

## FLUORIDE INDUCES APOPTOSIS IN MC3T3-E1 OSTEOBLASTS BY ALTERING ROS LEVELS AND MITOCHONDRIAL MEMBRANE POTENTIALS

Xiaoyan Yan,<sup>a</sup> Xiaolin Tian,<sup>a</sup> Lu Wang,<sup>a</sup> Wenli Zhou,<sup>b</sup> Wenyan Zhang,<sup>c</sup>  
Yi Lv,<sup>a</sup> Yulan Qiu,<sup>a</sup> Tong Wang<sup>a,\*</sup>

Taiyuan, Shanxi Province, and Guangzhou and Shenzhen, Guangdong Province,  
People's Republic of China

**ABSTRACT:** This research investigated the relationship between the levels of reactive oxygen species (ROS), mitochondrial membrane potentials, and apoptosis in MC3T3-E1 osteoblasts (OB) by administering varying concentrations of the fluoride ion. Sodium fluoride (NaF), at concentrations of 0, 1, 5, 10, and 30 mg/L, was administered to cultured OB. Hematoxylin and eosin (HE) and 4',6-diamidino-2-phenylindole (DAPI) staining were used to observe the effects of fluoride on OB apoptosis. In addition, the ROS levels and the mitochondrial membrane potentials of the OB were analyzed with a Fluorescence Activated Cell Sorter (FACS) using labeling probes DCFH-DA and JC-1 after fluoride treatment for 72 hr. There were pronounced negative effects on OB survival from long-term NaF treatment. These negative effects included apoptosis, increased ROS levels, and decreased mitochondrial membrane potentials. Our results demonstrated that fluoride-induced OB apoptosis is mediated by the direct effects of fluoride on ROS levels and mitochondrial membrane potentials.

Keywords: Apoptosis; Fluoride; ME3T3-E1 osteoblasts; Mitochondrial membrane potential; Reactive oxygen species.

### INTRODUCTION

The element fluorine is widely distributed in nature and reacts with most metal elements to generate fluoride compounds. The topical use of the fluoride ion helps to reduce tooth decay, but in excess, it is known to be toxic to both animals and humans. Fluorosis is a widespread endemic disease that is a major public health concern in the People's Republic of China, occurring in 29 provinces, municipalities, and autonomous regions. Fluoride can cause damage to multiple organs and tissues,<sup>1-3</sup> especially to the skeleton and teeth. Fluorosis is characterized by tooth discoloration and skeletal manifestations, such as crippling, osteoporosis, and osteosclerosis.<sup>4-6</sup>

Several investigations have suggested that fluoride could induce oxidative stress and modulate intracellular redox homeostasis, protein carbonyl content, and lipid peroxidation,<sup>7</sup> as well as alter gene expression and cause apoptosis.<sup>4,8</sup> Excessive fluoride may induce high levels of oxidative stress, which might be important in the pathogenesis of chronic fluorosis.<sup>8</sup>

Cell apoptosis can be initiated by the death receptor-mediated pathway or the mitochondria-mediated pathway.<sup>4,9</sup> Mitochondria are the key intracellular targets

---

<sup>a</sup>School of Public Health, Shanxi Medical University, Taiyuan 030001, Shanxi, People's Republic of China; <sup>b</sup>School of Life Science, Key Lab of Ecology and Environmental Science in Higher Education of Guangdong Province, South China Normal University, Guangzhou 510631, Guangdong, People's Republic of China; <sup>c</sup>Shenzhen University, Shenzhen 518060, Guangdong, People's Republic of China; \*For correspondence: Tong Wang, School of Public Health, Shanxi Medical University, Taiyuan 030001, Shanxi, People's Republic of China; E-mail: tongwang@sxmu.edu.cn; Phone: 86-351-4135397.

for different stressors, including fluoride. It is known that mitochondria are the major source of reactive oxygen species (ROS) production and the toxicity of fluoride is associated with ROS induction and reduction of cellular antioxidant defenses against oxidative damage. Failure to restore mitochondrial homeostasis and function can lead to a progressive increase in ROS and the ensuing oxidative stress.

