



Fluoride Inhibits Longitudinal Bone Growth by Acting Directly at the Growth Plate in Cultured Neonatal Rat Metatarsal Bones

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Abstract

Excessive intake of fluoride inhibits bone growth in both humans and animals. It is unknown whether fluoride acts directly on the growth plate to inhibit longitudinal bone growth, and its mechanism of action has not been elucidated. In this study, we used an organ culture system and SW1353 cells to evaluate the effects of fluoride on endochondral ossification. Neonatal rat metatarsal bones were dissected and cultured with or without fluoride for 7 days. The total length and width of the metatarsal rudiments and the length of the calcification zone were measured. Chondrocyte proliferation, differentiation, and apoptosis were analyzed by immunohistochemistry and TUNEL assay in sectioned bones. The apoptosis was detected by flow cytometry, and the expression of apoptosis-related proteins Bax, Bcl-2, and Caspase-3 were detected by western blotting in SW1353 cells. Linear measurements demonstrated that fluoride induced a biphasic effect on longitudinal bone growth in organ culture, with a significant growth inhibition at a high concentration (10^{-4} M) and a stimulatory action at low concentration (10^{-6} M) of fluoride. Histomorphometrical analysis of growth plate from fluoride-exposed metatarsal rudiments showed a significant reduction in the height of the proliferative and hypertrophic chondrocyte zones. Analysis of the *Col2α1* and *Col10α1* expression by immunohistochemistry revealed fluoride-suppressed metatarsal growth plate chondrocyte proliferation and differentiation. In addition, fluoride increased the number of apoptotic chondrocytes in the metatarsal growth plate. Western blotting showed an up-regulated expression of Caspase-3 and Bax and down-regulated expression of anti-apoptotic protein Bcl-2 after treatment with 5×10^{-4} M fluoride in SW1353 cells. Our findings indicated that fluoride inhibited longitudinal bone growth by acting directly at the growth plate in cultured neonatal rat metatarsal bones. Such growth inhibition was mediated by suppressing proliferation and differentiation, increasing apoptosis of resting chondrocytes and causing premature cell senescence in the growth plate.

Keywords Fluoride · Metatarsal organ culture · Bone growth · Endochondral ossification · Apoptosis

Abbreviations

<i>Col2α1</i>	Type II collagen
<i>Col10α1</i>	Type X collagen
RZ	Resting zone
PZ	Proliferative zone
HZ	Hypertrophic zone

Introduction

Fluoride is widespread in nature, and several studies have been reported that small amount of fluoride is beneficial to

stimulate new bone formation, but long-term excessive ingestion of fluoride can cause fluorosis [1]. Both animal studies and epidemiological investigations have demonstrated that high doses of fluoride inhibited the growth of long bones, enlarged growth plate, and delayed fusion of the growth plate [2–5].

In mammals, longitudinal bone growth occurs through a process of endochondral ossification within the growth plate, which gradually converts avascular cartilage into highly vascularized bony tissue [6]. The growth plate, a cartilaginous tissue with a unique morphological structure, comprises an array of chondrocytes organized into resting, proliferative and hypertrophic zones, which are important for endochondral ossification [7]. Endochondral ossification is initiated by condensation of mesenchymal cells, and subsequently the condensed mesenchymal cells proliferate and differentiate into chondrocytes. Proliferative chondrocytes express chondrogenic marker *Col2a1* and form characteristic columns [8]. Then they stop proliferating and further differentiate into

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hypertrophic chondrocytes and mineralize, express *Col10a1* and osteocalcin [9]. Finally, calcified chondrocytes that undergo apoptosis are replaced by the bone.

During endochondral ossification, chondrocytes in the hypertrophic zone of the growth plate exit their developmental pathways by apoptosis during terminal differentiation [10]. Previous studies have revealed that increased unscheduled apoptosis will disturb chondrocyte activity within the growth plate and lead to defective longitudinal bone growth [11]. There are two major apoptotic signaling pathways: the intrinsic pathway mediated by mitochondria and the extrinsic pathway induced by death signaling ligands that induce caspase-8 initiator protease, which activates the executioner proteases such as Caspase-3 [12]. Besides, changes in the level of pro-apoptotic (Bax) and pro-survival (Bcl-2) members of the Bcl-2 family are critical determinants of the intrinsic cell death pathway [13].

The formation of endochondral bone *in vivo* depends on a complex and concerted gene regulatory network; any disruption of this delicate network could lead to abnormal skeletal development and bring about skeletal disorders [14, 15]. Based on the evidence of previous studies, we hypothesized that high doses of fluoride may affect the process of endochondral ossification and cause longitudinal bone growth inhibition. Although there is ample evidence that fluoride affects the anabolic effects of the skeletal system, little is known about the direct effect and molecular mechanisms by which fluoride regulates endochondral ossification and linear growth.

To address these questions, the effects of fluoride on metatarsal longitudinal growth, chondrocyte proliferation, differentiation, and apoptosis were examined in cultured metatarsal, and the apoptosis-related protein expressions were detected by western blotting using SW1353 cells. *In vitro* organ culture model of neonatal rat metatarsal bones is a well-established physiological model that has been used extensively to study the effects of different types of factors on the process of endochondral ossification and postnatal growth during long bone development [16]. The human chondrosarcoma cell line SW1353 cells serve as a model for the development of chondrocytes, which has been reported to maintain the characteristics of chondrocytes and can be used to observe chondrocyte typical marker genes and the expression of the proteins [17]. The present study was designed to clarify whether fluoride could act a direct role on the growth plate to inhibit longitudinal growth, and the cellular events underlying fluoride-induced growth retardation.

