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Fluoride exposure induces myocardial injury through the Nrf2-mediated oxidative stress signaling pathway

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ABSTRACT

Purpose: Fluoride exposure in the natural environment has been shown to damage the cardiovascular system. This study aimed to systematically evaluate the potential effects of fluoride exposure on the cardiovascular system by treating rats with NaF (Sodium fluoride).

Methods: Eight rats (male to female ratio, 3:1) were randomly divided into two groups: a control group (distilled water) and a fluoride-exposed group (100 mg/L NaF). Male offspring rats from each group were randomly selected for the experiment.

Results: The experimental results suggest that fluoride exposure can cause mitochondrial damage in the myocardial tissue of rats. This is accompanied by a decrease in serum creatine kinase activity and a significant reduction in the levels of Ca^{2+}-ATPase. Moreover, there are notable changes in the expression of proteins associated with oxidative stress in the myocardial tissue, including KEAP1, HO-1, and Nrf2. These observations imply that fluoride may induce oxidative stress via the Nrf2 signaling pathway, leading to mitochondrial dysfunction in myocardial tissue and subsequently causing myocardial damage.

Conclusions: In summary, this study suggests that the Nrf2-mediated oxidative stress pathway plays an important role in NaF induced myocardial injury.

Key-words: *Sodium fluoride; Myocardial injury; Oxidative stress; Nrf2 signaling pathway.*

INTRODUCTION

Fluoride exposure poses a health threat and is a global public health issue.[1] Endemic fluorosis is widely distributed prevalent worldwide, particularly in Asia, the Americas, and Africa.[2] In China, Shanxi Province being one of the most affected areas.[3]

Endemic fluorosis is mainly characterized by bone-related damage, such as dental and skeletal lesions, but also affects non-bone-related organs, including the digestive, neurological, urinary, endocrine, and cardiovascular systems.[4,5] The mechanisms underlying the damage to the cardiovascular system by fluoride exposure are a current research hotspot. Subsequent studies have

found that fluoride exposure is also associated with hypertension, dyslipidaemia, and cardiomyopathy, further supporting the potential harm of fluoride to the cardiovascular system.[6] In recent years, numerous scientific investigations have gradually revealed the molecular mechanisms of fluoride exposure-induced cardiovascular diseases.[7] Existing studies have shown that fluoride exposure can induce cardiovascular dysfunction through a variety of pathways, including oxidative stress, inflammatory response, calcium (Ca^{2+}) disruption, mitochondrial damage, and apoptosis.[8-11]

Oxidative stress is a pathological condition characterized by an imbalance between the oxidative and antioxidant systems in the body, leading to excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that surpass the body's antioxidant capacity, ultimately causing cellular damage.[12] Fluoride can affect oxygen metabolism, induce oxygen radical generation, and enhance the level of oxidative stress in the blood.[11,13-18] Some studies have shown that oxidative stress-induced apoptosis can be reduced by the enhancement of Ca^{2}+ ATPase (SERCA) activity.[19] Studies have shown that the NRF2 signaling pathway has an important role in the maintenance of calcium homeostasis.[20] During oxidative stress, KEAP1 regulates intracellular redox homeostasis by regulating the activity of Nrf2. Its abnormal function may lead to oxidative stress-related diseases, such as cancer, Parkinson's disease, and atherosclerosis, etc. Nrf2-regulated genes include HO-1, NQO1, and GCLC, etc. which encode enzymes and proteins that can scavenge ROS, attenuate oxidative damage, and maintain cellular homeostasis. KEAP1 senses oxidative stress signals and releases Nrf2, which enters the nucleus to activate HO-1 and other antioxidant genes, thereby reducing oxidative damage and maintaining intracellular redox balance. This pathway plays an important role in cardiovascular system injury and is a potential therapeutic target. [21,22] In light of this, the present study established a fluoride-exposed male rat model spanning gestation, puberty, and adulthood. The aim was to preliminarily explore the mechanisms underlying fluoride exposure-induced damage to the myocardial tissue in male rats and to investigate the role of the Nrf2 oxidative stress signaling pathway in this context.

