Original

Treatment with 50 μM Sodium Fluoride Suppresses Aging-Induced Alveolar Bone Resorption in Mice

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Abstract: Ingestion of excess systemic fluoride results in physiological and pathological disturbances of bone homeostasis. We and others have established that treatment with 50 μM sodium fluoride (NaF) is safe and effective for bone remodeling in cellular and animal models. This study aimed to study the effects of treatment with 50 μM NaF on macrophage-driven osteoclastogenesis and to characterize the function of 50 μM NaF in alveolar bone resorption during aging. Murine RAW264.7 monocytic cells were treated with RANKL in the presence or absence of 50 μM NaF. The mRNA expression levels of Cathepsin K and nuclear factor-activated T-cell cytoplasmic 1 (NFATc1), which are involved in the mechanism of osteoclast induction, were measured using quantitative real time RT-PCR. An experimental 50 μM NaF-treated aging mouse model was used to confirm alveolar bone resorption. Micro-CT was used to assess bone loss and immunohistochemistry was performed to detect the protein expression levels of RANKL and Cathepsin K in periodontal tissues. Treatment of RAW264.7 cells with 50 μM NaF repressed osteoclastogenesis. Micro-CT results confirmed that treatment with 50 μM NaF alleviated alveolar bone resorption in aging. Immunohistochemical analysis revealed decreased expression levels of RANKL and cathepsin K in 50 μM NaF-treated mice during aging. Taken together, these results contribute fascinating experimental clues that 50 μM NaF has the potential to function as an anti-resorptive agent during alveolar bone aging.

Key words: Sodium fluoride, RANKL, Osteoclastogenesis, Alveolar bone resorption, Aging

Introduction

Osteoclasts are multinucleated giant cells specialized for mineralized bone matrix resorption. RANKL-stimulated active osteoclasts are differentiated from the monocyte/macrophage lineage and are responsible for calcium homeostasis to maintain bone remodeling1,2. The expression of RANKL is enhanced with age, but the mechanism of macrophage derived osteoclastogenesis during aging is unclear. Previous studies indicated that aged individuals have a highly increased fracture risk compared to young individuals3-6, and aged mice exhibit a decrease in bone elasticity and density7-12.

Macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) are two indispensable cytokines that stimulate osteoclasts13,14. Intercellular communications between osteoclasts and osteoblasts regulate proliferation and differentiation15,16 and consequently activate a transcription factor, nuclear factor-activated T-cell cytoplasmic 1 (NFATc1)17, that is necessary for osteoclast differentiation and function18. The loss of NFATc1 decreases osteoclast differentiation in stem cells19,20. Activated osteoclasts also secrete Cathepsin K to impair collagen and matrix proteins in osteoclastogenesis and the diminished activity of Cathepsin K can avoid bone loss without aggravating osteogenesis21-23.

Treatment with recombinant RANKL stimulates RAW264.7 cells, a classic mouse monocyte/macrophage cell line, to differentiate into functional osteoclasts24. In this study, we used macrophages since those cells are related to cortical porosity and thinning of cortical bones in old mice25-27. Among currently available pharmacologic agents, fluoride is the only agent that exerts a stimulatory effect on osteogenesis28,29 and dental health30, but excessive exposure to fluoride remains a major public health issue.

Fluorides are naturally occurring and are present ubiquitously in the environment. Certain levels of fluoride are favorable for the inhibition of dental caries and to trigger osteogenesis29,30. The mitogenic action of fluoride for osteoblasts31 and its inhibitory effect on osteoclasts32,33 is well documented. However, most of those fluoride studies were con-
ducted only in cell culture systems and there have been no reports on the involvement of aging in bone metabolism in animal models.

In this study, we established that treatment with 50 μM NaF functions as a competent inhibitor of osteoclastogenesis and ameliorates aging-induced bone loss in mice. Treatment with 50 μM NaF has a dominant suppressive effect on osteoclast function in RANKL-stimulated RAW264.7 cells, which suggests that NFATc1 signaling is a precondition for osteoclastogenesis. In addition, treatment with 50 μM NaF prevented bone loss in aging against monocyte/macrophage lineage-specific factors.

Material and Methods

Cell culture

RAW264.7 monocytic cells were purchased from the ATCC (Manassas, VA, USA). The cells were cultured in a humid environment under 5% CO2 at 37°C in DMEM (Wako, Tokyo, Japan) containing 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were sub-cultured when they reached a confluency of 70-80%. 10ng/ml murine sRANK-Ligand (PeproTech, Cranbury, USA) with or without 50 μM NaF was used to induce osteoclast differentiation for 9 days. TRAP (Wako, Tokyo, Japan) staining of cells was performed according to the instructions of the manufacturer.