Materials and Methods

Rat Neonate Metatarsal Organ Culture

Sprague-Dawley rats (weight of 150–200 g) were obtained from the Laboratory Animal Center of China Medical

University. All rats were provided regular diet and water *ad libitum* and raised under a 12-h light/dark cycle with a constant temperature (24°C) and relative humidity (45%–55%). Metatarsals were separated as previously described [18]. Briefly, the second, third, and fourth metatarsal bones were aseptically dissected from the hind paws of newborn Sprague-Dawley rats (within 24 h of birth) in phosphate buffered saline (1 × PBS) with 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Paisley). The metatarsals were intact and the perichondrium was not damaged. Bone rudiments were cultured in 24-well plates (Nunclon, Denmark). Each well contained 0.5 ml of α -modified minimal essential medium (α -MEM, Invitrogen, Paisley), supplemented with 0.05 mg/ml ascorbic acid (Sigma, Germany), 1 mmol/L β -sodium glycerophosphate (Sigma, Germany), 0.2% bovine serum albumin (BSA, Sigma, Germany), 100 U/ml penicillin, and 100 µg/ml streptomycin. The metatarsal bones were cultured for 7 days in a humidified incubator with 5% CO₂ at 37°C, and the medium was changed every other day. In the first set of experiments, metatarsals were cultured for 7 days in serum-free medium in the absence or presence of various concentrations of fluoride (10⁻⁷, 10⁻⁶, 10⁻⁵, 10⁻⁴ M, Wako Pure, Japan) to study the dose-response and select the dose which impaired bone development. In the second set of experiments, the metatarsal bones were cultured with 10⁻⁴ M fluoride for 7 days to study the effect of fluoride on morphometry and immunohistochemistry.

The experiments were set up for left-right paired observations, which the right metatarsals were treated with fluoride and the left metatarsals were served as controls. Controls were grown in the same amount of medium as treated cultures. Each experiment was carried out on the neonatal rats from a single mother, and rats significantly smaller or bigger compared with the average size were discarded. Each experiment was repeated at least three times. The study was approved by the local ethics committee at China Medical University.

Cell Culture

The human chondrosarcoma cell line SW1353 cells were obtained from the Cell Bank of Shanghai Institute of Biological Science of CAS (Shanghai, China). Cells were cultured in Leibovitz's L-15 Medium (Gibco, Australia) supplemented with 10% fetal bovine serum (FBS, Gibco, Australia), 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were grown at 37°C in a humidified incubator without CO₂, passaged two to three times per week, and exponentially growing cells were used for all described experiments.

Measurement of Longitudinal Bone Growth

To evaluate the longitudinal growth of the metatarsal bones *in vitro*, metatarsal bones were photographed at the dissection

day (designated as day 0) and at the end of the culture period (day 7) under a stereomicroscope (Nikon, Japan). The culture medium was briefly removed before each measurement. The total length of the bone and width through the center of the mineralizing zone and the length of the mineralizing zone (visible as a dark zone) in each metatarsal were measured by the SPOT Advanced Software (Sterling Heights, USA). The longitudinal growth of the metatarsal bone was expressed as the average percentage increase in length (% increase) relative to harvesting length. All measurements were performed by a single observer blinded to the treatment regimen.

Histomorphometry

After 7 days of incubation without or with fluoride, metatarsals were fixed with 4% phosphate-buffered paraformaldehyde for 24 h, decalcified in 10% EDTA overnight. After dehydrated through a graded series of ethanol solutions, metatarsals were embedded in paraffin wax, and three longitudinal 5- μm -thick sections were obtained from each metatarsal bone with a microtome (Leica, Germany). The sections were stained with 0.1% toluidine blue, counterstained with hematoxylin and eosin (HE) and evaluated at 10 \times magnification using microscope (Nikon, Japan) for general morphology. The heights (μm) of the resting zone, proliferative zone, and hypertrophic zone were measured, and the average value was calculated. The height of each zone was determined along the centerline of the axial sections. Measurements were performed using the SPOT Advanced Software. In the metatarsal growth plate, the resting zone is characterized by small and round cells. The proliferative zone comprises cells with a flattened shape, organized in columns parallel to the longitudinal axis of the bone. In the hypertrophic zone, large cells (defined by a height greater than 9 μm) form a layer adjacent to the calcified region of the metatarsal bone, the primary ossification center. All the measurements were performed by a single observer blinded to the treatment regimen.

Immunohistochemistry

Sections were deparaffinized in xylene and rehydrated in descending graded ethanol. Antigen retrieval was performed in 10-mM sodium citrate buffer (pH 6.0) at 98–100 $^{\circ}\text{C}$ for 10 min, and endogenous peroxidase activity was inactivated with 3% H_2O_2 for 20 min at room temperature. Sections then were blocked in blocking serum at room temperature for 30 min and incubated with primary antibody overnight at 4 $^{\circ}\text{C}$. After washing in PBS, sections were incubated with a biotinylated secondary antibody and then with an avidin-biotinylated horseradish peroxidase complex according to the manufacturer's directions. Finally, sections were developed with DAB and counterstained with hematoxylin

solution. The following primary antibodies were used: *Col2a1* (1:200, Boster, China) and *Col10a1* (1:200, Boster, China).

Apoptosis Assay (TUNEL)

Rat metatarsal bones cultured for 7 days were fixed in 4% phosphate-buffered paraformaldehyde, decalcified in 10% EDTA and 5- μm paraffin-embedded sections were obtained. Apoptotic cells in the growth plate were identified by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick end labeling (TUNEL) immunohistochemistry using the In Situ Cell Apoptosis Detection Kit I (POD) (Boster, China) according to the manufacturer's instructions. For each group, the number of apoptotic chondrocytes was determined in three metatarsals. Positive cells within the perichondrium and primary ossification center were excluded from the analysis. Apoptosis was quantitated by determining the apoptotic index that calculated as the number of apoptotic chondrocytes divided by the total number of chondrocytes. In each growth plate, the apoptotic index was calculated separately in three distinct locations of the resting, proliferative and hypertrophic zones, and then averaged. All determinations were made by the observer blinded to the treatment category.