MATERIAL AND METHODS

Animal grouping and interventions

Eight clean-grade rats (female-to-male ratio of 3:1, approximately 200g in weight) were used in this study. These animals were provided by the Laboratory Animal Center of Shanxi Medical University (License No: SCXK [Jin] 2015-0001). The animals were housed in an environment maintained at approximately 25°C, with 40%-70% humidity and a 12-hour light-dark cycle. All experiments were conducted in strict accordance with the operating procedures of the university's Animal Management Committee.

Eight healthy SD rats (6 females and 2 males, weighing 200-250 g) were selected and randomly divided into a control group (drinking distilled water) and a fluoride-exposed group (100 mg/L sodium fluoride) according to the female-to-male ratio of 3:1. After 10 days of fluoride exposure, the males and females were mated in a combined cage, with the detection of a vaginal plug being regarded as day 0 of pregnancy. Subsequently, the males and females were caged separately, and the pregnant rats were continuously exposed to the same fluoride environment from gestation day 1 to day 21 after delivery. Offspring were exposed to fluoride through the maternal uterus and breast milk from gestation day 1 to postnatal day 21. After weaning, they continued to be reared in the same environment until the age of three months. Male offspring rats from each group were randomly selected for the experiment.

Myocardial injury was observed by optical microscope

Optical microscopy observation of myocardial injury: Fresh myocardial tissue was processed through fixation, drying, clarification, and paraffin embedding, and then cut into paraffin sections with a thickness of 4μm. The sections were then spread, dried, dewaxed, and stained with a hematoxylin and eosin (HE) staining kit (Yongtai Biotechnology Research Institute, China). Finally, the stained sections of myocardial tissue were observed under an Olympus optical microscope (Olympus, Japan) to assess the degree of myocardial injury.

Observation of the ultrastructure of myocardial tissue by electron microscopy

Fresh myocardial tissues were harvested from rats and washed with physiological saline, then cut into small pieces of 1 mm^3. The tissue pieces were fixed in 2.5% glutaraldehyde at 4℃ for 2 hours, followed by graded dehydration in a series of acetone solutions and embedding in epoxy resin. After trimming, sectioning, and staining, the ultrastructure of the sections was observed and images were captured using a JEM-1011 transmission electron microscope.

Determination of CK in serum and Ca^{2+}-ATPase in heart tissue

(1) Serum preparation and determination of biochemical indices: Whole blood was collected from the rats and allowed to stand for 3 hours. The supernatant was obtained after centrifugation (performed three times) and then stored at -80°C after separation. The activity of creatine kinase (CK) in the serum was determined using a colorimetric method at 660 nm, according to the instructions provided in the kit (Jiancheng Bioengineering Institute, Nanjing, China).

(2) Myocardial Tissue Processing and Ca^{2+}-ATPase Activity: Fresh myocardial tissue was collected, frozen in liquid nitrogen, and stored at -80°C. An appropriate amount of tissue was weighed, mixed with pre-cooled saline, and homogenized using an ultrasonic tissue homogenizer. The homogenate was then centrifuged to obtain the supernatant for subsequent analysis. The activity of Ca^{2+}-ATPase was measured according to the instructions provided in the manual (Jiancheng Bioengineering Institute, Nanjing, China).

Western blot analysis

The heart tissues were disrupted using RIPA lysis buffer that included protease and phosphatase inhibitors. After centrifugation at 12,000×g for 10 minutes at 4°C, the resulting supernatant, which contained the total protein, was extracted. Protein concentrations were quantified using a BCA protein assay kit (Beyotime Institute of Biotechnology, China). For each sample, 30 μg of protein was loaded per lane and separated via 8% SDS-polyacrylamide gel electrophoresis. Subsequently, the proteins were transferred to a nitrocellulose (NC) membrane. The membrane was blocked with 5% (w/v) BSA in TBS containing 0.1% Tween-20 at room temperature for 2 hours, then incubated with primary antibodies at 4°C overnight and secondary antibodies at room temperature for 1 hour. The target proteins were ultimately detected using an ECL luminescence reagent (Boster, Wuhan, China).

