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# Exercise Mitigates Apoptosis Induced by Lipid Peroxidation in Ameloblasts of Fluorosis Mice

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## ABSTRACT

**Purpose:** Excessive fluoride ingestion induces dental fluorosis that is generated by oxidative stress-mediated apoptosis. Oppositely, regular exercise has been shown to ameliorate oxidative damages and alleviate cell apoptosis in various diseases. However, little attention is paid to the effect of exercise on dental fluorosis. Thus, we employed the exercise regime including forced treadmill running (FTR) in fluorosis mice to explore the effect.

**Methods:** Briefly, three-week-old Institute of Cancer Research (ICR) mice drinking water contained 100 mg/L sodium fluoride (NaF) and 0 mg/L NaF were compulsively submitted to treadmill running for 12 months.

**Results:** Results showed that 12 months of fluoride exposure markedly elevated the incisors fluorine concentrations. FTR restrained the elevation of cross-linked C-terminal telopetide of type I collagen (CTX-I) concentration in urine from mice after 12 months of fluoride exposure. The slightly reduced contents of reactive oxygen species (ROS), malondialdehyde (MDA) and observably enhanced activities of catalase (CAT), glutathione peroxidase (GSH-Px), total superoxide dismutase (T-SOD) and total antioxidant capacity (T-AOC) were found in the incisors of fluoride-toxic mice subjected to FTR. Furthermore, FTR restrained fluoride-caused ameloblasts apoptosis induced by MDA in incisors through reducing the mRNA expressions of p38 mitogen-activated protein kinase (p38MAPK), c-Jun N-terminal kinase (JNK), IkappaB kinase-beta (IKK-β), Caspase-3 and enhancing the mRNA expression of B-cell lymphoma 2 (Bcl-2), superoxide dismutase 1 (SOD1).

**Conclusions:** Above results suggested that regular exercise may be a convenient therapy for dental fluorosis.

***Key-words:*** *Exercise; Fluoride; Ameloblasts; Lipid peroxidation; Apoptosis.*

## INTRODUCTION

Fluorine generally exists in soil, water and minerals as fluorides in association with other chemical elements.[1] A low level of fluoride has been extensively added in mouth wash, drinking water and toothpaste for the prevention of dental caries. However, the boundary between efficacy and toxicity is narrow and chronic over-exposure to fluorine compounds can produce pathophysiological changes, affecting bone, teeth and various soft tissues (skeletal, dental, and nonskeletal fluorosis).[2] Among people and livestock,

beyond 1.5 mg/L of fluoride in drinkable water can cause enamel fluorosis in the phase of the enamel formation.[3,4] Over the last three decades, the morbidity rate of dental fluorosis in the United States of America (USA) has risen from 22% to 65%.[5] Additionally, more than 10 million people suffer from dental fluorosis in China, and approximately 50% of Chinese children are impacted by dental fluorosis in villages.[6] Hence, detecting the etiopathogenesis of dental fluorosis and adopting dependable prevention strategies are crucial.

Dental fluorosis is a pathological developmental deficiency of tooth enamel arising from the absorption of an excessively high level of fluoride during pivotal phases of amelogenesis.[7] The three crucial phases in the ameloblast differentiation: secretory, transition, and maturation, which are equivalent to different stages in enamel formation.[8] Excessive fluoride results in dental fluorosis primarily through impairment to ameloblasts.[9] Our laboratory and others discovered that excess fluoride induces oxidative stress in diverse rodent inner cells containing sperms and ameloblasts,[10,11] followed by ameloblast apoptosis. Oxidative stress is a disbalance between the emergence of reactive oxygen species (ROS) and the capacity of antioxidants to obliterate them.[11,12] Massive ROS could easily impair cell membranes and organelle membranes that possessed affluent polyunsaturated fatty acids (PUFAs), namely "lipid peroxidation". Lipid peroxides could activate signaling pathways such as mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-κB), and Caspase pathways related to apoptosis.[13,14] Prior reports have shown that high doses of fluoride restrains antioxidant activity,[15] leading to excess generation of ROS, making for lipid peroxidation, which causes apoptosis in ameloblasts by the activation of Caspase signaling pathways.

Suitable exercise can provide resistance to various diseases such as cancers, diabetes, and cardiovascular disease.[16] Diverse exercise parameters including intensity, duration, and type can all affect the ability of exercise to combat diseases. For instance, energetic jogging decreased body fat, body weight, and blood pressure in the sick, whereas medium intensity exercise did not have this influence.[17] Regular moderate exercise interventions has been proven to mitigate oxidative stress[16] via elevating antioxidant enzyme activity, repressing ROS generation and reducing lipid peroxides. In addition, exercise can enhance anti-apoptotic markers and shorten pro-apoptotic markers. To date, exercise ameliorates the detrimental effect of fluoride on central nervous system[18] and insulin-glucose system,[19] and current research from our group discovered that treadmill exercise alleviates fluoride-induced neurotoxicity, hepatic and renal toxicity, gastrointestinal toxicity, and bone injury in mice.[20-25] However, there are few reports that fluoride-caused oxidative stress and subsequent ameloblast apoptosis can be rescued via exercise.