Cell Viability Assay

The cell counting kit-8 (CCK-8, Beyotime, China) assay was used to assess cell viability. Briefly, to determine the appropriate dose, cells were seeded in 96-well plates at a density of 5×10^3 cells per well overnight and treated with 10^{-5} , 10^{-4} , 5×10^{-4} , and 10^{-3} M of sodium fluoride for 72 h. Then, we choose 5×10^{-4} M sodium fluoride treatment for 24 h, 48 h, and 72 h to select the optimal exposure time. After incubation for corresponding time, cells were incubated with CCK-8 for 3 h, and the absorbance of each well was determined at 450 nm using a microplate reader (Bio-Rad, USA). Cell viability (%) = $(\text{OD}_{\text{test group}} - \text{OD}_{\text{control group}}) / \text{OD}_{\text{control group}} \times 100\%$. Six wells per treatment group were measured, and the experiments were repeated three times. The viability curves were drawn based on the different concentrations with GraphPad Prism 7.

Flow Cytometry Analysis

The apoptosis was detected by FACS Calibur flow cytometry (BD Biosciences, USA) using the Annexin V-FITC/PI Detection Kit (KeyGEN, China) in SW1353 cells. Briefly, cells were exposed without or with fluoride for 72 h and digested with EDTA-free trypsin (KeyGEN, China) and washed three times with cold PBS, and then the SW1353 cells were resuspended at a density of 1×10^6 cells/ml in 500 μl

binding buffer. After stained with Annexin V-FITC (6 μg) and propidium iodide (PI, 5 μg) for 20 min incubated in the dark at room temperature, the cells were examined immediately by flow cytometry within 1 h, and the apoptotic cells were defined as the annexin FITC-positive and PI-negative (annexin FITC+/PI-) cells.

Western Blotting

Cells were harvested, washed twice with cold PBS, and lysed in RIPA lysis buffer (Beyotime, China). The total protein concentration was determined using a BCA Protein Assay kit (Beyotime, China). The proteins (15 μg) were separated by 15% SDS-polyacrylamide gel electrophoresis and transferred to the polyvinylidene difluoride (PVDF, 0.45 μM) membranes. The blots were blocked with 5% nonfat milk for 1 h at room temperature and were treated with primary antibodies against Bax (Abcam, 1:1000), Bcl-2 (Abcam, 1:1000), Caspase-3 (Abcam, 1:3000), and Gapdh (CST, 1:4000) for 1 h at room temperature and then incubated overnight at 4°C. After washed four times with PBST, the blots were incubated with fluorescent secondary antibody (Abcam, 1:1000) at room temperature for 1 h. Finally, the bands were visualized by scanning densitometry (Odyssey, USA), and the immunopositive band was analyzed using Image Studio software.

Statistical Analysis

Results are expressed as the mean \pm SD. Differences between groups were determined by the Student paired t-test or analysis of variance (ANOVA). Considered significant changes were classified as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Each experiment was performed at least three times, and the representative results were shown.

Results

Effects of Fluoride on Longitudinal Bone Growth

To evaluate the effects of fluoride on longitudinal bone growth, we cultured the neonatal rat metatarsal bones without or with graded concentrations of fluoride for 7 days. The total length of metatarsal bones and mineralized zone was measured on day 0 and day 7 in both fluoride-treated group and control group, and then the corresponding longitudinal growth rate was calculated. All neonatal rat metatarsals, even when maintained in fluoride-containing culture medium, underwent a steady growth in serum-free media during the course of 7 days culture and displayed the most central core of mineralized cartilage (dark zone) adjacent to the translucent

hypertrophic chondrocytes (light zone) on both sides (Fig. 1a), which suggested that the metatarsal bones were viable.

As shown in Fig. 1b, the mean percentage increase in the total length of the metatarsal rudiments containing 10^{-4} M fluoride was significantly reduced by approximately 14% after 7 days of culture compared with the control group ($p < 0.05$). Although treatment with 10^{-6} M fluoride for 7 days was increased in total longitudinal bone growth, these changes did not reach statistical significance, and other concentrations of fluoride (10^{-7} and 10^{-5} M) did not affect bone longitudinal growth. In the mineralized area, a similar alteration was observed in cultured rat metatarsals treated with 10^{-4} M fluoride compared with controls (Fig. 1c). The widths of the control and fluoride-treated metatarsals were not significantly different from each other during the culture (data was not shown).

Based on these results, particularly on the impairment of endochondral growth and mineralization, the significant inhibitory concentration of fluoride at 10^{-4} M was used for the following experiments.

The Time Course of Response to Fluoride

To further investigate the observed inhibitory effect of fluoride on bone growth, we cultured the rat metatarsals without or with 10^{-4} M fluoride for 7 days. At the beginning of the experiment, the mean length of metatarsals in the control and the fluoride-treated group was not statistically different. In the control group, the total longitudinal length of metatarsals increased time-dependently during the course of 7 days culture. However, metatarsals treated with 10^{-4} M fluoride showed a slower growth rate from the third day of culture. Then the difference between control and fluoride-treated metatarsals became larger with time. On day 5 and day 7, metatarsals cultured in the presence of 10^{-4} M fluoride grew significantly less than the control group. ($p < 0.05$, Fig. 2a). Similar changes of the mineralized area were observed in cultured rat metatarsals treated with 10^{-4} M fluoride, the mean percentage increase of the mineralized area in the fluoride-treated metatarsals was significantly less than the paired control group on day 5 and day 7 ($p < 0.05$, Fig. 2b).