Statistical analysis

This experiment was conducted using SPSS 26.0 software for between-group comparisons by two independent samples t-test, and ANOVA, and the data were statistically described using Mean ± SD, with a test criterion of $P<0.05$.

RESULTS

Effect of fluoride exposure on body weight in rats

As shown in Figure 1, there was no significant difference in body weight between the control group and the NaF exposed group of rats in the second month. However, in the third month, the body weight of the NaF exposed group was significantly lower than that of the control group $(P < 0.05)$.

Effect of fluoride exposure on the fluoride content of rat femurs

In the relevant article titled "Deregulation of Autophagy Is Involved in Nephrotoxicity of Arsenite and Fluoride Exposure from Gestation to Puberty in Rat Offspring", published in the 《*Archives of Toxicology*》, the fluoride ion content in the femur of rats exposed to NaF was found to be significantly higher than that in the control group, with a statistically significant difference.[23] This result indicates that NaF exposure successfully induced the accumulation of fluoride ions in the femur of rats, thereby confirming the successful construction of the animal model.[23]

Fluoride exposure induces pathological alterations in myocardial tissue

As illustrated in Figure 2, under low magnification microscopy, the myocardial cells in the control group are tightly arranged, exhibiting an elongated or ribbon-like morphology, with nuclei positioned centrally within the cells. Following exposure to fluoride, there is an observed phenomenon of cellular edema, accompanied by a noticeable increase in the intercellular spaces. Upon examination under high magnification, the morphology and internal structures of the myocardial cells are more distinctly visible, with the nuclear contours becoming more defined, and the

details of myofibrils and other organelles becoming more apparent, revealing the characteristic striated structure of myocardial cells. After exposure to fluoride, the edema of the myocardial cells becomes more pronounced, the intercellular spaces further widen, the nuclear staining becomes uneven, and the structure of the myofibrils exhibits disarray.

Fluoride exposure induces ultrastructural changes in the myocardial tissue of rats

As depicted in Figure 3, the myocardial cell nuclei in the control group are larger in size, exhibit regular morphology, possess distinct nuclear membranes, and display prominent nucleoli, with an abundance of mitochondria scattered around the nuclei. In contrast, the nuclei of myocardial cells exposed to fluoride demonstrate irregular shapes, the disappearance of nucleoli, and evident damage to the mitochondrial structures surrounding the nuclei.

Effect of fluoride exposure on serum CK enzyme levels in rats

As shown in Figure 4, serum CK enzyme activity was reduced in rats after NaF exposure, and the difference was not significant.

Results of fluoride exposure on Ca^{2+}-ATPase activity in rat heart tissue

As shown in Figure 5, Ca^{2+}-ATPase activity was significantly reduced *($P < 0.05$)* in rat femurs after NaF exposure compared with controls.

Fluoride Exposure Leads to Altered Protein Expression Levels of KEAP1, HO-1, and Nrf2, Key Proteins of Oxidative Stress, in Myocardial Tissues

As shown in Figures 6-b and 6-c, the expression of KEAP1 and Nrf2 was significantly reduced in the NaF group compared with the control group *($P < 0.05$)*

