Morphological changes and Mitochondrial alterations on Cardiomyocytes exposed to Fluoride

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Objective: To evaluate the morphological changes of cardiomyocytes exposed to different sodium fluoride (NaF) concentrations, as well as to evaluate the behavior of the mitochondria.

Methods: Rat H9c2 cardiomyocytes were exposed to NaF at concentrations of 0.5 to 5 mmol/L. The morphology and number of mitochondria in these cells were monitored, and the calcium ion (Ca^{2+}) concentration was determined.

Results: Morphological changes were evident in the cells treated with different NaF concentrations, and both the number of mitochondria and the Ca^{2+} concentration decreased in a dose-dependent manner.

Conclusion: Sodium fluoride induced morphological damage in cardiomyocytes, decreases the Ca^{2+} concentration and mitochondrial number. [*P R Health Sci J 2023;42(2):132-138*]

Key words: Fluoride, Morphological change, Cardiomyocytes, Mitochondria, Ca^{2+} concentration

Fluorine is a chemical element that is among the most abundant in nature; as the most electronegative chemical element, is present only in compounds formed with other elements (1). Fluorine is one of the main inorganic contaminants of drinking water, and its high concentration in drinking water may be due to natural processes (2), industrial processes, or the overexploitation of groundwater. These factors increase the risk of Fluorine exposure, and the groundwater of numerous geographical areas throughout the world presents high concentrations of fluoride that exceed the limits recommended by the World Health Organization.

Fluoride intoxication has been reported to have deleterious effects on bone, teeth, the liver, the kidneys, and the brain due to its ability to induce oxidative stress and initiate lipid peroxidation (3). Free radical generation, lipid peroxidation, and altered antioxidant defense systems have been documented to play critical roles in the toxicity of fluoride (4). It has been reported that fluorosis may induce nucleotide damage and mitochondrial dysfunction and inhibit protein synthesis (5).

It has been reported that exposure to fluoride induces hypertension and cardiac complications through the generation of reactive oxygen species (ROS); also, fluoride exposure has been associated with the mineralization of the myocardium and testicular degeneration (6). Fluoride is known to induce oxidative stress in mammalian tissues, alter gene expression, and intervene in distinct signaling pathways involved in cellular proliferation and division (7). Fluoride induces oxidative stress through the depletion of various antioxidants, such as glutathione. In addition, fluoride alters antioxidant defense mechanisms (8); causes mitochondrial damage due to decreased membrane potential (9); causes increased mitochondrial and endoplasmic reticulum volume; causes reductions in the numbers of organelles, such as mitochondria, microtubules, and synaptic vesicles (10); and induces apoptosis (11).

Mitochondria are the most important sources of energy in the heart, providing (through oxidative phosphorylation) over 90% of the adenosine triphosphate (ATP) used by the heart (9). It is generally accepted that mitochondria play crucial roles in the pathogeneses of multiple cardiac diseases, including ischemic heart disease and myocardial infarction, mainly due to imbalances in cellular bioenergetics (i.e., the loss of ATP synthesis and the increase of ATP hydrolysis), the impairment of ionic homeostasis (i.e., intracellular calcium ion [Ca^{2+}] level), and the formation of ROS (12). In addition, mitochondria play a key role in regulating apoptosis and cell growth and in generating ROS. Additionally, mitochondrial morphology is now recognized as an important factor closely associated with the energetic state of mitochondria (13).

In the cardiovascular system, Fluoride interacts with Ca^{2+} and magnesium in the bloodstream, causing disorders of the calcium metabolism and such manifestations as arteriosclerosis

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of the carotid artery, ventricular diastolic dysfunction, peripheral vascular disease, and ischemic heart disease (14–15). The incidence of coronary heart disease is varied and is attributed in part to the classic lifestyle risk factors such as: obesity, sedentary lifestyle, smoking; nonetheless, several environmental factors, including the presence of high concentrations of trace elements such as Fluorine in the drinking water, can also affect the incidence of coronary heart disease (14).

The exposure to and consumption of Fluorine through different products has been increasing in recent decades (16). Previous studies have reported that early exposure to fluoride is considered an important risk factor for increased susceptibility to various diseases, including cardiovascular disease, and have indicated the need for studies that contribute to the understanding of the mechanisms that might be involved (17).

