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Original Article

TGF-β2-modulating Anti-apoptosis Effect of Osteoblasts by Sodium Fluoride

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ABSTRACT

Objectives: This study aimed to investigate and discuss the mechanism of fluoride-induced osteoblast apoptosis in TGF-β2 signaling pathway. **Materials and Methods:** The osteoblasts were divided into 0 (control group), 5.0, 10.0, 20.0, and 40.0 mg/L sodium-fluoride induction groups, and the cells were collected after 24 hr and 48 hr induction (T1 and T2, respectively) to detect the changes in the mitochondrial-apoptosis pathway-related molecules (FoxO1, BAD, BCL-XL, BCL-2, and BAX). At the same time, the apoptosis index of fluoride-exposed osteoblasts was detected by flow cytometry. **Results:** The expression of TGF-β2 in the osteoblasts decreased with the increase in fluoride dose at T1 and T2. The expression levels of FoxO1 increased significantly at T1 and T2 in each dose group except for the 40 mg/L group (which had decreased expression). The expressions of p-BAD, BCL-XL, BCL-2, BAX, and other molecules increased with the increase in fluoride dose and exposure time. **Conclusion:** Fluoride can directly activate the TGF-β2 signaling molecules in osteoblasts and affect apoptosis. TGF-β2 plays an anti-apoptosis role in fluoride-exposed osteoblasts through the mitochondrial-apoptosis pathway.

Keywords: TGF-β2, Fluorosis, Mitochondrial Apoptosis, Osteoblasts, Signaling pathway.

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INTRODUCTION

The long-term excessive intake of fluoride can cause chronic fluorosis. The primary clinical manifestations are dental and skeletal fluorosis and pathological changes in the function and metabolism of various organs and tissues. The pathogenesis of this pathological damage is still unclear. The apoptosis of osteoblasts is the main pathological change of skeletal fluorosis.[1]

Our previous research shows that a variety of microRNAs in fluoride-stained osteocytes are associated with apoptosis. MiR212-3p is one of them, and the target gene of miR212-3p is TGF-β2.[2,3] TGF-β2 is a common subtype of TGF, and it has been reported that TGF-β2 has autocrine and/or paracrine effects in bone regeneration and remodeling.[4] TGF-β2 can reduce apoptosis in osteoblasts through downstream BCL-2, but the specific mechanism of TGF-β2 in osteoblasts with fluorosis is not clear.

Mitochondrial-apoptosis pathway is involved in the regulation of cell proliferation, differentiation; apoptosis, ATP production and utilization, and can promote apoptosis in a variety of ways, thus making the inhibition of this pathway an important condition to maintain cell survival.[5] It has been reported that bone damage in animal models of skeletal fluorosis involves the regulation of the mitochondrial-apoptosis pathway.[6,7] In addition, fluoride can affect the functions of liver cells through this pathway.[8] However, the relationship between TGF-β2 and the mitochondrial-apoptosis pathway is unclear in fluoride-induced osteoblast apoptosis. Tan established an *in vitro* model of fluoride exposure and found, with the help of a light microscope and transmission electron microscope, that the mitochondrial-apoptosis pathway was involved in the process of cellular fluorosis.[9] However, the above studies did not involve TGF-β2, and there are no relevant reports on how TGF-β2 affects the apoptosis of fluoride-exposed osteoblasts. Therefore, further investigation of the change rules of the mitochondrial-apoptosis pathway and mitochondrial apoptosis-related signal molecules (FoxO1, BDA, BCL-XL, BCL-2, or BAX) in the bone tissue of endemic fluorosis can facilitate the understanding of the pathogenesis of fluorosis bone injury and the role of these molecules in its pathogenesis, thus providing a theoretical basis for the prevention and early diagnosis of endemic fluorosis. In this experiment, the osteoblasts were divided into the 0 (control group), 5.0, 10.0, 20.0, and 40.0 mg/L sodium-fluoride induction groups, and the cells were collected after 24 and 48 hr induction (T1 and T2, respectively) to detect the changes in the mitochondrial-apoptosis pathway and mitochondrial apoptosis pathway-related molecules (FoxO1, BAD, BCL-XL, BCL-2, and

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BAX). At the same time, the apoptosis index of fluoride-exposed osteoblasts was detected by flow cytometry.