Animals

Ten-week-old male Senescence-resistant (SAMR1) mice (n=12) were obtained from Japan SLC (Shizuoka, Japan) and were randomly divided into a control group (n=6) and a 50 μM NaF-treated group (n=6). The mice were maintained for 2 weeks and were fed with standard chow. Water was then supplemented with or without 50 μM NaF for 40 weeks. Three-dimensional (3D) images were captured using micro-computed tomography (micro-CT) (R_mCT2, Rigaku Corp., Tokyo, Japan). Seven sites on the buccal side of the mandibular bone of each mouse were chosen to assess the distance from the cemento-enamel junction to the alveolar bone crest. The exposure conditions of micro-CT were tube voltage, 90 kV; tube current, 200 μA; voxel size, 20 × 20 × 20 μm. All animal experiments were performed after approval by the Institute’s Ethics Committee at Nihon University School of Dentistry at Matsudo (AP17MD015).

RNA extraction and Real-time Polymerase Chain Reaction (PCR)

A real Time PCR System (Applied Biosystems, CA, USA) was used for total RNA extraction and quantitative real-time PCR. One μg aliquots of RNA were synthesized and transcribed to cDNA. TaqMan probes for mCTSK (Mm00484039_m1; Applied Biosystems, CA, USA), mNFATc1 (Mm01265944_m1; Applied Biosystems, CA, USA) and mACTB (Mm02619580_g1; Applied Biosystems, CA, USA) were used for RT-PCR.

Immunohistochemistry

Sections of upper and lower jaws were immersed at pH 6.0 in citrate buffer solution (Abcam, Cambridge, MA, USA) for antigen unmasking followed by endogenous peroxidase activity blocking (Dako, Carpinteria, CA, USA) for 10 min. Specimens were incubated with antibodies to Cathepsin K (1:50; LS-B2512; LifeSpan BioSciences, Inc., Seattle, WA, USA) and RANKL (1:75; NB100-80849; Novus Biologicals, LLC, Centennial, CO, USA) overnight at 4°C. Each specimen was then covered by secondary antibodies conjugated to peroxidase (Nichirei Biosciences, Tokyo, Japan) for 30 min at room temperature. Images were obtained using an optical microscope (DP72; Olympus, Tokyo, Japan).

Statistical analysis

SPSS 19.0 software was used for statistical analysis. The quantitative variables were assessed by an independent two-tailed Student’s t-test or analysis of variance (ANOVA). A p-value of less than 0.05 is considered statistically significant.

Results

Treatment with 50 μM NaF inhibits the expression of osteoclast markers

To understand the effects of treatment with a low level of NaF on RANKL-induced osteoclasts, the expression of osteoclast markers was characterized. RT-PCR analysis showed that the mRNA expression levels of Cathepsin K and Nfatc1 were inhibited after treatment of RANKL-induced RAW264.7 cells with 50 μM NaF for 9 days (Fig. 1, p<0.05).

![Figure 1](image-url)
Treatment with 50 μM NaF suppresses osteoclast differentiation

Trap staining was applied to determine the differentiation of osteoclasts. Trap staining revealed that the differentiation of RANKL-induced osteoclasts was inhibited by treatment with 50 μM NaF (Fig. 2, *p<0.05, **p<0.01). Those results revealed that treatment with 50 μM NaF suppressed osteoclast differentiation.

Alveolar bone resorption is reduced by treatment with 50 μM NaF

Mice ingested water with or without 50 μM NaF for 40 weeks. Micro-CT analysis showed that the resorption of alveolar bone was significantly reduced after treatment of mice with 50 μM NaF compared to untreated mice (Fig. 3A, B, ***p<0.001). Consistently, the expression of RANKL and Cathepsin K were both suppressed in the 50 μM NaF treated group (Fig. 3C).

Figure 2. Low level NaF suppresses osteoclast differentiation. Trap staining confirmed that the differentiation of osteoclasts was inhibited after treatment with 50 μM NaF for 9 days. Data shown represent means ± SD; *p<0.05, **p<0.01. All results are representative of at least three independent experiments. The arrow indicates TRAP-positive multinucleated osteoclasts. An inverted microscope (OLYMPUS, Tokyo, Japan) was used to capture images. A 10x objective lens was used to select areas with the highest fraction of stained cells. Subsequently, a 60x objective lens was used to count the fraction of positive cells in that region. Original magnification: 20x, scale bars = 100 μm.