The role of oxidative stress, ROS in particular, in the induction of apoptosis seems to be concentration-dependent. Many works have concluded that fluoride induces apoptosis by elevating oxidative stress-induced lipid peroxidation, thus causing mitochondrial dysfunction and the loss of mitochondrial membrane potential ( $\Delta\psi_m$ ).<sup>10</sup>

However, the relationship between fluoride-induced and mitochondria-mediated apoptosis in OB has not been addressed. In this study, we explored the intracellular mechanisms of fluoride-induced cell apoptosis in mouse osteoblastic MC3T3-E1 cell lines. Furthermore, we investigated whether mitochondria are involved in the apoptosis process.

#### MATERIALS AND METHODS

*Reagents:* MC3T3-E1 cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Biological Engineering Material Company (Hangzhou, China). Dulbecco's Modified Eagle Medium (DMEM) and trypsin were obtained from Gibco (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO), EDTA, and the Cell Proliferation Kit, with thiazolyl blue tetrazolium blue (MTT) for assessing mitochondrial function, were purchased from Sigma (St. Louis, MO, USA). The ROS Assay Kit, the Mitochondrial Membrane Potential Assay Kit with JC-1, and the 4',6-diamidino-2-phenylindole (DAPI) Assay Kit were obtained from Beyotime Biological Engineering Material Company (Shanghai, China).

*Cell culture:* Osteoblastic MC3T3-E1 cells (ATCC, subclone-14, China) were cultured in DMEM with 10% FBS at 37 °C in a 5% CO<sub>2</sub> incubator. Cells were subcultured every 3 days using 0.2% trypsin, plus 0.02% EDTA. For the experiments described below, cells were seeded in 96-well plates for cell proliferation studies or in 24-well plates for apoptosis, intracellular ROS, and mitochondrial membrane potential expression.

*MTT assay:* To determine the effects of fluoride on cell proliferation, OB were cultured in the presence of increasing concentrations of sodium fluoride (NaF; 0, 1, 5, 10, and 30 mg/L) for 24, 48, 72, and 96 hr. The viability of cells was evaluated by MTT assay. At the end of each treatment period, MTT stock solution was added (1 part to 10 parts medium) to the cells in each well of a 96-well tissue culture plate and incubated at 37 °C for 4 hr. Later, 100  $\mu$ L of formazan dissolving 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.

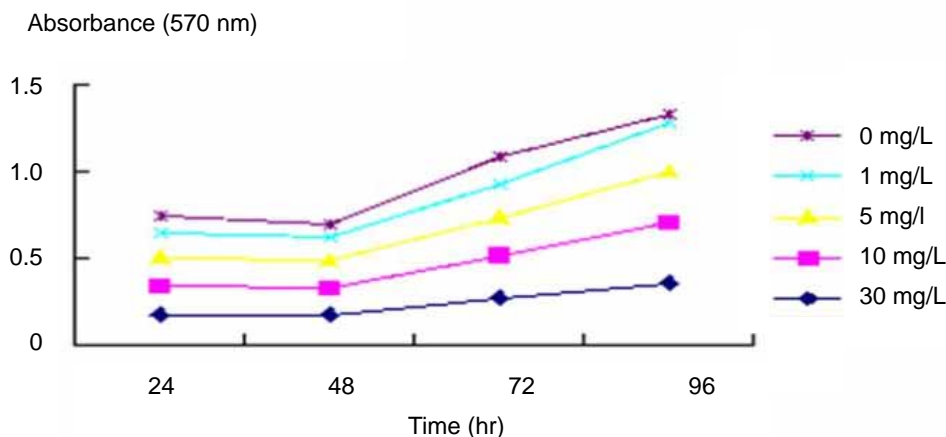
*Detection of intracellular ROS levels:* After treatment for 72 hr with NaF, to determine the ROS generation within the fluoride-treated cells, fluorescence-activated cell sorting (FACS) analysis was performed. Cells were stained with 5  $\mu\text{g}/\text{mL}$  2,7-dichlorofluorescein-diacetate (DCF-DA) for 30 min, subjected to flow cytometry using a Becton-Dickinson FACS Caliber, and analyzed by Cell Quest software (Becton-Dickinson, San Jose, CA).

*Detection of intracellular mitochondrial membrane potential levels:* To determine mitochondrial membrane potentials ( $\Delta\psi\text{m}$ ) in OB after fluoride treatment for 72 hr, FACS analysis was performed according to the instructions provided. The cells were stained with 0.5 mL JC-1 (50 $\times$ ) work buffer for 20 min in 37  $^{\circ}\text{C}$  in order to load the JC-1 probe, then subjected to flow cytometry using a Becton-Dickinson FACS Caliber, and analyzed by Cell Quest software (Becton-Dickinson, San Jose, CA).