MATERIALS AND METHODS

Cell culture

The human osteosarcoma Saos-2 cells were purchased from the Shanghai Cell Bank of the CAS and were cultured in DMEM containing 10% FBS at 37°C and 5% CO_2 (v/v); the medium was replaced every two to three days according to the cell density and pH of the culture medium. Count the number of cells with a counter under the microscope and resume the growth function of the number of cells. When the cell confluence reached 80%, the cells were sub-cultured.

In vitro model of fluoride exposure and antibody molecules

The osteoblasts in the logarithmic growth phase were digested with 0.25% trypsin and inoculated into six-well plates while maintaining the cell concentration at 2×10^5/mL. Next, 2 mL of the cell-culture medium was added to each well and incubated for 24 hr at 37°C, 5% CO_2 (v/v), and saturated humidity. According to the fluoride doses, the osteoblasts were divided into the 0 (control group, CON), 5.0, 10.0, 20.0, and 40.0 mg/L sodium fluoride-induction groups with three replicates in each group.

The primary antibodies used in the study were TGF-β2 (ab102118A), FoxO1 (cst2880S), BAD (cst9268S), p-BAD (cst9291), BCL-XL (cst24780), BCL-2 (cst15071), BAX (cst5023), and GAPDH (cst5174S), all purchased from Cell Signaling and Abcam, with a dilution ratio of 1:1,000. The secondary antibody (Goat anti-rabbit HRP) was purchased from Wuhan Boster Biological Technology, Ltd. (ba1054).

Detection of the expressions of TGF-β2, FoxO1 BAD, p-BAD, BCL-XL, BCL-2, BAX, and GAPDH by western blotting

After culturing the osteoblasts *in vitro* for 24 and 48 hr (T1 and T2, respectively), the cells were collected and lysed with a Radio-Immunoprecipitation assay (RIPA) protein lysis solution (BioTeke Corporation, Beijing, China) to obtain the whole-cell proteins. The proteins were denatured by adding a 4x loading buffer and undergoing a metal bath at 100°C for 5 min. After SDS-PAGE, the proteins were transferred onto a Polyvinylidene Difluoride (PVDF) membrane using a wet-transfer method to block phosphorylated proteins with 1-3% BSA. The corresponding primary antibody incubation solution (TGF-β2, FoxO1, BAD, p-BAD, BCL-XL, BCL-2, BAX, and GAPDH) diluted with blocking solution was then used to soak the PVDF membrane overnight at 4°C. This was followed by rinsing to remove the excess primary antibodies. The PVDF membrane was incubated for 2 hr in a corresponding HRP-labeled secondary antibody incubation solution (1:50,000) at 37°C on a shaker. It was then rinsed with TBST to remove the excess secondary antibodies, with several minutes of coloration of the PVDF membrane by the dropwise addition of the enhancement solution mixture into the ECL reagent and the stabilization peroxidase solution (ratio=1:1) until the fluorescence bands became apparent, absorbing the excess substrate solution with filter paper, and finally, X-ray-film development. A GE imaging analysis system was used for imaging. GAPDH was used as the internal reference for the semi-quantitative analysis of the comparison of the gray scales of the target proteins to GAPDH. The blots were visualized with a Western Breeze kit (catalog no. WB7105; Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol.

Detection of early cell apoptosis by the Annexin V-FITC/PI double-labeling method (flow cytometry)

Falcon test tubes were numbered according to the order of the negative control and samples. The cells were rinsed twice with cold PBS buffer and prepared in a suspension of 1×10^6 cells/mL using 1x binding buffer; 100 µL of the cell suspension was then added to each Falcon test tube. The Annexin V and nucleic acid dye were added, and the tubes were mixed gently and placed in darkness at room temperature (20~25°C) for 15 min. When using the Annexin V-Biotin reagent for detection, the cells were rinsed once with 1x binding buffer, and the supernatant was removed. Next, 0.5 µg of SAV-FITC reagent dissolved in 100 µL of 1x binding buffer was added to the tubes and mixed gently. Then, 5 µL of PI was added and placed in darkness at room temperature (20~25°C) for 15 min; 400 µL of 1x binding buffer was added to each test tube, and flow cytometry was performed within one hour.

Statistical analysis

The data were analyzed using SPSS 22.0 statistical software. The statistical description was expressed by comparing the expressions at T1 and T2 between groups ($x \pm s$), and the statistical inference was expressed by ANOVA with $P<0.05$ considered statistically significant.

