EFFECT OF FLUORIDE ON OSTEOCLAST FORMATION AT VARIOUS LEVELS OF RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA-B LIGAND (RANKL)

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SUMMARY: Receptor activator of nuclear factor kappa-B ligand (RANKL) acting on osteoblasts is an essential cytokine for osteoclast formation. Recent studies have shown that fluoride (F) can stimulate RANKL expression of osteoblasts. However, the effect of F under various levels of RANKL on osteoclast formation is not clear. In this study, stem cell technology was used to observe the effect of F on osteoclast formation at different levels of treatment with RANKL. Our results show that F significantly inhibits the formation of osteoclasts treated with 50 ng/mL RANKL. The number of osteoclasts in the 0.5 mg/L F group fell to about 60% of the control group with no further significant change at the higher fluoride concentrations used (2 and 8 mg/L). On the other hand, F had no effect on the formation of osteoclasts treated with 100 ng/mL RANKL. In fact, the number of osteoclasts formed at 100 ng/mL RANKL was significantly higher than at the 50 ng/mL RANKL level. However, F significantly decreased the activity of osteoclast bone resorption even at a low level of 0.5 mg/L F, and the higher the F concentration was, the lower the activity of bone resorption became. Therefore we conclude that the main effect of F on osteoclasts was the inhibition of their capacity for bone resorption, thus resulting in osteosclerosis as the major clinical manifestation in patients with skeletal fluorosis.

Keywords: Bone resorption; Fluoride effect on osteoblasts; Fluoride effect on osteoclasts; Osteoclast formation; RANKL (Receptor activator of nuclear factor kappa-B ligand).

INTRODUCTION

Skeletal fluorosis, the most serious consequence of fluorosis, is a bone disease with osteosclerosis as the major clinical outcome, mostly involving bone joints, causing ligament calcifications, accompanied in various degrees by osteopenia, osteoporosis and osteomalacia.[1,2] The diversity of clinical manifestations of skeletal fluorosis is associated with the complexity of bone metabolism. The process of bone metabolism includes bone resorption by osteoclasts and bone formation by osteoblasts. A tight interplay between these two kinds of cells is essential to maintain homeostasis of bone.[3] One study found that osteoblasts, while active, secrete more cytokines such as receptor activator of nuclear factor kappa-B ligand (RANKL), which can stimulate formation of osteoclasts, thereby increasing bone resorption and maintaining homeostasis of bone metabolism.[4] Furthermore, osteoblasts treated with a low dose of F show enhanced proliferation with increase in bone formation.[5,6] Moreover, recent studies indicate that F can stimulate the RANKL expression of osteoblasts.[7,8] However, the effect of F under various levels of RANKL on osteoclast formation as part of bone resorption remains unknown. In order to provide some profiles for further clarifying the pathogenesis of skeletal fluorosis, stem cell technology was used in the present study to observe the effect of F on osteoclast formation by treatment with different levels of RANKL.

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MATERIALS AND METHODS

Animals: Six- to eight-week-old male C57BL/6 mice were obtained from Vital River Laboratories (Beijing, China) and housed in a pathogen-free animal facility at Harbin Medical University (Harbin, China). All animal protocols and procedures used in the present study were approved by the Medical Ethics Committee of Harbin Medical University.

Reagents: Macrophage colony-stimulating factor (M-CSF) and RANKL were purchased from PeproTech (Rocky Hill, NJ, USA). α-Minimal essential medium (α-MEM), antibiotics (10000U/mL penicillin G and 10000 μg/mL gentamycin), and fetal bovine serum (FBS) were obtained from Thermo Scientific HyClone Company (Beijing, China). Sodium fluoride (NaF) was purchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China) and a tartrate-resistant acid phosphatase (TRAP) staining kit was obtained from SunBio Company (Shanghai, China). Bone slices were purchased from IDS Company (Herlev, Denmark). Toluidine blue was purchased from Sigma Company (St.Louis, MO, USA) and the water- soluble tetrazolium (WST)-8 dye kit was acquired from Beyotime Inst Biotech (Haimen, China).

