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Original Article

Excessive fluoride stimulated osteoclast formation through up-regulation of receptor activator for nuclear factor-κB Ligand (RANKL) in C57BL/6 mice

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Abstract: Skeletal fluorosis is a metabolic bone disease caused by excessive consumption of fluoride. However, the pathogenesis of skeletal fluorosis remains unclear. Recent vitro studies have demonstrated that fluoride could increase RANKL mRNA expression in osteoblast. In this study, the relationship between RANKL and osteoclast formation was investigated in fluorosis mice. Twenty male C57BL/6 mice were randomly divided into two groups: control group (drinking distilled water) and 100 mg/L fluoride group (drinking distilled water with 100 mg/L fluoride ion). Mice were fed for 15 weeks and allowed to eat and drink freely. The occurrence of dental fluorosis in mice was observed during the fed period. Bone fluoride content was detected by ion selective electrode method. The effect of fluoride on bone tissue was assessed with hematoxylin and eosin (HE) staining. Tartrate-resistant acid phosphatase (TRAP) staining was used to evaluate osteoclast formation in bone tissue. The mRNA and protein expression and distribution of RANKL were separately detected using real-time quantitative PCR (RT-PCR), Western-blot and immunohistochemistry. By the end of the animal experiment, all mice in the fluoride group had dental fluorosis with different severities, while none dental fluorosis was found in the control group. Bone fluoride content in fluoride group was about 4.6 times higher than that in the control group. Compared with control group, the trabecular bone in experimental group was expanded and fused, which lead to an increase of bone mass in fluoride group. An increase of osteoclast number was found in bone tissue of fluorosis mice. The expression of RANKL mRNA and protein in bone tissue of fluorosis mice were significantly higher than that of control group, however, the distribution of RANKL between the two groups did not differ. Excessive fluoride could stimulate osteoclastogenesis in bone tissue of mice, and the molecular mechanism of fluoride action may be through up-regulation of RANKL expression. It is suggested that RANKL may play a vital role in the pathogenesis of skeletal fluorosis.

Keywords: Skeletal fluorosis, fluoride, osteoclast, RANKL

Introduction

Endemic fluorosis is caused by excessive intake of fluoride through drinking water, tea, burning high-fluoride coal and industrial processes [1]. It is estimated that about 200 million people from 25 countries may suffer from fluorosis caused by fluoride contamination in groundwater [2]. China is one of the most serious epidemic countries of endemic fluorosis in the world. Endemic fluorosis is prevalent in 28 provinces in China [3]. It was estimated that there are 28.82 million dental fluorosis and 5.63 million skeletal fluorosis in China (data not published). Therefore, endemic fluorosis is still a serious problem of public health in China.

Skeletal fluorosis, caused by excessive accumulation of fluoride in bones, leads to joint pains, physical limitation, and even disability [4]. The process of bone metabolism includes osteoblasts, forming bone and osteoclasts, degrading bone. Osteoblasts and osteoclasts are responsible for regulating the balance of bone metabolism [5]. Previous studies have shown that fluoride at the physiological level can promote osteoblasts proliferation, and excessive fluoride induces osteoblasts apoptosis and death [6, 7]. However the effect of fluoride on osteoclasts is still controversial. Some studies showed excessive fluoride stimulates osteoclasts formation [8, 9]. However other research showed excessive fluoride reduces