RESULTS

Impact of fluoride on the expression of TGF-β2 in osteoblasts

The results of western blotting showed that the expression of TGF-β2 in the osteoblasts decreased with the increase in fluoride dose at T1 and T2, especially in the 10-40 mg/L group at T1 and the 20-40 mg/L group at T2 ($p=0.008$) (Figures 1a and 1c). It peaked when the fluoride dose was 2.5 mg/L (both at T1 and T2), which was close to the CON group ($p>0.05$). Later, with the increase in fluoride dose, the expression of TGF-β2 was reduced and reached its lowest level at 40.0 mg/L (both at T1 and T2) (Figures 1b and 1d).

Impact of fluoride on mitochondrial apoptosis signaling molecules

Expression of mitochondrial apoptosis signal molecules at T1

After 24 hr of fluoride exposure, the expression of BAD decreased slightly compared with the CON group reaching its lowest level in the 10 mg/L group, and the difference was not statistically significant($p>0.05$) When the concentration of sodium fluoride was between 10 mg/L and 40 mg/L, the expression of BAD was directly proportional to the concentration of sodium fluoride (Figures 2a and 2b).The expressions of p-BAD and BAX increased with the increase in fluoride dose ($p<0.05$) and peaked in the 40 mg/L group (Figures 2c and 2d), while the expression of BCL-XL was similar to that of BCL-2, which both increased ($p<0.05$) and peaked in the 10 mg/L group. The expression of BCL-XL and BCL-2 is proportional to sodium fluoride concentration when sodium fluoride concentration is less than 10 mg/L; The expression of BCL-XL and BCL-2 is inversely proportional to sodium fluoride concentration when sodium fluoride concentration is between 10 mg/L and 40 mg/L (Figures 2d and 2e).

Expression of mitochondrial-apoptosis signaling molecules at T2

After 48 hr of fluoride exposure, compared with the CON group, the expression of BAD decreased only in the 40 mg/L group, while it increased in the other groups and reached its highest level in the 10 mg/L group, but the difference was not statistically significant($p>0.05$) (Figures 3a and 3b). The expressions of p-BAD and BAX were proportional to the fluoride dose and peaked in the 40 mg/L group (Figures 3c and 3d), but BCL-2 and BCL-XL only decreased in the 40 mg/L group and increased in the other groups, except the CON group, reaching their highest level in the 10 mg/L group. The expression of BCL-XL and BCL-2 is proportional to sodium fluoride concentration when sodium fluoride concentration is between 5 mg/L and 10 mg/L. The expression of BCL-XL and BCL-2 is inversely proportional to sodium fluoride concentration when sodium fluoride concentration is between 10 mg/L and 20 mg/L . (Figures 3d and 3e).

After fluoride exposure for 24 and 48 hr, the expressions of p-BAD and BAX were proportional to the fluoride concentration and reached their highest level at a concentration of 40 mg/L. The expressions of BCL-2 and BCL-XL became uniform. At T1, the expressions of BCL-2 and BCL-XL were higher than in the CON group. At T2, the expressions of BCL-2 and BCL-XL in the 40 mg/L group were lower than in the CON group, while the other groups all showed higher expressions than the CON group. At T1 and T2, the highest expression appeared in the 10 mg/L group (Figures 2 and 3).

Expression of the FoxO1 molecule

The level of FoxO1 protein became enhanced in a dose- and time-dependent manner, especially in the 10-40 mg/L groups at T2, but not in the CON group ($p<0.05$) (Figure 4a). FoxO1 was in direct proportion to fluoride concentration at T1 and T2 (Figures 4b and 4c).

Detection of apoptosis of fluoride-exposed osteoblasts by flow cytometry

Flow cytometry was used to detect the apoptosis of osteoblasts at T1 and T2, and the results revealed that the apoptosis index increased with the increase in dose and time (Figure 5). At T1, the apoptosis index in each group was 2.40, 3.65, 3.88, 5.43, and 7.04%, respectively, and the highest apoptosis index appeared in the 40 mg/L group, but the difference was not statistically significant. ($p>0.05$). At T2, the apoptosis index was 3.41, 4.29, 5.36, 13.37, and 20.94%, respectively. The apoptosis indexes in the 20 mg/L group and 40 mg/L group were significantly higher than in the CON group ($p<0.05$).