Osteoclast cultures: After the mice were fatally euthanized for dissection, the femora and tibiae were taken out aseptically, and the adherent soft tissue was removed. The bone distal ends were cut away, and the marrow was flushed out from one end using α-MEM in a disposable 5 mL syringe. The bone marrow suspension was carefully agitated with a glass pipette to obtain single cells, washed twice and resuspended in α-MEM containing 10% FBS and 100 U/mL of antibiotics. The suspension was then incubated in α-MEM supplemented with M-CSF (30 ng/mL) in plates at 37ºC, in a 5% CO_2 atmosphere for 24 hr. Non-adherent cells were harvested in α-MEM with M-CSF (30 ng/mL) and 1×10^5 and 2×10^6 cells were incubated in a 96-well plate and a 24-well plate, respectively, for 3 days. The harvested cells were bone marrow macrophages (BMMs), which when treated with M-CSF (30 ng/mL) and RANKL (50 ng/mL or 100 ng/mL, respectively), can differentiate into osteoclasts in 3 days.

Determination of cell viability: The WST-8 dye kit was employed to quantitatively evaluate the cell viability according to manufacturer's instructions. Briefly, 3×10^4 BMMs/well were seeded in a 96-well plate, and cultured as described above. The cells were incubated in α-MEM medium with M-CSF for 3 days and then replaced with α-MEM containing M-CSF (30 ng/mL), RANKL (100 ng/mL) and various concentrations of F. After culturing for 24, 48, and 72 hr, the cells were subsequently incubated with 0.2 mL of α-MEM containing 10% WST-8 dye solution at 37ºC for 2 hr. The absorbance was measured at 450 nm using a microplate reader. The experiment was repeated five times.

TRAP staining and osteoclast counting: BMMs (1×10^5/well) were seeded in a 96-well plate and cultured for 3 days with α-MEM containing M-CSF (30ng/mL). The medium was then replaced with α-MEM containing M-CSF (30 ng/mL), RANKL (50 ng/mL or 100 ng/mL), and different concentrations of F (0, 0.5, 2, and 8 mg/L). This time point was designated as day 0 of F treatment in the

experiments. The cell culture was terminated on days 1, 3 and 5 to observe the impact of F on osteoclast formation. The cells were fixed and dyed with the TRAP staining kit according to the manufacturer's instructions. TRAP-positive cells contained red granular material. TRAP-positive multinucleated cells containing ≥3 nuclei were counted as osteoclasts.[9] The cells were microscopically observed using a 10×/0.30 objective lens (Plan Flour, Nikon) on a microscope (TE2000, Nikon). Images were captured with a digital camera (DS-Fi1, Nikon). Four images were merged into one photo using the Image-software (NIS-Elements, Nikon). The experiment was repeated five times.

Bone slice resorption: Osteoclasts were grown on bone slices and cultured as described above. Briefly, 1×10^5 BMMs/well were seeded on bone slices in a 96-well plate and cultured for 3 days in α-MEM containing M-CSF(30ng/mL). The medium was then replaced with α-MEM containing M-CSF (30 ng/mL), RANKL (100 ng/mL) and different concentrations of F (0, 0.5, 2 and 8 mg/L). After F treatment for 5 days, bone slices were washed with 0.25 M ammonium hydroxide, sonicated 3 times for 5 min each and then stained with 1% (w/v) toluidine blue solution. The bone slices were washed with distilled water, dried at room temperature (RT)[10], and microscopically observed using a 4× objective lens (UPlanSApo, Olympus) on a microscope (BX51, Olympus). Images were captured with a digital camera (DP72, Olympus). The resorption area was analyzed with Olympus Image-Pro Plus 6.0 software. Bone resorption activity was expressed as a percentage (resorption area/statistics vision area × 100%). The experiment was repeated five times.

Statistical analysis: The data area expressed as mean ± SD. SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. Comparisons between groups were evaluated using one-way ANOVA followed by a post-hoc Student-Newman-Keul (SNK) test. A p-value < 0.05 was considered statistically significant.

RESULTS

Effect of F on the viability of osteoclast precursors: Prior to investigating the effect of F on osteoclast formation, osteoclast precursors were prepared as described previously.[11] Here, no significant differences were observed in the viability of osteoclast precursors on exposure to different F concentrations (0, 0.5, 2, and 8 mg/L) for 24, 48, and 72 hr (Figure 1).