Effects of Fluoride on Growth Plate Histology

We then evaluated the effects of fluoride on the metatarsal growth plate morphology. As shown in Fig. 3a, the growth plate was composed of round resting chondrocytes, columnar proliferating chondrocytes, and mature hypertrophic chondrocytes. In control group, the round resting chondrocytes were closely arranged. The orderly column of proliferating chondrocytes undergone differentiation to pre-hypertrophic and hypertrophic cells in control metatarsals. In contrast, the growth plate of fluoride-treated metatarsals

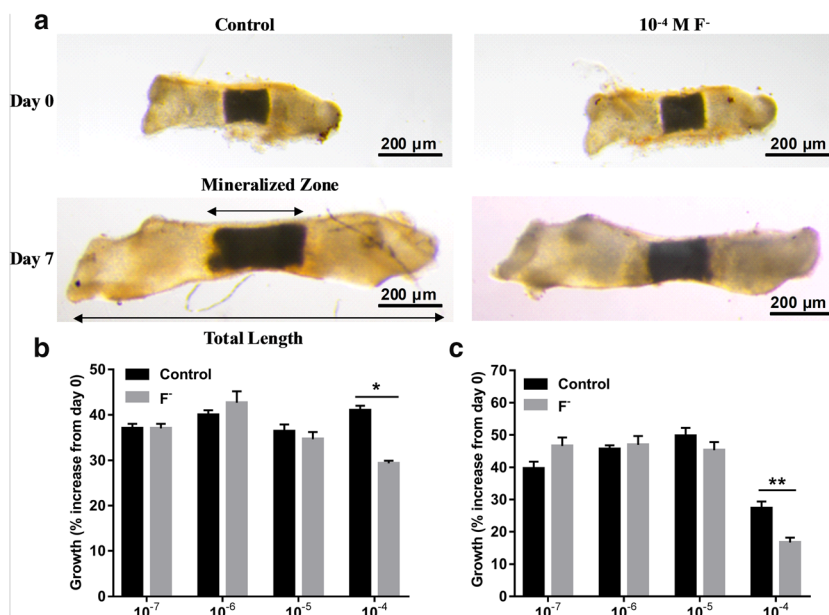


Fig. 1 Effects of fluoride on longitudinal bone growth of rat metatarsal bone rudiments. **(a)** Neonatal rat metatarsals were cultured without or with 10^{-4} M fluoride for 7 days and observed under stereomicroscope. Noticed the increased length of the metatarsals and the dark zone of mineralized matrix in the center of each bone rudiment after 7 days (Bar = 200 μ m). **(b)** Dose-dependent effect of fluoride on total length of rat metatarsals. Neonatal rat metatarsal bones were cultured for 7 days in serum-free medium containing 0, 10^{-7} , 10^{-6} , 10^{-5} , or 10^{-4} M fluoride. Total length of metatarsals was measured at day 0 and day 7 of culture under stereomicroscope. Growth was expressed as percent increase in

metatarsal bone length from day 0 of culture. **(c)** Dose-dependent effect of fluoride on the length of mineralized area of rat metatarsals. Neonatal rat metatarsal bones were cultured for 7 days in serum-free medium containing 0, 10^{-7} , 10^{-6} , 10^{-5} , or 10^{-4} M fluoride. The length of mineralization of neonatal rat metatarsal bones was measured at day 0 and day 7 of culture in stereomicroscope. Growth was expressed as percent increase in metatarsal mineralization area length from day 0 of culture. All data was expressed as the mean \pm SD for at least three independent experiments ($n = 6$). * $p < 0.05$ and ** $p < 0.01$ vs. control group

exhibited some morphological abnormalities, including disorganized resting chondrocyte, proliferative chondrocytes loss of the normal columnar arrangement, and hardly defined transition zone from proliferative to hypertrophic chondrocyte. Besides, the morphometric analysis of the metatarsals revealed that the heights of the resting, proliferative and hypertrophic zone in fluoride-treated metatarsals were decreased significantly by 16%, 13%, and 22% relative to the control ($p < 0.05$, Fig. 3b).

Effect of Fluoride on *Col2a1* and *Col10a1* Expression in Metatarsals

To further define the domains of proliferative and hypertrophic chondrocytes in the rat metatarsals, we performed immunohistochemistry to detect the expression of *Col2a1* and *Col10a1* (the specific markers of chondrocyte proliferation and differentiation) at the end of the culture period. Both *Col2a1* and *Col10a1* are mainly expressed in the chondrocyte

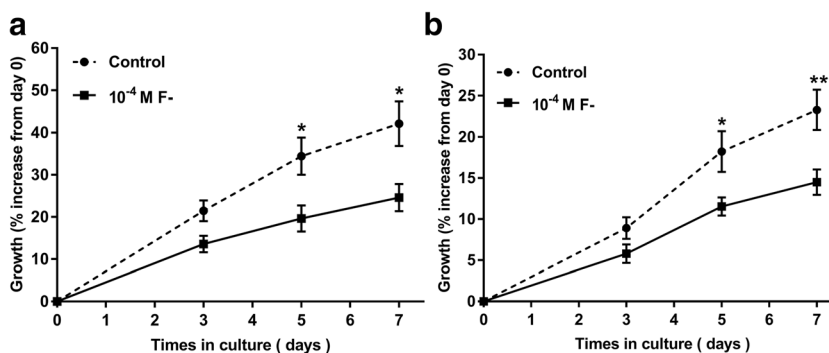


Fig. 2 Time course of the longitudinal growth of rat metatarsal bones treated without or with fluoride (10^{-4} M). Rat metatarsals were cultured in the absence (dashed line, \bullet) or presence of fluoride at 10^{-4} M (solid line, \blacksquare) for 7 days. **(a)** The total length and **(b)** length of mineralization area of neonatal rat metatarsal bones were measured on day 0, 3, 5, and 7

in culture as described in Materials and Methods. Growth was expressed as percent increase in metatarsal bone length from day 0 of culture. All data was expressed as the mean \pm SD for at least three independent experiments ($n = 6$). * $p < 0.05$ and ** $p < 0.01$ vs. control group

