

## EFFECTS OF FLUORIDE ON THE EXPRESSION OF BMP-2 AND SMAD1 IN RAT OSTEOBLASTS *IN VITRO*

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**ABSTRACT:** The expression of BMP-2 and SMAD1 and their roles in osteoblast differentiation were investigated in rat osteoblasts exposed to fluoride for 24 and 72 hr. The gene and protein expression levels of BMP-2 and SMAD1 were quantified using QRT-PCR and immunohistochemistry, respectively. The results showed that after 24 and 72 hr of exposure to fluoride at 0.01 and 0.1 mM osteoblast proliferation was enhanced while with exposure to fluoride at 2.5 mM it was inhibited. The expression of BMP-2 and SMAD1 in the 0.5 and 1.0 mM fluoride groups showed a tendency to increase at 24 hr and to reduce at 72hr. In the group with exposure to 2.5 mM NaF, the formation of osteoblasts and the gene expression were significantly inhibited and the protein levels of BMP-2 and SMAD1 decreased ( $p < 0.01$ ). Therefore, low (0.01 and 0.1 mM) and intermediate (0.5 and 1 mM) concentrations of fluoride initially stimulated the expression of BMP-2 and SMAD1 for a short time (24 hr) but inhibited them after a longer period (72 hr). In contrast, with a high concentration (2.5 mM) of fluoride their expression was reduced at both 24 and 72 hr.

**Keywords:** BMP-2; Fluoride; Osteoblasts; SMAD1.

### INTRODUCTION

Osteoblasts are located in the deeper layers of the periosteum and the bone marrow and arise from osteoprogenitors. They play a major role in bone formation<sup>1-3</sup> and differentiate under the influence of growth factors.<sup>4</sup> Transforming growth factor beta (TGF- $\beta$ ) is a protein that controls cellular proliferation and differentiation in most cells.<sup>5</sup> As the largest part of the TGF- $\beta$  superfamily, bone morphogenetic proteins (BMPs) are known to be essential regulators in orthotopic bone formation and determinants of bone mineral content.<sup>6</sup> Bone morphogenetic protein 2 (BMP-2), like other BMPs, is considered to be a powerful bone morphogen for inducing osteoblastic differentiation.<sup>7-9</sup> Its expression may trigger the elaboration of osteogenic transcriptional regulatory programs. The signals of BMPs are mediated by SMAD proteins, which are homologs of the *Drosophila* protein “mothers against decapentaplegic” (MAD) and the *Caenorhabditis elegans* protein SMA. In response to BMP ligands, mothers against decapentaplegic homolog 1 (SMAD1), as a receptor regulated SMAD (R-SMAD) protein, can be phosphorylated and activated by the BMP receptor kinase to act as a transcription factor for cell growth, apoptosis, morphogenesis, development, and immune responses.<sup>6,8</sup> In particular, for the signal transduction pathways of BMP-2, only R-SMAD 1 and 5 can be ligand-dependently phosphorylated.<sup>10</sup>

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For many years, fluoride advocates believed that fluoride was beneficial for bone due to its ability, under certain circumstances, to increase bone mass.<sup>11</sup> Attempts to use fluoride as an experimental treatment for osteoporosis, however, resulted in more fractures.<sup>12</sup> Meanwhile, it became increasingly appreciated that fluoride was a highly toxic substance because of the tens of millions of people throughout China and India who suffered from skeletal fluorosis, a serious crippling bone disease resulting from drinking water with high levels of fluoride.<sup>13</sup> More recently, studies of human populations have reported increased fracture rates in communities with 4 mg/L fluoride in the water, and animal studies have consistently found reductions in bone strength from exposure to fluoride.<sup>14</sup> Consistent with the clinical trials, numerous animal and *in vitro* studies have reported that bone strength declines with increased fluoride exposure.<sup>15</sup> Although many osteogenic molecules reside in bone and have the potential to mediate fracture repair, BMP-2 activity, acting on bone formation, is required for the initiation of fracture healing.<sup>16</sup> Prospective studies showed that fluoride at low concentrations, such as those that occur following fluoride modification of dental implants, could influence the expression of cell growth factors and affect osteoblastic proliferation and differentiation.<sup>3,17-20</sup> A report also confirmed that excessive fluoride *in vivo* stimulated the activation of rat osteoblasts and increased their proliferation and that the protein expression of BMP-2 and BMP-4 correlated with the expression of SMAD4.<sup>21</sup> However, no studies have been made on whether fluoride has negative effects on the expression of BMP-2 and SMAD1 *in vitro*.