At T1, the expressions of p-BAD and BAX were the highest in the 40 mg/L group, and the apoptosis index was also the highest; at T2, the expressions of p-BAD and BAX were the highest in the

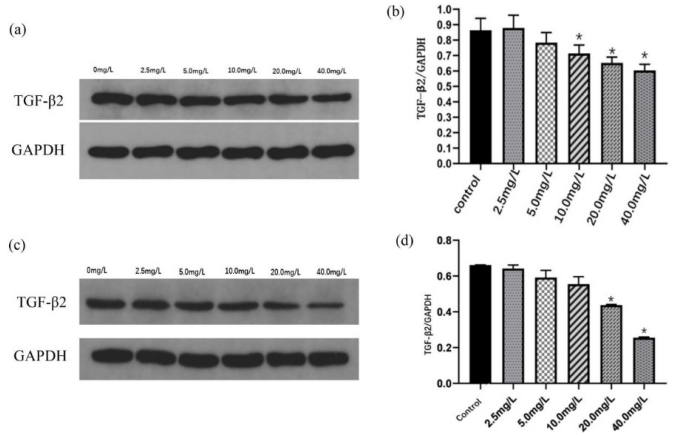

Figure 1: Impact of fluoride on the expression of TGF-β2 in osteoblasts.

*Compared with control group, the difference was significant ($p<0.05$).

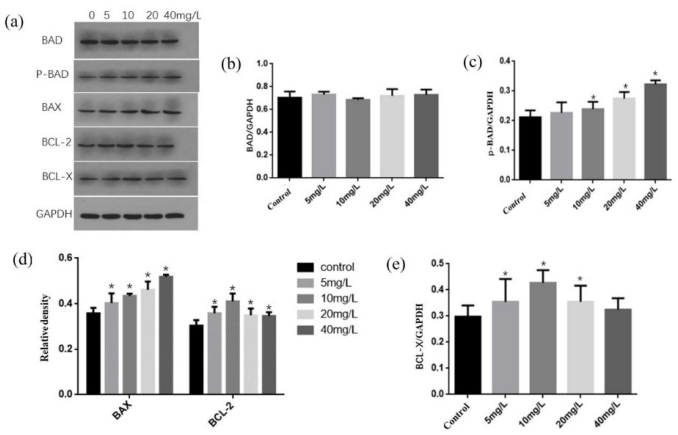

Figure 2: Expression of mitochondrial apoptosis signal molecules at T1.

*Compared with control group, the difference was significant ($p<0.05$).

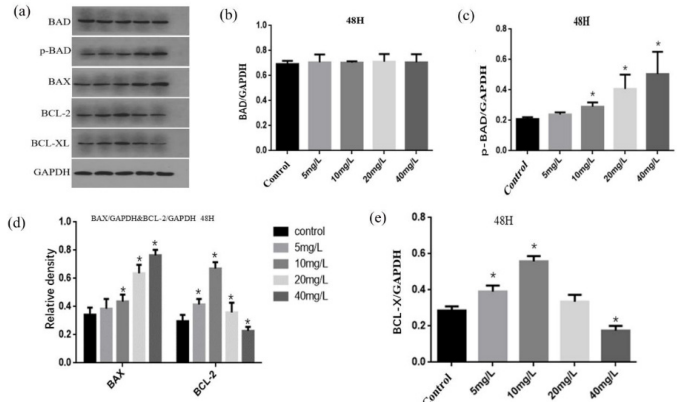

Figure 3: Expression of mitochondrial-apoptosis signaling molecules at T2.

*Compared with control group, the difference was significant (p<0.05).

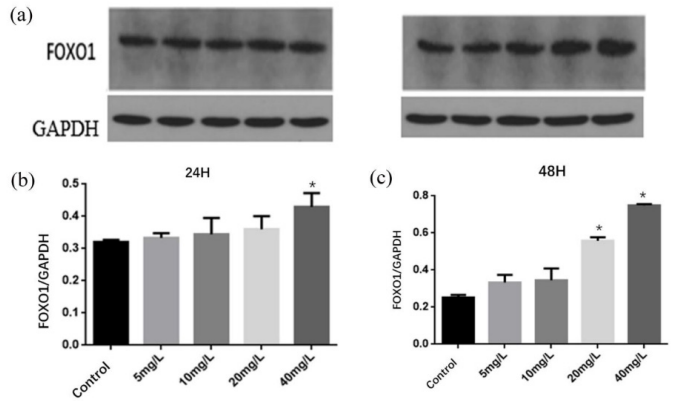

Figure 4: Expression of the FoxO1 molecule.