Therefore, in this research, we formulated an exercise regimen containing forced treadmill running (FTR) in fluorotic mice, and detected the effects of exercise on fluoride-induced variations in histological structure, oxidative stress markers, and genes associated with apoptosis in incisors of mice. The aim of the study was to examine the effect of exercise on dental fluorosis resulting from fluoride-induced oxidative stress causing apoptosis and the possibility that this might lead to the recommendation of a suitable method for the prevention or treatment of dental fluorosis.

## MATERIAL AND METHODS

***Animals:*** Three-week old female and male Institute of Cancer Research (ICR) mice and the required normative diet were offered by the Experimental Animal Center of Shanxi Medical University (Taiyuan, Shanxi, China). All mice were kept in animal houses at regulatory temperature (22-23 °C), relative humidity (55-60%) on a 12 h/12 h light-dark circle and received water and chow ad libitum. Following one-week acclimation, all experiments including fluoride treatment, forced treadmill running (FTR), and so on were conducted under the approval of animal ethics committee in Shanxi Agricultural University (No. SAXU-EAW-2021M0429)(Jinzhong, Shanxi, China).

***FTR administration and fluoride treatments:*** Sixty mice were equally and randomly assigned to four groups: group control (C), mice were provided water with 0 mg/L sodium fluoride (NaF);

group fluoride (F), mice were provided water with 100 mg/L NaF, according to the $LD_{50}$ of fluorine ion[26] and the pharmacokinetics of fluoride in the mouse and human;[27] group exercise (E), mice were provided water with 0 mg/L NaF and submitted to FTR; group fluoride plus exercise (F+E), mice were provided water with 100 mg/L NaF and submitted to FTR. The FTR protocol in present experiment based on the researches of Daniele et al. (2017)[28] and Zhao et al. (2018)[29], with proper modifications. The FTR intensity of current experiment was a moderate exercise intensity of long time aerobic exercise. During the FTR experiment, the adaptive FTR conducted on the treadmill (SANS Biological Technology Co. Ltd, Jiangsu, China) of 0° inclination at the speed of 5 m/min for 10 min during the first week. After the finish of adaptive phase, the formal FTR, the mice from group E and group F+E were exercised for 30 min/day and 5 days/week, first 5 min at the speed of 5 m/min was the warm-up stage; middle 20 min at the speed of 10 m/min was the training stage; last 5 min at the speed of 5 m/min was cool-down stage. Until the fifth week of the formal FTR, the speed of training stage was adjusted to 12 m/min for the middle 20 min. After 12 months of FTR and fluoride exposure,[30,31] mice were sacrificed and their urine, incisors were obtained and preserved in -80 °C. Fractional mice incisors fixed in 4% paraformaldehyde were detected by the morphological observation methods.

***Incisors fluorine determination:*** The upper incisors were removed from mice head and separated of total soft tissue. Incisors were desiccated at 105 °C for 4 h, and then weighed. The desiccative incisors were next incinerated at 550 °C for 5 h. Weighed approximately 20 mg of incisors powder deliquesced overnight with 0.25 mol/L hydrochloric acid (HCl) followed by the neutralization of 0.5 mol/L sodium hydrate (NaOH) was intermingled with same volume of total ion strength adjustment buffer (TISAB). The fluorine level in upper incisors from mice and fluoride standards was determined with the fluoride ion-selective electrode potentiometric method.

***Bone resorption marker and oxidative damage parameters analysis:*** When the mouse models were established, their urine and lower incisors were obtained and preserved at -80 °C until analysis. The lower incisors supernatant was collected after homogenate for further measure. The levels of cross-linked C-terminal telopeptide of type I collagen (CTX-I) possessed predictive value in teeth properties[32] in urine and the contents of ROS, MDA in lower incisors homogenate supernatant were detected employing the commercial enzyme-linked immunosorbent assay (ELISA) kits (MEIMIAN, Jiangsu, China), following the experimental kit specifications.

***Biochemical assays:*** The incisors of mice were ground applying the autoclave mortars and pestles. Subsequently, milled incisors were homogenized in ice-cold 0.9% normal saline and then were centrifuged at 3000 rpm for 15 min at 4 °C for obtaining the supernatant. The activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) and total antioxidant capacity (T-AOC) were detected by Thermo Scientific Varioskan Flash Multimode Reader (Thermo Fisher Scientific, MA, USA) following the specifications in corresponding commercial biochemical kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

***Hematoxylin and eosin (HE) staining:*** Lower incisors were fixed with 4% paraformaldehyde, and then decalcified with ethylenediaminetetraacetic acid (EDTA) solution. Decalcifying incisors were embedded in paraffin. Incisors embedding in paraffin blocks were sagittally sliced for obtaining 5 micrometer-thick sections. The incisors sections were stained applying conventional HE staining steps (Solarbio, Beijing, China). The variations in lower incisors were observed and photographed with a light microscope (Olympus, Tokyo, Japan).

***Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining:*** The ameloblasts apoptosis in lower incisors was assessed employing commercial TUNEL assay kit (KeyGen Biotech, Jiangsu, China) following the protocol of manufacturer. The apoptotic ameloblasts were captured under the light microscope (Olympus, Tokyo, Japan) and quantified utilizing Image Pro Plus 6.0 software (Media Cybernetics, Maryland, USA).

