SODIUM FLUORIDE INDUCES APOPTOSIS AND ALTERS THE CARDIAC ARREST RATE IN PRIMARY CARDIOMYOCYTES

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SUMMARY: The objectives of this study were to determine the influence of sodium fluoride (NaF) in different concentrations on morphological change, cell viability, cardiac arrest rate, and apoptosis in primary cardiomyocytes of neonatal rats. NaF at concentrations of 0, 2, 4, 8, and 16 mg/L was administered to cultured cardiomyocytes. The results showed that, NaF, in a concentration dependent-manner and even at the low concentration of 2 mg/L, changed the morphology of the cardiomyocytes, reduced cell viability, increased the cardiac arrest rate, and enhanced the levels of apoptosis.

Keywords: Apoptosis; Cardiomyocytes; Heart structure; Sodium fluoride.

INTRODUCTION

Although many studies have been made of the various effects of chronic fluorosis on the different organ systems, only a few have been on the cardiovascular system. Fluorine is a lively nonmetal element, and the fluoride ion (F) combines avidly with blood calcium and magnesium with resultant metabolic disorder and cardiovascular system damage.[1] F toxicity can cause atherosclerosis at the molecular level and aortic stiffness and disturbed ventricular distensibility clinically.[2-5] The mechanism of F toxicity on the cardiovascular system is complex. Our investigations have previously demonstrated that F is a cytotoxic agent inducing damage in myocardial tissues by oxidative stress through the Bcl-2/Bax signal pathway, which also contributes to atherosclerosis, vascular stiffness, inflammatory mechanisms, and myocardial cell damage.[6-8] Using *in vitro* cultured cardiomyocytes to study the toxic effects of F allows a direct evaluation to be made of the effect of F on the heart. In this study, we investigated the effect of F on cell viability, the cardiac arrest rate, and apoptosis of cardiomyocytes, to provide basic data for further elucidation of the molecular mechanisms of damage induced by F in cardiomyocytes.

MATERIALS AND METHODS

Cardiomyocyte culture: Cardiomyocytes were collected and purified from cardiac tissue of neonatal rats (The Experimental Animal Center of Shanxi Medical University) which was digested with pancreatin (Sigma Aldrich, St. Louis, MO, USA). The cardiomyocytes were cultured in the Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS; Sijiqing, Hangzhou, China) at 37ºC in a humid atmosphere with 5% CO_2 for 72 hr. The cells were treated with NaF

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(guaranteed reagent; China Pharmaceutical Group, Shanghai Chemical Reagent Company, China) solution which was used in final concentrations of 0, 2, 4, 8, and 16 mg NaF/L after culturing.

MTT assay for cell viability: The cells were cultured in 96-well plates. After 24, 48, 72, and 96 hr NaF solution exposure, 200 μL DMEM and 20 μL of the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma Aldrich. St. Louis, MO, USA) (5 mg/mL) were re-added, respectively. After another 4 hr at 37ºC in an incubator in the dark, 150 μL DMSO (dimethylsulfoxide; Salorbio, China) per well were added to stop the reaction and the culture plate was shaken for 10 min. The optical density (OD) of each well was measured at 490 nm with a microplate reader (Shenzhen Rayto Co. Ltd, China).

Cardiac arrest rate assay: The cells were cultured in 24-well plates. After 24, 48, 72, and 96 hr NaF exposure, the beating frequencies of cardiomyocytes were counted with an inverted microscope. The results were calculated by the formula:

$$F = \frac{f_{\text{frequency of beating before the treatment}} - f_{\text{frequency of beating after the treatment}}}{f_{\text{frequency of beating before the treatment}}} \times 100\ \%$$

where F is the cardiac arrest rate (%).