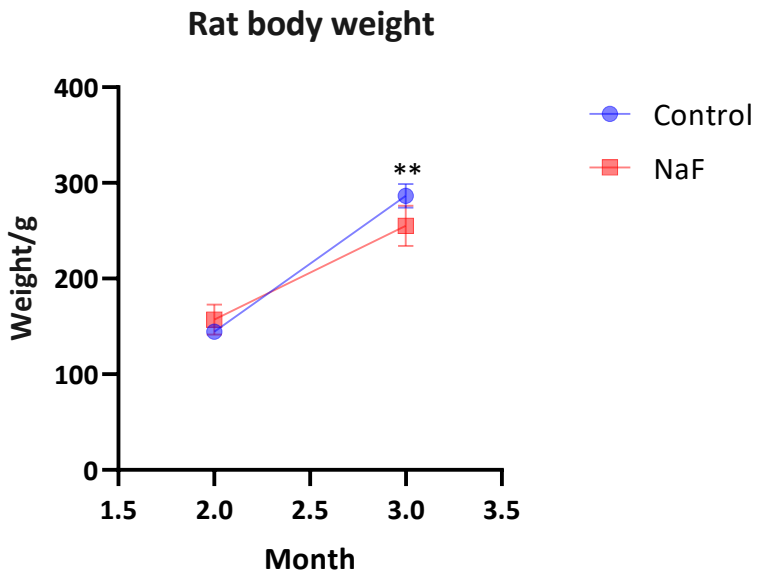

Figure 1. Changes in body weight of rats (n=4). ** indicates $P < 0.01$.

