

Fluoride Stimulates the Proliferation of Osteoclasts *in vitro* by Upregulating MCM3

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Abstract: We have previously shown that the expression of the minichromosome maintenance protein 3 (MCM3) gene was upregulated in lymphocytes of patients with skeletal fluorosis. We speculated that increased MCM3 expression may contribute to osteopathy in patients with skeletal fluorosis. Here, we investigated the effect of fluoride on the proliferation of osteoclasts derived from RAW264.7 cells and the involvement of MCM3. Our MTT assays showed that 0.25 mM NaF markedly stimulated the proliferation of RAW264.7 cells. The RT-PCR and immunoblotting assays revealed that 0.25 mM NaF upregulated MCM3 expression in RAW264.7 cells. The MTT assays additionally demonstrated that stimulation with MCM3 potentiated the effect of fluorine on the proliferation of RAW264.7 cells. These results demonstrated that fluoride at clinical relevant concentration upregulates MCM3 expression in osteoclasts *in vitro*. We are currently conducting a series of experiments to examine whether increased MCM3 in osteoclasts indeed contributes to osteopathy in skeletal fluorosis.

Keywords: Fluoride, Osteoclasts, Proliferation, Minichromosome maintenance protein 3, Expression.

Introduction

Fluoride is essential in the development and maintenance of teeth and bone [9]. Low doses of fluoride increase the trabecular bone mass [16] as well as spinal bone density [14]. Long-term exposure to excessive fluoride, on the other hand, may result in detrimental effects, e.g., skeletal and dental fluorosis [2, 7]. As estimated by the World Health Organization (WHO), there are approximate 40 million dental fluorosis victims in China; this is compared with a total of 70 million patients with the same disease all over the world. Endemic skeletal fluorosis, which is characterized by immobilization of joints of the axial skeleton and of the major joints of the extremities, is a chronic metabolic bone and joint disease caused by ingestion of large amounts of fluoride either through water or rarely from foods of endemic areas. The bone lesions in skeletal fluorosis are manifested by osteosclerosis, osteomalacia and osteoporosis to various extents. Endemic fluorosis is widely distributed, particularly in the northwest of China. Recent data from the Ministry of Health of China show that there are

around 2.8 million patients with skeletal fluorosis in China, indicating that the prevalent situation of the disease in China is as serious as that in Bengal and India.

Previous studies have established that fluoride at concentrations that are relevant to endemic skeletal fluorosis could stimulate the proliferation of osteoclasts, which are involved in bone resorption [8]. Furthermore, fluoride overdose activates osteocytes and accelerates bone turnover involving active bone formation and absorption, which leads to various pathological skeletal impairments including osteosclerosis, osteoporosis and osteomalacia [18]. The effects of fluoride on the cells have been used to stimulate bone formation in several animal models [13]. Animal studies demonstrated that fluoride given by water intake can lead to increased bone mass by enhancing ossification in skeletal fluorosis [3]. Besides ossification, bone absorption is also enhanced during fluorosis as characterized by the increased number of cells with positive tartrate-resistant acid phosphatase, a biomarker for activated osteoclasts, and osteoporosis.

The protein minichromosome maintenance 3 (*MCM3*), which is an important member of the *MCMs* family, is closely associated with DNA replication [17] and, as a participant in cell division cycle, protein *MCM3* is a sensitive biomarker for the cellular proliferation [12]. *MCM3* is positively correlated to the proliferation and growth ability of cells and *MCM3* level is low in the G_0 phase and terminally differentiated cells, suggesting that the *MCM3* level could represent the cellular proliferation status. We have shown earlier that the expression of the *MCM3* gene was increased in lymphocytes in patients with skeletal fluorosis [14]. In the current study, we have tested the effect of fluoride on the proliferation of osteoclasts which derived from RAW264.7 cells. Furthermore we have examined the potential role of *MCM3* in the proliferative action of pathological concentration of fluoride on osteoclasts.

Materials and methods

Cell culture

RAW264.7 cells were purchased from ATCC (Manassas, VA) and osteoclasts were obtained by treating RAW264.7 cells with sRANKL as previously reported [4, 11, 15]. Briefly, RAW264.7 cells were maintained at 37°C in a humidified incubator with 5% CO₂ and 95% air and cultured in Dulbecco's modified Eagle's Medium (DMEM, high glucose type) cell culture medium (H-DMEM) supplemented with 20% fetal bovine serum (FBS). Sub-confluent cells were treated with 100 ng/mL sRANKL (Sigma, St. Louis, MO) for 10 d.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

reduction assays

Cell proliferation was assessed by the tetrazolium-based semi-automated colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. Logarithmically growing osteoclasts were plated in 96-well plates at a density 1×10^5 cells/well and after an overnight growth were incubated with NaF at 0.05 or 0.25 mM in triplicates for three times independently. After 24-h incubation, 20 μ L MTT (5 mg/mL) was added to the medium and MTT assays were carried out as instructed by the manufacturer (Promega, Madison, WI). Absorbance was read at 490 nm.