Effect of F on osteoclast formation: Over the five-day incubation period, the effect of F on osteoclast formation and morphology was very revealing. During the first 2 days of culture, TRAP-positive cells were absent (data not shown); however, on day 3, TRAP-positive cells suddenly appeared in large numbers. The osteoclasts contained red granular substances and had irregular, oval and omelette-like shapes, and some contained one or more outstretched pseudopodia and vacuoles throughout the cell (Figure 2). The cell morphologies were consistent among all groups, but the osteoclasts treated with 100 ng/mL of RANKL were larger than those treated with 50 ng/mL of RANKL. F significantly inhibited the formation of osteoclasts treated with 50 ng/mL of RANKL. The number of

osteoclasts in the 0.5 mg/L F treatment group fell to about 60% of the control group, but was not different among the 0.5, 2 and 8 mg/L F treatment groups (Table). When the treatment level of RANKL was increased to 100 ng/mL, F had no effect on osteoclast formation, although the number of newly-formed osteoclasts was significantly higher than those treated with 50 ng/mL of RANKL. The number of osteoclasts and giant osteoclasts (≥20 nuclei) among all groups was similar (Table).

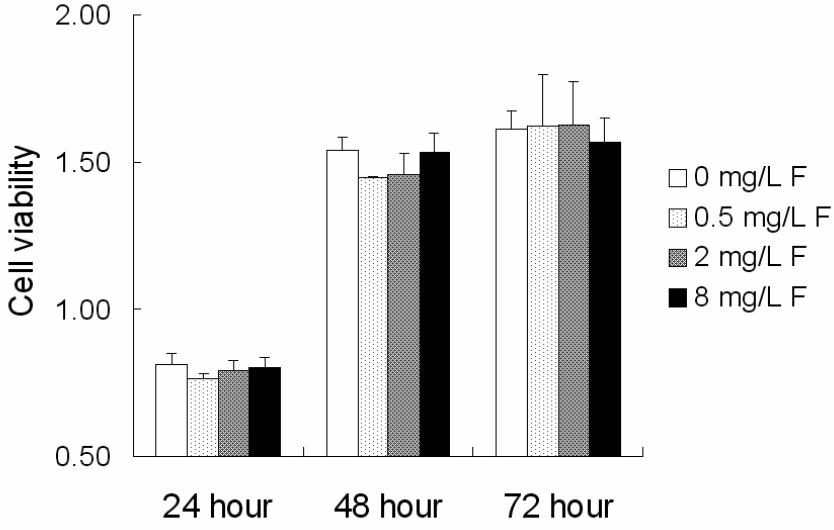

Figure 1. Effect of F on osteoclast precursor viability. Note that the viability of osteoclast precursors was not significantly different among the groups with different F concentrations.

Table. Results of osteoclast count (n=5, mean±SD)

RANKL concentration	F concentration (mg/L)	Number of nuclei	
		≥3	≥20
50 ng/mL	0	246±20	25±4
	0.5	156±39*	13±5*
	2	128±4*	3±2*
	8	124±8*	8±8*
100 ng/mL	0	268±12	92±24
	0.5	231±15	100±9
	2	241±24	95±11
	8	270±30	100±12

*Compared with 0 mg/L group ($p<0.05$).