HE staining for assessing morphological changes: Approximately 1×10^5 cells were cultured and the cover-slips were coated with poly-L-lysine. The cell slides were collected after 48 hr incubation with different concentrations NaF and fixed in 4.0% paraformaldehyde after washing twice with phosphate buffer saline (PBS). For the HE staining, we followed our previous protocol.[9,10]

Cell apoptosis assay: The cells were cultured in 6-well plates and after 72 hr incubation with NaF they were collected with 0.25% trypsin and 0.02% EDTA (Sigma, USA). They were washed with HANK'S, filtered twice with a 48 μm millipore filter, and centrifuged at 1,000 rpm for 5 min. The supernatant was then collected. 1 μg/mL propidium iodide (PI) and 5 μL Annexin-V (BD, USA) were added for incubation away from light for 15 min at room temperature, and then 200 μL of binding buffer were added. The cell apoptotic rate was measured with flow cytometry (Beckman, USA).

Statistical analysis: All the results are represented as mean±SEM. Data were analyzed by one-way ANOVA. A p value of <0.05 was considered as a significant difference.

RESULTS

Effects of NaF treatment on cardiomyocyte morphology: After treatment with various concentrations of NaF for 48 hr, the cells adhered to the cover-slips and were stained with hematoxylin and eosin (H&E). While the control group cells showed the classical morphological characteristics of cardiomyocytes, including spindle, triangular and polyangular shapes (Figure 1A), the high dose NaF treatment (16 mg/L) was toxic to the cardiomyocytes, resulting in decreased cell

density and the majority of the cells becoming curled, flaked, and contracted (Figure 1D).

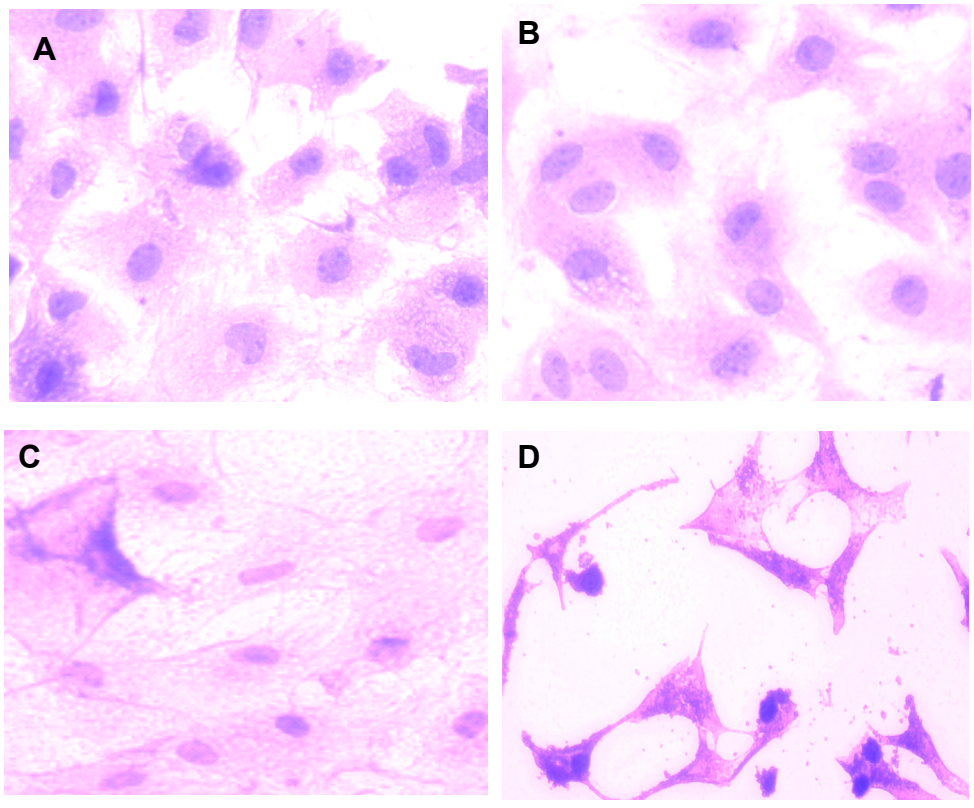

Figure 1. The result of HE staining of cardiomyocytes after fluoride treatment for 48 hr. A: Normal cells in the control group after 48 hr (40×); B: Cells with the 2 mg/L NaF treatment for 48 hr (40×); C: Cells with the 8 mg/L NaF treatment for 48 hr (40×); D: Cells with the 16 mg/L NaF treatment for 48 hr (40×).