The aim of this study was to evaluate the morphological changes in cardiomyocytes exposed to NaF at millimolar concentrations as well as to evaluate the behavior of the mitochondria under such conditions.

Materials and methods

Cell cultures

Rat H9c2 cardiomyocytes were purchased from the cell bank of the Chinese Academy of Science (Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (Gibco Laboratories, USA) enriched with 10% fetal bovine serum and 1% antibiotic (1000 units of penicillin, 10 mg of streptomycin, and 25 µg of amphotericin B per mL, obtained from Sigma-Aldrich, St. Louis, MO, USA) with NaF concentrations of 0.5, 0.75, 1.5, 2.5, and 5.0 mmol/L (18),. the rat equivalents of the human doses of 0.05 to 0.5 nmol/L. Due to the high metabolic rate of rodents, fluoride is quickly removed from their system; the literature reports that plasma fluorine concentrations in rodents that have been treated with doses of 50 and 100 mmol/L of fluoride range from 0.04 to 0.08 nmol/L respectively (19). Because we were working with a cell culture model lacking complete metabolic systems, we decided to treat the cells with the proposed concentrations for 7 days (to mimic a sub-chronic exposure) at 37°C with 95% humidity and 5% carbon dioxide; the medium was replaced every third day.

Qualitative morphological grading

The treated cells were evaluated by optical microscopy according to the specifications of International Organization for Standardization 10993-5-2009 (20). The following parameters were evaluated: (i) morphology, (ii) vacuolization, (iii) detachment, (iv) cell lysis, and (v) membrane integrity.

MitoTracker mitochondrion-selective probes

The test was performed using the rat H9c2 cardiomyocytes cultured in the presence of different NaF concentrations. MitoTracker Deep Red FM (Invitrogen by Thermo Fisher Scientific) was used at 25 to 500 nmol/L concentrations. The solution was preheated to 37°C, added to the treated cells, and incubated for a period of 15 to 45 minutes at 37 °C, at which point the dye was replaced with phosphate-buffered saline. Finally, the samples were evaluated under a laser confocal microscope (DMI4000 B, Leica Microsystems, Wetzlar, Germany). The results are reported in fluorescence units and as representative images.

Determination of the intracellular and extracellular Ca^{2+} concentrations

The Ca^{2+} concentrations were determined by means of the calcium Arsenazo spectrophotometric technique; 100 µL of Arsenazo reagent was added to 15 µL of sample and incubated for 15 minutes. The samples were read in a microplate reader at 650 nm (Thermo Fisher Scientific). The determination of the extracellular Ca^{2+} concentrations was carried out in the culture medium in which the cells were cultured with the different treatments. The intracellular Ca^{2+} concentration was measured in lysates obtained by the lysis (with an ultrasonic tip) of the NaF treated cells. For both intra- and extracellular Ca^{2+} measurements, the number of cells per experiment was adjusted to 20,000/mL. The Ca^{2+} concentration was reported in mg/dL.

Statistical analysis

All the values are expressed as the mean plus or minus standard deviation of each group. The data were analyzed with 1-way analysis of variance followed by Tukey's multiple comparison test using the SigmaPlot (11.0) software package (Systat Software, Inc., San Jose, CA, USA). A P value less than .05 was considered statistically significant. Tests were performed in triplicate.

Results

Fluoride-induced morphological changes in cardiomyocytes

The control group (Fig. 1A) cardiomyocytes showed a characteristic morphology of trapezoidal cells growing in the shape of a hive with defined cell walls as well as extensive cell–cell contact. Several cellular and subcellular changes were observed with optical microscopy. The morphologic changes occurred beginning at the lowest NaF concentration and included alterations in the focal contacts (Figs. 1B and 1C), membrane integrity loss, cell swelling (Fig. 1D), cytoplasmic vacuolization (Fig. 1E), and a totally altered cell morphology, with both membrane integrity loss and the presence of vacuoles (Fig. 1F).