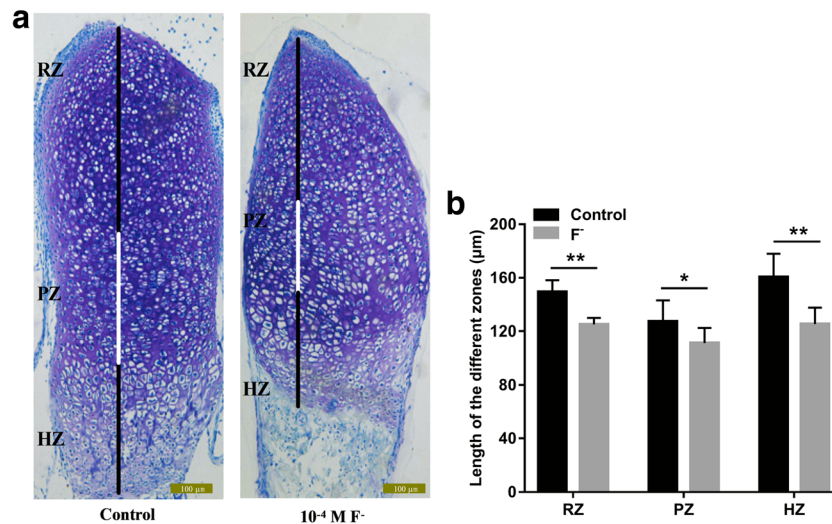


Fig. 3 Histological analysis in cultured rat metatarsals. Metatarsals were cultured without or with 10^{-4} M fluoride for 7 days. After routine histological processing, the metatarsal bones were embedded in paraffin, and 5- μ m-thick longitudinal sections were obtained and stained with 0.1% toluidine blue and counterstained with hematoxylin. (a)

Representative photographs were obtained from each treatment of metatarsal bones stained with toluidine blue (magnification $10\times$). (b) The bar graph showed analysis of the length of different zones ($n = 6$). RZ, resting zone; PZ, proliferative zone; HZ, hypertrophic zone

extracellular matrix with brown staining. As shown in Fig. 4a, *Col2a1* was highly expressed throughout all zones of the growth plate except for the area of calcified cartilage. In the resting, proliferative, and hypertrophic zone, the expression of *Col2a1* in the control group was more intense than that of the fluoride-treated group. Similarly, the staining in the control group was stronger than that of the fluoride-treated group for *Col10a1*, which was expression predominantly restricted to the hypertrophic zone of the rat metatarsals (Fig. 4b). This demonstrated that except for inhibiting proliferation, hypertrophic differentiation was also inhibited by fluoride.

Fluoride-Induced Apoptosis of Chondrocytes

To explore the reason for fluoride-inhibited longitudinal bone growth, we analyzed apoptosis in the metatarsal bones firstly (Fig. 5a, b). Quantitative analysis revealed that 10^{-4} M

fluoride caused a dramatic increase in apoptosis which was three fold in the resting zone and two fold in the proliferative zone ($p < 0.05$). There was no significant change in the level of apoptosis in the hypertrophic zone (Fig. 5e, f). These results suggested that stem-like chondrocytes in the growth plate were more susceptible to apoptosis compared with hypertrophic chondrocytes. Secondly, to investigate the potential effects of fluoride on cell growth, cell viability was examined by CCK-8 assay. The cell viability of SW1353 cells was significantly decreased at 5×10^{-4} M fluoride for 72 h ($p < 0.05$, Fig. 6a). Next, to determine the optimal exposure time, cells were treatment with 5×10^{-4} M fluoride for 24 h, 48 h, and 72 h. The result showed fluoride significantly reduced cell proliferation at 72 h compared with control ($p < 0.05$, Fig. 6b). Therefore, the 5×10^{-4} M fluoride treatment for 72 h was used in subsequent experiments. Then, we investigated whether fluoride-induced cell apoptosis by annexin V-

Fig. 4 Effect of fluoride on *Col2a1* and *Col10a1* expression in the metatarsals. *Col2a1* and *Col10a1* expression were analyzed in the metatarsal of control (left) and fluoride-treated (right) by immunohistochemistry (magnification $10\times$). (a) Staining of *Col2a1* protein using a rabbit polyclonal antibody detected against rat *Col2a1* at a dilution of 1:200. (b) Staining of *Col10a1* protein using a rabbit polyclonal antibody detected against rat *Col10a1* at a dilution of 1:200. Bar = 100 μ m ($n = 6$)

