Combined Effect of Fluoride and Arsenate on Gene Expression of Osteoclast Differentiation Factor and Osteoprotegerin

LIN JIA \(^1\) AND TAI-YI JIN

Department of Toxicology, School of Public Health, Fudan University, Shanghai 200032, China

**Objective** To study the combined effect of fluoride and arsenate on the expression of SD rat osteoblastic osteoclast differentiation factor (ODF) mRNA and osteoprotegerin (OPG) mRNA. **Methods** Osteoblasts were obtained by enzymatic isolation from newborn SD rats. A factorial experiment was performed. Osteoblasts were exposed to NaF (0.5 mmolF/L, 4 molF/L) and Na\(_3\)AsH\(_2\) (12.5 µmolAs/L and 200 µmolAs/L) separately or F plus As and cultured for 48 h. The gene expression of osteoblastic ODF and OPG was observed by RT-PCR. **Results** The expression of ODF mRNA increased in F0.5, F4 groups compared with control group and two groups of F0.5As200, F4As200 compared with As200 group, and decreased significantly in groups of F4As12.5, F0.5As200, and F4As200. The expression of OPG mRNA decreased in groups of F4, As200, F4As12.5, F0.5As200, and F4As200. **Conclusion** The joint effect of fluoride and arsenate on the gene expression of ODF is antagonistic, while the combined effect on the gene expression of OPG is synergistic. F4, F4As12.5, and F0.5As200 promote bone resorption of rat osteoclasts, whereas F0.5As12.5 inhibits osteolytic effect of rat osteoclasts.

**Key words:** Fluoride; Arsenate; ODF; OPG; Combined effect

**INTRODUCTION**

Fluoride is an essential trace element in human bodies and is highly correlated with the metabolism of bone and tooth. But excessive exposure to fluoride for a long term leads to bone damage with complicated pathological changes such as osteoporosis and osteopetrosis. Arsenide is an environmental toxicant and a known carcinogen\(^1\). Inorganic arsenide is highly accumulated in vivo. Arsenate deposits in skeleton because it takes the place of phosphate in the apatite crystal of bone. Fluoride and arsenide in groundwater, air, and food in some areas of China as a result of specific geographical and geological environment and living habits of local people have tremendous impact on health of local residents. Great attention has been paid to the possible combined effect of fluoride and arsenide\(^3\). There are different reports about the role of arsenide in the bone damage caused by fluoride\(^4\). Both osteoblasts and osteoclasts are involved in bone damage. Studies indicate that the proliferation, differentiation, and maturity of osteoclasts are dependent on the existence of osteoblasts\(^5\).

\(^1\)Correspondence should be addressed to Lin JIA, School of Public Health, Fudan University, Box 288#, 130 Dongan Road, Shanghai 200032, China. Tel: 08-021-54237214 or 08-021-64178160; Fax: 08-021-64178160; E-mail: linjia_1978@yahoo.com or xinerjl@yahoo.com.cn

Biographical note of the first author: Lin JIA, born in 1978, female, master candidate, majoring in toxicology.
were then cut into scraps of 1 mm$^3$ and incubated in 0.25% trypsin for 15-20 min at 37°C to remove the fibroblasts. The cells were digested twice in the solution of 0.1% type II collagenase (Sigma, USA), each for one hour, and centrifuged at 1500 g for 15 min. Then the cell conglomérates were mixed up and seeded into culture flasks in DMEM (Gibco, USA) containing 10% heat-inactivated newborn calf serum and 100 U/mL sodium penicillin and 100 U/mL streptomycin in a controlled atmosphere (5% CO$_2$/95% air, 37°C). When the cells reached confluence after 7-10 days, they were released with 0.25% trypsin solution and implanted in culture plates according to required density.

The fluoride and arsenate were divided into 9 groups according to the factorial design (Table 1).

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>As (µmol/L)</th>
<th>F (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>As$_{12.5}$</td>
<td>F$_{0.5}$</td>
</tr>
<tr>
<td>0.5 F</td>
<td>F$_{0.5}$</td>
<td>F$_{0.5}$</td>
</tr>
<tr>
<td>4 F</td>
<td>F$_{4}$</td>
<td>F$_{4}$</td>
</tr>
</tbody>
</table>

**MTT Colorimetric Assay**

Released osteoblasts were implanted in 96-well plates (Falcon, Germany) at a density of 5000-6000/well. Four hours before measurement, 20 μL MTT was added to each well and incubated for additional 4 h at 37°C. DMSO (150 μL) was then added and the plates were shaken for 10 min. Finally the optical density (OD) of each well was measured using an ELISA plate reader, and the cell relative viability (= OD of experimental group/OD of control group) was assessed.