Fluoride-induced decreases of mitochondrial numbers in cardiomyocytes

The number of mitochondria in the cardiomyocytes treated with NaF decreased in a concentration-dependent manner, as indicated by the green circles (Fig. 2A); this decrease was quantified in fluorescence units (Fig. 2B). The differences in the numbers of mitochondria were statistically significant ($P < .05$).

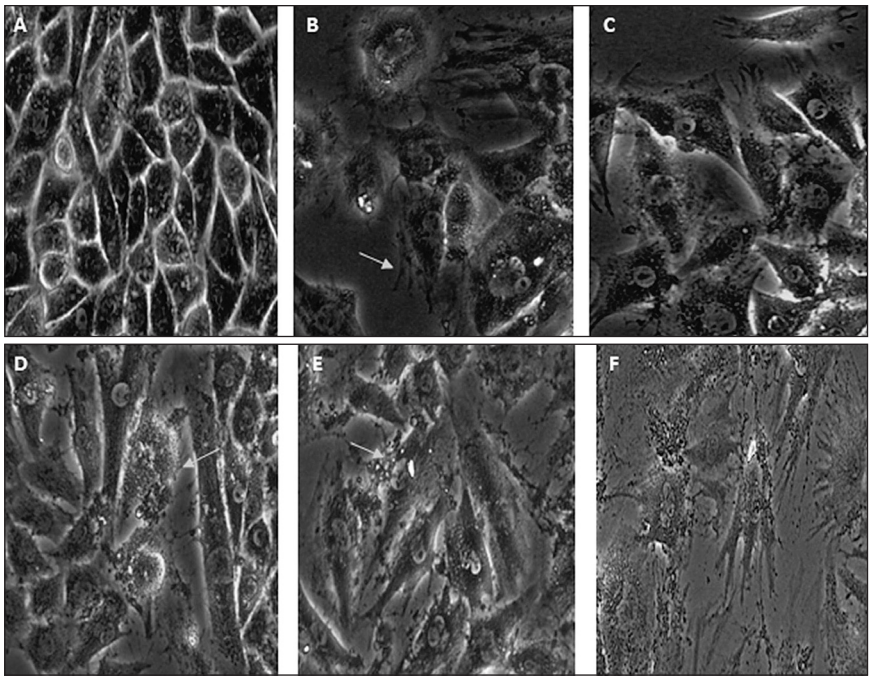

Figure 1. Morphological changes in H9c2 cells after stimulation with NaF for 7 days: A) control, B) 0.5 mM, C) 0.75 mM, D) 1.5 mM, E) 2.5 mM, and F) 5 mM. The yellow arrow indicates the changes observed in the cells treated with different NaF concentrations. Changes in the size of focal contacts (Figs. 1B and 1C), membrane integrity loss, cell swelling (Fig. 1D), and cytoplasmic vacuolization (Fig. 1E). Total alteration of cell morphology with membrane integrity loss and the presence of vacuoles (Fig. 1F).

The experiment was carried out with 5 images per treatment being captured at random of the cells subjected to the different concentrations. Compared to the untreated cardiomyocytes in the control group, differences were statistically significant in cardiomyocytes treated with concentrations higher than 1.5 mmol/L.

Intracellular and extracellular Ca^{2+} concentration

The concentration of intracellular and extracellular Ca^{2+} was considerably decreased. The intracellular Ca^{2+} concentration exhibited decreases ranging from a minimum of 46% (cells treated with 3 mmol/L of NaF) to a maximum of 90% (cells treated with 5 mM of NaF), as shown in Fig. 3A. The extracellular Ca^{2+} concentration also decreased substantially by at least 75% (cells treated with 1 mmol/L of NaF) (Fig. 3B). In both experiments, the Ca^{2+} concentration of the control group was 100%. The differences in Ca^{2+} concentration was statistically significant.

Discussion

Human activity and different natural processes release large amounts of Fluorine into the environment and water; this contamination is considered a serious public health problem, worldwide (21). The biological effect of chronic fluoride ingestion depends on the total dosage and duration of the exposure; fluoride in low concentrations has been proven to be beneficial for the development of bones and teeth, but excessive amounts in drinking water have adverse effects (22). Cardiovascular disease is the most common cause of death, worldwide; in addition to genetic and lifestyle risk factors, environmental exposure to toxicants during childhood has been associated with cardiovascular dysfunction (23).