Our study focused on the expression of BMP-2 and its downstream signal transducers in order to understand better the effect of fluoride in rat osteoblast cell culture and to provide basic data for further elucidating the molecular mechanisms of skeletal damage induced by fluoride toxicity.

#### MATERIALS AND METHODS

*Cell culture:* The study design was approved by the Institutional Animal Care and Use Committee of China. One-day-old neonatal Wistar rats were provided by the Experimental Animal Center of Shanxi Medical University of China. Primary osteoblasts were isolated from the skull of newborn Wistar rats and cultured with DMEM medium supplemented with 10% fetal calf serum (FCS), 100 IU mL<sup>-1</sup> penicillin, and 100g mL<sup>-1</sup> streptomycin at 37°C in air with 5% CO<sub>2</sub>. Meanwhile, the morphosis of osteoblasts was observed under a phase-contrast microscope. Alkaline phosphatase (ALP) and calcification nodule staining were used to identify the cells. When the osteoblast cells were grown to 90% confluence in T25 cell culture flasks, the cells were passaged to new culture flasks for the experiments.

*Detection of cell proliferation:* Cells were seeded (2 M cells per well) into 6-well plates and allowed to attach for 24 hr. They were then treated with DMEM containing 2.5, 1.0, 0.5, 0.1, 0.01, and 0 mM sodium fluoride (NaF). At the end of 24 and 72 hr, 20 µL MTT (5 mg/mL, Sigma, US) per well was added to the medium. Four hours later, the cell culture was terminated to observe the impact of

fluoride on osteoblast proliferation with the MTT colormetric method in each group at 570 nm.

According to the data analysis, the groups with significant changes were chosen to do the following experiment.

*Total RNA extraction and QRT-PCR:* Total RNA was extracted from cells at 24 and 72 hr using TRIzol reagent (Invitrogen, US). Based on the available rat sequences in Genbank for BMP-2, SMAD1, and  $\beta$ -actin, primers for quantitative real-time polymerase chain reaction (QRT-PCR) were designed using Primer 5.0 software (Table1). The three pairs of primers were tested for their specificity by the conventional reverse transcription polymerase chain reaction (RT-PCR) before being used in the QRT-PCR studies. Melting curve analysis carried out following QRT-PCR.

**Table 1.** Primer sequences, corresponding PCR product sizes, and positions

Gene	Primers (5'→3')	Primer location	Product (bp)	Genbank Accession No
$\beta$ -actin	AGCCATGTACGTAGCCATCC ACCTCATAGATGGGCACAG	471-585	115	NM_031144.2
BMP-2	TGAACACAGCTGGTCTCAGG TGACGCTTTTCTCGTTTGTG	814-903	90	NM_017178.1
Smad1	ATGAGCTCAACAACCGTGTG TCCGGTTAACATTGGAGAGC	1173-1301	129	NM_013130.2