*Statistical Analysis:* Data is expressed as mean $\pm$ standard error (SE). One-way analysis of variance (ANOVA) was performed by SPSS 17.0 statistical software (SPSS Inc., USA).  $p < 0.05$  was considered statistically significant.

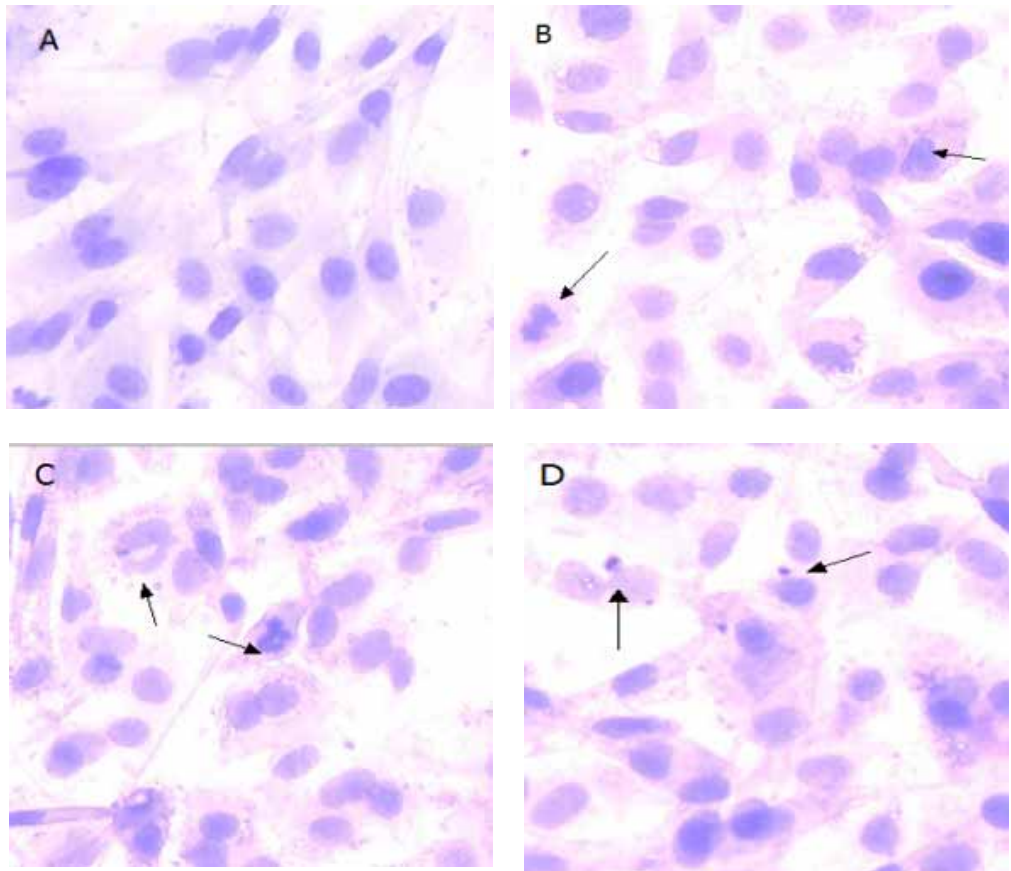
## RESULTS

*Effects of NaF treatment on OB proliferation:* MTT assays were performed to determine if fluoride treatment influences OB proliferation during 24–96 hr of culture. As depicted in Figure 1, the numbers of viable OB were slightly affected (relative to control) following 48 hr exposure to 1 mg/L NaF, but the increase was not statistically significant ( $p > 0.05$ ). However, higher concentrations of NaF (10 and 30 mg/L) suppressed OB proliferation through 72 hr of culture ( $p < 0.01$ ). Cell proliferation was reduced following culture of OB for 72 hr and 96 hr in the presence of all concentrations of NaF tested ( $p < 0.05$ ; Figure 1). These results supported the proposal that cultures with higher concentrations of fluoride produced more potent cytotoxic effects for longer amounts of time.