The NaF concentrations used in this study were chosen because rodents experience a high metabolic rate that favors the rapid removal of fluoride from their body. Quadri et al. reported that fluoride concentration of 10 nmol/L in rodents is equivalent to 1 to 2 nmol/L in humans; therefore, in this study, a range of 0.05 to 0.5 nmol/L was used (7). Another factor to consider is the levels of fluoride in the plasma after its introduction into the body. Some authors reported that after administering 50 and 100 nmol/L to rodents, they obtained plasma concentrations of fluoride of 0.04 and 0.08 nmol/L, respectively (18–19). In the present study, a mouse cardiomyocyte cell culture model was used; it is well known that cell cultures lack complex metabolic systems that are necessary to eliminate fluoride from the medium in which the cells are growing.

The NaF concentrations used in this study are above those concentrations detected in individuals exposed to high levels of Fluorine in drinking water. In this report, it was intended to explore the effects (*in vitro*) of this chemical compound on cardiomyocyte cell cultures, as mentioned above, as this type of experimental model is limited in its ability to reproduce conditions related to plasma levels and to chronic exposure. Therefore, the present study provides evidence of the possible effects of fluoride on cardiac cells, bolstering studies showing a strong association between endemic Fluorine exposure and an increased prevalence of cardiac complications (24). Although the exact mechanism of soft-tissue fluorosis has not been fully clarified (25), it is understood that fluoride is involved in the inhibition of protein synthesis, the disruption of cell metabolism, and the increase of ROS production, with all 3 processes contributing to the generation of oxidative stress. The activities of fluoride are widely accepted as the most common phenomena in the toxicity of same.

The accumulation of fluoride in the cell can cause significant damage to the cytoskeleton and have an impact on the formation, in muscle cells, of the dynamic cytoskeleton and its

binding proteins (26). In the present study, the morphology of the H9c2 cells changed in a dose-dependent manner and included an increase in size, the loss of membrane integrity, and the presence of vacuoles; each of these changes in cell morphology may have been an indication that the cells treated with different NaF concentrations were in the initial stages of apoptosis (27, 28). The assays carried out in this study focused mainly on analyzing the effect of NaF on mitochondria; further studies analyzing other cellular organelles that may also be affected by the presence of fluoride should be carried out under the necessary conditions, thus complementing the results obtained.

Qualitative evaluation is one of the methods used to estimate the cytotoxicity of a biomaterial or chemical compound such as NaF (20, 29). Examining cells under an optical microscope is considered to be an essential step in evaluating damaged cells; through this process, changes in morphology, vacuolization, detachment, cell lysis, and membrane integrity can be assessed, which is the starting point in evaluating the cytotoxic effect of a chemical compound or biological material. Further, this kind of examination is done *in vivo*, so the treated cells can be used in other experiments. Additionally, the information provided by the qualitative analysis of cytotoxicity reveals the effects of a given treatment on the cell, since the cell can undergo important morphological changes (that, nevertheless, do not lead to its death) after being in contact with the biomaterial, which would not be reflected in cytotoxicity tests such as the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), or lactate dehydrogenase (LDH) assay (30).

Cardiomyocytes are enriched with mitochondria, and many functional mitochondria are required in these cells if they are to produce enough ATP for normal cardiac contractile function (31). Mitochondria regulate the operation of intracellular signaling cascades, mitochondria generate ROS, mitochondria activate fatty acid β-oxidation, and mitochondria participate in amino acid metabolism, mitochondria participate in pyridine synthesis, mitochondria participate in phospholipid modifications, mitochondria participate in calcium regulation, and mitochondria participate in cell survival, cell senescence, and cell death. (32). Likewise, the evaluation of the mitochondrial state after the cells were treated with different NaF concentrations provided important evidence regarding the effect of fluoride on one of the cellular organelles. Since the major functions of mitochondria are producing aerobic energy and ROS, maintaining calcium