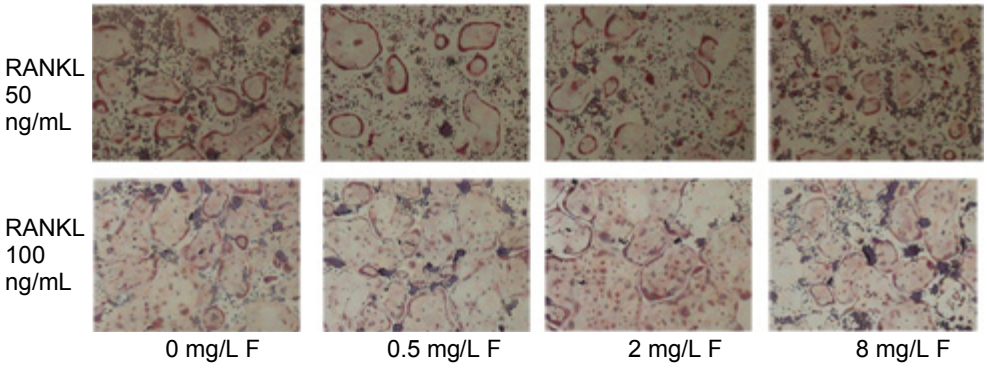

Figure 2. Effect of F on osteoclast morphology treated with different levels of RANKL. The number and size of osteoclasts are both larger in the 100 ng/mL of RANKL groups than that in the 50 ng/mL of RANKL groups.

Effect of F on osteoclast bone resorption activity: Resorption lacunae were dyed purple and round, oval or sausage-shaped, with clear outlines and various sizes (Figure 3). Even at 0.5 mg/L, F significantly decreased bone resorption as compared to the control group. Although bone resorption was similar in the 0.5 and 2 mg/L F treatment groups, it was significantly higher in these groups than in the 8 mg/L F treatment group. These results indicate that F can significantly reduce bone resorption by osteoclasts; and the higher the F concentration, the lower the degree of bone resorption (Figure 4).

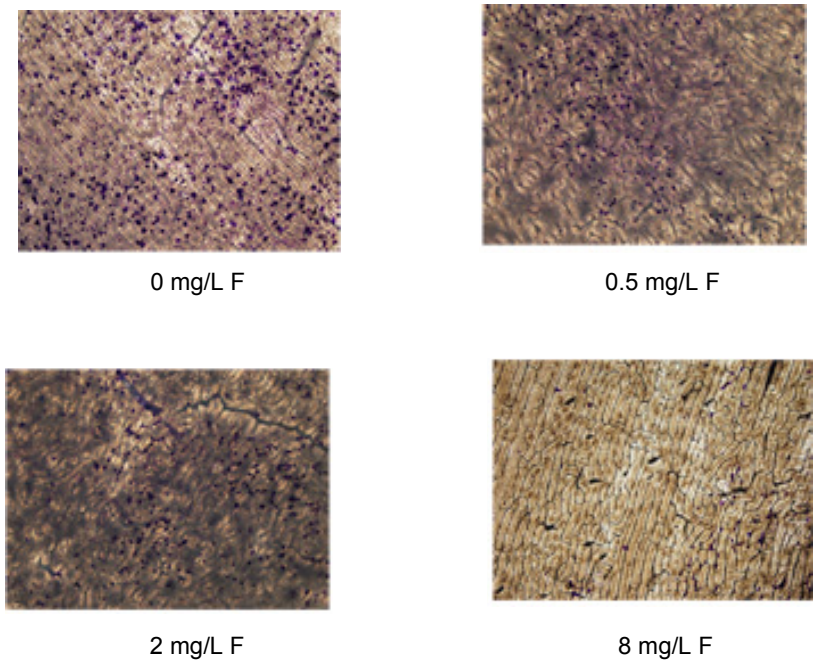

Figure 3. Effect of F on bone resorption by osteoclasts formed with 100 ng/mL of RANKL. Resorption lacunae were dyed purple and round, oval or sausage-shaped, with clear outlines and various sizes.