*Compared with control group, the difference was significant (p<0.05).

40 mg/L group, and the apoptosis index was significantly higher than for the CON group. No relationship was found between the expressions of p-BAD and BAX and the expression of TGF-β2.

DISCUSSION

Some of the experimental concentrations in this study can be found in the living environment, and some are far higher than the usual fluoride concentration in the human body. It is mainly an *in vitro* experiment. This study has not yet entered the category of an animal experiment; it is a cell-based experimental study. The primary purpose is to explore the possible effects (harmful aspects) on the body's bone cells.

The mitochondrial-apoptosis pathway is involved in regulating bone metabolism and function, and it can mediate the differentiation and proliferation of osteoblasts and induce bone formation. As a key regulator of apoptosis, Bcl-2 family proteins mainly act on mitochondrial OMM,[10] mainly by regulating mitochondrial membrane pore forming protein, and then regulate the release of some mitochondrial pro-apoptotic factors, involved in mitochondria-mediated apoptosis. Mitochondria regulate the process of apoptosis through apoptosis-related proteins, and a

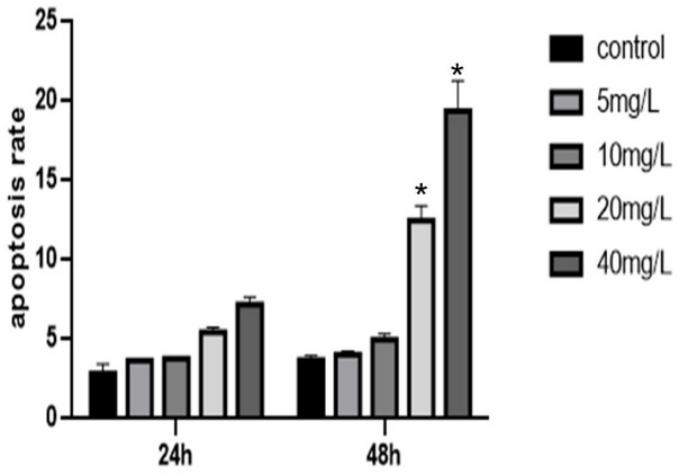

Figure 5: Detection of apoptosis of fluoride-exposed osteoblasts by flow cytometry.

*Compared with control group, the difference was significant (p<0.05).

variety of factors can regulate mitochondria-mediated apoptosis by affecting mitochondrial apoptosis-related proteins.[9] Fluoride has been reported to be able to activate the mitochondrial-apoptosis pathway. Previous studies have shown that TGF-β2 can reduce the apoptosis of osteoblasts through downstream BCL-2.[11] The signaling pathway mediated by TGF-β2 receptors affects the differentiation and cell viability of fluoride-exposed osteoblasts.[12] Therefore, we propose that TGF-β2 has an anti-apoptotic effect through the mitochondrial-apoptosis pathway *in vitro* under fluoride exposure.

This study finds that under the action dose of sodium fluoride (5-40 mg/L, the highest dose reaches mM sodium fluoride, the recognized harmful dose, μM can promote the growth of osteoblasts. The international drinking water standard is that the fluoride in water does not exceed 1 ppm), the expressions of phosphorylated BAD and BAX in the osteoblasts gradually rise with the increase in fluoride dose and the prolongation of fluoride exposure (except for the 40 mg/L groups at T2), indicating that the mitochondrial-apoptosis pathway has been activated and enhances the anti-apoptotic effects. However, this mechanism no longer functions in the 40 mg/L group at T2, and it is unable to resist the apoptosis-inducing effects of a high fluoride concentration. It may be associated with prolonged, high concentrations of sodium fluoride causing other intracellular signaling pathways to actively exert anti-apoptotic effects.

FoxO1, also known as FoxO1a, FKH1, or FKHR, belongs to the O subclass of the forkhead protein family and mediates signal transduction pathways, including IGFR1, TGF-β,PI3K, and Akt. After the FoxO1 protein is activated, it can promote cell-cycle arrest and apoptosis. Highly expressed forkhead-family proteins will lead to the activation of the cell-cycle inhibitory protein p27Kip1. FoxO1 can up-regulate AQP2 expression by binding to the promoter of AQP2, while up-regulated AQP2 can inhibit the