***Reverse transcription quantitative PCR (RT-qPCR):*** Frozen lower incisors from mice, were immersed in Trizol reagent (Takara Biological

Engineering Company, Liaoning, China) and triturated for obtaining the total RNA. Acquired total RNA was submitted to the reverse transcription applying the Prime Script™ RT Master Mix (Takara Biological Engineering Company, Liaoning, China) for converting to cDNA. Subsequently, RT-qPCR was executed on the Mx3000P™ QPCR system (Stratagene, California, USA) employing TB Green™ Premix Ex Taq™ II (Takara Biological Engineering Company, Liaoning, China). The protocols of RT-qPCR were 95 °C/30 sec and 40 cycles of 95 °C/5 sec, 58 °C/30 sec, and 72 °C/30 sec. The melting curve was analysed at 95 °C/15 sec, 60 °C/1 min, and 95 °C/15 sec. The relative gene expression levels of superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), CAT, GSH-Px, p38MAPK, c-Jun N-terminal kinase (JNK), NF-κBp65, IkappaB kinase-beta (IKK-β), B-cell lymphoma 2 (Bcl-2), Caspase-3 were calculated via the $2^{-\Delta\Delta CT}$ method. The primers sequences of these target genes and β-actin as reference gene are shown in Table 1.

***Statistical analysis:*** The experimental results were presented as the mean ± the standard error of the mean (SEM). The homoscedasticity and normality of all laboratorial data were verified. Statistical analysis of these results was conducted via GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, USA). The unpaired Student's two-tailed t-test was employed for the detection of differences in measured data between two groups. In all tests, statistical significance was defined as the p less than 0.05.

## RESULTS

***Incisors fluorine content:*** Fig. 1a shows the fluorine content of upper incisors from mice (mg/kg). The incisors fluorine concentrations of mice drinking water with 100 mg/L NaF were markedly increased when compared to group C after 12 months of fluoride exposure ($p < 0.05$); nevertheless, there was no variation between mice from group F and fluorosis mice subjected to FTR ($p > 0.05$).

***Urinary CTX-I concentrations:*** As Fig. 1b shown, the urinary concentrations of CTX-I were detected to elevate observably in mice from group F compared with group C ($p < 0.05$). Besides, in the group F+E, urinary CTX-I levels were significantly declined versus the group F ($p < 0.05$). According to these experimental consequences, it can be speculated that FTR improves the teeth properties in fluorosis mice.

***The contents of ROS and MDA and activities of GSH-Px, CAT, T-SOD and T-AOC in lower incisors:*** As shown in Fig. 2, the contents of ROS and MDA in lower incisors of mice from group F were dramatically higher than group C ($p < 0.05$) (Fig. 2a and 2b), while the activities of GSH-Px, CAT, T-SOD, and T-AOC were lower ($p < 0.05$) (Fig. 2c, 2d, 2e and 2f). Consistently, when compared to group C, the mRNA expressions of CAT and SOD1 were also markedly decreased in incisors from fluorosis mice ($p < 0.05$) (Fig. 3). Nevertheless, no difference in the contents of ROS and MDA were discovered between fluorosis mice and FTR fluorosis mice ($p > 0.05$) (Fig. 2a and 2b). After FTR, the activities of GSH-Px, CAT, T-SOD, and T-AOC were remarkably elevated in FTR fluorosis mice with comparison to group F ($p < 0.05$) (Fig. 2c, 2d, 2e and 2f). Additionally, compared to the group F, FTR dramatically enhanced the gene expression of SOD1 in lower incisors of group F+E ($p < 0.05$) (Fig. 3). These results indicated that FTR could mitigate fluoride-induced oxidative damages in incisors form mice.

***The morphology and apoptosis of ameloblasts in lower incisors:*** Mice were treated with 100 mg/L NaF in drinking water and subjected to FTR for 12 months. After 12 months, HE staining and TUNEL staining were respectively conducted on lower incisors sections for evaluating the incisors injury. HE staining (Fig. 4) revealed that the ameloblasts in lower incisors presented as columnar cells that were neatly arranged, with no distinct morphological variation in ameloblasts between all groups.

As appeared in Fig. 5a, after TUNEL staining, the normal ameloblasts in lower incisors were dyed blue, the apoptotic ameloblasts in lower incisors were dyed brown. In comparison of group C, fluoride treatment for 12 months markedly elevated the apoptotic index of ameloblasts (Fig. 5b, $p < 0.05$). Inversely, the dramatically reduced apoptotic index of ameloblasts was discovered in fluorosis mice subjected to FTR when compared to those in group F (Fig. 5b, $p < 0.05$). Congruously, RT-qPCR results of lower incisors manifested that fluoride induced an obvious elevation

in Caspase-3 expression and a significant recession in Bcl-2 expression at gene level, and FTR partly reversed these alterations (Fig. 5c, $p < 0.05$). In short, the above results demonstrated that FTR ameliorated fluoride-caused ameloblasts apoptosis in lower incisors.