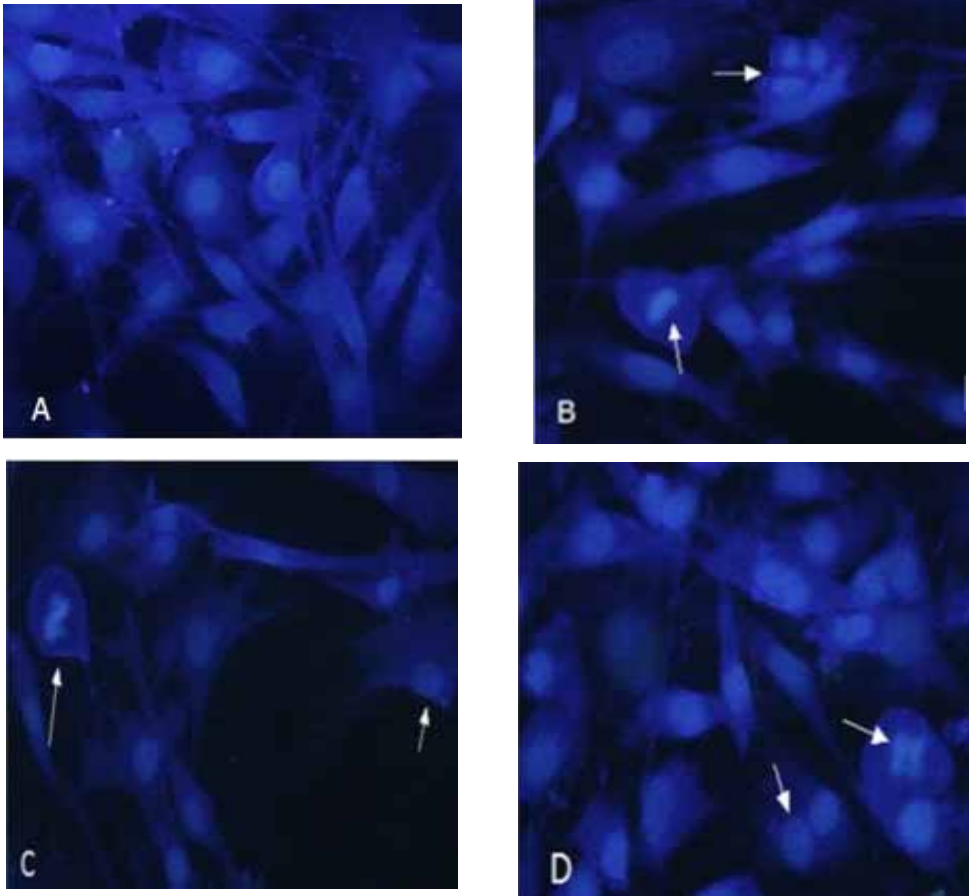
**Figure 1.** Effects of sodium fluoride treatment on MC3T3-E1 proliferation.

*Effect of NaF on OB cell morphology:* HE staining clearly showed an increase in the classical morphological characteristics of apoptosis in the fluoride-treated groups (Figures 2A, 2B, 2C, and 2D). Oval nuclei and obvious cytoplasm were observed in the normal OB cells without fluoride treatment (Figure 2A). After 24 hr in the culture, cells adhered to the flasks and showed the classical morphological characteristics of apoptosis, including spindle, triangle, or polyangular shapes as revealed by HE staining (Figures 2B, 2C, and 2D). Increasing the concentrations of NaF causes more significant apoptotic cell characteristics, including chromatin condensation, marginalization, nuclear membrane lysis, chromatin divided into blocks, and apoptotic bodies.



