



## Technical note

Strontium stimulates alkaline phosphatase and bone morphogenetic protein-4 expression in rat chondrocytes cultured *in vitro*Jinfeng Zhang<sup>a,b,1</sup>, Xiaoyan Zhu<sup>a,1</sup>, Yezi Kong<sup>a</sup>, Yan Huang<sup>a</sup>, Xukun Dang<sup>a</sup>, Linshan Mei<sup>a</sup>, Baoyu Zhao<sup>a</sup>, Qing Lin<sup>a,b,\*</sup>, Jianguo Wang<sup>a,\*</sup><sup>a</sup> College of Veterinary Medicine, Northwest A&F University, Yangling 712100, Shaanxi, China<sup>b</sup> State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University, Xining 810016, Qinghai, China

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## ABSTRACT

The trace element strontium has a significant impact on cartilage metabolism. However, the direct effects of strontium on alkaline phosphatase (ALP), a marker of bone growth, and bone morphogenetic protein-4 (BMP-4), which plays a key role in the regulation of bone and cartilage development, are not entirely clear. In order to understand the mechanisms involved in these processes, the chondrocytes were isolated from Wistar rat articular cartilage by enzymatic digestion and cultured under standard conditions. They were then treated with strontium at 0.5, 1.0, 2.0, 5.0, 20.0 and 100.0  $\mu\text{g}/\text{mL}$  for 72 h. The mRNA abundance and protein expression levels of ALP and BMP-4 were measured using real-time polymerase chain reaction (real-time PCR) and Western blot analysis. The results showed that the levels of expression of ALP and BMP-4 in chondrocytes increased as the concentration of strontium increased relative to the control group, and the difference became significant at 1.0  $\mu\text{g}/\text{mL}$  strontium ( $P < 0.05$ ). These results indicated that strontium could be involved in cartilage development via regulating ALP and BMP-4 expression.

## 1. Introduction

Chondrocytes can synthesize and maintain the extracellular matrix [1,2]. They can also control the structure and function of articular cartilage and maintain tissue homeostasis [3]. Loss of function due to injury to the articular cartilage may lead to osteoarthritis, since the ability of the joint to heal naturally is limited [4]. The symptoms are often characterized by cartilage degradation, stiffness, and loss of mobility [5,6].

ALP is a membrane-bound glycoprotein, widely distributed in most organisms. Tissue-nonspecific alkaline phosphatase (TNAP), which is expressed abundantly in the bone, liver, and kidneys, is involved in bone growth [7]. It is a marker of osteoblastic differentiation, expressed during the initial phases of the process [8]. It is a marker of the terminal phase of chondrogenesis, as chondrocyte hypertrophy [9,10]. The level of ALP expression has been regarded as a reliable indication of the chondrocytic phenotype [11].

Bone morphogenetic proteins (BMPs) are growth factors involving in the induction of bone formation. They belong to the transforming growth factor  $\beta$  (TGF- $\beta$ ) superfamily [12,13]. BMPs are synthesized by chondrocytes and osteoblasts. They are known to induce

chondrogenesis through the differentiation of mesenchymal stem cells (MSCs) toward the osteoblastic lineage. BMP-4 is secreted from the extracellular matrix and induces cartilage formation by induction of MSCs toward chondroprogenitor cells and maturation of chondrocytes. BMP-4 can also improve articular cartilage repair [14–19].

Strontium is a natural component of food [20]. Once absorbed, it tends to become deposition in bone [21]. Animal experiments indicate that strontium can increase bone formation [22,23]. Strontium administration significantly increases osteoblast-related gene expression and ALP expression in the osteogenic lineage differentiation of MSCs [24]. Although previous clinical study suggests that strontium has pronounced effects on cartilage formation, the effect of strontium on rat chondrocytes has not been elaborated [25]. Thus the aim of the present study was to investigate the effects of strontium on ALP and BMP-4 expression pattern in rat chondrocytes cultured *in vitro*.

## 2. Materials and methods

## 2.1. Reagents and chondrocyte culture medium

There were collagenase II, Hyaluronidase, trypsin, phosphate

\* Corresponding authors at: College of Veterinary Medicine, Northwest A&amp;F University, Yangling 712100, Shaanxi, China.

E-mail addresses: [yllinqing@126.com](mailto:yllinqing@126.com) (Q. Lin), [jgwang0625@nwsuaf.edu.cn](mailto:jgwang0625@nwsuaf.edu.cn) (J. Wang).<sup>1</sup> These authors contributed equally to this study.

buffered saline (PBS), Iscove's modified Dulbecco's medium (IMDM), supplemented with 15% fetal bovine serum (FBS; Gibco; ThermoFisher Scientific, Inc.) and 1% penicillin-streptomycin (Sigma Chemical, St. Louis, MO, U.S.A.). Strontium chloride ( $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ , Sigma) is the resource of strontium. All other chemicals were purchased from Sigma Aldrich (Sigma Chemical, St Louis, MO, USA).