RT-PCR assays

Total cellular RNA was extracted using the TRIzol Reagent (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was carried out using reverse transcriptase (Invitrogen) by incubation at 25 °C for 10 min, 37 °C for 60 min and 95 °C for 5 min. Primers for *MCM3* and

GAPDH were designed using Premier 5.0 software (PREMIER Biosoft International, Palo Alto, CA), and are listed in Supplemental Table 1.

Table 1. Primer sequences for RT-PCR

Name	Gene bank accession number	Fragment size, (pb)	Sequence	T _m , (°C)
<i>MCM3</i>	NM_008563.2	275	Fw 5'-TCTGACCTCACCCACCTA-3'	58
			Rv 5'-TAGCAGCCCTTCTTCCCT-3'	
<i>GAPDH</i>	NM_008084.2	289	Fw 5'-CGGTGCTGAGTATGTCGT-3'	58
			Rv 5'-CTTCTGGGTGGCAGTGAT-3'	

Western blotting studies

Cell lysates were prepared using a lysis buffer containing: 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 10% glycerol, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL aprotinin, 1 µg/mL leupeptin and 1 µg/mL pepstatin A. After incubation on ice for 30 min, the samples were centrifuged at $15,493 \times g$ for 10 min at 4 °C. The supernatant was re-suspended in $2 \times$ loading buffer containing 187.5 mol/L Tris (pH 6.8), 6% SDS, 50% glycerol, 150 mmol/L DTT and 0.03% bromophenol blue. The samples were heated for 5 min at 95 °C prior to electrophoretic separation by 12% SDS-PAGE. Immunoblotting procedure was performed as previously depicted [13]. Briefly, proteins were then electroblotted onto nitrocellulose membranes. Polyclonal antibodies against *MCM3* and *GAPDH* (Cell Signaling Technology, Danvers, MA) were used and incubated with the membranes for 8 h at 4 °C. Thereafter, the membranes were washed in Tris borate saline containing 1% Tween 20 (TBST), and further incubated with a peroxidase-conjugated antibody (Boster, Wuhan, China). Proteins were visualized by the substrate chemical luminescence method. The bands were semi-quantitatively evaluated by densitometric analysis (America Bio-Red Gel Doc 2000 Quantity One Gel Image Analysis System). Protein expression levels of *MCM3* were normalized against *GAPDH*.

Immunofluorescence studies

For immunofluorescence staining, previously prepared 4% PF was fixed for more than 15 min and rinsed twice in phosphate buffered saline, each for 3-5 min. The plate was then added with 500 µL 0.3% of triton and let stand for one h to block the antigens. Primary antibodies were added and incubated at room temperature for 5-6 h and then placed at 4 °C overnight. The product was then rinsed for three times in phosphate buffered saline, each for 3-5 min. Secondary antibodies were then added followed by incubation at room temperature for 2 h. Then, it was rinsed in phosphate buffered saline for three additional times, each for 3-5 min, and fluorescent antibodies were added and kept in the dark. The products were then rinsed in phosphate buffered saline for three more times, each for 3-5 min, placed at 4 °C for 30 min before it was sealed and photographed under a confocal microscope (90i C1Si Nikon, Japan).

Statistical analysis

Data were expressed as mean \pm S.E.M. Statistical analysis was carried out using analysis of variance (ANOVA), followed by Dunnett's test for post-hoc pairwise comparisons, with the Statistical Package for Social Science (SPSS11.5). A value of $p < 0.05$ was considered statistically significant.

Results and discussion

Fluoride exerts a dose-dependent effect on the growth of RAW264.7 cells

We induced the formation of osteoclasts by treating RAW264.7 cells with sRANKL. TRAP staining of undifferentiated and differentiated osteoclasts derived from sRANKL-stimulated RAW264.7 cells showed formation of TRAP-positive mature osteoclasts (Fig. 1A and 1B). We then examined the effect of NaF exposure on the proliferation of osteoclasts. Our MTT assays showed that exposure to 0.25 mM NaF markedly increased the proliferation of RAW264.7 cells ($p < 0.05$ vs. controls) (Fig. 1C) while increase to the concentration of NaF to 1.25 resulted in a significant decline in the viabilities of RAW264.7 cells ($p < 0.05$ vs. controls), suggesting a dose-dependent effect of NaF on the growth of RAW264.7 cells.