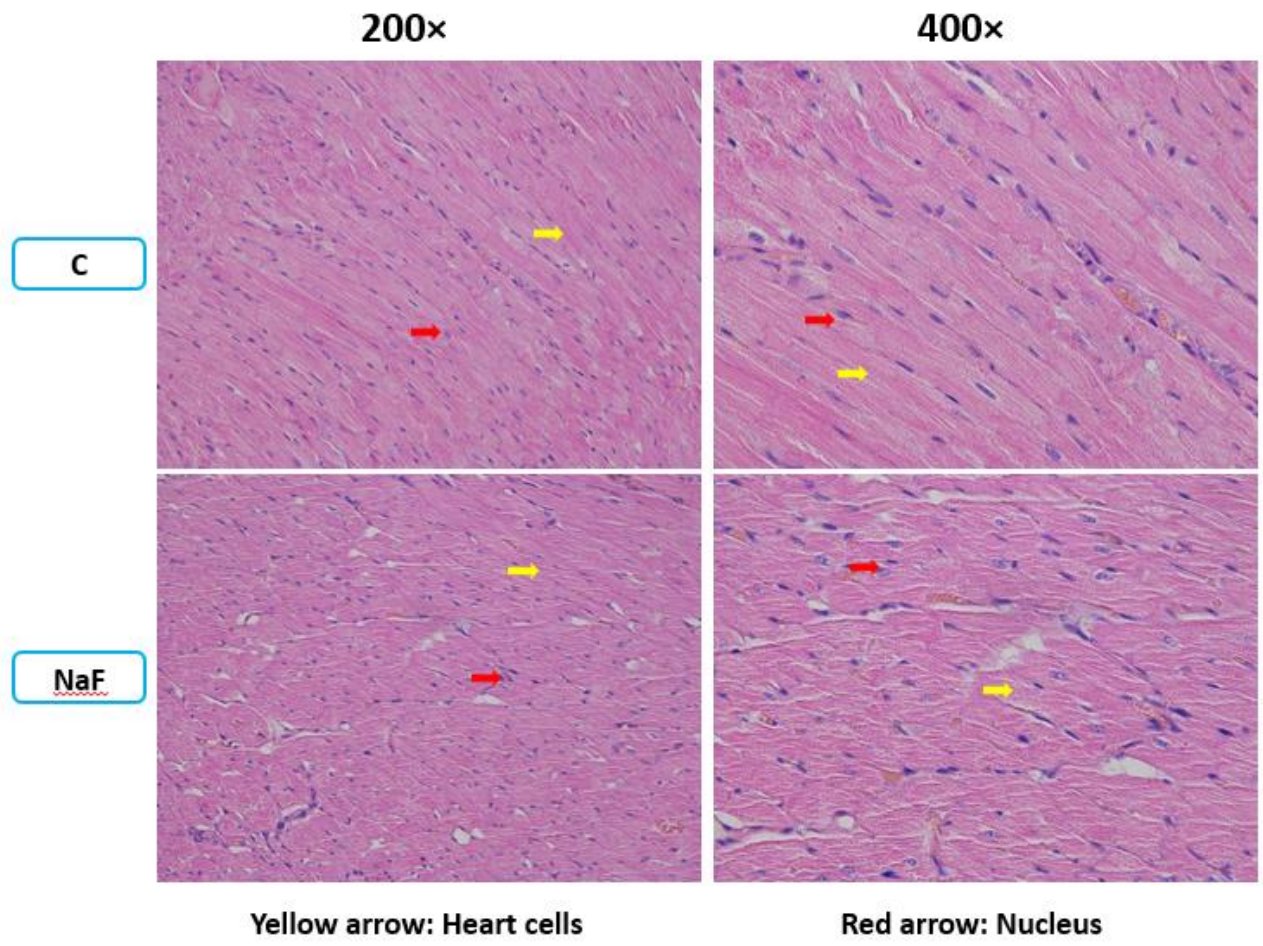

Figure 2. Fluoride exposure induces pathological alterations in myocardial tissue. (Yellow arrow: Myocardial Cells， Red arrow: Nucleus).