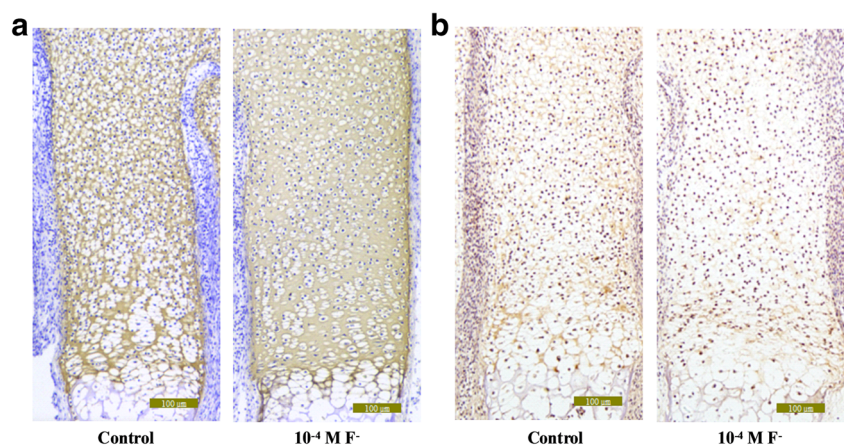
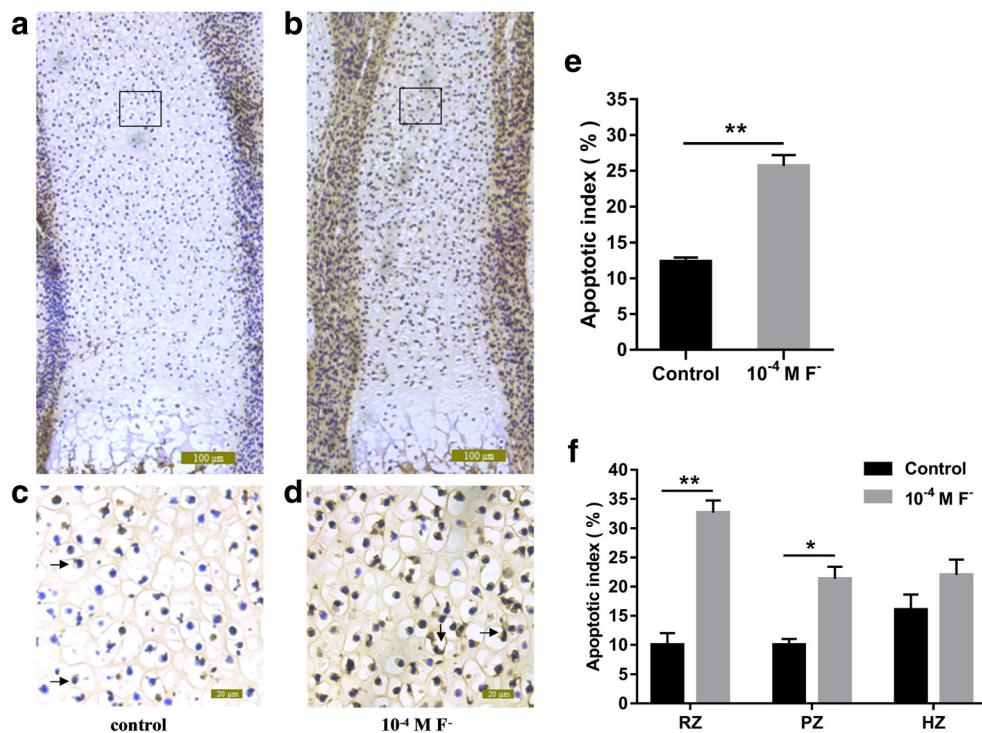


Fig. 5 Effect of fluoride on chondrocyte apoptosis in metatarsal bones. Apoptotic cells in the metatarsal were analyzed by TUNEL assay. Representative photomicrograph showed apoptosis in the metatarsals culture. **(a and b)** Rat metatarsal bones were cultured without (left) or with (right) 10^{-4} M fluoride for 7 days. **(c and d)** The apoptosis chondrocytes were shown in higher magnification (arrows indicate apoptotic chondrocytes, original magnification, $20\times$). Bar = $100\ \mu\text{M}$. **(e)** Total apoptotic index in control and 10^{-4} M fluoride-treated metatarsals. **(f)** The bar graph showed apoptotic index in resting zone, proliferative zone and hypertrophic zone. Data was expressed as the mean \pm SD for at least three independent experiments ($n = 6$). $*p < 0.05$ and $**p < 0.01$ vs. control group



FITC/PI double staining, which was used to detect PS externalization, a feature of the early phase of apoptosis. According to the results of flow cytometry analysis, treatment with fluoride significantly increased the apoptosis rates, which was three fold compared with control groups ($p < 0.05$, Fig. 7a, b). The western blotting assay showed that the protein level of Bax and Caspase-3 were markedly increased, while the level of Bcl-2 was dramatically declined after treated with fluoride for 72 h, compared with controls ($p < 0.05$, Fig. 7c, d).

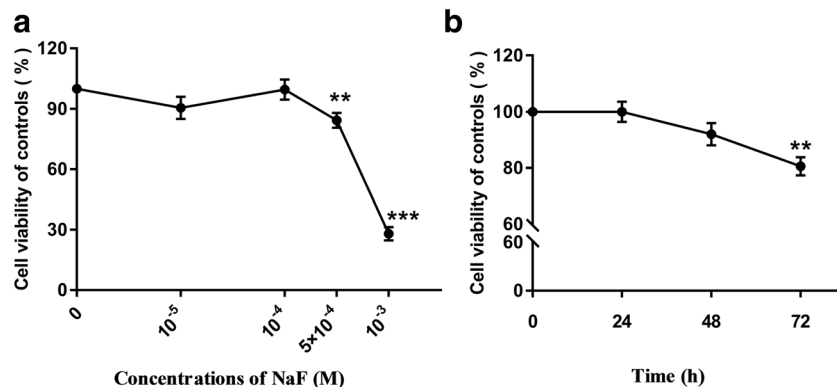
Discussion

Although previous studies have raised the possibility that fluoride has an inhibitory effect on longitudinal bone growth, the molecular mechanisms of fluoride on endochondral bone

formation still remain elusive. The observed inhibitory effect of fluoride on longitudinal bone growth could result from indirect action mediated by the circulating systemic hormones or direct action on the growth plate mediated by local humoral factors in a paracrine manner. In this study, to examine the direct effect of fluoride on bone growth in vitro, we used a previously reported neonatal rat metatarsal organ culture model to exclude the influence of systemic hormones. We found that fluoride impaired longitudinal bone growth by decreasing growth plate chondrocyte proliferation and differentiation and increasing chondrocytes apoptosis in the neonatal rat metatarsals. Fluoride also promoted cell apoptosis and altered the normal expression of apoptosis-related proteins in SW1353 cells.