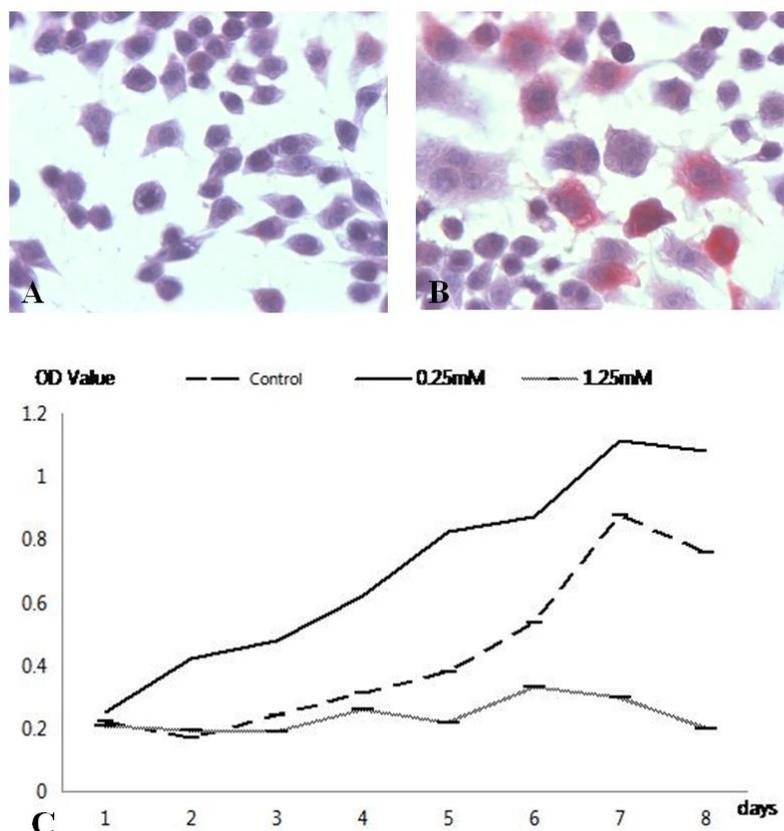
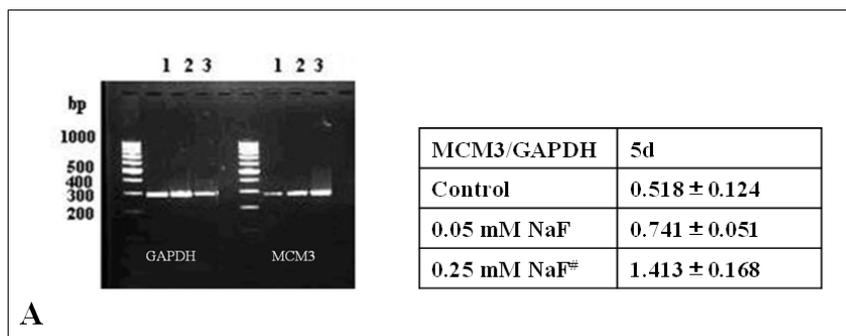


Fig. 1 RAW264.7 cells were treated with sRANKL as described in Methods to induce the formation of mature osteoclasts.

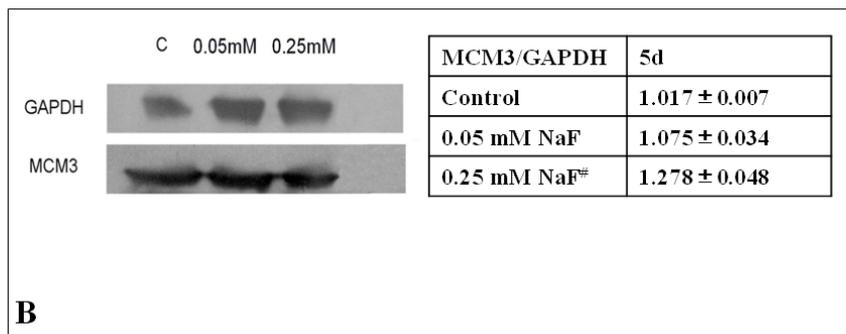
TRAP staining of undifferentiated (A) and differentiated (B) osteoclasts derived from sRANKL-stimulated RAW264.7 cells. Magnification of the cell is 400 \times . (C) Vertical coordinate is OD value, Horizontal coordinate is time.