The RT-PCR assays were performed in a 10  $\mu$ L reaction mixture on the Mx3000PTM QRT-PCR system (Stratagene, USA). The mixture contained 2  $\mu$ L 5 $\times$ Prime ScriptTM Buffer, 0.5  $\mu$ L PrimeScriptTM RT Enzyme Mix I, 0.5  $\mu$ L 50  $\mu$ M Oligo dT Primer, 0.5  $\mu$ L 100  $\mu$ M Random 6 Mers, 0.5  $\mu$ L Total RNA and 6  $\mu$ L RNase Free dH<sub>2</sub>O. QRT-PCR was performed in a total reaction volume of 25  $\mu$ L with SYBR Premix Ex TaqTM (TaKaRa, Japan) per well. The system was composed of 12.5 $\mu$ L 2 $\times$ SYBR Premix Ex TaqTM, 0.5  $\mu$ L PCR Forward Primer, 0.5 $\mu$ L PCR Reverse Primer, 0.5 $\mu$ L 50 $\times$ ROX Reference Dye II, 2  $\mu$ L cDNA, and 9  $\mu$ L RNase Free dH<sub>2</sub>O. The relative quantification of mRNA abundance for BMP-2 and SMAD1 was performed using the comparative  $\Delta\Delta$ CT method expression with the house-keeping gene  $\beta$ -actin as a calibrator. The reaction conditions were as follows: an initial reverse transcription step of 10 sec at 95°C and 40 cycles at 95°C (denaturation) for 5 sec, 59/60°C (annealing, 59°C for BMP-2 and 60°C for SMAD1) for 20 sec, and 72°C (extension) for 6 sec. Finally, the melting curve analysis was performed at 95°C for 1 min, at 55°C for 30 sec, and at 95°C for 3 sec as in the protocol for the three reaction steps. The amplified products were analyzed by agarose gel electrophoresis.

*Immunohistochemistry:* The procedures were processed according to the protocol recommended for the BMP-2/SMAD1 immunohistochemistry kit (Strept Avidin-Biotin Complex kit, Wuhan BosterBiotechnology, Co., Ltd.). After being

detached from the culture flask by trypsin solution, the cells were re-suspended in culture medium and transferred to culture dishes with the cover slips in DMEM including NaF for 24 and 72 hr. Once cultured and grown to semi-confluence, the cells were rinsed with PBS at room temperature and fixed in 4% paraformaldehyde in PBS for 30 min. This was then followed by three washes with PBS for 3 min. The cells were then put in a mixture of 30% H<sub>2</sub>O<sub>2</sub> and pure methanol (at a ratio of 1:50) for 10 min and drops of goat serum albumin were then added as the confining liquid at 37°C for 10 min.

The cells were incubated separately with the Rabbit Anti-BMP-2/Anti-SMAD1 polyclonal antibody (1:100, Usen Sciences Co. Ltd., Wuhan, China) for 2.5 hr at 37°C and washed 3 times for 2 min in PBS. Next, biotin-labeled anti-rabbit secondary antibody (Usen Sciences Co. Ltd., Wuhan, China) were introduced for 25 min at 37°C. After washing in PBS, cells were incubated with strept avidin-biotin complex for 25 min at 37°C. At the end of the incubation time, the cells were visualized with DAB (diaminobenzidine) and counterstained with haematoxylin. After infusion in xylene, the sections were mounted on glass slides under cover slips for microscopic examination.

*Statistical Analysis:* The data area expressed as mean±SD. T-tests, performed by SPSS13.0 software (SPSS, Inc., Chicago, IL, USA) were used to analyze the BMP-2 and SMAD1 protein expression levels in the rat osteoblasts by administering different concentrations of NaF. Differences with  $p < 0.05$  were considered statistically significant.

## RESULTS

*Isolation and identification of osteoblasts:* Osteoblasts was successfully isolated from the rats and identified with alkaline phosphatase (ALP) staining and alizarin red staining of calcified nodules. Before the cell attachment process, osteoblast cells showed spheric formation. After the spheric formation process, the cells spread much more and 80% cells became spindle shaped and attached to the surface. At 4–5 days, osteoblasts grew to a single cell layer and covered the whole bottom during cytomixis. The result of the ALP test and calcification nodule staining were positive, which was triggered by ALP in the cytoplasm and calcium deposits, respectively.