***Detections of p38MAPK/JNK signaling pathways and NF-κB/IKK-β signaling pathways in the lower incisors:*** Fig. 6 presented the effects of FTR on p38MAPK/JNK signaling pathways and NF-κB/IKK-β signaling pathways associated with apoptosis induced by MDA in lower incisors from mice during the fluoride treatment. The gene expressions of p38MAPK and JNK were distinctly increased in lower incisors from group F when compared with group C ($p < 0.05$). Oppositely, compared to group F, the gene expressions of p38MAPK and JNK were dramatically declined in group F+E ($p < 0.05$). In addition, fluoride-exposed mice showed up-regulated gene expression of IKK-β in lower incisors compared to group C, which was reversed by FTR ($p < 0.05$). However, no significant difference in the gene expression of NF-κBp65 was observed between all groups ($p > 0.05$). These suggested that exercise rescued damaged ameloblasts in lower incisors from fluorosis mice, partly attributed to the inhibition of fluoride-caused MDA inducing apoptosis through adjusting p38MAPK/JNK signaling pathway and IKK-β independent of NF-κB signaling pathway.

**Table 1.** List of primer sequences applied for RT-qPCR

| Gene name | Primer sequence (5'→3') | Product size (bp) | GenBank number |
|---|---|---|---|
| β-actin | F: GCCTTCCTTCTTGGGTATGG<br>R: GCACTGTGTTGGCATAGAGG | 107 | NM_007393.5 |
| CAT | F: CCAACAAGATTGCCTTCTCC<br>R: GCTCCTTCCACTGCTTCATC | 142 | NM_009804.2 |
| SOD1 | F: AGATGACTTGGGCAAAGGTG<br>R: AATCCCAATCACTCCACAGG | 85 | NM_011434.2 |
| SOD2 | F: GCGTGACTTTGGGTCTTTTG<br>R: AGCGACCTTGCTCCTTATTG | 110 | NM_013671.3 |
| GSH-Px | F: CCAGGAGAATGGCAAGAATG<br>R: CATTCCGCAGGAAGGTAAAG | 146 | NM_001329528.1 |
| p38MAPK | F: GGAGAAGATGCTCGTTTTGG<br>R: TCATCAGGGTCGTGGTACTG | 93 | NM_001357724.1 |
| JNK | F: ACAGCTCGGAACACCTTGTC<br>R: GAGTCAGCTGGGAAAAGCAC | 135 | AJ315338.1 |
| NF-κBp65 | F: CATTGTGTTCCGGACTCCTC<br>R: GTGGCGATCATCTGTGTCTG | 142 | AK089489.1 |
| IKK-β | F: ATAAATTGCTGCTGGCTTGG<br>R: AGTGCCATCATCCGCTCTAC | 97 | NM_001159774.1 |
| Bcl-2 | F: CTTCTGCAAATGCTGGACTG<br>R: CGTAGGAATCCCAACCAGAG | 105 | NM_177410.3 |
| Caspase-3 | F: ACATGGGAGCAAGTCAGTGG<br>R: ATCCGTACCAGAGCGAGATG | 142 | NM_009810.3 |

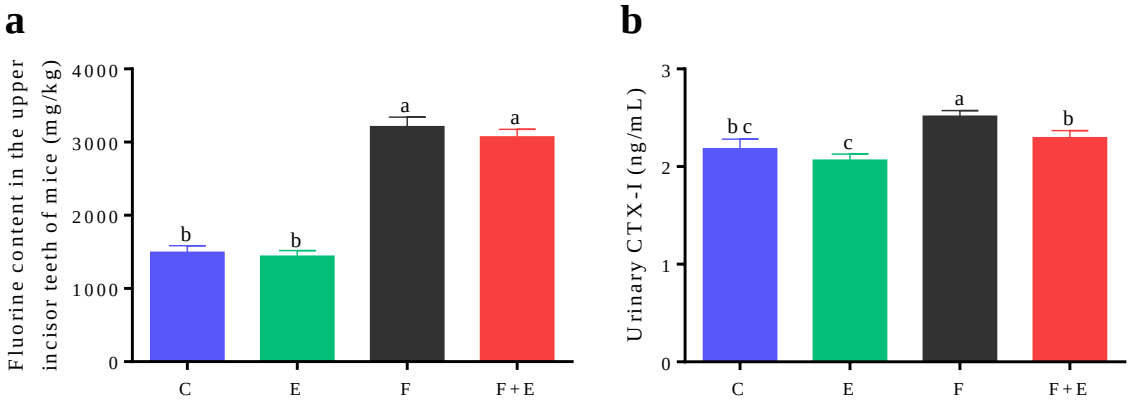


**Figure 1.** Fluorine content in upper incisor teeth (a) of mice from group C, E, F, F+E (n = 13 mice in each group) were measured by the fluoride ion-selective electrode potentiometric method, and the ELISA determination results of Urinary CTX-I levels (b) in mice from group C, E, F, F+E (n = 8 mice in each group). C: control group; E: exercise group; F: fluoride group; F+E: fluoride plus exercise. Data are shown as mean ± SEM. Different lowercase letters manifest significantly different between any two groups ($p < 0.05$), while occurring same lowercase letters manifest no difference ($p > 0.05$).