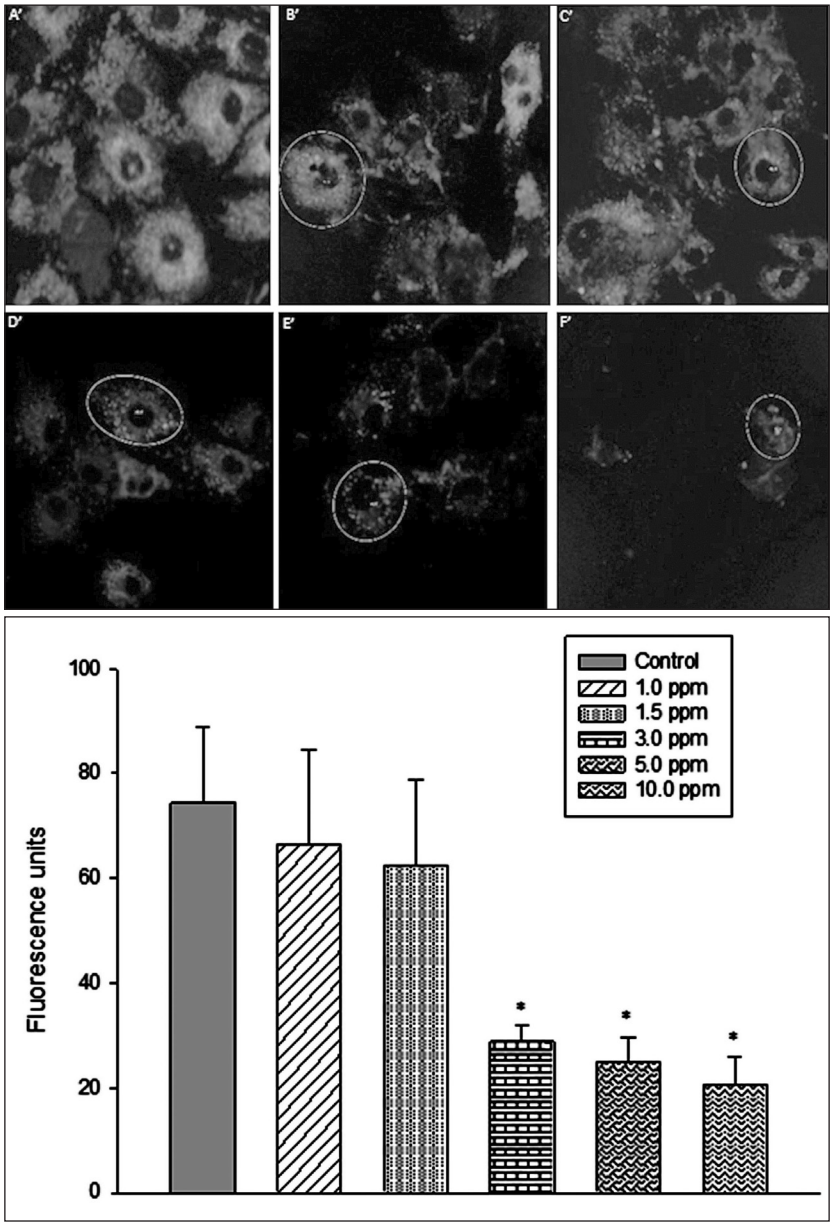

Figure 2. MitoTracker mitochondrion-selective probes: A) The changes in the mitochondria of H9c2 cells after stimulation with NaF for 7 days were analyzed with confocal microscopy. A') control, B') 0.5 mM, C') 0.75 mM, D') 1.5 p pm, E') 2.5 mM, and F') 5 mM. Green circles highlight the decreased presence of mitochondria in cardiomyocytes treated with different concentrations of fluoride. B) Fluorescence units. The changes in the mitochondria of H9C2 cells after stimulation with different NaF concentrations for 7 days, expressed in fluorescence units. **P* < .05.