Effects of NaF treatment on cardiomyocyte viability: When compared to the control groups, all the concentrations of NaF (2, 4, 8, and 16 mg/L) resulted in a significant loss of cell viability which increased with increasing duration of the exposure (Figure 2).

Effects of NaF treatment on cardiomyocyte cardiac arrest rate: The cardiac arrest rate at different time points (24, 48, 72, and 96 hr) in the experimental and control groups are presented in Figure 3. Compared to the control group, increasing both the NaF dose and the duration of exposure increased the cardiac arrest rate ($p< 0.05$ and $p<0.01$).

Effects of NaF treatment on cardiomyocyte apoptosis: Confirmation of the effects of 72 hr of NaF exposure on cardiomyocyte apoptosis was obtained by Annexin V/PI staining and flow cytometry analysis. The number of cells corresponding to the early or late apoptotic/dead phenotype was increased after exposure to the highest concentration of NaF (16 mg/L, Figure 4C). The cell

apoptosis rate in the cardiomyocytes of the NaF treated groups was higher than in the control group, and the apoptosis rate increased with increasing doses of NaF. At the end of the 72 hr incubation, the apoptosis rate of the two groups, containing 2 and 16 mg NaF/L, increased by 3.28- and 3.40-fold ($p<0.01$), respectively (Figure 4D).

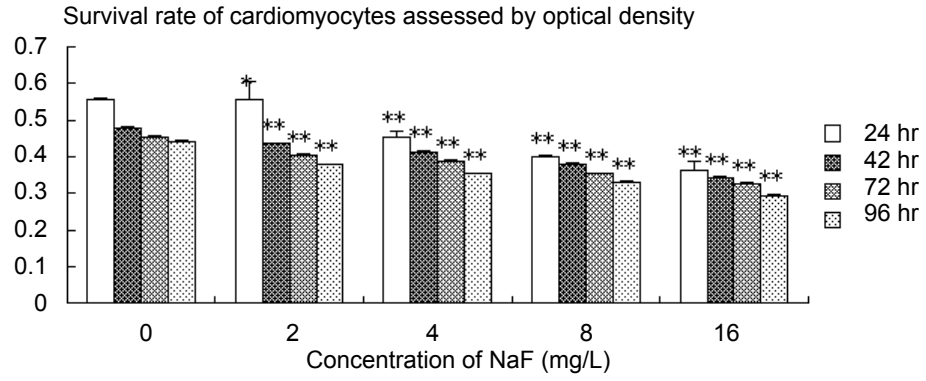

Figure 2. Dose- and time-dependent effects of NaF on the survival rate of cardiomyocytes measured by the mean±SEM optical density absorbance value. The cells were treated with 0, 2, 4, 8, and 16 mg NaF/L for 24–96 hr. Each bar denotes the mean±SEM of four independent experiments carried out in duplicate. Compared to the control: *p<0.05, **p<0.01.

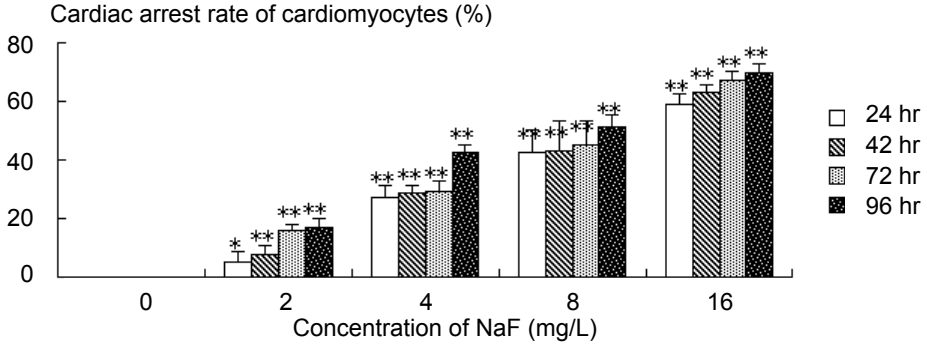

Figure 3. Dose- and time-dependent effects of NaF on the cardiac arrest rate measured by counting with an inverted microscope. The cells were treated with 0, 2, 4, 8, and 16 mg NaF/L for 24–96 hr. Each bar denotes the mean±SEM of four independent experiments carried out in duplicate. Compared to the control: *p<0.05, **p<0.01.