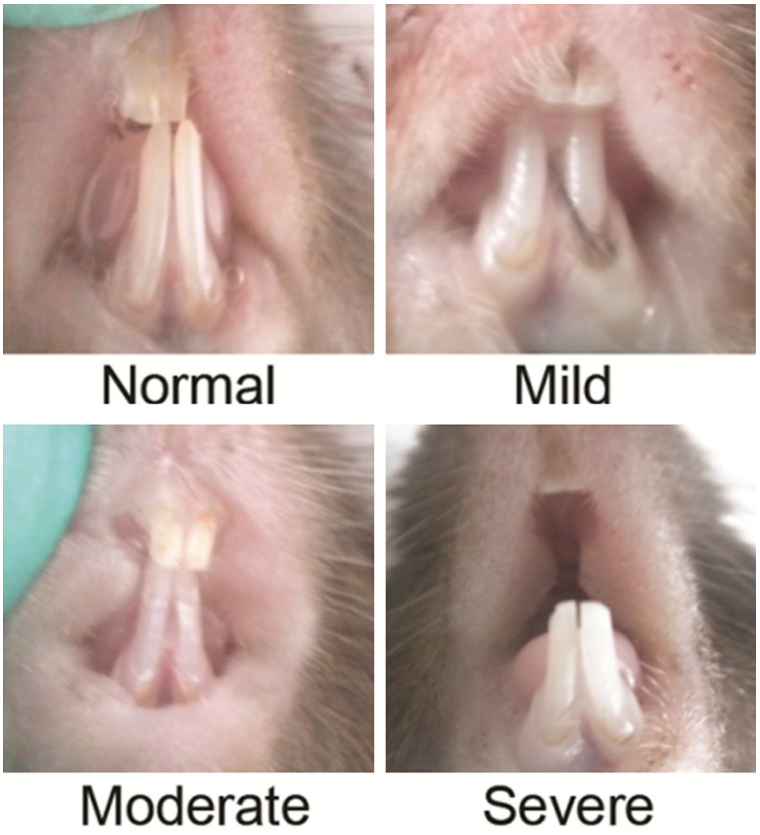

Figure 1. Classification standard of dental fluorosis in mice.

the number of osteoclasts and decreases the bone absorption capacity [10]. Therefore, more evidence is needed to clarify this issue.

Moreover, the molecular mechanism of fluoride on osteoclasts remains largely unknown. Recent studies have shown that fluoride increases mRNA expression of receptor activator for nuclear factor-κB ligand (RANKL) in osteoblasts [11-13]. RANKL, the indispensable cytokines of osteoclast formation, is a member of TNF ligand super family and plays an essential role in osteoclast differentiation, development and resorption. It is mainly expressed on oseteobasts, mesenchymal cells and hypertrophic chondrocytes [14]. RANKL has a dose-response relationship with the number of osteoclast formed, the higher the concentration of RANKL, the more osteoclasts are formed [15]. Thus, we speculate that fluoride may affect osteoclasts formation through regulation of RANKL expression in bone tissue.

Our previous study found that fluoride has different effects on osteoclast formation under various concentrations of receptor activator for nuclear factor-κB Ligand (RANKL) in vitro [16]. In this study, the effect of fluoride on osteoclasts formed and RANKL expression in bone tissue of mice was observed. We found that excessive fluoride can stimulate osteoclasts formation through increasing the expression of RANKL at the mRNA and protein levels in bone tissue.

Materials and methods

Animal model

Twenty three-week healthy SPF male C57BL/6 mice (Vital River Laboratories, Beijing, China) were randomly divided into two groups: control group (drinking distilled water) and 100 mg/L fluoride group (drinking distilled water with 100 mg/L fluoride ion). Sodium Fluoride was purchased from Tianjin Guangfu Fine Chemical Research Institute, Tianjin, China. Mice were raised for 15 weeks and allowed to eat and drink freely. All mice were housed in a specific pathogen-free animal facility of Harbin Medical University.

Dental fluorosis in mice

According to Dean's method, dental fluorosis of mice was classified into four degree in this study. Normal: Teeth were yellow-orange color and glossy. Mild: Thin white opaque lines were seen on all part of the teeth surface. Moderate: The white opaque lines were merged to form cloudy areas. Severe: The entire surface of the teeth was opaque and chalky white. Some parts of teeth were lost and chipped off (see **Figure 1**).

Bone fluoride analysis

The femur was cut off and dissected free of soft tissues. The bones were dried at 105°C for 4 hours, and then were weighed. The dried bones were ashed at 550°C for 5~6 hours and weighed again. The bone ashes were dis-

Table 1. Primers used for real-time RT-PCR

The name of gene	Genbank accession number		Primer sequence	Annealing temperature (°C)	Product length (bp)
β-actin	NM007393	Forward	CTGGCTGGCCGGGACCTGAC	61.8	226
		Reverse	ACCGCTCGTTGCCAATAGTGATGA		
RANKL	AF019048	Forward	CCATCGGGTTCCCATAAAGTCAC	61	407
		Reverse	AAAGCCCCAAAGTACGTCGCATCT		

solved in 1 M HCl, and neutralized by 1 M NaOH. Fluoride concentration in bone were analyzed by fluoride ion selective electrode method.