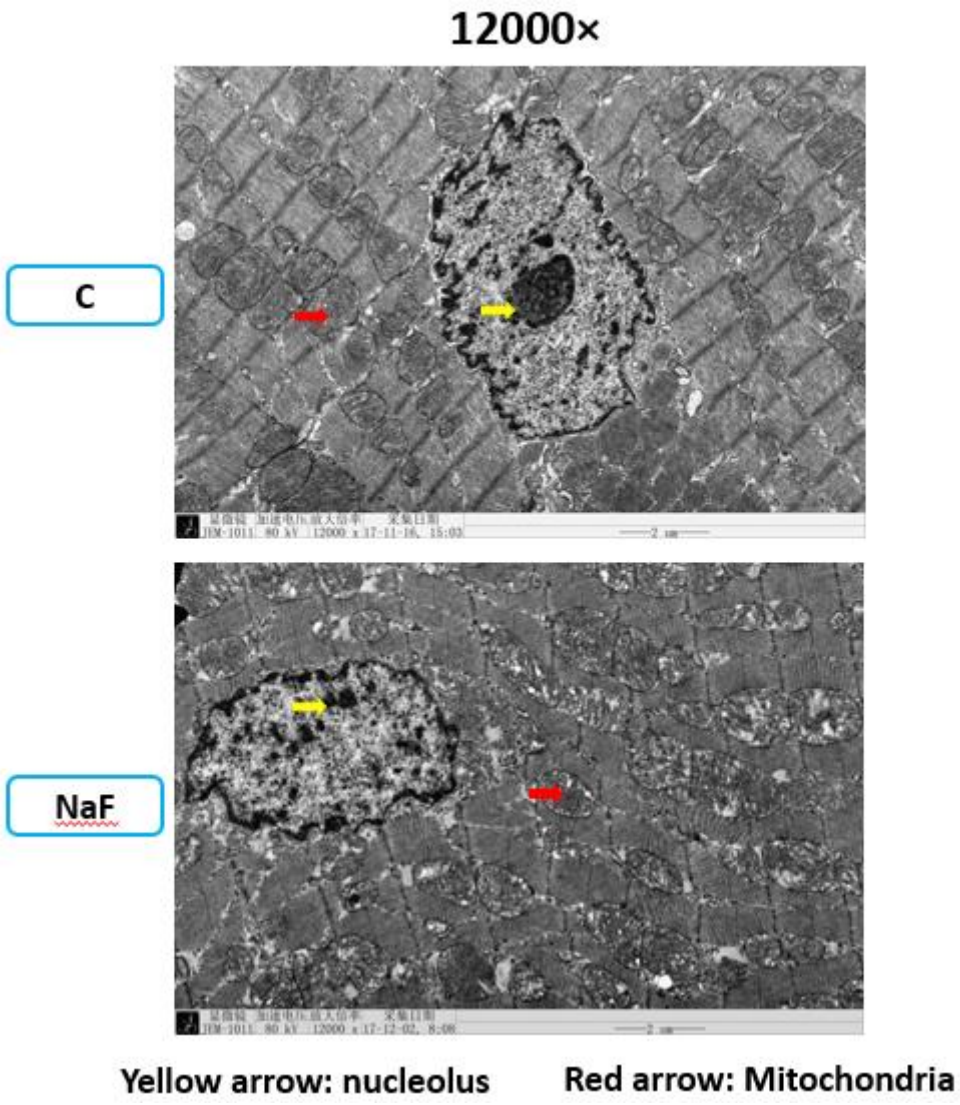

Figure 3. Fluoride exposure induces ultrastructural changes in the myocardial tissue of rats. (Yellow arrow: nucleolus， Red arrow: Mitochondria).

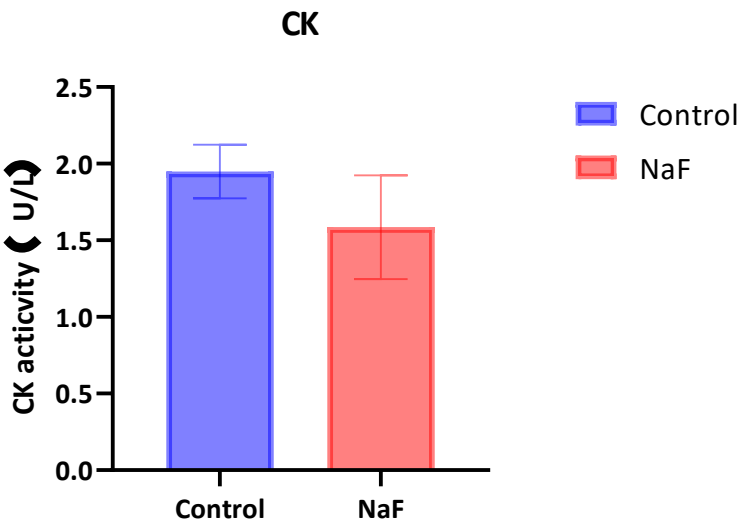

Figure 4. CK enzyme levels in rat serum (n=4).

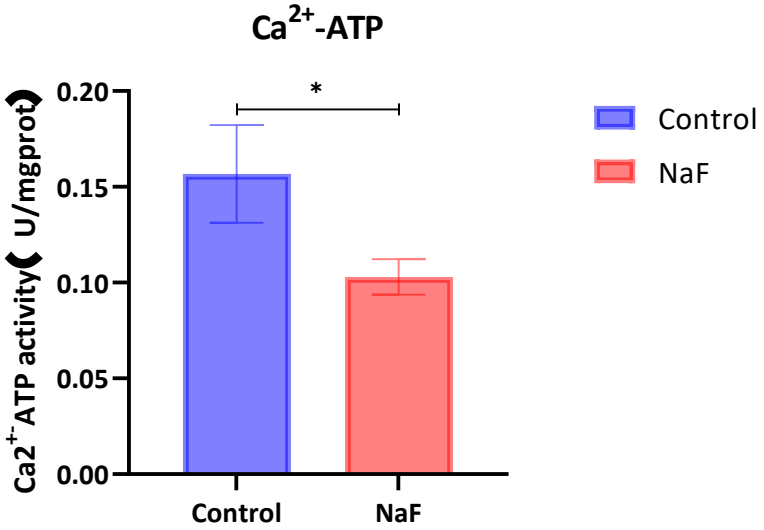

Figure 5. Ca^{2+}-ATPase content in rat myocardial tissue (n=3). * indicates $P < 0.05$.