Figure 3. Alveolar bone resorption was reduced by treatment of mice with 50 μM NaF. (A, B) Micro-CT results show that the resorption of alveolar bone is reduced after treating the mice with 50 μM NaF for 40 days. a: Parasagittal slice image; b: Cross-sectional slice image. The arrow and arrowhead indicate volumetric changes in alveolar bone. (C) The expression of RANKL and Cathepsin K were both suppressed in the mice treated with 50 μM NaF. Data shown represent means ± SD; *p<0.05, **p<0.01. All results are representative of at least three independent experiments. Original magnification: 60x, scale bars = 20 μm.
Bone metabolism requires a continuous balance between bone resorption and bone formation to maintain homeostasis. Disproportionate bone resorption and defective bone microstructure occurs due to excessive bone degradation by active osteoclasts in various bone diseases\(^{40}\). Aging is linked to drastic increases in non-collagenous matrix proteins while less resorbed bone is restored during bone remodeling\(^{39,36}\). Aging in humans involves decreased bone renewal and healing efficiency, and age-induced bone loss is also seen in mouse models\(^{37,39}\). Thus, preventing osteoclastic bone resorption might be an effective approach for the prevention of bone loss in pathological bone diseases.

This study was performed using an age-related experimental mouse model of alveolar bone resorption. Indeed, 50 μM NaF-treated aged mice revealed a seminal relationship between RANKL and Cathepsin K expression and age-related loss of alveolar bone assessed using histopathology and microradiography (Fig. 3). The expression of RANK receptors and colony-stimulating factor-1 (c-fms) receptors is increased in aging\(^{75}\). Compared to stromal cells of the bone marrow in young mice, old mice revealed decreases in osteoclast differentiation\(^{39,40}\). In this study, we demonstrated that treatment of mice with 50 μM NaF attenuates bone loss with aging. We examined the effects of long-term exposure of mice to NaF and show that 40 weeks of treating mice with NaF curtails the expression of osteoclast markers and increases cortical thickness. Conceptually, the changes in the control mice might occur due to dysfunctional bone remodeling. Furthermore, our data showed that the degree of deterioration in bone formation is not significant in the presence of 50 μM NaF. Fluoride has been well-known to retain a therapeutic impact to expedite the healing and mechanical function of bone. Nonetheless, the molecular mediators in skeletal aging are likely very complex. Advanced recognition of cell-intrinsic regulation might establish therapeutic approaches to maintain skeletal changes during aging.

Fluoride is mostly found in mineralized tissues in the bodies of adults\(^{41}\). We previously treated bone marrow mesenchymal stem cells in culture with different concentrations (50 μM, 500 μM and 5 mM) of NaF for various times and noticed a significant upregulation of osteogenesis in 50 μM NaF-treated cells\(^{39}\). Our previous study also confirmed 50 μM NaF as a candidate to induce osteogenesis including stimulation of the expression of mineralization-associated genes\(^{41}\). Yu et al. reported stimulatory and/or inhibitory effects in 250 μM to 8 mM NaF-treated osteoclasts and osteocytes\(^{46}\). In line with those reports, this study analyzed the effect of 50 μM NaF on macrophage-driven osteoclastogenesis.

Intercellular signaling crosstalk between immune cells and macrophages is indispensable for physiological bone remodeling\(^{46}\). Upon RANKL stimulation, RAW264.7 monocyte cells differentiate into bone-resorbing osteoclasts\(^{46,47}\). Accordingly, we selected RAW264.7 cells that exhibited no toxic effects on cell viability at 50 μM NaF (data not shown), to explore the potential effects of NaF in the regulation of osteoclastogenesis. In this study, treatment with 50 μM NaF robustly repressed RANKL-induced RAW264.7 cell differentiation into mature osteoclasts. The disruption of RANKL/RANK signaling is considered a competent method to restrain and mitigate bone loss\(^{40}\). Accumulating evidence suggests that the aggregation of macrophages commits to the enhanced expression of RANKL, resulting in aggressive alveolar bone resorption in aging. Disrupted expression of Cathepsin K and Nfatc1 had a marked constrained action on the physiological function of osteoclasts following treatment with 50 μM NaF.

In conclusion, our data indicate that 50 μM NaF is a potential therapeutic target to mediate alveolar bone resorption in aging. This study has an important limitation in that it only examined the effects of 50 μM NaF. These results support supplementary vigorous exploration of the effective therapeutic application of NaF against alveolar bone resorption during aging.

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Conflict of Interest
The authors declared no conflicts of interest.

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