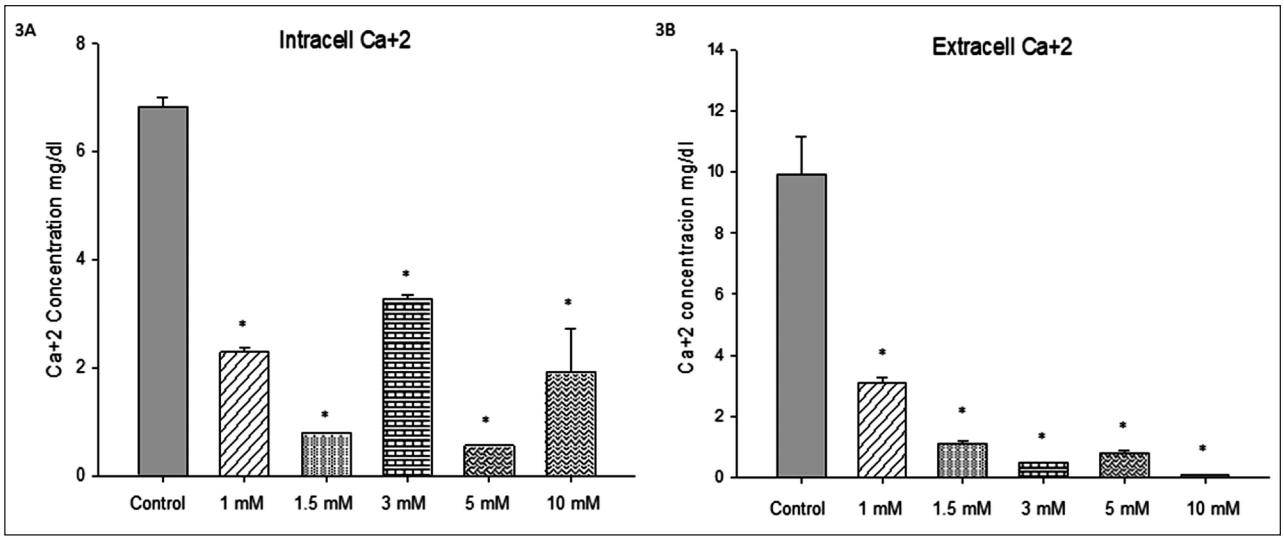

Figure 3. The intracellular and extracellular concentrations of Ca2+ in H9c2 cardiomyocytes exposed to different NaF concentrations: A) intracellular Ca2+ and B) extracellular Ca2+. Compared with the control group, $*P < .05$.

homeostasis, and mediating cellular signaling pathways (33), mitochondria also play an important role in cellular aging and cell death, which are associated with necrosis, apoptosis, autophagy, and mitophagy, through the modulation of redox homeostasis by reduction reactions (34).

Aging may be associated with the accumulation of damage precipitated by such processes as the production of metabolic byproducts and ROS, the accumulation of biological waste products, the shortening of telomeres, and the dysregulation of metabolic pathways (31). Most of these aging factors are associated with mitochondrial dynamics and function, indicating a close relationship between aging and mitochondria (32). Cells treated with different NaF concentrations showed a concentration-dependent reduction in the number of mitochondria. Mitochondrial homeostasis is preserved by the fine coordination of 2 opposing processes: the generation of new mitochondria by mitochondrial biogenesis and the elimination of damaged mitochondria by a process known as mitophagy (35). The cardiac muscle formed by cardiomyocytes is rich in mitochondria, and this type of tissue has the fundamental function of contracting muscle, a process requiring significant amounts of ATP, itself generated by this organelle. Therefore, a decrease in the number of mitochondria brings with it a decrease in the production of this energetic molecule par excellence, ATP, and makes the contraction of skeletal muscle weak (19, 31).

This is possibly due to a dysregulation of mitochondrial dynamics that causes autophagy as a consequence of mitochondrial damage. At high concentrations of fluoride, the mitochondrial membrane potential in H9c2 cells is altered and inhibits oxidative phosphorylation in cardiac mitochondria (36), and these high concentrations also cause cardiac dysfunction through (i) increased myocardial oxidants and the inhibition of antioxidant machinery, (ii) the induction of apoptotic and necrotic stimuli, and (iii) the impairment of myocardial cytoskeletons and depletion of ATP, both of which latter lead to the activation of adenosine monophosphate-activated protein kinase signaling molecules in the rat heart (26). Mitochondria play an important role in the apoptotic process and have been suggested to be the center of the apoptotic pathway.