**RNA Extraction**

The procedure of RNA extraction was carefully carried out according to the manual (Molecular Research Center, Inc.). In brief, media were poured off and wells were washed with PBS after cells were cultured in 6-well plates with fluoride or arsenate and in combination for 48 h. Then 1 mL TRI reagent was added to every well and the cell lysate was passed several times through a pipette. The homogenates were stored for 5 min at room temperature, to which 0.2 mL chloroform was added, and were vigorously shaken for 15 s. The mixture was stored at room temperature for 10 min and centrifuged at 12 000 g for 15 min at 4°C. Then the aqueous phase was carefully transferred to a fresh tube and the RNA was precipitated by mixing with 0.5 mL isopropanol. After storage at room temperature for 10 min, the samples were centrifuged at 12 000 g (4°C) for 10 min. RNA precipitates formed a white pellet on the side or the bottom of the tube. The supernatant was removed carefully and RNA pellet was washed with 75% ethanol by vortexing, and then centrifuged at 7500 g for 5 min at 4°C. At the end of the procedure the ethanol was removed and the RNA pellet was air-dried for 20 min. The RNA pellet was dissolved with 20 μL DEPC-treated water for 20 min at 55°C and stored at -80°C.

**Semi-quantitative RT-PCR**

The preparation of full-length first strand cDNA from RNA templates and the PCR amplification carefully followed the protocol described in the RevertAid™ first strand cDNA synthesis kit (Fermentas) and 2×PCR Master Mix (Fermentas), respectively. The PCR primers (Table 2) of β-actin and ODF were designed with the Primer 5 software according to the cDNA sequences from http://www.ncbi.nlm.nih.gov/. Using the standard protocol of the manufacturer, after denaturation at 94°C for 5 min, 35 cycles of amplification were performed, each consisting of denaturation at 94°C for 30 s, annealing at 56°C for 1 min for β-actin while at 54°C for 1 min for ODF, and extension at 72°C for 50 s, then a final extension at 72°C for 10 min. OPG[6] (Table 2) underwent 5 min of denaturation at 94°C with 30 cycles of denaturation for 1 min at 94°C, annealing elongation at 55°C for 2 min and extension at 70°C for 1 min, then a final extension at 72°C for 7 min. After PCR, 5 μL products and 1 μL 6×loading dye were subjected to electrophoresis on 1.7% agarose gel with 0.01% ethidium bromide. The image was visualized and photographed under UV transillumination.
The intensity of each band was measured and analysed using the software of Quantity One and Bandscan. The relative abundance of each target band was normalized according to the housekeeping gene β-actin, calculated as the ratio of each target product to β-actin.

*Statistical Analysis*

The analysis of interaction was carried out with the software SPSS11.0. If there existed interaction, the one-way ANOVA was performed to test for the statistical significance of the means of all groups. *P*<0.05 was considered statistically significant.

**RESULTS**

*Morphology*

Osteoblasts first released from bones were round, suspending in the medium. After 24 h, most of them adhered tightly to the surface of the culture dishes and exhibited a variety of forms such as triangle and polygon. When the cells reached confluence, they connected with each other in the form of slabsstones and even grew overlapped (Figs. 1 and 2).

**Cell Relative Viability**

After exposure to fluoride or arsenate alone or their combination for 72 h and 120 h (Table 3), F0.5 and As12.5 enhanced the proliferation of osteoblasts, while the group of F4 and As200 inhibited osteoblast proliferation (*P*<0.05). Compared with F0.5, F0.5As12.5 and F0.5As200 decreased cell viability (*P*<0.05). In addition, after treatment for 72 h, the group of F4As200 compared with As200 group and the groups of F0.5As12.5 and F4As12.5 compared with As12.5, inhibited osteoblast proliferation (*P*<0.05). Table 3 also demonstrates that after osteoblasts were exposed to F4As12.5 for 120 h, compared with the group of As12.5, cell viability decreased significantly (*P*<0.05).