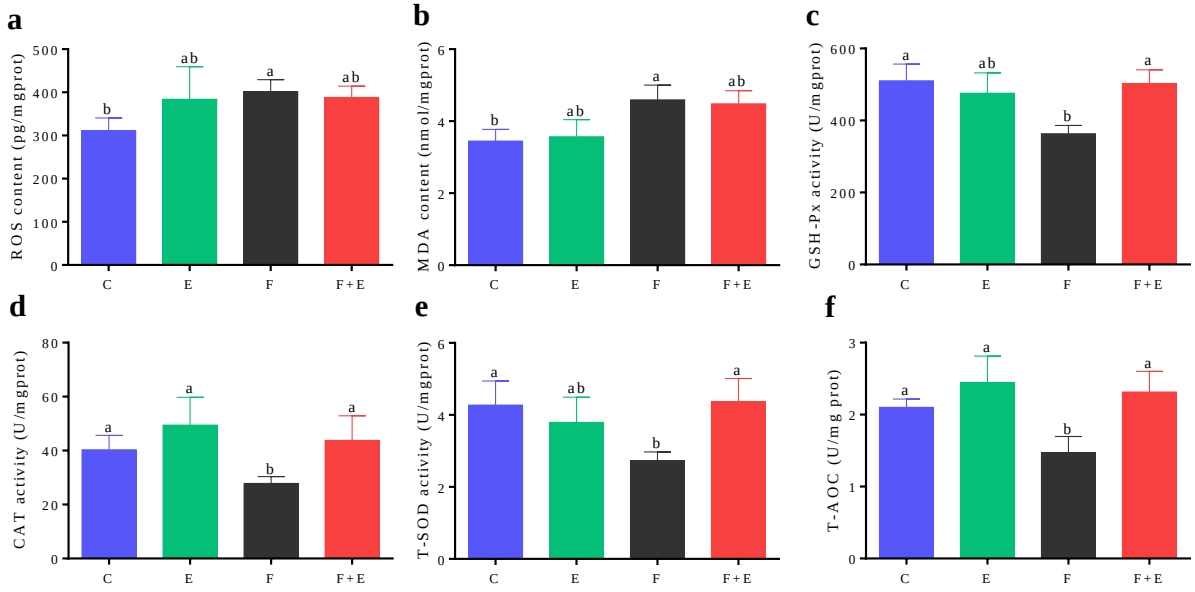


**Figure 2.** The ROS content (a), MDA content (b), GSH-Px activity (c), CAT activity (d), T-SOD activity (e), and T-AOC (f) in lower incisor teeth of mice from group C, E, F, F+E (n = 5 mice in each group). C: control group; E: exercise group; F: fluoride group; F+E: fluoride plus exercise. Data presented are mean ± SEM. Diverse lower-case letters denote statistically significant difference between any two groups ($p < 0.05$), whereas the existence of same lower-case letters denote no difference ($p > 0.05$).

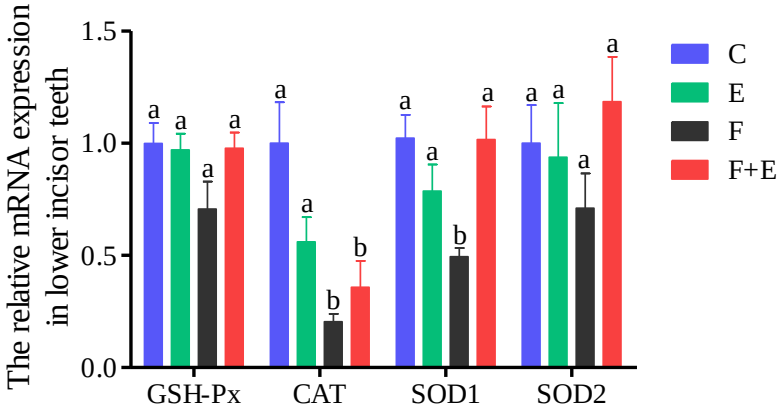


**Figure 3.** Antioxidant enzymes-related genes such as GSH-Px, CAT, SOD1 and SOD2 mRNA levels in lower incisor teeth of mice from group C, E, F, F+E (G) (n = 4 mice in each group). C: control group; E: exercise group; F: fluoride group; F+E: fluoride plus exercise. Statistic data are represented as mean ± SEM. Distinct characters indicate statistically significant between any two groups ($p < 0.05$), while emerging same characters indicate not statistically significant ($p > 0.05$).

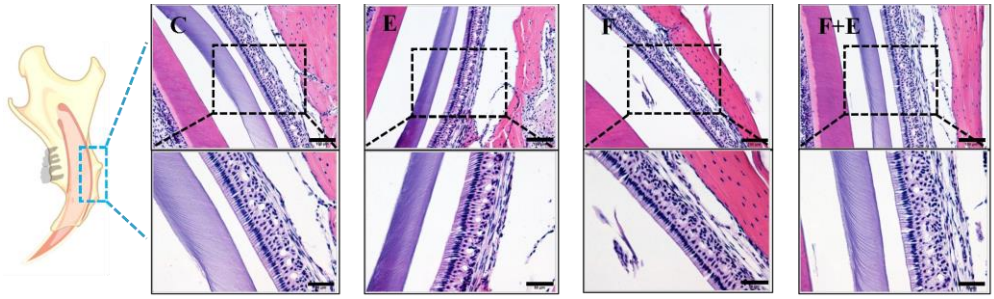


**Figure 4.** Typical sections of HE staining for lower incisor teeth in mice from group C, E, F, F+E (n = 3 mice in each group). Scale bar: 100 μm or 50 μm. C: control group; E: exercise group; F: fluoride group; F+E: fluoride plus exercise.