Fluoride upregulates MCM3 expression in the nuclei of RAW264.7 cells

We then examined the effect of fluoride on the expression of *MCM3* in RAW264.7 cells. Our RT-PCR assays revealed that treatment of RAW264.7 cells with 0.25 NaF for 5 d upregulated the expression of *MCM3* (Fig. 2A). Furthermore, our immunoblotting studies showed that treatment of RAW264.7 cells with 0.25 NaF for 5 d also resulted in the upregulation of the protein levels of *MCM3* (Fig. 2B). These findings indicated that NaF could upregulate the expression of *MCM3* both the translational and transcriptional level.



$p < 0.05$ compared with controls. $p = 0.003$



$p < 0.05$ compared with controls. $p = 0.04$

Fig. 2 Fluoride upregulates the expression of *MCM3*: (A) RAW264.7 cells were treated with 0, 0.05 or 0.25 mM NaF (line 1 to 3, respectively) and RT-PCR was performed at d 5 of NaF treatment *GAPDH* was used as a loading control. (B) RAW264.7 cells were treated as in (A) and Western blotting assays were performed using lysates of RAW264.7 cells at d 5 of NaF treatment *GAPDH* was used as a loading control.

We further examined the localization of *MCM3* following NaF treatment in RAW264.7 cells. Our immunofluorescent staining showed that *MCM3* was located in the nucleus (Fig. 3A to 3C). Treatment of RAW264.7 cells with 0.25 mM NaF showed apparent increase in the expression of *MCM3* in the nuclei.

Stimulation with MCM3 antigen potentiates the proliferation of RAW264.7 cells by fluoride

We further investigated the effect of *MCM3* on the proliferation of RAW264.7 cells. We stimulated RAW264.7 cells with *MCM3* antigen or the blocking antibody, anti-*MCM3* antibody. We found that treatment with 0.25 mM fluoride or stimulation with *MCM3* antigen markedly increased the proliferation of RAW264.7 cells at 24 h post treatment ($p < 0.05$) (Table 2). Furthermore, combination treatment with fluoride and *MCM3* antigen increased the proliferation of RAW264.7 cells to a greater extent compared with fluoride or *MCM3* alone. On the other hand, treatment with anti-*MCM3* antibodies caused a marked suppression of the proliferation of RAW264.7 cells compared with controls ($p < 0.05$).

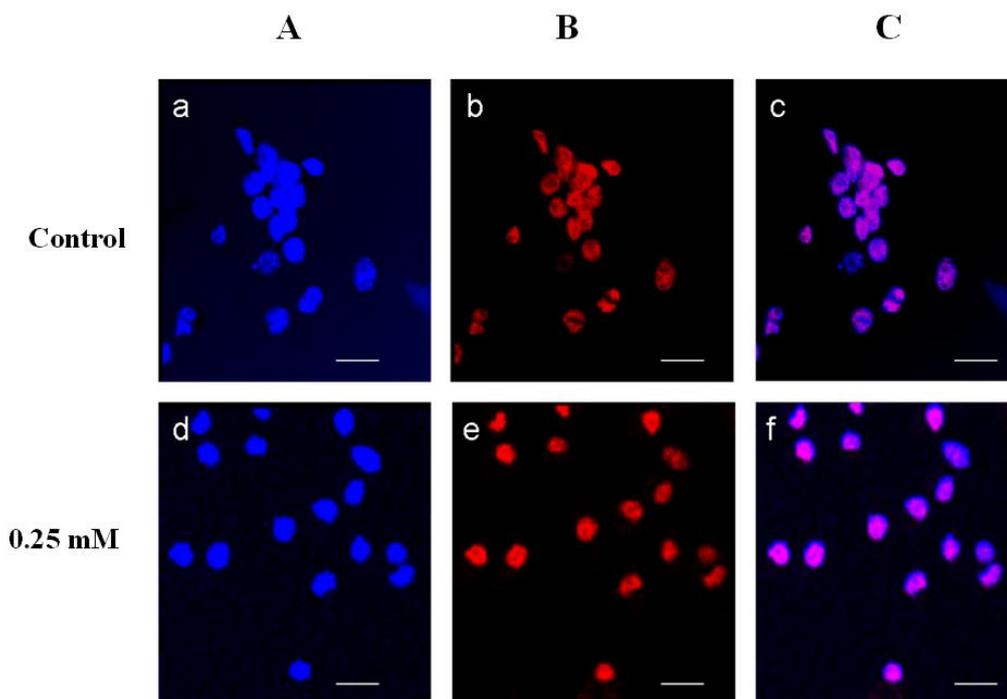


Fig. 3 RAW264.7 cells were treated with NaF at the doses indicated for 5 d and immunofluorescent staining with anti-*MCM3* antibody was carried out and the cells were examined under a fluorescent microscope.