Bone histological analysis

Tibiae were decalcified with 0.25 M EDTA (pH 7.5) and 4% formaldehyde solution at room temperature for 5 weeks. The solution was changed every week. The decalcified bone was processed for standard paraffin embedding and sectioning, and stained with hematoxylin and eosin (HE). The observation was performed with a microscope (BX51, Olympus Corporation, Tokyo, Japan) and the images were captured with a digital camera (DP72, Olympus Corporation, Tokyo, Japan).

TRAP staining and osteoclast count

Identification and quantitation of osteoclasts was done in demineralized bone sections according to previously described procedures [17-19]. Fixed, demineralized, and paraffin embedded tibiae were sectioned and dyed with TRAP staining kit (D023, Nanjing Jiancheng Bioengineering Institute, Shanghai, China.) according to manufacturer's protocol. The positive cells for TRAP staining contain red granular material in cells. TRAP-positive multinucleated cells containing greater than or equal to three nuclei were identified as osteoclasts. TRAP content of bone tissue was expressed as integral optical density (IOD). Images were captured with a digital camera (DP72, Olympus Corporation, Tokyo, Japan) and analyzed by Image-Pro Plus 6.0 software (Media Cybernetics Corporation, Warrendale, USA).

RT-PCR

Femora of mice were removed and dissected free of adherent soft tissue. The bones were snap frozen with liquid nitrogen, and then grinded to powder. Total RNA was extracted from the bone powder with RNAiso plus (TaKaRa, Dalian, China). cDNA was synthesized from 1 μg of RNA with the PrimeScript RT reagent Kit (TaKaRa, Dalian, China). Quantitative polymerase chain reaction (q-PCR) was performed using the SYBR Premix Ex Taq kit (TaKaRa, Dalian, China) on ABI 7500 Fast Light Cycler (Thermo Fisher Scientific Corporation, Waltham, USA). **Table 1** shows the primers used in PCR reactions. Results were calculated using $2^{-\Delta\Delta CT}$ method normalizing to β-actin expression for each sample [20]. PCR product specificity was measured by gel electrophoresis and melting curve. The experiment was repeated three times, with triplicate samples.

Western-blot analysis

The protein was also extracted from the prepared bones powder using a protein extraction kit (Beyotime Institute of Biotechnology, Shanghai, People's Republic of China). The protein concentrations were analyzed by an enhanced BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, People's Republic of China). Equal amounts of protein from each sample in loading buffer was heated at 100°C for 5 min and loaded onto 10% poly acrylamide gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes. Subsequently, the membranes were blocked by incubating in TBST plus 5% fat-free milk at room temperature for 2 h, probed with Rabbit anti-RANKL antibody (1:300) or Rabbit anti-β-actin antibody (1:800) overnight at 4°C, and then incubated for 1 h with the secondary antibody (Anti-Rabbit IgG, 1:5000). Signals were detected by Odyssey Infrared Imaging System (Li-COR). Data are expressed as normalized ratios of RANKL to β-actin.

Immunohistochemistry experiment

The decalcified bone tissue sections of 5 μm thickness were deparaffinized and hydrated. Endogenous peroxidase was inactivated by co-

Table 2. Dental fluorosis in mice

Group	Number	Normal	Mild	Moderate	Severe
Control group	10	10	0	0	0
100 mg/L F group	10	0	3	2	5

Table 3. Bone fluoride content in mice

Group	Number	Bone fluoride content (mg/kg)	*t*	P
Control group	10	876.258±14.462	-92.112	<0.001
100 mg/L F group	10	4098.645±58.842*		

Note: *, compared with the control group, $P < 0.05$.