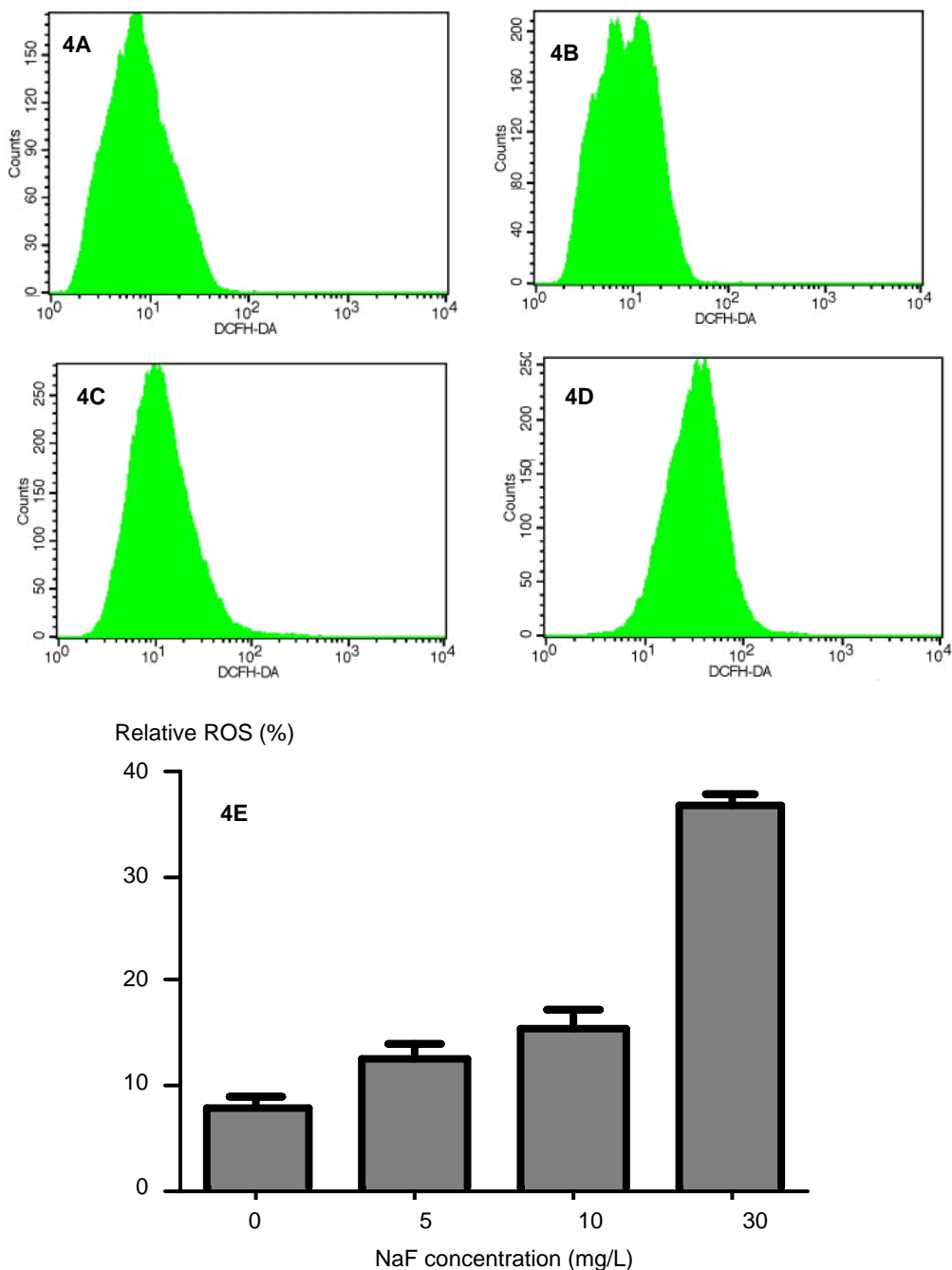
**Figures 2A, 2B, 2C, and 2D.** HE staining morphological of the MC3T3-E1 cells. 2A: control group; 2B, 2C, and 2D: NaF-treated groups.

*Detection of the effect of NaF on OB apoptosis:* As can be seen in Figure 3A, our results indicated that the control groups' OB nuclei were oval and showed blue fluorescence. Abnormal nucleus morphology was observed in all the NaF-treated groups (Figures 3B, 3C, and 3D). The chromatin appeared to be in a concentrated state and was highly condensed and marginalized, the DNA was fragmented, and nucleus cleavage fragments were present, resulting in apoptotic bodies.



**Figures 3A, 3B, 3C, and 3D.** DAPI staining morphological of the MC3T3-E1 cells. 3A: control group; 3B, 3C, and 3D: NaF-treated groups.