DISCUSSION

Cardiomyocytes in *in vitro* culture have been widely used in the screening and evaluation of cardiac poisons, so *in vitro* cultured cardiomyocytes can be used to directly study the cardiac toxicity of F. We found that F exerted diverse cellular effects in a time-, concentration- and cell-type-dependent manner.[11-14] Our study disclosed a negative effect of NaF on cardiomyocyte survival, in a concentration dependent-manner and even at the low concentration of 2 mg/L, which was associated with morphological changes, the induction of apoptosis, and an increased cardiac arrest rate.

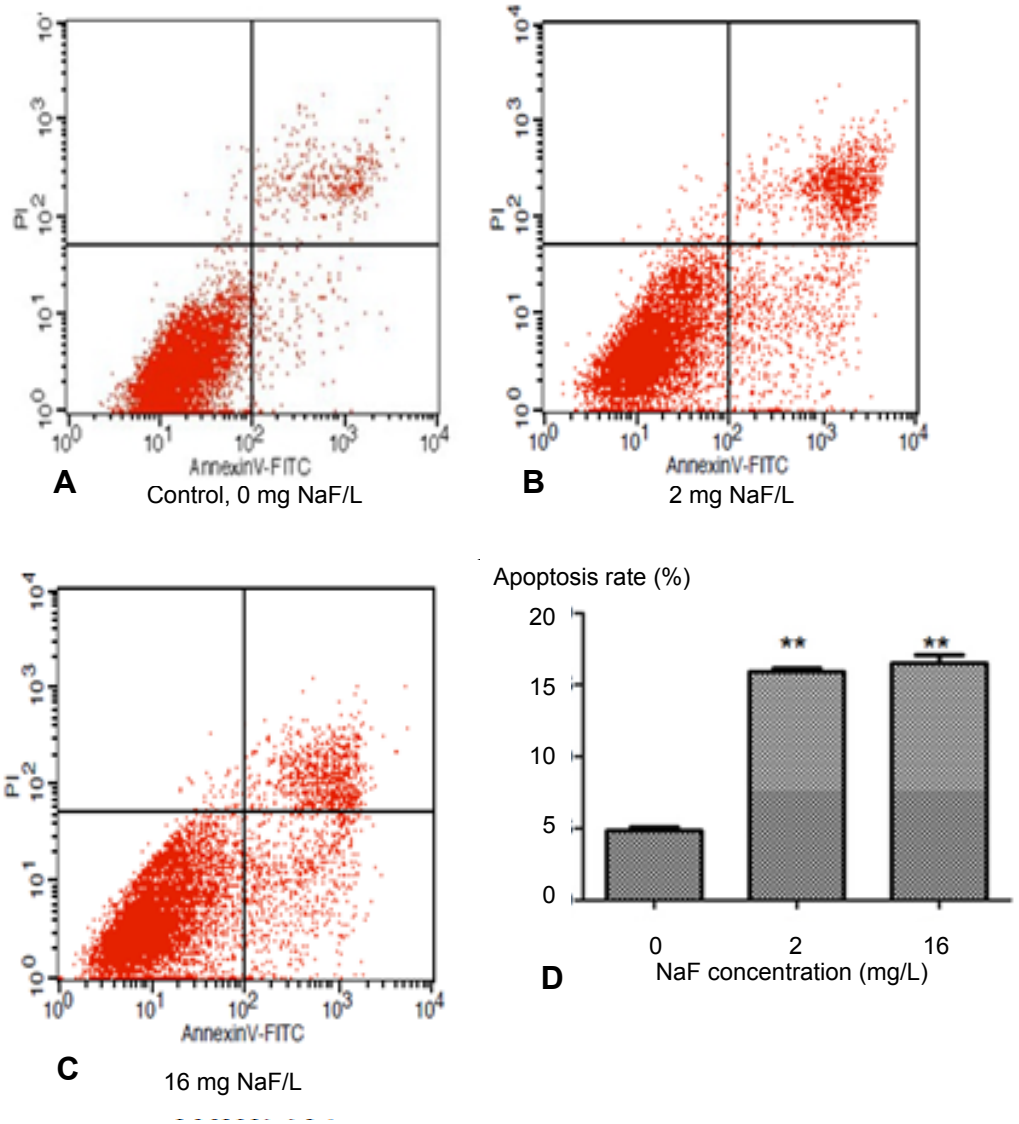

Figure 4A-D. Effects of NaF on cell apoptosis in cardiomyocytes measured by flow cytometry assay. A-C: Cell apoptosis was measured by Annexin V-FITC and PI double-staining after 72 hr exposure to 2 and 16 mg NaF/L. The cells in the lower left quadrant of each picture correspond to normal cells (Annexin V/PI). The cells in the right lower quadrant correspond to early apoptotic cells (Annexin V+/PI). The cells in the right upper quadrant correspond to late apoptotic/dead cells (Annexin V+/PI+). D: Quantification of the effects of treatment with various concentrations of NaF on apoptosis (percent of late apoptotic/dead cardiomyocytes, Annexin V+/PI+). Comparing the NaF-treated cardiomyocytes with the control group: *p<0.05, **p<0.01.

It is well known that a significant number of cardiac myocytes die by apoptosis, in myocardial infarction and heart failure, as well as in other conditions of stress.[13] The apoptosis is mediated by three central pathways involving the mitochondria, the death receptor, and the endoplasmic reticulum.[14,15] Mitochondria are accepted as being the key organelles and that they play a crucial role in the determination of

