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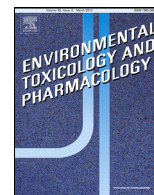

# Relationship between fluoride exposure and osteoclast markers during RANKL-induced osteoclast differentiation

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ABSTRACT

Skeletal fluorosis is a metabolic bone disease caused by excessive accumulation of fluoride. Although the cause of this disease is known, the mechanism by which fluoride accumulates on the bone has not been clearly defined, thus there are no markers that can be used for screening skeletal fluorosis in epidemiology. In this study, osteoclasts were formed from bone marrow cells of C57BL/6 mice-treated with macrophage colony stimulating factor and receptor activator of nuclear factor kappa-B ligand. The mRNA expression of tartrate-resistant acid phosphatase 5b (TRAP5b), osteoclast-associated receptor (OSCAR), calcitonin receptor (CTR), matrix metalloproteinase 9 (MMP9) and cathepsin K (CK) were detected using real-time PCR (RT-PCR). Results showed that fluoride between 0.5 and 8 mg/l had no effect on osteoclast formation. However fluoride at 0.5 mg/l level significantly decreased the activity of osteoclast bone resorption. Fluoride concentration was negatively correlated with the activity of osteoclast bone resorption. On day 5 of osteoclast differentiation maturity, MMP9 and CK mRNA expression were not only negatively correlated with fluoride concentration, but directly correlated with the activity of osteoclast bone resorption. TRAP5b, CTR and OSCAR mRNA expression were positively correlated with the number of osteoclast and they had no correlation with the activity of osteoclast bone resorption. Thus, it can be seen that MMP9 and CK may reflect the change of activity of bone resorption as well the degree of fluoride exposure. TRAP5b, CTR and OSCAR can represent the change of number of osteoclast formed.

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## 1. Introduction

Skeletal fluorosis is a metabolic bone disease with osteosclerosis as the major clinical sign, mostly involving bone joints. It results in ligament calcifications, accompanied by osteopenia, osteoporosis and osteomalaciato varying degrees (Boivin et al., 1990). Epidemiological surveys have demonstrated that the onset of skeletal fluorosis takes more time before symptoms are observed. Skeletal fluorosis may occur in an individual whose daily fluoride intake ranges between 36 and 54 mg for up to 10 years or more(Sarala Kumari and Ramakrishna Rao, 1993). This means there is enough time to prevent the progression and full development of skeletal fluorosis. However to accurately detect and monitor the changes of bone metabolism induced by the exposure of fluoride, is one of the main issues faced by researchers for many years. Research has shown that when a patient with bone disease receives therapeutic interventions, resorption markers of bone such as acid phosphatase etc. respond approximately 1–3 months after the intervention and formation markers of bone such as bone-specific alkaline phosphatase respond after 6–9 months (Christenson, 1997). On the other hand, bone mineral density, which is a traditional indicator of bone metabolism, respond more slowly to intervention than biochemical markers of bone, typically after about 2–3 years (Christenson, 1997). Thus implying resorption markers of bone are the most rapid indicators of bone metabolism.

Bone metabolism includes the process of osteoblasts forming bone and the osteoclasts degrading bone. The effect of fluoride on osteoblast has been well studied. In vitro studies have demonstrated that fluoride at physiological levels promoteosteoblast proliferation, increase bone mass, as well as increase osteoblast activity via the up-regulation of markers such as alkaline phosphatase (ALP), bone morphogenetic protein (BMP) and bone gla protein (BGP) (Bellows et al., 1990; Song et al., 2011; Xu et al., 2000).Epidemiological survey also showed that the level of ALP and BGP in patients with skeletal fluorosis were higher than the control group (Zhou and Yuan, 2003). However, the effect of fluoride on osteoclasts is still not well-understood. Some results showed high fluoride concentrations increased the activity of tartrate-resistant acid phosphatase (TRAP), an osteoclast biomarker promoted the

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formation of osteoclasts and enhanced the capacity of bone resorption (Debinski and Nowicka, 2004; Hua et al., 2003). However, others found such high concentrations of fluoride reduced the number of osteoclasts and decreased their bone resorption ability (Ma et al., 2004; Okuda et al., 1990).

In recent years, during the development of osteoclast research, researchers discovered TRAP5b as the newest addition to the TRAP family.This enzyme is specially secreted by osteoclasts during bone resorption and became a new biomarker (Tian et al., 2013). Several markers related to differentiation and function of osteoclasts are also found to be expressed at the same time including osteoclast-associated receptor (OSCAR), calcitonin receptor(CTR), matrix metalloproteinase 9 (MMP9) and cathepsin K(CK) (Herman et al., 2008; Meier et al., 2006; Ohshiba et al., 2003; Takahashi et al., 1995). These findings made it difficult to clearly map out the relationship between fluoride and markers of osteoclasts.

In this study, osteoclasts were formed from bone marrow cells of C57BL/6 mice-treated with macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL). The effect of fluoride on the total number and function of osteoclasts were observed, and the relationship between fluoride and markers of osteoclasts were analysed. This was aimed at finding sensitive markers that could reflect the effect of fluoride on osteoclast bone resorption ability.