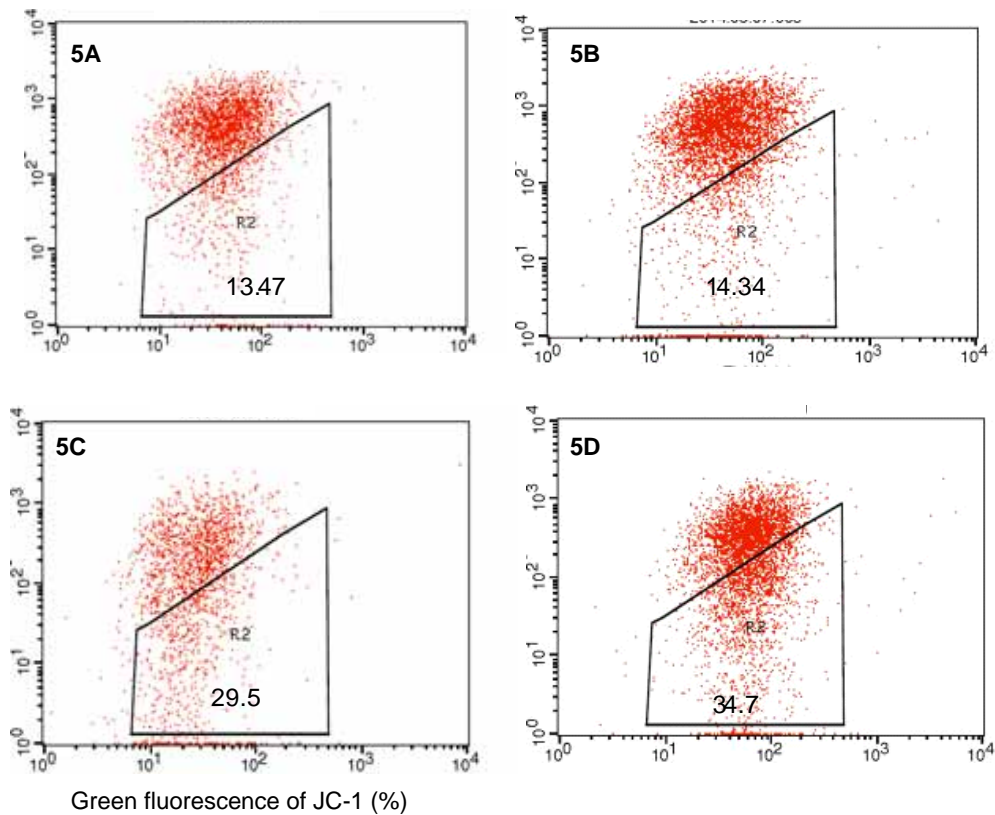
*Effects of NaF on the ROS Levels in the MC3T3-E1 cells:* We measured the intracellular ROS levels in the MC3T3-E1 cells with DCF-DA staining. As can be seen in Figures 4A, 4B, 4C, 4D, and 4E, our results indicate that ROS production increased after fluoride treatment.



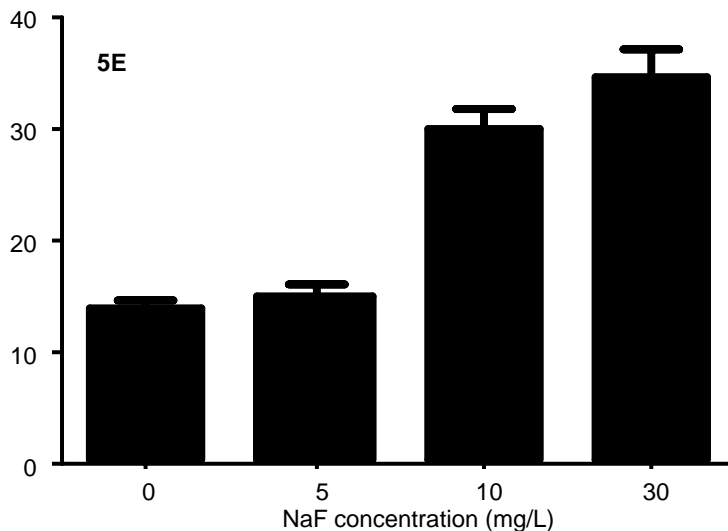
**Figures 4A, 4B, 4C, 4D, and 4E.** Effects of NaF on the ROS level in the MC3T3-E1 cells. 4A: control group 0 mg NaF/L; 4B: 5 mg NaF/L; 4C: 10 mg NaF/L; 4D: 30 mg NaF/L; 4E: vertical bar graph of the relative ROS (%).

*Effects of NaF on the mitochondrial membrane potential in the MC3T3-E1 cells:*  
The R2 zone in each picture shows the green fluorescence of OB; a higher proportion of this zone indicates a lower  $\Delta\psi_m$  and number of normal OB. As can

be seen in Figures 5A, 5B, 5C, 5D, and 5E, our results indicated that  $\Delta\psi_m$  decreased after NaF treatment.