The nuclei were counter stained with DAPI. Bar =10 μm .

Table 2. Viability of RAW264.7 cells after exposure to fluoride, *MCM3* antigen and anti-*MCM3* antibody determined by MTT assays (mean \pm s.d.)

Group [#]	24 h
Control* [#]	0.778 \pm 0.131
Fluoride + 0.25 mM NaF [#]	0.898 \pm 0.056
Fluoride + <i>MCM3</i> * [#]	0.960 \pm 0.018
<i>MCM3</i> [#]	0.923 \pm 0.053
Anti- <i>MCM3</i> antibody	0.689 \pm 0.006

[#]*p* < 0.05 compared with anti-*MCM3* antibody group; **p* < 0.05 compared with controls.

Discussion

Fluoride can induce diverse orthopedic pathologies, involving various cells that participate in bone turnover simultaneously, including osteoclasts, which are crucial to the development of skeletal fluorosis. We induced the formation of osteoclasts by treating RAW264.7 cells with sRANKL. TRAP is “tartrate resistant acid phosphatase” staining of undifferentiated and differentiated osteoclasts derived from sRANKL-stimulated RAW264.7 cells showed formation of TRAP-positive mature osteoclasts. We and others have demonstrated that sodium fluoride can promote the growth of osteoclasts in vivo at certain concentrations, suggesting that fluoride could change the original growing status of the cell. This change may be associated with the regulation of the cellular growth related genes.

MCM3 is closely associated with DNA replication. There are 7 members in *MCMs* family. *MCM* is formed as an intranuclear hexamer, which is a key component of the pre-replication complex and is closely associated with the formation of replicating fork [5] and the

recruitment of other replication-related and DNA repair-related proteins [6, 10, 17]. *MCM* binds to and separates from the chromatin respectively at G1/S phase and S phase and is regulated according to the cell cycle [1]. The *MCM3* level could represent the cellular proliferation status with low *MCM3* levels in the G_0 phase and terminally differentiated cells.

We have previously shown that *MCM3* was upregulated in the lymphocytes of patients with skeletal fluorosis. In this study, we further demonstrated that the protein and mRNA of *MCM3* were upregulated in osteoclasts exposed to 0.25 mM of sodium fluoride and the protein was mainly distributed in the nucleus. Previous studies revealed that *MCM3* were mainly distributed in the cytoplasm and nucleoplasm in a relatively stable manner except a small portion binding to the chromatin at late M and S phases [2]. Although these findings were different from ours, our study also showed that increased aggregation of *MCM3* in the nuclei of osteoclasts after exposure to fluoride may represent the enhancement of *MCM3* expression as a result of fluoride treatment.

The semi-quantitative determination showed that 0.25 mM sodium fluoride increased the mRNA transcript levels of *MCM3*. Our previous gene array evaluation also showed that *MCM3* was expressed at higher levels in patients with severe fluorosis than healthy volunteers [14]. The findings from both studies indicated that sodium fluoride could function to activate and promote the proliferation of osteoclasts. This may be because that fluoride can induce a persistent expression of *MCM3*, leading to sustained DNA replication in osteoclasts, which would subsequently proliferate and be activated. Meanwhile, it can be concluded that the dose of fluoride is related to the expression level of *MCM3*, that is, while short-term high dose of fluoride cannot upregulate *MCM3* expression, long-term exposure to low doses of fluoride can achieve this, resulting in diverse orthopedic pathologies such as osteoporosis by facilitating the proliferation and activation of osteoclasts. Moreover, an intervention using *MCM3* antigen and antibody to osteoclasts showed that *MCM3* antigen could promote the proliferation of these cells while the anti-*MCM3* antibody aborted the increase in the proliferation of these cells, suggesting that highly expressed *MCM3* may play a role in inducing the proliferation of osteoclasts during fluorosis.

Currently, it remains unclear what exact roles *MCM3* play in skeletal fluorosis, and the length of fluoride exposure and the number of doses of fluoride that can affect *MCM3* expression in osteoclasts also need further research. In future studies, we will further define the localization of *MCM3* in the nucleus and cytoplasm of osteoclasts and the possible means of transport by NMR and MS analysis so as to provide some experimental basis of the development of skeletal fluorosis.

Conclusion

These results demonstrated that fluoride at clinical relevant concentration upregulates *MCM3* expression in osteoclasts in vitro. We are currently conducting a series of experiments to examine whether increased *MCM3* in osteoclasts indeed contributes to osteopathy in skeletal fluorosis.

Acknowledgements

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