The rate of longitudinal bone growth is determined by the complex interaction of proliferation kinetics, proliferation pool size, matrix synthesis, and hypertrophic chondrocyte

Fig. 6 Effects of various concentrations of sodium fluoride on the viability of SW1353 cells. **(a)** SW1353 cells were treated with indicated concentrations of sodium fluoride for 72 h. **(b)** SW1353 cells were treated with 5×10^{-4} M sodium fluoride for 24, 48, and 72 h. Cell viability was examined by CCK-8 assay. Data was expressed as the mean \pm SD from three separate experiments ($n = 6$). $**p < 0.01$, $***p < 0.001$ vs. control group



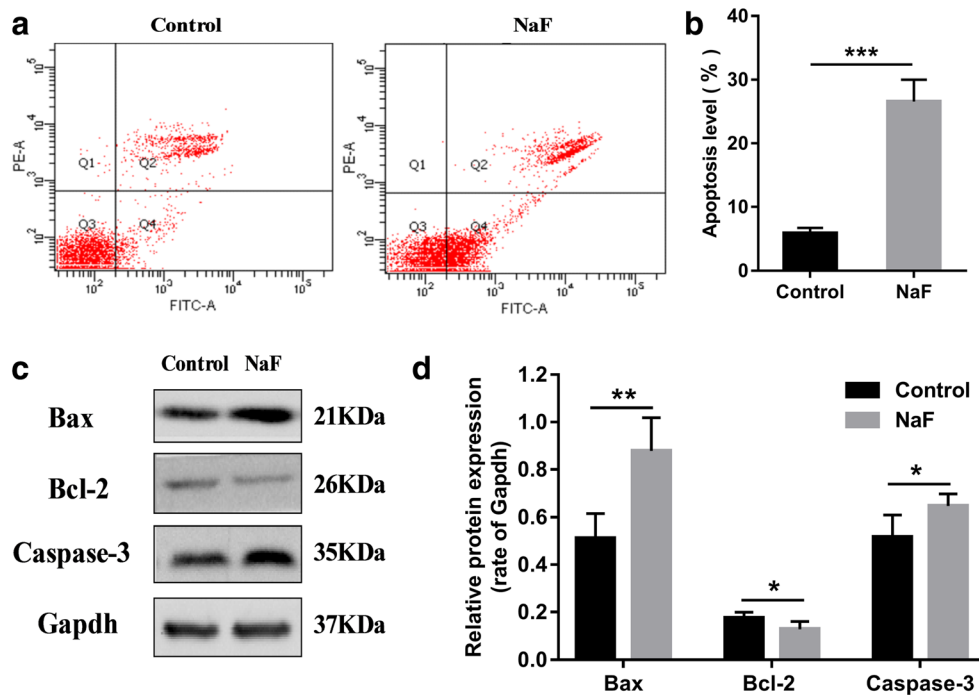


Fig. 7 Fluoride-induced apoptosis of SW1353 cells. **(a)** SW1353 cells were treated with or without 5×10^{-4} M sodium fluoride for 72 h. The percentage of apoptotic cells was analyzed by flow cytometry of annexin FITC/PI double staining. **(b)** The histogram illustrated the apoptosis rate in control and 5×10^{-4} M fluoride treatment, and the apoptotic cells were defined as the annexin V-positive and PI-negative (annexin V+/PI-) cells. **(c)** After treated without or with 5×10^{-4} M sodium fluoride for 72 h, the

protein expression of Bax, Bcl-2, and Caspase-3 were examined by western blotting. **(d)** Quantification of protein expression in control and 5×10^{-4} M fluoride-treated SW1353 cells. Equivalent protein loading was determined by detection of Gapdh. Data was expressed as the mean \pm SD of three independent experiments ($n = 5$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group

enlargement [19, 20]. However, those control processes still remain a matter of debate, each variable has a different effect on the growth rate of the bone, and it is not concordant for all bones [21]. In those harmonized variables, any disorder caused by fluoride could lead to changes in bone growth. We performed longitudinal measurements during in vitro culture as previously described. The results showed that fluoride exhibited a biphasic manner on longitudinal bone growth in organ culture, with a significant growth inhibition at high concentrations and a stimulatory action at low concentrations of fluoride. This finding is consistent with the effects elicited by fluoride in vivo [3]. Our results definitely showed that high concentration of fluoride inhibited metatarsal longitudinal growth, since the total length of metatarsal bones were significantly reduced compared with the control groups after 7 days cultured. Besides, metatarsals treated with 10^{-4} M fluoride showed a slower growth rate of mineralized area from the third day of culture, and the difference became larger with the extension of time. On day 5 and day 7, the metatarsals cultured in 10^{-4} M fluoride grew significantly less than the control.