cell fate with respect to survival and apoptosis in heart injury.[16] As an important intrinsic regulator of apoptosis, the Bcl-2 family resides upstream of the pathway for irreversible cellular damage, and plays a pivotal role in deciding cell survival.[17] Evidence indicates that Bcl-2 protein has the ability of forming homodimers and heterodimers. Bax is the homologous gene of Bcl-2, forming a heterodimer initiating signal pathway of apoptosis. The assignment of the ratio of Bcl-2 to Bax (Bcl-2/Bax) is important in deciding the prognosis of the cell experiencing harmful insults.[18] A previous study found that treatment with norepinephrine decreased the ratio of Bcl-2/Bax, which was correlated with an increase of apoptosis of cardiomyocytes. [19] It has been reported that in the presence of NaF, human gingival fibroblasts show a down-regulation of the Bcl-2/ Bax ratio followed by activation of the mitochondrial cell death pathway.[20,21] Similarly, NaF-induced apoptosis and S-phase arrest are linked to altered expression of Bcl-2 family members and trigger the release of downstream caspases.[9,11,16] Our previous report demonstrated that the signal pathway of Bax and Bcl-2 might be one of the molecular mechanisms by which fluorosis affects myocardial apoptosis or the cardiovascular system.[6]

CONCLUSION

In conclusion, this study demonstrated that NaF, in a concentration dependent-manner and even at the low concentration of 2 mg/L, changed the morphology of the cardiomyocytes, reduced cell viability, increased the cardiac arrest rate, and enhanced the levels of apoptosis. These effects are mediated by a direct effect of F on the mitochondria and nucleus. The cellular mechanisms underlying the cytotoxicity of F in cardiomyocytes are very complex. Further studies are warranted to elucidate the complex signaling pathways of all of the three major apoptosis pathways: the mitochondrial, the death receptor, and the endoplasmic reticulum pathways. Understanding the mechanisms of F-induced cardiotoxicity may provide novel approaches for attenuating fluorosis.

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