*Effect of fluoride on osteoblast proliferation:* The cell proliferative ability of the fluoride treated groups was less than that of the control group, and the cell proliferation rates decreased with the increasing doses of NaF (Table2). A significant inhibition in osteoblast proliferation was observed after exposure to 2.5 mM NaF at 24 and 72 hr, and after exposure to 1.0 mM NaF at 72 hr ( $p < 0.01$ ). At 72 hr, the group exposed to 0.5 mM NaF also showed a measurable reduction ( $p < 0.05$ ). However, there is no significant changes in the two low dose groups (0.01 and 0.1m M NaF).

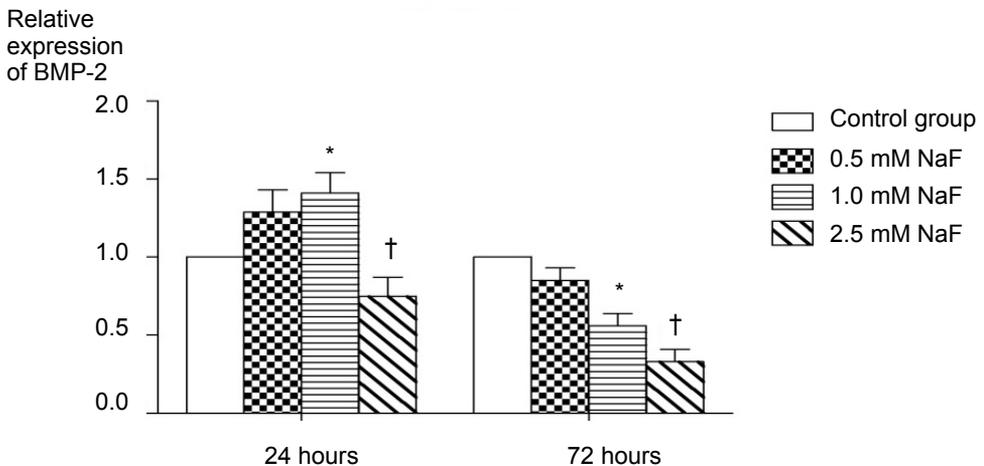
After a series of comparisons, the three experimental groups (2.5, 1.0, and 0.5 mM NaF) and the control group were chosen to determine BMP-2 and SMAD1 expression.

**Table 2.** The effects of different concentrations of sodium fluoride (NaF) on osteoblast proliferative ability at 24 and 72 hours using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method at 570 nm (Values are mean±SD, N=6)

NaF (mM)	Osteoblast proliferative ability (colormetric value at 570 nm)	
	24 hours	72 hours
0	0.278±0.050	0.601±0.050
0.01	0.282±0.016	0.620±0.010
0.1	0.286±0.039	0.625±0.017
0.5	0.269±0.037	0.507±0.052*
1.0	0.239±0.026 <sup>†</sup>	0.178±0.050 <sup>†</sup>
2.5	0.203±0.011 <sup>†</sup>	0.061±0.007 <sup>†</sup>

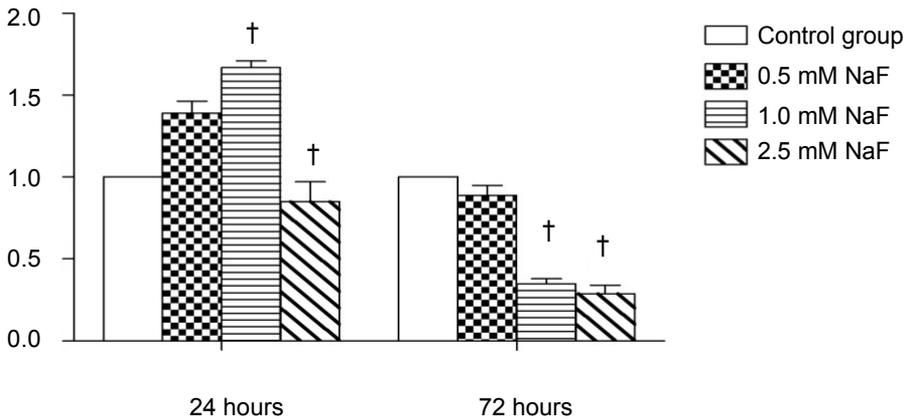
Compared to the control group: \*p<0.05; <sup>†</sup>p<0.01.