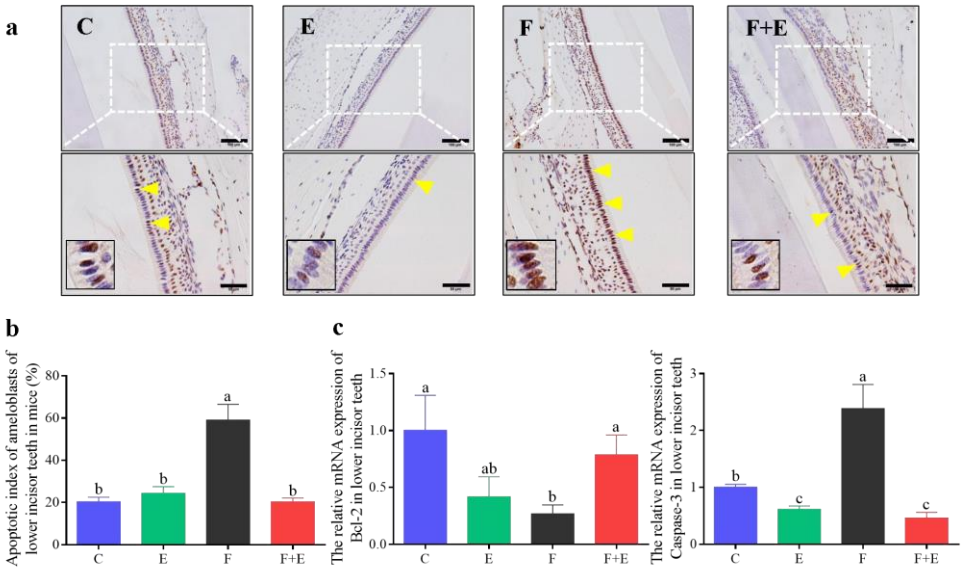


**Figure 5.** Representative TUNEL staining micrographs (a) from lower incisor teeth sections in mice of group C, E, F, F+E (n = 3 mice in each group). Yellow triangles indicate apoptotic-positive ameloblasts. Scale bar: 100 μm or 50 μm. C: control group; E: exercise group; F: fluoride group; F+E: fluoride plus exercise. The apoptotic index (b) of ameloblasts was calculated as positive ameloblasts/total ameloblasts. RT-qPCR analysis results of Bcl-2 and Caspase3 mRNA expressions (c) in lower incisor teeth of mice from group C, E, F, F+E (n = 4 mice in each group). Statistical data are presented as mean ± SEM. Different lower case letters demonstrate significant differences between any two groups ($p < 0.05$), however identical lower case letters demonstrate non-significant differences ($p > 0.05$).

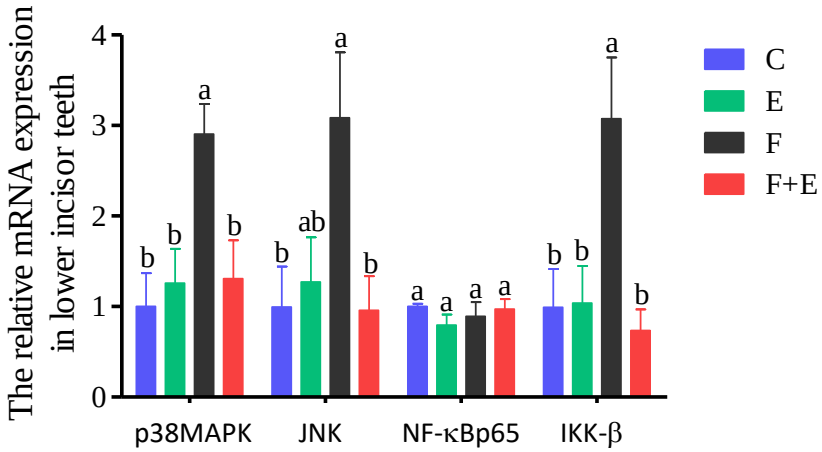


**Figure 6.** The expression levels of genes associated with p38MAPK/JNK signaling pathways and NF-κB/IKK-β signaling pathways in lower incisor teeth from mice of group C, E, F, F+E (n = 4 mice in per group). β-actin was employed to normalize these genes mRNA expression levels. C: control group; E: exercise group; F: fluoride group; F+E: fluoride plus exercise. All bar graphs are showed as mean ± SEM. Diverse letters indicate statistical significance between any two groups ($p < 0.05$), but same letters indicate no statistical significance ($p > 0.05$).