vering the tissue with 3% hydrogen-peroxide for 10 min. The slides were washed three times with phosphate buffer (pH 7.2; 3 min each), and then incubated in 0.2% Triton-100 solution for 20 min at room temperature. Subsequently, the slides were washed with phosphate buffer (pH 7.2) 3 times (3 min each). The slides were blocked with 5% bull serum albumin solution at room temperature for 20 min. Tissue sections were then incubated overnight with Rabbit anti-RANKL antibody (1:100) or phosphate buffer (negative control) in a humidified chamber at 4°C. After three washes with phosphate buffer, tissue sections were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Zhong Shan Golden Bridge) at 37°C for 20 min. The slides were then washed five times with phosphate buffer (3 min each). Freshly prepared diaminobenzidine substrate was added to the sections at room temperature for 3 min. The sections were then rinsed with water and counterstained with hematoxylin. Samples were dehydrated using a general protocol and sealed with neutral balsam. Immunohistochemical staining was examined by two pathologists blinded to the array composition.

Statistical analysis

The data were expressed as mean ± SD. The comparisons of groups were evaluated using Student's t-test. Stata 12.0 software was used for all statistical analyses. *P* values less than 0.05 were considered statistically significant.

Results

Dental fluorosis in mice

Dental fluorosis is a sensitive biomarker of fluorosis in human and animals [21, 22]. It was used to identify whether fluorosis mice model were prepared successfully in this study. At 6 weeks, thin white opaque lines were found on the teeth surface in some mice of 100 mg/L F group. By the end of the animal experiment, all mice in the fluoride group had dental fluorosis with different severities. However the mice from the control group had no dental fluorosis (**Table 2**). These results demonstrated that our fluorosis mice model was made successfully.

Bone fluoride in mice

Bone fluoride content is closely associated with fluoride exposure [22]. The content of fluoride in mice bone was detected for reflecting fluoride exposure in the present study. **Table 3** showed that bone fluoride content in fluoride treatment group was about 4.6 times higher than that in control group. It was indicated that mice with 100 mg/L F treatment had excessive fluoride accumulation in bone tissue.

Bone histological analysis

The effect of fluoride on pathological changes of bone tissue was evaluated by HE staining of the femur. **Figure 2** showed, compared with the control group, the trabecular bone was expanded and fused, which lead to an increase of bone mass in the fluoride group. In addition, the chondrocytes of growth plate were less and irregular in fluoride treatment group. These results indicated that the bone tissues were significantly damaged by excessive fluoride exposure in mice. Therefore, a skeletal fluorosis mouse model was made successfully in this study.

TRAP staining and osteoclast count in bone tissue

In this study, the effect of fluoride on osteoclasts formation in bone tissue was evaluated by TRAP staining. **Figure 3A** and **3B** showed that the red signals of TRAP staining in the experimental group were significantly more than that in control group.

The images of TRAP staining was processed by Image-Pro Plus 6.0 software, and the level of TRAP was expressed by IOD. **Figure 3C** showed that the value of IOD in experimental group was