In mammals, the rate of longitudinal bone growth depends primarily on the rate of chondrogenesis in the growth plate, which is generally divided into three regions, including the resting zone, the proliferative zone and the hypertrophic zone

[22]. The resting zone chondrocytes are the reserve forces of cartilage, also known as the reserve zone chondrocytes since the splitting is lower and can be converted into the proliferation chondrocytes [23]. The function of proliferative zone chondrocytes is to stimulate the longitudinal bone growth by promoting cell proliferation and secreting extracellular matrix [24]. The chondrocytes become calcified and are replaced with bone in the hypertrophic zone terminally [25]. Briefly, the growth of the long bone requires the coordinate development and maturity of the three zones mentioned above. To determine the mechanism underlying the fluoride-induced growth deceleration, we evaluated the effects of fluoride on the height of the resting, proliferative, and hypertrophic zones. Our results showed that fluoride decreased the height of the three zones of the metatarsal growth plate, and the ratio of the proliferation zone to the hypertrophic zone was unbalanced. This result suggested that fluoride inhibited the longitudinal growth by decreasing the height of the resting, proliferative, and hypertrophic zones and altering the balance between proliferating and hypertrophic chondrocytes within the metatarsals, but the specific regulation mechanism still needs further research. Collagen is a fibrous protein that is important for maintaining the normal structure and shape of cartilage, while various chondrocytes express diverse collagen phenotypes at different stages of differentiation [26, 27]. Fluoride is an

essential trace element closely related to collagen metabolism, and excessive fluoride can cause abnormal metabolism of collagen in animal bones and cartilages [28]. We found that the markers of chondrocyte proliferation (*Col2a1*) and hypertrophic differentiation (*Col10a1*) were decreased upon fluoride treatment. These results indicated that fluoride inhibited chondrocyte proliferation and differentiation and decreased the thickness of the proliferative zone and hypertrophic zone. These data suggested an inhibitory role for fluoride in bone growth, decreased chondrocyte proliferation presumably results in fewer chondrocytes that were available to differentiate to hypertrophic cells, which was consistent with the results of morphometrics.

It has been reported that apoptosis plays a critical role in growth plate homeostasis during development. Therefore, to further understand the mechanisms of fluoride-induced growth retardation, we assayed chondrocyte apoptosis in neonatal rat metatarsal bones treated with fluoride after 7 days culture by TUNEL staining. The results showed that treatment with fluoride caused a dramatic increase in apoptosis, especially in the resting zone, which was three fold of the control groups. In addition, the results of annexin FITC/PI double staining revealed that treatment with fluoride significantly increased the early apoptosis rate in SW1353 cells. Hence, we speculated fluoride inhibited metatarsal longitudinal growth by increasing apoptosis. To date, the apoptosis-inducing pathway can be divided into two main pathways: the death receptor-mediated extrinsic and the mitochondrial-dependent intrinsic pathway [29]. Both the pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 belong to Bcl-2 family proteins, which regulate mitochondria-dependent apoptosis. Previous study showed that silence of Bax expression using siRNA efficiently blocked Dexamethasone-induced apoptosis and prevented from Dexamethasone-induced bone growth retardation [13]. A recent study showed that a small peptide humanin possessing anti-Bax and anti-apoptotic effects prevents bone growth retardation [30]. Besides, one of the biomarkers of the apoptotic process is the activation of cysteine proteases [31]. The group of intracellular cysteine enzyme caspases can destroy essential cellular proteins and arbitrate cell death including both initiators and executors. Caspase-3 is an imperative executor among them [32, 33]. We detected the protein expression of Bax, Bcl-2, and Caspase-3 by western blotting in SW1353 cells and found that fluoride treatment markedly increased the expression of Bax and Caspase-3 and decreased the expression of Bcl-2. Based on the above results, we speculated that combination of the changes of proliferation, differentiation, and apoptosis could explain fluoride inhibited metatarsal longitudinal growth. Cell proliferation, differentiation, and apoptosis are three optimal parameters for the development of growth in every dynamic model, which lead to cell senescence eventually [34]. The cell senescence is associated with the growth rate and the number of the growth plate chondrocytes.

We observed that apoptosis was significantly increased in the resting zone and the proliferative zone. The resting chondrocytes are also known as the stem-like chondrocytes, which are essential to product proliferative chondrocytes and provide the basis for normal longitudinal bone growth in the growth plate [35]. Loss of stem-like chondrocytes by apoptosis upon fluoride treatment will decrease the number of chondrocyte columns and the total number of chondrocytes, cause premature cell senescence and early cessation of bone growth, and diminish the growth potential. Besides, premature loss of resting or early proliferative cells could reduce the production of hypertrophic cells, decrease the number of calcified chondrocytes which are soon replaced by bone, destroy the growth potential, accelerate cellular senescence, and eventually result in longitudinal bone growth inhibition.

In conclusion, this study provided the in vitro evidence that fluoride at high concentration directly inhibited longitudinal bone growth, which was associated with decreased cell proliferation, differentiation, and increased apoptosis. Inhibition of proteasome function has been shown to sensitize the stem-like chondrocytes to undesired apoptosis [36]. Similarly, local inflammation can also impair proliferation and differentiation of chondrocytes causing growth retardation [37]. Our research revealed the inhibitory mechanism of fluoride on the longitudinal bone growth and provides new ideas for the prevention and control of fluorosis. However, additional studies are still needed to explore the potential ways to rescue fluoride-induced growth retardation, by using small molecules/peptides. Besides, whether there is catch-up growth of metatarsal bones cultured in vitro after discontinuing fluoride treatment is also worth further study.

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Compliance with Ethical Standards

All experiments were performed according to the norms of the local ethics committee at China Medical University in accordance with the national guidelines for animal use and care.

Conflict of Interest The authors declare that there is no conflict of interest.

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