TGF-β signaling pathway.[13] This study shows that as the dose of fluoride increases, the osteoblastic apoptosis index increases, and the expression of FoxO1 also increases. Li reported that FoxO1 in fluoride-exposed ameloblasts mainly played a role in inhibiting cell apoptosis, and the expression level of non-phosphorylated FoxO1 remained unchanged in each dose group.[14] Phosphorylated FoxO1 increased with the increase in fluoride dose (1-8 mM). In Gao's study, incisors were sampled from Sprague Dawley rats after three months of fluoride exposure (50, 100 ppm), and the results revealed that as the concentration of fluoride increased, the expression level of FoxO1 showed a downward trend.[15] The fluoride doses in the two experiments were quite different from this study (this study mainly maintained the fluoride dose at the μM level), and the cells tested were also different, so the protein expression results were not similar. The expression level of phosphorylated FoxO1 in fluoride-exposed osteoblasts still needs further study.

There have been reports confirming that under the condition of fluorosis, BIM (BCL-2 interacting mediator of cell death, a pro-apoptotic protein belonging to the subfamily of the BCL-2 family containing only BH3) is activated, and mitochondrial instability leads to the apoptosis of osteoblasts.[16,17] BAD is an important downstream targeting molecule of TGF-β and also belongs to the pro-apoptotic gene of the BCL-2 family. Because TGF-β can down-regulate BAD mRNA expression, it exerts an anti-apoptotic effect and effectively inhibits BAD-induced apoptosis.[17] This study shows that the expression change of BAD has no apparent relationship with the dose or time of fluoride exposure, but, within the same period of fluoride exposure, the expression of p-BAD increases with the increase in dose. The phosphorylation of BAD plays an essential role in the fluoride-induced apoptosis of osteoblasts.

Members of the BCL-2 family are important regulators of the mitochondrial pathway of apoptosis, and their family members mainly include BCL-2, BCL-XL, BAX, and BAD. As an anti-apoptotic regulator, the BCL-2 protein is widely involved in cell activities. The BAX protein is a pro-apoptotic protein that induces cell death through homodimerization and heterodimerization with BCL-2. This study finds that the expression changes of BCL-XL and BCL-2 are consistent under different fluoride-exposure concentrations and time, which only decrease in the 40 mg/L group at T2, but their expressions in the other groups increase and reach their highest level in the 10 mg/L group. The possible mechanism is that low-dose fluoride has strong promoting effects on their expressions. The apoptosis index in each experimental group is higher than in the CON group under the same conditions, and the change rules are similar to those of BCL-XL and BCL-2. The changes in BCL-XL and BCL-2 are similar to the reported results of apoptosis in recent years.[18,19] In addition, in this study, it has been found that the expression change of BCL-XL is similar to p-BAD and FoxO1, and all the experimental groups exhibit stronger expression than the CON group, except for the 40 mg/L group at T2. FoxO1 decreased in the 40 mg/L group at T2, which may be caused by the toxic effects of high fluoride concentrations.

In summary, TGF-β2 can inhibit the apoptosis of osteoblasts through the mitochondrial-apoptosis pathway and downstream mitochondrial apoptotic molecules, such as BAD, FoxO1, BCL-XL, BCL-2, and BAX. Fluorosis can accelerate the apoptosis of osteoblasts. The discovery of the anti-apoptotic effects of TGF-β2 will help increase the understanding of the mechanism of bone cell apoptosis in fluorosis. The use of the anti-apoptotic effects of TGF-β2 for the treatment of skeletal fluorosis still requires further study.

CONCLUSION

Fluoride can directly activate the TGF-β2 signaling molecules in osteoblasts and affect apoptosis. TGF-β2 plays an anti-apoptosis role in fluoride-exposed osteoblasts through the mitochondrial-apoptosis pathway.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

Osteofluorosis is a global public health problem, and its final pathological result is osteocyte apoptosis. Although the cause of the disease is clear, its pathogenesis is unclear, which leads to the disease being preventable but incurable. To explore the differential expression of apoptosis-related miRNA in osteoblasts under excessive fluoride, to study the anti-apoptosis effect of TGF-β2 in fluoride-stained osteoblasts, and to explore the therapeutic effect of puerarin on skeletal fluorosis. Cell models and animal models of fluorosis were established, and puerarin was given at different concentrations to detect the expression changes of phase miRNA and signal pathway. The selected miRNA was involved in inhibiting apoptosis and promoting apoptosis, and fluoride could directly activate TGF-β signaling pathway in osteoblasts. Puerarin can regulate bone metabolism and reduce blood fluoride in fluorosis rats.

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