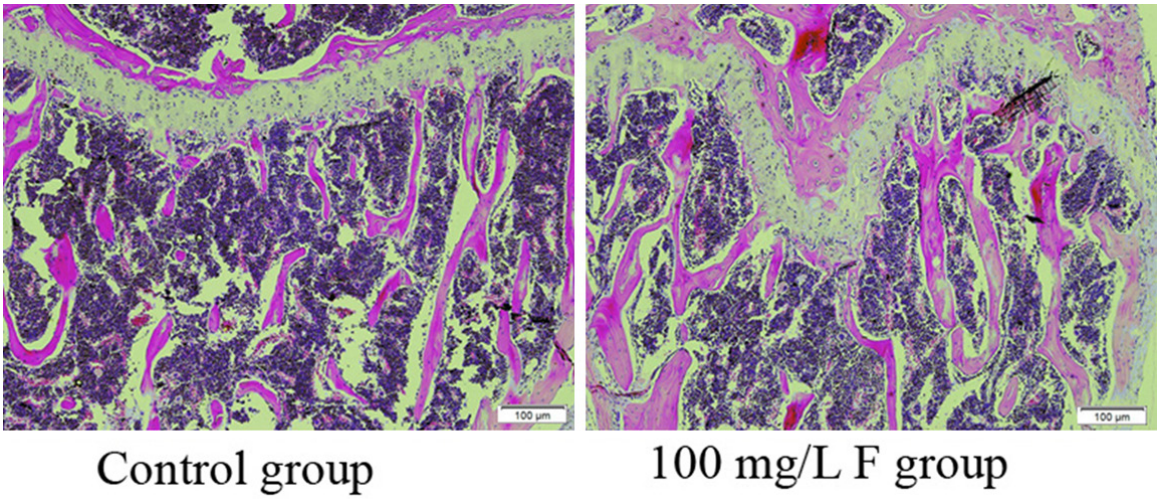

Figure 2. HE staining of bone in mice. Compared with control group, the trabecular bone was expanded and fused leading to an increase of bone mass in fluoride group. In addition, the chondrocytes of growth plate were less and irregular in fluoride treatment group.

about two times higher than that in control group (t = -2.465, P = 0.027). The results of osteoclast count showed that the number of osteoclasts in experimental group was significantly higher than that in control group (t = -2.886, P = 0.015) (see **Figure 3D**). The above results demonstrated that fluoride could stimulate osteoclasts formation in bone tissue.

RANKL expression in bone tissue

Quantitative real-time RT-PCR was used to detect the expression of RANKL mRNA. As is shown in **Figure 4A**, the expression of RANKL mRNA in experimental group was significantly higher than that in control group (t = -3.563, P = 0.018). Western-blot was used in this study to evaluate the effect of fluoride on the expression of RANKL protein in bone tissue. **Figure 4B** showed that the protein bands in the experimental group were significantly more intense and thicker than that in the control group. The electrophoresis bands were quantitatively analyzed by the software. The expression of RANKL protein in the experimental group was also significantly higher than that in the control group (t = -2.365, P = 0.046) (see **Figure 4C**). These results indicated that fluoride increased the expression of RANKL at mRNA and protein levels.

The effect of fluoride on the distribution of RANKL in bone tissue was detected by immunohistochemical technique. The positive signal of RANKL was brownish-yellow. **Figure 4D** showed that the brownish-yellow signal was mainly located in osteoblast, hypertrophied chondrocytes and macrophages. The negative control sections had no positive brown-yellow signals of RANKL. The brown-yellow faintly appeared in control group, more and darker brown-yellow was found in experimental group. Above results showed that RANKL expression of bone tissue in fluoride group was higher than that in control group, however the distribution of RANKL between the two groups had no difference.

Discussion

Excessive exposure of fluoride has an adverse effect on teeth, bone, nervous system and other organs [23]. In the present study, excessive accumulation of fluoride in bone, dental fluorosis and the significant change of bone histology were found in mice treated with excessive fluoride (100 mg/L F ion). These results demonstrated that the fluorosis mice model were made successfully and can be used to study the effect of fluoride on bone tissue.

Although skeletal fluorosis is a bone disease of known cause, its pathogenesis remains unclear. Pathological manifestations of skeletal fluorosis are complicated including osteosclerosis (increased bone mass), osteoporosis (reduced bone mass), interosseous membrane calcification [24]. In the process of bone metab-