**Combined Effects of Fluoride and Arsenate on Expressions of Osteoblastic ODF mRNA and OPG mRNA**

The expression of ODF mRNA was induced when osteoblasts were exposed to fluoride or arsenate alone or their combination (Table 4, Fig. 3). When treated with fluoride alone, both F0.5 and F4 increased the expression of ODF mRNA (*P*<0.05). In addition, F0.5As200 and F4As200 compared with As200 also enhanced the gene expression of ODF (*P*<0.05). However the

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**TABLE 3**

<table>
<thead>
<tr>
<th></th>
<th>F0.5</th>
<th>F4</th>
<th>F0.5As200</th>
<th>F4As200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>1.00±0.14</td>
<td>1.34±0.07a</td>
<td>0.19±0.06c</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>1.00±0.18</td>
<td>2.11±0.06c</td>
<td>0.18±0.09c</td>
<td></td>
</tr>
<tr>
<td>As12.5</td>
<td>1.27±0.06c</td>
<td>0.91±0.04ab</td>
<td>0.19±0.05c</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>1.55±0.09c</td>
<td>1.14±0.11ab</td>
<td>0.20±0.24b</td>
<td></td>
</tr>
<tr>
<td>As200</td>
<td>0.4±0.06c</td>
<td>0.39±0.08ab</td>
<td>0.18±0.07cd</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>0.18±0.11c</td>
<td>0.20±0.09bc</td>
<td>0.20±0.17</td>
<td></td>
</tr>
</tbody>
</table>

*Note.* *P*<0.05 vs F0.5, *P*<0.05 vs As12.5, *P*<0.05 vs control group, *P*<0.05 vs As200.
decreased gene expressions of ODF were found in the groups of F0.5As12.5 and F0.5As200 (compared with F0.5, P<0.05) and in the groups of F4As12.5 and F4As200 (compared with F4, P<0.05).

TABLE 4
Effects of Different Groups on the Gene Expression of Osteoblastic ODF and OPG (T±s)

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>ODF / β-actin</th>
<th>OPG / β-actin</th>
<th>ODFmRNA/OPGmRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>0.33±0.17</td>
<td>0.45±0.18</td>
<td>0.74±0.24</td>
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<tr>
<td>As12.5</td>
<td>3</td>
<td>0.32±0.05</td>
<td>0.72±0.14</td>
<td>0.45±0.18</td>
</tr>
<tr>
<td>As200</td>
<td>3</td>
<td>0.19±0.12</td>
<td>0.18±0.12</td>
<td>1.09±0.22</td>
</tr>
<tr>
<td>F4</td>
<td>3</td>
<td>0.74±0.05</td>
<td>0.68±0.08</td>
<td>1.09±0.08</td>
</tr>
<tr>
<td>F4As12.5</td>
<td>3</td>
<td>0.35±0.17</td>
<td>0.70±0.09</td>
<td>0.40±0.01</td>
</tr>
<tr>
<td>F4As200</td>
<td>3</td>
<td>0.33±0.01</td>
<td>0.07±0.05</td>
<td>4.59±0.04</td>
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<tr>
<td>F4As12.5</td>
<td>3</td>
<td>0.84±0.06</td>
<td>0.46±0.08</td>
<td>1.80±0.12</td>
</tr>
<tr>
<td>F4As12.5</td>
<td>3</td>
<td>0.12±0.36</td>
<td>0.12±0.36</td>
<td>3.04±0.39</td>
</tr>
<tr>
<td>F4As200</td>
<td>3</td>
<td>0.29±0.03</td>
<td>0.22±0.12</td>
<td>1.30±0.14</td>
</tr>
</tbody>
</table>

Note. *P<0.05 vs F0.5, †P<0.05 vs F4, ‡P<0.05 vs control group, §P<0.05 vs As12.5, ‹P<0.05 vs As200.

The expression of OPG mRNA was observed when osteoblasts were treated with fluoride or arsenate alone or their combination (Table 4, Fig. 4). When treated with arsenate alone, osteoblasts exposed to As200 had low gene expression of OPG (P<0.05). In the combined groups, F0.5As200 could inhibit the gene expression in osteoblasts when compared with F0.5 and As200, respectively (P<0.05). The same situation could be found in the group of F4As12.5 compared with the groups of F4 and As12.5 treated with fluoride or arsenate alone (P<0.05).

