs. control; ##P<0.01 vs. control)
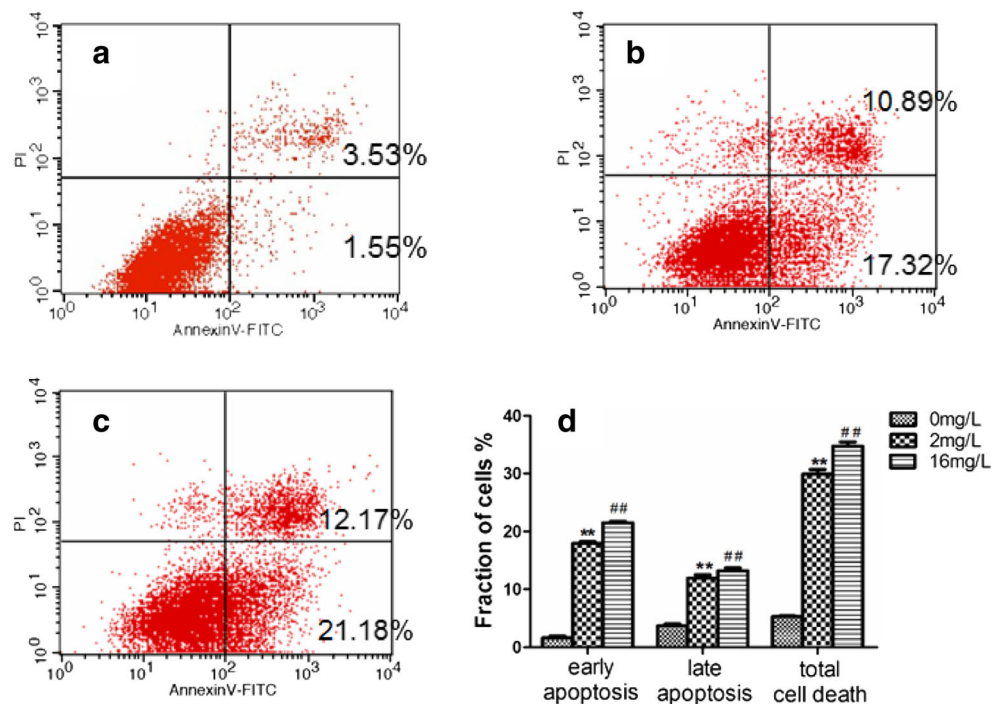
complexity and severity of fluoride's effects on cellular functions because the effects of fluoride are closely related to exposure time and concentration. In addition, a more precise action mechanism of fluoride on these molecular targets should be delineated in further studies.

Conflicts of Interest There are no conflicts of interest.

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