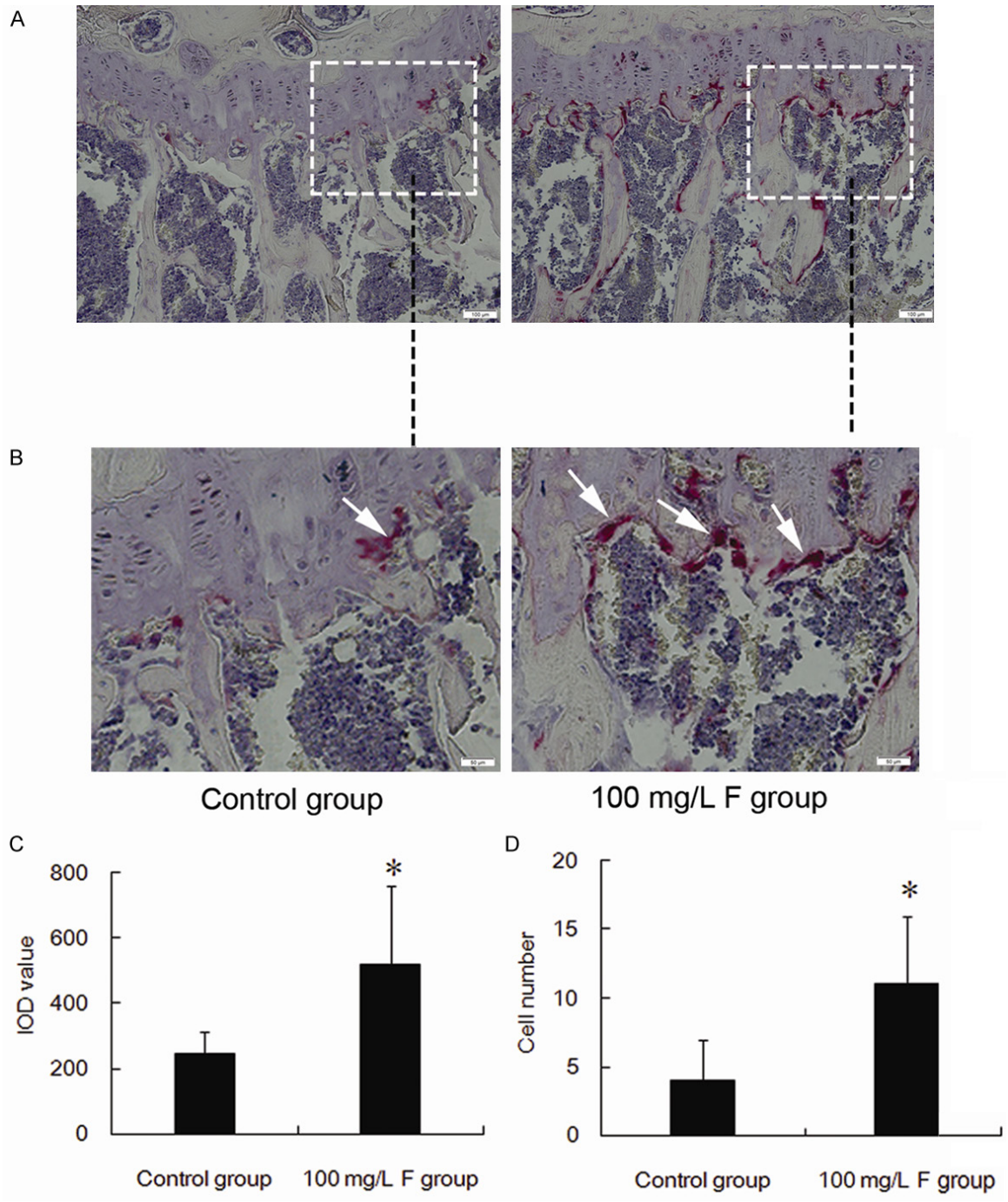

Figure 3. TRAP stainning of mice bone tissue. (A) The images of TRAP staining. TRAP was red and located on the surface of cartilage and trabecula. (B) The images of high magnification of rectangle in (A). The white arrows indicate osteoclast. (C) The comparison of TRAP expression between the two groups. TRAP content of bone tissue was expressed as IOD. (D) The comparison of osteoclast number between the two groups. *, compared with the control group, $P < 0.05$.

olism, osteoblasts are responsible for bone formation and osteoclasts are responsible for bone resorption [25]. At present, fluoride-induced increased bone formation has been proven *in vitro* and *in vivo* studies [26, 27]. However there is not a consensus for the effect of fluoride on bone resorption. *In vitro* studies, Okuda *et al.* study showed fluoride concentrations of 0.5-1.0 mM (converting mM to mg/L: 9.5-19 mg/L) decreased the activity of isolat-