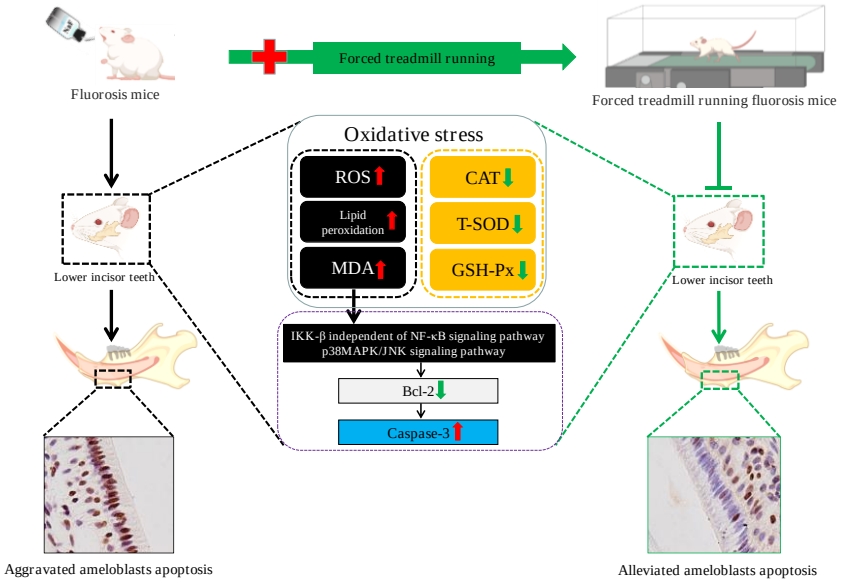


**Figure 7.** Potential mechanisms of forced treadmill running ameliorated oxidative stress and ameloblasts apoptosis in incisor of fluorosis mice.

## DISCUSSION

Dental fluorosis is a widespread endemic fluorosis induced by excessive fluoride in the consuming water, which results in the accumulation of fluoride in teeth and causes ameloblasts apoptosis by increasing oxidative stress produced lipid peroxides.[11] Inversely, physical exercise fulfils antioxidation through numerous approaches containing enhancing the capacity of antioxidant enzymes, reducing the production of ROS, restraining lipoprotein oxidation, and prohibiting oxidative stress-caused apoptosis.[33] Meanwhile, moderate exercise alleviates the detrimental toxic effect induced fluoride.[20] However, there is a vacancy behind the influencing mechanisms of exercise on fluoride-induced oxidative stress causing ameloblasts apoptosis in spite of the rise in prevalence of dental fluorosis. Besides, to date, there is still no specific and effective exercise regime for the therapeutic of dental fluorosis. For these objectives,

we employed the exercise strategy involving 12 months of FTR to fluorosis mice that possess consecutively growing incisors throughout its lifetime, which can reveal the developmental phases of amelogenesis. Our experimental findings demonstrated that FTR alleviated fluoride-caused oxidative stress inducing apoptosis in incisors of mice during 12 months of exercise coexisting fluoride exposure.

Increasing fluoride exposure resulted in the accumulation of fluorine ions in teeth and bones, the fluorine ions concentration in teeth is identified as a fluoride exposure indication. Our researches indicate a remarkable enhancement in fluorine ions concentration in teeth of mice subjected to 12-month fluoride exposure. Similar experimental result was found by Li et al. (2017)[11]. The accumulation of fluorine ions in teeth is closely associated with fluorine metabolism.[34] The knowledge of diverse affecting factors of fluorine metabolism is vital for the avoidance of fluoride toxicity. Although exercise is known to be a factor influencing fluorine metabolism, the mechanism the mechanism for this is not clear. Fluorine pharmacokinetics may be affected by the alteration of physiological reaction to exercise. Up to now, only a study in human shown that moderate exercise obviously elevated plasma fluorine concentrations when compared with sedentary control group.[34] On the contrary, two rat experimental studies have revealed a remarkable decrease in plasma F concentration of rats submitted to treadmill running exercise.[19] Inconsistently, our study manifested no influence of exercise intervention including FTR on teeth fluorine concentrations in fluoride-toxic mice, which was similar to our prior research[20]. These various experimental observations in the intervention action of exercise on plasma, teeth, and bone fluorine concentrations among these researches may be explained via the deviations of fluoride exposure dose, fluoride exposure duration, exercise type, exercise intensity, exercise duration, and subject species. Hence, conducting further investigations are necessary to explore the possible mechanism of action of exercise on fluorine metabolism for the prevention and treatment of fluorosis. It is commonly admitted that the severity of dental fluorosis is positively correlated with the duration of fluoride exposure. Additionally, an experimental study on pigs found that the concentration of CTX-I as a bone resorption marker negatively correlated with tooth properties such as morphology, dentin volume and its mineral content.[32] That is, the assessment of CTX-I concentration is very suitable for determining the prognosis of tooth properties. Accordingly, the concentration of CTX-I in urine of mouse model was measured in current research. We found that 12 months of fluoride exposure results in the remarkable elevation of CTX-I concentration in urine. Inversely, FTR markedly reduced the concentration of CTX-I in urine from mice who suffered fluoride exposure of 12 months, which implied that FTR may mitigate the detriment of dental fluorosis.