Green fluorescence of JC-1 (%)



**Figures 5A, 5B, 5C, 5D, and 5E.** Effects of NaF on the  $\Delta\psi_m$  level in the MC3T3-E1 cells. 5A: control group 0 mg NaF/L; 5B: 5 mg NaF/L; 5C: 10 mg NaF/L; 5D: 30 mg NaF/L; 5E: vertical bar graph of the green fluorescence of JC-1 (%).



## DISCUSSION

Metabolic, functional, and structural damage caused by chronic fluorosis have been reported in many tissues.<sup>11-14</sup> Research data strongly suggested that fluoride inhibits protein secretion and/or synthesis and that it influences distinct signaling pathways involved in proliferation and apoptosis.<sup>13</sup> In the present study, we described the toxic effects of NaF treatment in MC3T3-E1 cells. The observations clearly demonstrated that high doses of NaF inhibited cell proliferation and induced cell apoptosis, which were confirmed by cellular and molecular evidence. The cytotoxicity assays employed were MTT assay, HE staining, and DAPI staining. The MTT assay showed that decreased NaF exposure (<1 mg/L) had stimulation functions to promote OB cell proliferation. Our results indicated that a higher-level of NaF treatment (>5 mg/L) induced obvious apoptosis by triggering signal pathways. This apoptosis can be observed from the morphology of the HE and DAPI staining on OB cells after fluoride treatment. The results showed that there are significant apoptotic cell characteristics, including chromatin condensation, marginalization, nuclear membrane lysis, and blocked chromatin.

Mitochondria represent key organelles for cell survival and their role in programmed cell death has been known for several years.<sup>15,16</sup> Mitochondria dysfunction is considered an early event in apoptosis, which could cause oxidative stress and decreased  $\Delta\psi_m$ .

The role of oxidative stress in general, and of ROS in particular, in the induction of apoptosis seems to be concentration-dependent and many studies have concluded that fluoride induces apoptosis by elevating oxidative stress-induced lipid peroxidation, thus causing mitochondrial dysfunction and the activation of downstream pathways.<sup>17,18</sup> In the present study, we hypothesized that the fluoride-induced apoptosis response in MC3T3-E1 cells was through ROS generation. Cells have a number of mechanisms of protection against oxidative damage, including direct interaction with anti-oxidants.

Mitochondria are conspicuous producers of ROS, such as superoxide radical, hydrogen peroxide, hydroxyl radical, and singlet oxygen, under pathological as well as normal physiological conditions.<sup>10</sup> Concurrently, these organelles are also the key targets of ROS. The rate of mitochondrial ROS generation has a significant positive correlation to the steady-state levels of oxidative stress to mtDNA.<sup>19</sup>

ROS also damaged mitochondrial proteins and lipids, thereby upsetting consistent mitochondrial functional and structural attributes. This inhibition resulted in an elevation in the production of ROS, thereby causing damage to mitochondrial DNA, lipids, and proteins and a collapsing of the  $\Delta\psi_m$ , which opens up the mitochondrial permeability transition pores, and leads to the release of pro-apoptotic proteins into the cytoplasm. An impairment of mitochondrial function leading to a decrease in  $\Delta\psi_m$  and the consequent permeability transition are considered hallmarks of apoptosis.<sup>20</sup> Thus, it is possible to conceive that, even



in the models in which mitochondrial function is involved, a decrease in  $\Delta\psi_m$  would be an ancillary event of the apoptotic process. In the present study, we observed that  $\Delta\psi_m$  decreased with high levels of fluoride concentration by JC-1 staining and FCM analysis. We hypothesized that the decreased  $\Delta\psi_m$  was related to the increase of ROS, and that both were responsible for the fluoride-induced apoptosis.

### CONCLUSIONS

In conclusion, our study suggested that the mitochondrial pathway is involved in apoptosis induced by fluoride in MC3T3-E1 cells. Fluoride might initiate the generation of ROS in the mitochondrial membrane and cause the formation of membrane pores, which leads to the decrease in the  $\Delta\psi_m$ . However, the specific molecular mechanism of this pathway remains controversial.

### ACKNOWLEDGEMENT

This research was sponsored by the China National Natural Science Foundation (Grant Nos. 31240009, 31302158, 81473073), the Postdoctoral Science Foundation of China (2016M600199), the PhD Start-up Fund of Shanxi Medical University (BS03201647), and the Shanxi Scholarship Council of China (Grant No. 2016–056).