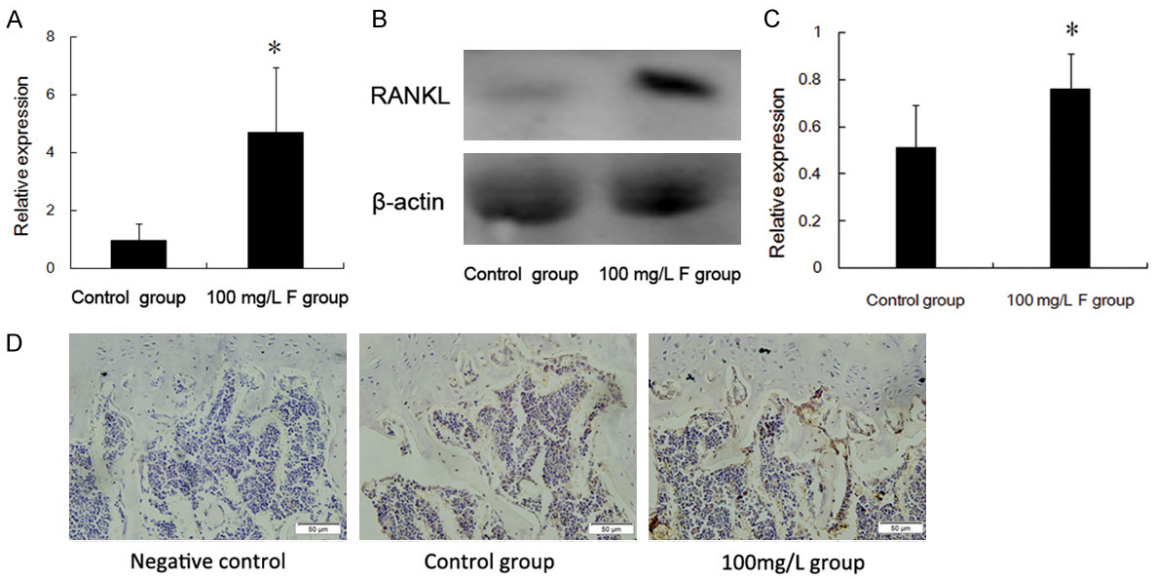

Figure 4. Expression of RANKL in bone tissue of mice and immunohistochemical images of RANKL. *, compared with the control group, $P < 0.05$.

ed rabbit osteoclasts [10]. Hua *et al.* reported that number of isolated rats osteoclasts treated with 1.00, 2.00 and 4.00 mg/L of fluoride exhibited dose-dependent increase [8]. These studies suggest that fluoride below 4 mg/L levels stimulate osteoclast formation while unusually high exposure to fluoride had opposing effects *in vitro*. *In vivo* studies, Debinski *et al.* found that fluoride increased osteoclast number in bone tissue of rats received 100 ppm fluoride in water for 8 weeks [9]. However Yan *et al.* found that fluoride had no effect on osteoclast formation in bone tissue of C57BL mice treated with fluoride in the drinking water (0 ppm, 50 ppm and 100 ppm F ion) for 3 weeks [18]. In humans, the prevalence and severity of skeletal fluorosis increases with the period of fluoride exposure [28]. So feeding time of C57BL mice was extended to 15 weeks in this study. Osteoclast number of 100 ppm fluoride group in bone tissue was significantly higher than that in the control group. Above results suggest that excessive exposure to fluoride lead to an increase of osteoclast formed in bone tissue.

At present the molecular mechanism of fluoride effect on osteoclast is largely unknown. The relationship between RANKL and skeletal fluorosis has recently drawn more attention. RANKL, a membrane protein expressed by osteoblasts, plays an essential role in osteoclast differentiation [29]. *In vitro* studies found that fluoride increased RANKL mRNA expression in osteoblasts isolated from mice and sheep [11, 12]. Our previous study showed that fluoride increased RANKL mRNA in bone tissue of rats [30]. The above results indicated that fluoride could increase RANKL mRNA expression, however the relationship between fluoride, RANKL and osteoclast formation remains unclear. In this study, we further investigated the effects of fluoride on RANKL and osteoclast formation in bone tissue of mice. The results showed that fluoride not only increase osteoclast number, but RANKL mRNA and protein levels in mice. In addition, the effect of fluoride on RANKL expression in bone tissue was observed by immunohistochemical technique. Fluoride also stimulated RANKL expression, but had no effect on the distribution of RANKL in bone tissue.

In summary, our study suggested that fluoride has a stimulating effect on osteoclast formation in mice and the molecular mechanism of fluoride action maybe through up-regulation of RANKL expression in bone tissue.

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Disclosure of conflict of interest

None.

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