Oxidative stress is a comprehensive term that represents the stress condition when the balance of ROS generation and scavenging activities of antioxidant enzymes was destroyed in the cells. Increasing reports revealed that fluoride-caused oxidative stress may be a pathogenesis mechanism of dental fluorosis.[11] Chronic fluoride exposure causes the excessive production of ROS followed by lipid peroxidation and restrains the removal ROS capacity of antioxidants in dental maturational ameloblasts that seem to be vulnerable in continuous fluoride treatment. ROS, containing free radicals, peroxides, and oxygen ions, have a detrimental affection on cells. MDA, a primary product in lipid peroxidation, can circuitously reflect the degree of free radical impairment of cell components. Inversely, principal antioxidative guard systems such as SOD, CAT, and GSH-Px can defend proteins and deoxyribonucleic acid (DNA) against oxidative stress injury. In present study, we found that high doses of fluoride dramatically upregulated ROS and MDA contents but markedly downregulated SOD, CAT, and GSH-Px activities in the incisors in line with the preceding research.[11] Earlier researches suggested that fluoride could change gene expressions in ameloblasts.[35] So we further conducted the analyses of gene expressions related antioxidant enzymes, which indicated that incisors in fluorosis mice had observably lower mRNA expressions of SOD1 and CAT in accordance with our prior study,[36] indicating that oxidative stress levels were raised in the fluoride groups. Interestingly, several reports have proved that exercise reduces oxidative stress via varying the mRNA expressions and activities of antioxidant enzymes.[37] Previously, Hoffman-Goetz et al found that voluntary wheel running enhanced the levels of CAT and GSH-Px

in mice.[38] Other experiment researches on mice have indicated that regular exercise restricted the production of ROS and MDA for coordinating the balance of oxidative and antioxidative systems.[39] Accordance with these, mice in the group of F+E owned observably higher activities in CAT, GSH-Px, T-SOD, and T-AOC but slightly lower levels of ROS and MDA in incisors of mice from group F, showing the antioxidant effects induced by FTR on incisors in fluorosis mice. Thus, these results convincingly demonstrated that 12 months of FTR can ameliorate the oxidative stress in incisors of fluoride-toxic mice via enhancing the antioxidant defenses.

The emergence of oxidative stress is followed by apoptosis. Apoptosis is a cellular event of programmed cell death, which is nicely adjusted at gene level leading to the ordered and effective elimination of impaired cells, for instance, those arising from DNA lesion or during development. ROS generation can result in cell death through initiating apoptosis process. Noticeably, ROS-caused lipid peroxidation also plays a vital part in apoptosis. Lipid peroxidation appears when ROS such as free radicals assault carbon double bonds of lipids, which destroys lipid membranes containing the mitochondrial membranes and plasma membranes in the cell. Lipid peroxides damage enzyme activities, proteins, and DNA as well as activate the intrinsic and extrinsic pathways associated with apoptosis.[40] Previous researches have indicated that lipid peroxides form compounds with p38 and JNK, and then activate the Caspase pathway involved apoptosis.[14] Caspase-3 is in charge of the cleavage of pivotal cellulate proteins, and subsequent results in characteristic morphological alterations in cells experiencing apoptosis. The study of Bodur et al showed that IKK can straightly inactivate Bcl-2 via phosphorylation in lipid peroxidation regulated apoptosis, which is independent of the NF-κB pathway.[41] Bcl-2 belongs to an apoptosis inhibitor that is one of the members of the bcl-2 family in apoptosis. A large amount of studies have demonstrated that fluoride causes apoptosis via increasing ROS-induced lipid peroxidation, consequently activating various signaling pathways.[42] Thus, in current research, the apoptosis indexes of ameloblasts were assessed in incisors. TUNEL staining and RT-qPCR results suggested that 100 mg/L NaF dramatically increased the mRNA expression of Caspase-3 but remarkably decreased the mRNA expression of Bcl-2 in incisors, subsequently leaded to the elevatory apoptotic index of ameloblasts of incisors in mice, consistent with the experimental results of Li et al.[22] Moreover, fluoride markedly upregulated the gene expressions levels of p38MAPK, JNK and IKK-β in incisors. As mentioned above, our research identified the event that fluoride contributed to ameloblasts apoptosis through increasing the level of MDA, and then activating the pathway associated with apoptosis including p38MAPK/JNK signaling pathway and IKK-β independent of NFκB signaling pathway. Oppositely, We detected that 12 months of FTR observably downregulated the gene expressions of Caspase-3, p38MAPK, JNK and IKK-β but prominently upregulated the mRNA expression of Bcl-2 in injured incisors of fluorosis mice, followed by the reduction of ameloblasts apoptotic index. In accordance with our results, growing explorations have observed the prohibitive effects of exercise on lipid peroxidation-induced apoptosis.[33] Collectively, our entire data indicated that exercise suppressed the fluoride-induced lipid peroxidation linked apoptosis through mediating p38MAPK/JNK signaling pathway and IKK-β independent of NFκB signaling pathway.

Although our present research makes clear that the effect of suitable exercise on oxidative stress related ameloblasts apoptosis in fluorosis mice incisors, there are still some limitations in our study. Firstly, owing to the deficiency of in vitro experimental validation, the explanation of these experimental results remains hypothetical. Besides, our research was completed employing animal model of mouse and it is very importantl to investigate the mechanism of the effect ofexercise on patients with dental fluorosis.

## CONCLUSIONS

Taken together, the current research indicated that moderate exercise can rescue injured ameloblasts in incisors of fluorosis mice against ROS production induced lipid peroxidation related apoptosis via ameliorating oxidative damage and adjusting MDA mediated p38MAPK/JNK signaling pathway and IKK-β independent of NF-κB signaling pathway (Fig. 7). Consequently, regular exercise regime seems to be a novel and inexpensive therapeutic tactics for resisting dental fluorosis.

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## CONFLICT OF INTERESTS

The authors declare no competing interests.

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