## 2.2. Isolation and culture of chondrocytes

The articular cartilage samples of two adult Wistar rats (male, 150 g) were aseptically separated and cut into pieces, and primary chondrocytes were isolated from the extracellular matrix by enzymatic digestion, by means of a previously described method [26]. Cartilage fragments were first digested with 0.1% hyaluronidase (Sigma) for 20 min at 37 °C, then treated with 2 mg/mL trypsin (Sigma) in serum-free IMDM at 37 °C for 1 h in 5%  $\text{CO}_2$ , followed by 4 h digestion with 0.25 mg/mL collagenase II (Gibco) dissolved in serum-free IMDM at 37 °C. The cell suspensions were filtered through a nylon mesh (45  $\mu\text{m}$ ) and washed three times with 10 mM PBS to remove undigested extracellular matrix. Isolated chondrocytes were resuspended in IMDM with 15% FBS, 1% penicillin/streptomycin, then the density was adjusted to  $4.0 \times 10^4$  cells/mL. Cells were seeded in 6-well culture plates and incubated in a humidified atmosphere of 5%  $\text{CO}_2$  in air at 37 °C. Cell growth was observed by inverted microscope every day and the culture medium was changed every 3 days. The cells were maintained in culture medium until they reached confluency. Subsequently, the cells were detached by 0.25% trypsin-EDTA, replicated in 6-well cell culture plates (3 mL per well,  $5.0 \times 10^5$  cells/mL) and cultured in a humidified 37 °C, 5%  $\text{CO}_2$  incubator. After passage, cells were used for the experiments.

Strontium chloride ( $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ , Sigma) was added to culture media at different final concentrations (0.5, 1.0, 2.0, 5.0, 20 and 100  $\mu\text{g}/\text{mL}$ ) sterilized by filtration before used. PBS was used as control group, Strontium chloride of different final concentrations were used as treatment group in triplicate, respectively. After incubation (37 °C, 5%  $\text{CO}_2$ ) for 72 h, cells were collected for further analysis. The choice of the strontium doses was based on the results of preliminary study and concentration range that was confirmed by literature report [27].

## 2.3. Total RNA extraction and complementary DNA synthesis

The total RNA in chondrocytes was extracted using Minibest Universal RNA Extraction Kit (TaKaRa, Dalian, China). Then RNA concentration was analyzed by spectrophotometry at 260 and 280 nm, and only samples with an optical density ratio at 260/280 nm > 1.9 were used in further analyses. Approximately 5  $\mu\text{g}$  of total RNA was reverse transcribed to cDNA in 20  $\mu\text{L}$  reactions using a FastQuant RT kit (With gDNase) (Tiangen, Beijing, China), according to manufacturer's instructions.

## 2.4. Primer design and real-time PCR

The primer sequences (Table 1) were designed with Primer Designer 5.0 (Scientific and Educational Software, Durham, USA), according to the Coding sequences of ALP, BMP-4 and GAPDH obtained from

GenBank. The mRNA expression was evaluated by real-time PCR analysis using the SYBR Green QuantiTect RT-PCR Kit (TaKaRa Biotechnology Co, Ltd, Tokyo, Japan). RT-PCR reactions were performed in triplicate on a Real-Time PCR System (Applied Biosystems) and the relative expression of genes was calculated by the comparative cycle threshold (CT) method (using the formula  $2^{-\Delta\Delta CT}$ ,  $\Delta\Delta CT = (\text{CT. Target} - \text{CT. GAPDH})_{\text{Treatment}} - (\text{CT. Target} - \text{CT. GAPDH})_{\text{Control}}$ ) and was normalized to abundance of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Coefficient of determination ( $r^2$ ) and efficiency (Ex) of PCR amplification were validated before quantification (Table 1). The  $r^2$  confirmed the linearity, and Ex was calculated using the equation  $\text{Ex} = (100^{-1/\text{slope}}) - 1$ .

## 2.5. Protein extraction and western blot analysis

Total cell protein was extracted from chondrocytes incubated for 72 h, using a M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA), according to the supplier's protocol [28]. Protein concentration was determined using a BCA Protein Assay Kit (Thermo Scientific). Protein was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then electroblotted onto polyvinylidene fluoride (PVDF) membranes for immunoblot analysis. After blocking for 2 h in TBST buffer containing 5% nonfat milk, the membranes were washed three times with TBST and hybridized overnight at 4 °C with primary antibodies at 1:500 dilution (Santa Cruz Biotechnology). Then membranes were washed three times with TBST at room temperature with shaking, 10 min per time. Afterward, the blots were incubated with secondary antibody (goat anti-rabbit IgG-HRP conjugated, Santa Cruz, 1:5000) for 1.5 h at room temperature, then washed three times for 10 min with TBST at room temperature. The blots were detected by chemiluminescence using an ECL kit (Jingcai, Xi'an, China).

## 2.6. Statistical analyses

Statistical analysis was performed using GraphPad Prism 5, version 5.01 (GraphPad Software Inc., USA). Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's test. The results were expressed as the means  $\pm$  standard deviations (SD).  $P < 0.05$  was considered statistically significant, and  $P < 0.01$  was considered highly statistically significant.