*Effect of fluoride on BMP-2 and SMAD1 gene expression:* After treatment with fluoride for 24 hr, the level of BMP-2 and SMAD1 mRNA increased in the 1.0 mM NaF group, compared with the control group, but after 72 hr treatment the expression of both proteins in this group declined markedly (Figures 1 and 2). The levels of BMP-2 and SMAD1 mRNA in the high fluoride concentration group of 2.5 mM decreased dramatically at both 24 and 72 hr, while no significant change occurred in the 0.5 mM fluoride group (Figures 1 and 2).



**Figure 1.** Quantitative real time PCR analysis of the effect of 24 and 72 hr treatment with increasing doses of NaF on osteoblast BMP-2 mRNA expression *in vitro*. The relative mRNA abundance was calculated using the  $\Delta\Delta CT$  method. The expression levels of BMP-2 mRNA were normalized relative to that of  $\beta$ -actin mRNA. Compared to the control group: \*p<0.05; <sup>†</sup>p<0.01.

Relative expression of SMAD1



**Figure 2.** Quantitative real time PCR analysis of the effect of 24 and 72 hr of treatment with increasing doses of NaF on osteoblast SMAD1 mRNA expression *in vitro*. The relative mRNA abundance was calculated using the  $\Delta\Delta CT$  method. The expression levels of SMAD1 mRNA were normalized relative to that of  $\beta$ -actin mRNA. Compared to the control group: † $p < 0.01$ .

*Effect of fluoride on BMP-2 and SMAD1 protein levels:* Tables 3 and 4 show the expression levels of BMP-2 and SMAD1 protein induced by NaF at 24 and 72 hr. After treatment with fluoride for 24 hr, the protein expression of BMP-2 and SMAD1 in the 0.5 and 1.0 mM groups showed a tendency to increase but both protein levels decreased significantly in the 2.5 mM group ( $p < 0.01$ ). After 72 hr, there was a downward trend in the protein expressions of BMP-2 and SMAD1 in all the treatment groups, compared to the control group, with the decreases in the 1.0 and 2.5 mM concentration groups being significant ( $p < 0.01$ ).

**Table 3.** The effects of different concentrations of sodium fluoride (NaF) on inducing the expression of BMP-2 protein at 24 and 72 hours. (Values are mean  $\pm$  SD, N=6)

NaF (mM)	Expression of BMP-2 protein	
	24 hours	72 hours
0	0.307 $\pm$ 0.010	0.517 $\pm$ 0.007
0.5	0.315 $\pm$ 0.011	0.508 $\pm$ 0.013
1.0	0.320 $\pm$ 0.011	0.272 $\pm$ 0.018†
2.5	0.261 $\pm$ 0.008†	0.208 $\pm$ 0.010†

Compared to the control group: † $p < 0.01$ .

**Table 4.** The effects of different concentrations of sodium fluoride (NaF) on inducing the expression of SMADA1 protein at 24 and 72 hours. (Values are mean±SD, N=6)

NaF (mM)	Expression of SMADA1 protein	
	24 hours	72 hours
0	0.514±0.011	0.632±0.005
0.5	0.521±0.010	0.623±0.006
1.0	0.529±0.022	0.568±0.007 <sup>†</sup>
2.5	0.365±0.061 <sup>†</sup>	0.329±0.005 <sup>†</sup>

Compared to the control group: <sup>†</sup>p < 0.01.