Under normal conditions, intracellular Ca^{2+} is sequestered in organelles such as the mitochondria, endoplasmic reticulum (ER), and nucleus (37). The mitochondria and ER have been considered the primary reservoir of Ca^{2+} inside the cell; the mitochondria and the ER are organelles that work together and share membranous structures. The reduction of the number of mitochondria resulting from the activity of fluoride probably also leads to decreases of ER and the levels of Ca^{2+}. This would explain the dose-dependent decreases in the amounts of intra- and extracellular Ca^{2+} in cardiomyocytes treated with different NaF concentrations. With that in mind, it is likely that an analysis using electron transfer microscopy would provide a useful assessment of these 2 organelles.

In the present study, cells treated with different NaF concentrations were subjected to ultrasound to release Ca^{2+} and thus be quantified. Calcium ion is one of the most important elements in cellular signaling. It participates in numerous cellular processes in all cell types. Additionally, this trace element plays an important role in muscle contraction, glycogenolysis, and the activation of the mitochondrial Krebs cycle (38), just as it plays a central role in the excitation–contraction coupling process that takes place in cardiac muscles (39); the contraction and relaxation of the heart result from cyclical changes of intracellular Ca^{2+} concentration (40). The low concentrations of intracellular and extracellular calcium in the cells treated with the different NaF concentrations were related to changes in the mitochondrial state; because treatment with NaF decreased levels of Ca^{2+} and the number of mitochondria, as shown in the MitoTracker trial (Figures 2 and 3). Compared to other

regulatory mechanisms, such as phosphorylation, Ca^{2+} signaling is very fast. However, since Ca^{2+} cannot be destroyed, Ca^{2+} signaling can be controlled only by pumping the ion through membranes. Also, mitochondrial Ca^{2+} plays an important role in the regulation of various responses, such as apoptosis; different factors may be involved in the regulation of the size and number of mitochondria to manage the movement and concentration of Ca^{2+}.

Finally, the results described in this study are intended to encourage the development of new studies that will enrich the knowledge of the effect of fluoride in organelles, such as mitochondria, which are vital for function in all types of cells. Through a detailed study of the alterations in the processes of mitochondrial biogenesis and mitophagy to understand the mechanisms that cause the mitochondrial decrease in cardiomyocytes treated with the different concentrations of NaF. In addition, this work has generated new interest in conducting experiments in which NaF concentrations that are closer to those found in the plasma of populations exposed to high concentrations of Fluorine in drinking water are evaluated.

Conclusions

Overall, the results demonstrate the marked toxic effect of NaF on H9c2 cells. Moreover, they suggest that NaF concentrations above 1.5 nmol/L induce significant morphological damage and decrease the Ca^{2+} concentration in H9c2 cells. In addition to the morphological alterations and the notable decrease in the number of mitochondria dependent on the concentration of NaF, the decrease in the concentration of Ca^{2+} is an important finding since Ca^{2+} is involved in numerous activities essential for the cell; however, the mechanisms of fluoride in the cardiovascular system are very intricate, and further investigation is required to develop a complete theoretical basis for the prevention and treatment of cardiovascular disorders mediated by fluoride

Resumen

Objetivo: Evaluar cambios morfológicos de cardiomiocitos expuestos a diferentes concentraciones de fluoruro de sodio (NaF, por sus siglas en inglés) así como evaluar el estado de las mitocondrias. Metodología: Cardiomiocitos H9c2 de rata fueron expuestos a concentraciones de NaF entre 0.5-5 mmol/L. Resultados: La morfología y número de mitocondrias en estas células fueron monitoreadas y la concentración de iones de calcio (Ca^{2+}, por sus siglas en inglés) fue determinada. Fueron evidentes los cambios morfológicos en las células tratadas con las diferentes concentraciones de NaF; el número de mitocondrias y la concentración de Ca^{2+} disminuyó de manera dosis dependiente. Conclusión: El fluoruro de sodio a las concentraciones evaluadas induce cambios morfológicos en los cardiomiocitos, disminuye la concentración de Ca^{2+} y el número de mitocondrias.

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