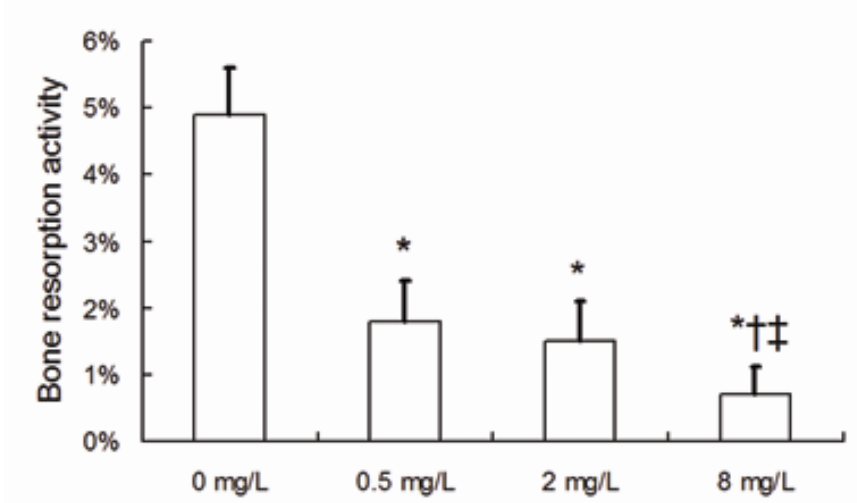

Figure 4. Effect of F on osteoclast bone resorption activity with 100 ng/mL of RANKL. Bone resorption activity expressed as a percentage (resorption area/statistics vision area×100%). The experiment was repeated 5 times. *Compared with 0 mg/L F group ($p<0.05$); †compared with 0.5 mg/L F group ($p<0.05$); ‡compared with 2 mg/L fluoride group ($p<0.05$).

DISCUSSION

Osteoclasts are multinucleated cells derived from hematopoietic progenitors in bone marrow. They are responsible for bone resorption, play a critical role in normal skeletal development, and help maintain bone integrity throughout life.[12] Osteoclast numbers are usually low in bone and are terminally differentiated cells; therefore, they are typically not isolated to create osteoclast cell lines.[13] However, with the development of stem cell techniques, RANKL and M-CSF have been found to be essential cytokines for induction of osteoclast formation.[14,15] *In vitro* studies indicate that osteoclasts are induced from bone marrow macrophages by these two cytokines.[16]

In our previous study, the density of bone trabeculae in rats treated with 150 mg/L of F in their drinking water was markedly higher than those in the control group. Additionally, abnormal osteoblast proliferation and osteoclast formation was observed and the number of lysosomes and synthesis of lysosomal enzymes in osteoclasts declined.[17] The number and function of lysosomes were closely associated with osteoclast bone resorption.[18] Therefore, we conclude that F promoted osteoclast formation, but decreased the capacity of osteoclast bone resorption. However, the regulatory mechanisms involving F remain unclear.

Recent studies found that osteoblasts have an important role in osteoclast formation and regulation. When osteoblasts function actively, their secretion of cytokines such as RANKL is enhanced and increases formation of osteoclasts, which keep bone turnover balanced.[19] In F-supplemented rats, RANKL expression in serum and bone tissue increased significantly compared to the control group,[20,21] indicating that F could stimulate osteoblast secretion of

RANKL. However, the effect of F on osteoclast formation and function under various levels of RANKL remains unclear.

The present study showed that the number and volume of osteoclasts increased with a rise in RANKL concentration, which was consistent with results of a previous study.[22] However, the effect of F on osteoclast formation was different at various levels of RANKL. At 50 ng/mL of RANKL, F significantly inhibited osteoclast formation, whereas it had no such effect at 100 ng/mL RANKL. The number and size of osteoclasts increased in 100 ng/mL RANKL groups treated with the same concentration of F as used in the 50 ng/mL RANKL group, suggesting that increased RANKL concentrations could stimulate osteoclast formation despite F treatment.

Another study has shown that the number of osteoclasts is not necessarily proportional to the level of their function. When chloride channel 7, a membrane transport protein, was knocked-out in mice, osteoclast formation was the same as that of controls; however, bone resorption by osteoclasts decreased significantly.[23] Similarly, we observed that the 100 ng/mL RANKL treatment groups had the largest number and size of osteoclasts. However, our data also show that 0.5 mg/L of F significantly decreases osteoclast bone resorption. To summarize our above results, we found that although a rise in RANKL level increased osteoclast formation, treatment with the same concentration of F significantly decreased bone resorption by osteoclasts.

In conclusion, we propose that when the level of RANKL in humans is low, F can decrease the capacity of bone resorption through the inhibition of osteoclast formation. Conversely, when the level of RANKL increases, F has little or no effect on the number of osteoclasts formed, yet bone resorption by newly formed osteoclasts is significantly reduced by F. As a consequence, patients with skeletal fluorosis often exhibit osteosclerosis as the major clinical manifestation.

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