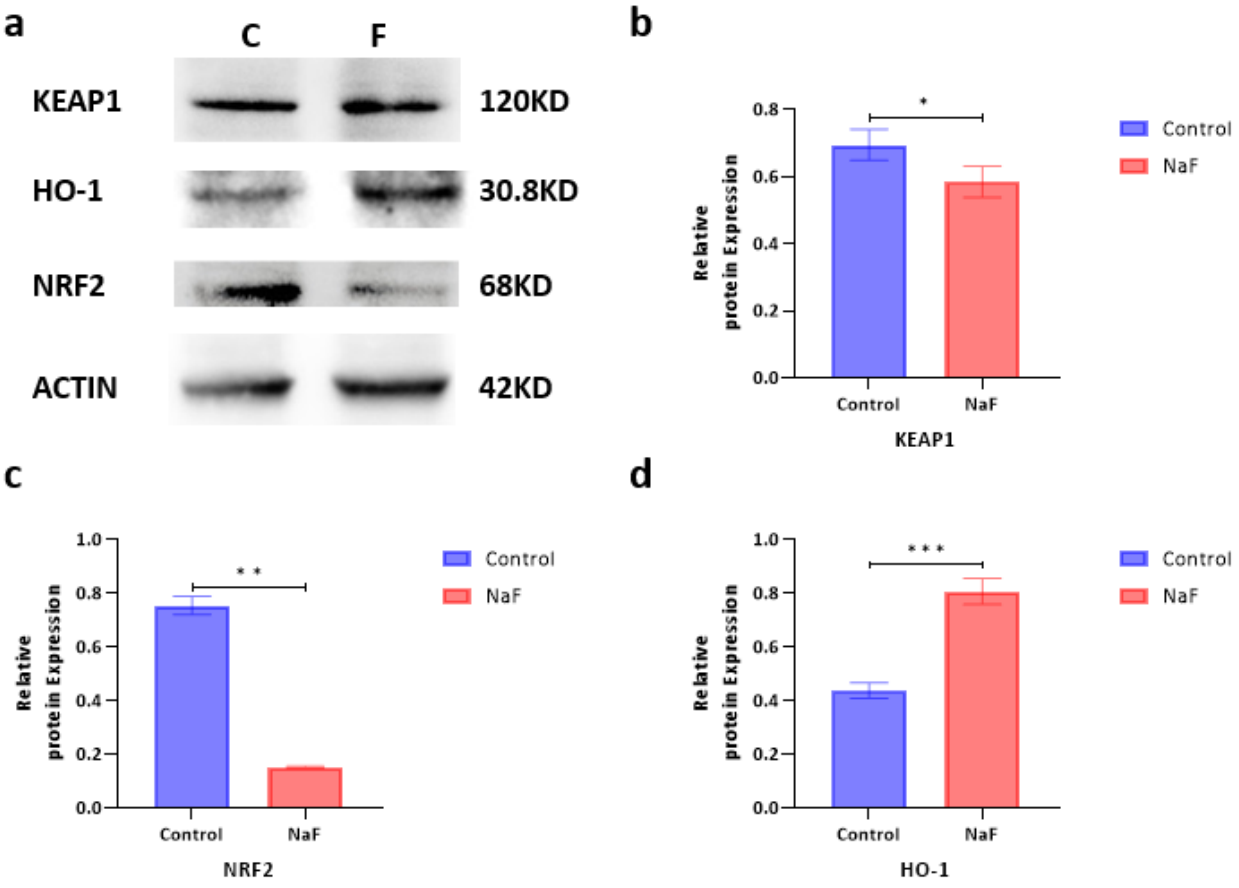

Figure 6. Changes in key proteins of oxidative stress in rat myocardial tissues. (b) KEAP1 protein level (n=3); (c) Nrf2 protein level (n=3); (d) HO-1 protein level (n=3); * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$.