## 2. Materialsand methods

### 2.1. Materials

Six to eight weeks old male C57BL/6 mice were obtained from Vital River Laboratories (Beijing, China).M-CSF and RANKL were purchased from PeproTech (USA). α-Minimal essential medium (α-MEM), antibiotics (penicillin G and gentamycin), and fetal bovine serum (FBS) were obtained from Thermo Scientific HyClone Company (USA). Sodium fluoride waspurchased from Tianjin Guangfu Fine Chemical Research Institute (Tianjin, China). Tartrate-resistant acid phosphatase (TRAP) staining kit was from SunBio Company (Shanghai, China). RNA extraction, cDNA synthesis and PCR kits were obtained from Takara Biotechnology Company (Dalian, China). Bone slices were purchased from IDS Company, U.K. Toluidine blue was purchased from Sigma Company in USA. A WST-8 dye kit was purchased from Beyotime Inst. Biotech (China).

### 2.2. Culture of osteoclasts

The average blood F level is 1.5 mg/l in patients with skeletal fluorosis, but this varies greatly between 0.5–6.1 mg/l (Singh and Jolly, 1961). The fluoride concentration in bone marrow is unknown, however, the bone marrow cavity is connected with the venous system (Draenert, 1989). We speculate that the fluoride concentration of bone marrow is similar to the blood fluoride concentration in patients with skeletal fluorosis. Therefore, the fluoride concentrations chosen were 0, 0.5, 2, and 8 mg/l in this experiment.

All the mice were housed in a specific pathogen-free animal facility of Harbin Medical University. The animal protocols and procedures used in this study were approved by the Medical Ethics Committeeof Harbin Medical University.

Femora and tibiae were removed aseptically and dissected free of adherent soft tissue. The bone ends were cut away, and the marrow mass was flushed out from one end of the bone with α-MEM using a disposable 5 ml syringe. The bone marrow suspension was carefully pipette several times with a glass pipette to obtain single cells, which were washed twice and resuspended with α-MEM containing 10% FBS and 100U/ml of antibiotics. The suspension was then incubated in a medium with M-CSF (30 ng/ml) in plates at 37 °C, 5% $CO_2$ for 24 h. Non-adherent cells were subsequently harvested in medium with M-CSF (30 ng/ml), and The harvested cells were incubatedin for 3 days. The harvested cells are bone marrow macrophages(BMMs). BMMs treated with M-CSF (30 ng/ml) and RANKL (100 ng/ml) differentiate into osteoclasts after 3 days.

### 2.3. TRAP staining and osteoclast count

$1 \times 10^5$ BMMs/well were seeded in a 96well plate, and cultured for 3 days with medium containing M-CSF (30 ng/ml). The medium was then replaced with the medium containing M-CSF (30 ng/ml), RANKL (100 ng/ml) and different concentrations of fluoride (0, 0.5, 2 and 8 mg/l). This time point was designated as day 0 of fluoride treatment in the experiments.The cell culture was terminated at day 1, day 2, day 3 and day 5, respectively, to observe the impact of fluoride on osteoclast formation.

The cells were fixed and dyed with TRAP staining kit (SunBio, China) according to the manufacture's instructions. Cells that stain positive for TRAP contain red granular substances in their cytoplasm. TRAP-positive multinucleated cells containing 3 or more nuclei were identified as osteoclasts. The cells were also observed with a light microscope (TE2000, Nikon) under the10 × /0.30 objective lens(Plan Flour, Nikon). Images were captured with a digital camera (DS-Fi1, Nikon) andfour images were merged into one photo with the Image software (NIS-Elements, Nikon). The experiment was repeated 5 times.

### 2.4. Bone slice resorption

Osteoclasts were grown on bone slices and cultured as previously described. $1 \times 10^5$ BMMs/well were seeded on bone slices in a 96 well plate, and were cultured for 3 days with medium containing M-CSF (30 ng/ml). The medium was then replaced with medium containing M-CSF (30 ng/ml), RANKL (100 ng/ml), and different concentrations of fluoride (0, 0.5, 2 and 8 mg/l). After fluoride treatment for 5 days, bone slices were washed with 0.25 M ammonium hydroxide, sonicated for 5 min,3 times and then stained with 1% (wt/vol) toluidine blue solution. The bone slices were then washed with distilled water and dried at room temperature according to the method of Wu et al. (Wu et al., 2009). These were observed with a microscope (BX51, Olympus) using a 4× objective lens (UPlanSApo, Olympus) and images captured with a digital camera (DP72, Olympus). The resorption area (staining area) was analysed using the Olympus Image-Pro Plus6.0 software and bone resorption activity was expressed as a percentage (resorption area/statistics vision area × 100%). The experiment was repeated 5 times.

### 2.5. cDNA synthesis and quantitative RT-PCR

$1 \times 10^6$ BMMs/well were seeded into a 12 well plate, and cultured for 3 days with medium containing M-CSF (30 ng/ml). The medium was then replaced with the medium containing M-CSF (30 ng/ml), RANKL (100 ng/ml) and different concentrations of fluoride (0, 0.5, 2 and 8 mg/l, respectively). This time point was designated as day 0 of fluoride treatment in the experiments. The cell culture was terminated at day 1 and day 5, respectively, to analyze the impact of F on gene expression.

Total RNA was extracted from cells with RNAiso plus (TaKaRa, Dalian, China). cDNA was synthesized from 1 μg ofRNA with the PrimeScript RTreagent Kit (TaKaRa, Dalian, China). Quantitative PCR was performed using the SYBR Premix Ex Taq kit (TaKaRa, Dalian, China) and Chromo4$^{TM}$ (Biorad,USA). Table 1 shows the primers used in the PCR reactions. The primers were designed and synthesized by TaKaRa Company (Dalian, China). Results were calculated using $2^{-\triangle\triangle CT}$ method and normalizing to β−actin expression for each sample (Livak and Schmittgen, 2001). PCR prod-

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