## DISCUSSION

Fluoride is not considered to be an essential nutrient for mammals and humans, and, despite some claims to the contrary, not necessary for the development of healthy teeth and bones.<sup>17,22-25</sup> Excessive exposure to high concentrations of fluoride can cause skeletal fluorosis with effects on bone cells including osteoblasts. Previous findings suggest that there are two dose-related aspects to the effect of fluoride on undifferentiated osteoblast proliferation: a low concentration of fluoride stimulates osteoblast proliferation and differentiation while a high concentration of fluoride has the opposite effect.<sup>3,23,26-31</sup> In our study, after 24 and 72 hr of treatment, the number of osteoblasts increased in the lower dosage groups (0.01 and 0.1 mM of fluoride) but decreased in the highest dose group (2.5 mM). The variation in the effects of fluoride on osteoblast proliferation may reflect dose-effect relationships and the differences in cell differentiation with time.<sup>3,32</sup>

BMPs are the strongest inducers and stimulators for enhancing the differentiation of osteoprogenitors into mature osteoblasts. To study the effects of fluoride on the biological functions of BMPs, some investigators implanted a model containing BMP and sodium fluoride into the abdominal skin of rats.<sup>33</sup> Their results revealed that fibroblast cells treated with both BMP and fluoride transformed into osteoblast cells more frequently than did fibroblasts treated only by BMP. This finding indicates that fluoride has a direct effect on the biological function of BMP.

The SMAD proteins are located in the canonical signaling pathway of TGF- $\beta$  and the main signal transduction pathways of BMPs.<sup>6,34</sup> Research has shown that R-SMADs, including SMAD1, as the downstream molecules of BMP receptors, play critical roles in the signal transduction pathways of BMPs, which could mediate several different subsequent biological effects. It is well-known that BMP-2, a widely studied member of the BMP family, plays a key role in osteoblast differentiation.<sup>8,35</sup> After BMP-2 dimeric ligands bind to receptors, the

intracellular transducers, SMAD1 and SMAD5, are phosphorylated, which results in the inhibition of the differentiation of myoblasts and the induction of osteoblast differentiation.<sup>10</sup> In addition, some researchers contend that sustained BMP signaling in osteoblasts of mice can increase the expression level of phosphorylated SMAD1 which stimulates bone formation by promoting osteoblast differentiation.<sup>8</sup> During the period of osteogenesis, the BMP/SMAD signaling pathway can be altered and the activities of p-SMAD1/5 would be required for osteoblast-like Saos-2 cells viability and differentiation induced by fluoride.<sup>36</sup>

In the present study, the results showed not only that fluoride in low and intermediate doses could induce an increase in the gene expression and protein levels of BMP-2 in osteoblast lineage cells in the short term (24 hr), but also that longer term (72 hr) fluoride treatment could inhibit the expression of BMP-2, which is consistent with the results of measuring the effect of fluoride on osteoblast proliferative ability. Thus, fluoride could inhibit osteoblast proliferation by controlling BMP-2 activity or delaying BMP-2 bioinformation. In addition, the variation of the gene expression and protein levels of SMAD1 consistently matched the changes in BMP-2 activity at the same fluoride concentrations and treatment times. This may occur through effects on phosphorylation in the signal transduction pathways of BMP-2.

Some earlier research produced similar findings, with fluoride inhibiting the expression of SMAD1 in the inner enamel epithelia of human teeth germs cultured *in vitro*.<sup>37</sup>

## CONCLUSIONS

Our overall findings revealed that fluoride could alter the expression of BMP-2 and SMAD1 in rat osteoblast culture. In the short term, after 24 hr, a low concentration (0.01–0.1 mM) of fluoride may stimulate the expression of BMP-2 and SMAD1 and strengthen osteoblast proliferative ability. However, with increases in the fluoride concentration (2.5 mM) and reaction time (72 hr), significant reductions occurred in the gene expression and protein levels of both BMP-2 and SMAD1, the downstream signal of BMP-2.

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