### CONFLICTS OF INTEREST

There are no conflicts of interest.

### ETHICAL STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

### REFERENCES

- 1 Dhar V, Bhatnagar M. Physiology and toxicity of fluoride. *Indian Journal of Dental Research* 2009;20(20):350-5.
- 2 Perumal E, Paul V, Govindarajan V, Panneerselvam, L. A brief review on experimental fluorosis. *Toxicol Lett* 2013;223(2):236-51.
- 3 Wang H, Zhao W, Tan P, Liu J, Zhao J, Zhou B. The MMP-9/TIMP-1 system is involved in fluoride-induced reproductive dysfunctions in female mice. *Biol Trace Elem Res* 2017; DOI 10.1007/s12011-016-0929-3.
- 4 Barbier O, Arreola-Mendoza L, Del Razo L M. Molecular mechanisms of fluoride toxicity. *Chemico-Biological Interactions* 2010;188(2):319-33.
- 5 Yu J, Gao Y, Sun D. Effect of fluoride and low versus high levels of dietary calcium on mRNA expression of osteoprotegerin and osteoprotegerin ligand in the bone of rats. *Biol Trace Elem Res* 2013;152(3):387-95.
- 6 Yan X, Yan X, Morrison A, Han T, Chen Q, Li J. Fluoride induces apoptosis and alters collagen I expression in rat osteoblasts. *Toxicol Lett* 2011;200(3):133-8.
- 7 Shivashankara A R, Shivarajashankara Y M, Bhat P G, Rao S H. Lipid peroxidation and antioxidant defense systems in liver of rats in chronic fluoride toxicity. *Bulletin of Environmental Contamination and Toxicology* 2002;68(4):612-6.

- 8 Varol E, Varol S. Effect of fluoride toxicity on cardiovascular systems: role of oxidative stress. *Arch Toxicol* 2012;86(10):1627.
- 9 Crow M T, Mani K, Nam Y J, Kitsis R N. The mitochondrial death pathway and cardiac myocyte apoptosis. *Circ Res* 2004;95(95):957-70.
- 10 Ly J D, Grubb D R, Lawen A. The mitochondrial membrane potential ( $\Delta\psi_m$ ) in apoptosis; an update. *Apoptosis* 2003;8(2):115-28.
- 11 Cheng RY Nie QL, Sun HF, Zhang YJ, Wu LH, Ma YG, Yan XY. Fluoride-induced oxidative stress in rat myocardium through the Bax/Bcl-2 signalling pathway. *Fluoride* 2013;46(4):198-203.
- 12 Ge Y, Niu R, Zhang J, Wang J. Proteomic analysis of brain proteins of rats exposed to high fluoride and low iodine. *Arch Toxicol* 2011;85(1):27-33.
- 13 Song G H, Gao J P, Wang C F, Chen C Y, Yan X Y, Guo M. Sodium fluoride induces apoptosis in the kidney of rats through caspase-mediated pathways and DNA damage. *Journal of Physiology and Biochemistry* 2014;70(3):857-68.
- 14 Sun Z, Niu R, Wang B, Jiao Z, Wang J, Zhang J. Fluoride-induced apoptosis and gene expression profiling in mice sperm in vivo. *Arch Toxicol* 2011;85(11):1441-52.
- 15 Green D R, Kroemer G. The pathophysiology of mitochondrial cell death. *Science* 2004;305(5684):626-9.
- 16 Green D R, Reed J C. Mitochondria and apoptosis. *Journal of Anhui Institute of Education* 1998;252(1):1-15.
- 17 Mukhopadhyay D, Chattopadhyay A. Induction of oxidative stress and related transcriptional effects of sodium fluoride in female zebra fish liver. *Bulletin of Environmental Contamination and Toxicology* 2014;93(1):64-70.
- 18 Yang Y, Huang H, Yuan W, Feng D, Wang Z, Cheng X. Effects of fluoride on oxidative stress and apoptosis in primary culture of rat sertoli cells. *Wei Sheng Yan Jiu (Journal of Hygiene Research)* 2013;42(6):1004-7.
- 19 Chomyn A, Attardi G. MtDNA mutations in aging and apoptosis. *Biochemical & Biophysical Research Communications* 2003;304(3):519-29.
- 20 Kroemer G, Zamzami N, Susin S A. Introduction: mitochondrial control of apoptosis. *Biochimie* 2002;84(2–3):103-4.