We also calculated the ratio of ODF mRNA to OPG mRNA (Table 4, Fig. 5). In the groups treated with fluoride and arsenate, the ratio declined only in the group of F0.5As12.5. The increased ratio could be seen in the group of F0.5As200 compared with F0.5 and As200. In addition, osteoblasts exposed to F4As12.5 had a significantly higher ratio than those exposed to As12.5 (P<0.05).

DISCUSSION

Lindgren et al. found that the concentration of arsenic in organs tends to be higher after administration of arsenite than that of arsenate at all time points, and there is a significant accumulation of arsenate in bone with prolonging time. This is ascribed to arsenate having a structure analogous to phosphate which is substituted in the apatite crystal of bone. Therefore we used arsenate to study the effect of arsenide on bone in our experiments.

ODF is also called OPGL, which is an important osteoclast differentiation/activation factor responsible for bone modeling. Lacey et al. found that ODF is
expressed in various cells such as osteoblasts and lymphocytes. Further experiments found that the mutant mice with OPGL exhibit severe osteoporosis, delay of growth, and defect in tooth eruption.[8] However, OPGL in these mice with hematopoietic precursors differentiate into functionally mature osteoclasts in vitro in the presence of recombinant OPGL and CSF-1, suggesting that ODF plays an important role in promoting the differentiation and activation of osteoclasts. In 1997, Simonet et al.[9] first identified a novel member of the tumor necrosis factor receptor (TNFR) superfamily during a cDNA-sequencing project of a fetal rat, and named this factor osteoprotegerin (OPG) because they found that it decreases osteoclastogenesis in vitro and blocks the loss of bone induced by ovariectomy. The OPG transgenic mice suffered severe yet nonlethal osteoporosis with a defect of osteoclast differentiation in later stages. In vitro, recombinant OPG protein inhibits differentiation of osteoclasts in a dose-dependent manner. In the same year, Eisuke Tsuda et al.[10] isolated a factor from the conditioned medium of human embryonic fibroblasts (IMR-90), which could inhibit osteoclast development and was named osteoclastogenesis inhibitory factor (OCIF). All these findings show that OCIF is a new factor that specifically inhibits osteoclastogenesis. Sequence analysis of OCIF cDNA revealed that OCIF is identical with OPG.[9]

In our experiment, osteoblasts exposed to fluoride or arsenide alone or their combination induced the gene expression of ODF and OPG. But fluoride stimulated the expression of ODF mRNA and promoted the function of osteoclasts, whereas arsenate played an important role in decreasing the expression of ODF mRNA and inhibiting the function of osteoclasts. On the whole, the inhibitory role of arsenate exceeded the promoting role of fluoride, so in the combined groups, the gene expression of ODF decreased with declining bone resorption of osteoclasts. The combined effect of fluoride and arsenate was antagonistic. The gene expression of OPG decreased significantly in the groups treated with higher doses of fluoride and arsenate. The expression of OPG mRNA was markedly decreased when osteoblasts were treated with F0.5 or As200 alone and F0.5As200, F0.5As200, F0.5As200 in combination, indicating that the combined effect of fluoride and arsenate is mainly synergistic.

Some scholars believe that the ratio of ODF mRNA to OPG mRNA directly influences bone remodeling. The increased ratio shows that the role of osteoclasts enhanced by ODF predominates in bone resorption[11]. We found that in the groups of F4, F4As12.5, and F0.5As200, the ratios increased significantly, suggesting that the resorption function of osteoclasts is promoted. In contrast, osteoblasts exposed to the group of F0.5As12.5 had a remarkably decreased ratio value, which could protect osteoblasts against bone injury. All these show that F0.5As12.5 increases the gene expression of OPG while F0.5As12.5 decreases the expression of ODF mRNA, thus reducing the resorption of osteoclasts.

In summary, fluoride and arsenate may influence the process of bone remodeling by changing the gene expressions of OPG and ODF of osteoblasts, which cause bone damages. However, in vivo, a number of factors are involved in bone metabolism including transforming growth factor (TGF)-β, colony-stimulating factor-1 (CSF-1 or MCSF), interleukin (IL)-1, tumor-necrosis factor (TNF)-α besides ODF and OPG, all of which may influence the dynamic balance between formation and resorption of bone. The roles of fluoride and arsenide in the osteoporosis and fluorosis need to be further studied.

REFERENCES


(Received January 20, 2005 Accepted December 10, 2005)