DISCUSSION

Numerous studies have demonstrated that exposure to high levels of fluoride not only triggers typical skeletal damage, such as dental fluorosis and skeletal fluorosis, but also contributes to cardiovascular damage.[6]

During the morphological assessment of myocardial tissue, it was observed that exposure to fluoride resulted in edema of the myocardial cells, an increase in intercellular spaces, and uneven staining of the cell nuclei. Additionally, there was disorder in the arrangement and structure of the myofibrils. These findings suggest that fluoride exposure may induce damage to the myocardial tissue. To further investigate the mechanisms underlying this damage, we conducted a detailed examination of the ultrastructural changes in the myocardial tissue using transmission electron microscopy. The results revealed that fluoride exposure led to irregularly shaped cell nuclei, disappearance of nucleoli, and significant damage to the mitochondrial structures surrounding the nuclei. Based on these observations, it is reasonable to hypothesize that fluoride exposure may cause myocardial tissue damage through mitochondrial dysfunction.

CK is a key marker of myocardial injury, and changes in its serum levels reflect the degree of damage to cardiomyocytes. In the present study, we observed a decrease in CK enzyme activity in rat serum after NaF exposure.[24-26] Collectively, the results of the experiments reveal that NaF exposure can damage the myocardial tissue.

As indicated by experimental result 4, fluoride exposure induces mitochondrial damage in cardiomyocytes. Extensive research has demonstrated that impaired mitochondrial function significantly increases the production of reactive oxygen species (ROS), thereby triggering oxidative stress.[27] Our published research suggests that key biochemical markers, including glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), malondialdehyde (MDA) and Na^+/K^+-ATPase, among others, may exceed the threshold of the oxidative stress pathway, thereby leading to myocardial injury.[28]

Studies have shown that Ca^{2+}-ATPase is another essential biomarker for oxidative stress, with elevated ROS levels inhibit Ca^{2+}-ATPase activity.[29,30] In the present study, Ca^{2+}-ATPase activity was found to be reduced in rat myocardial tissues after NaF exposure, suggesting that oxidative stress occurs and leads to myocardial injury.

The NADPH oxidase pathway, TLR4/MAPK pathway, JAK/STAT pathway, PI3K/AKT pathway, and other signaling pathways have been implicated in the induction of oxidative stress within myocardial tissue, which subsequently contributes to myocardial injury.[31-34] Given that the Nrf2 signaling pathway plays a crucial role in orchestrating the response of cardiomyocytes to oxidative stress, we chose to investigate the protein expression levels of key pathway-related proteins, namely KEAP1, Nrf2, and HO-1. This approach was intended to elucidate the alterations in the Nrf2 signaling pathway that occur under oxidative stress conditions.[35-37] As demonstrated in Result 7, the protein expression levels of KEAP1 and Nrf2 were significantly reduced in the NaF exposed group. Under normal physiological conditions, Nrf2 binds to KEAP1 in the cytoplasm, where it is inhibited and degraded. However, under conditions of oxidative stress, the function of KEAP1 is inhibited, allowing Nrf2 to escape inhibition and be transported to the nucleus. In some cases, the rate of Nrf2 degradation may exceed its synthesis, resulting in a decrease in protein levels.[38] A substantial augmentation in the expression level of HO-1 protein downstream of the Nrf2 signaling pathway during the onset of oxidative stress has been demonstrated to facilitate the neutralisation of ROS and the attenuation of the damage caused by oxidative stress to cardiomyocytes.[37] This finding is consistent with the results of our study, as illustrated in Result 7 (Figure d).

CONCLUSIONS

Based on the aforementioned findings, it is reasonable to infer that fluoride exposure induces oxidative stress in cardiac mitochondria, thereby causing myocardial injury. The Nrf2 oxidative stress signaling pathway is likely to play a pivotal role in this process.

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CONFLICT OF INTERESTS

None

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