## 3. Results

### 3.1. Effect of strontium on ALP and BMP-4 mRNA levels in chondrocytes

As shown in Fig. 1, when chondrocytes were treated with gradient doses of strontium chloride, relative mRNA levels of ALP and BMP-4 were found to be higher than those in the control group in a dose-dependent manner. At doses of strontium concentration over 1  $\mu\text{g}/\text{mL}$ , the BMP-4 mRNA levels were significantly higher than in the control group ( $P < 0.05$ ). Strontium of 0.5  $\mu\text{g}/\text{mL}$  showed no significant effect on BMP-4 mRNA levels ( $P > 0.05$ ). However, the effect of strontium on ALP mRNA levels was significantly higher than that of the control group ( $P < 0.05$ ) at doses of 0.5–100  $\mu\text{g}/\text{mL}$ .

**Table 1**  
The primers sequences of the genes.

Gene	Primer sequences (5'-3')	Length	GenBank accession No.	$r^2$	Ex
GAPDH	For GGCAAGTTCAACGGCACAG Rev CGCCAGTAGACTCCACGACAT	142 bp	NM_017008.3	0.997	0.97
ALP	For CGACACGGACAAGAAGCCCTT Rev ACTTCTGTTCTGCTCGAGGTTG	485 bp	J03572.1	0.998	0.98
BMP-4	For GTGGGAAACTTTCGATGTGAGC Rev GGGACGGCAGTTCATTACT	296 bp	NM_012827.2	0.998	0.96

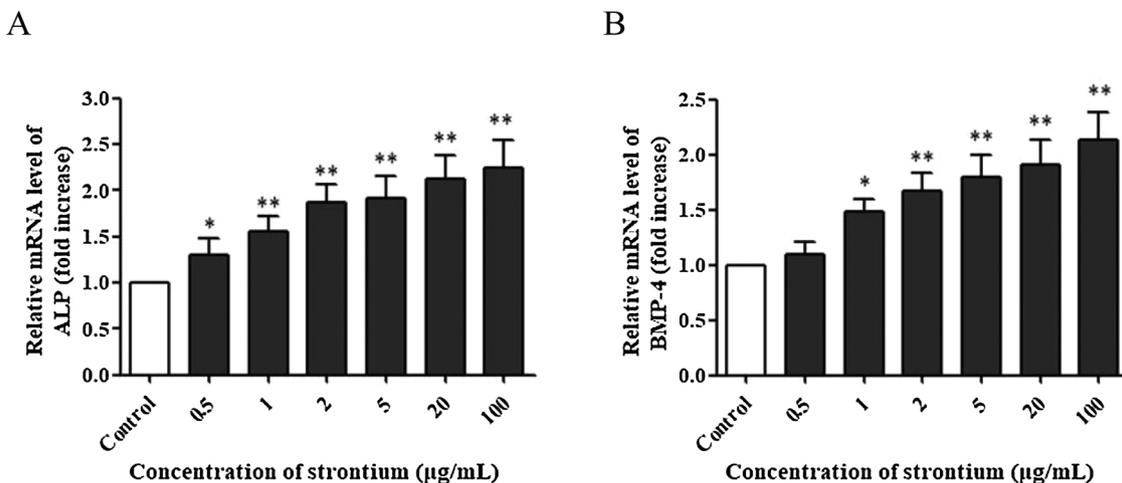


Fig. 1. Effect of strontium on ALP and BMP-4 mRNA levels in treated chondrocytes. A is the relative mRNA level of ALP. B is the relative mRNA level of BMP-4. \*  $p < 0.05$ , \*\*  $p < 0.01$  versus the control group.

### 3.2. Effect of strontium on ALP and BMP-4 protein expression in chondrocytes

As shown in Fig. 2, similarly, the protein expression of ALP and BMP-4 in chondrocytes went up in a dose-dependent manner, as the strontium concentrations increased. As the strontium concentration was over 1 µg/mL, the levels of ALP and BMP-4 protein expression were significantly higher than in the control group ( $P < 0.05$ ).

## 4. Discussion

Chondrocytes are the exclusive component cell of cartilage. They are responsible for the maintenance of the extracellular matrix, which is able to withstand physical deformation and promote tissue function

[5]. The most common degenerative conditions associated with the chondrocytes are osteoarthritis. Various clinical trials and experimental models have been utilized for cartilage repair and regeneration, including the administration of growth factors [4].

The newly discovered antiosteoporosis drug strontium ranelate has a dual influence, decreasing bone resorption and increasing bone formation [29]. Studies have shown that strontium increases cartilage matrix formation by promoting collagen synthesis and inhibiting collagen degradation in rat chondrocytes cultured *in vitro* [26]. The potentially favorable effect of strontium ranelate on reducing osteoarthritis progression has been reported, though the actual effect and mechanism of action remain to be fully elucidated [30].

Related studies have focused on the turnover of extracellular matrix components such as collagen, glycoproteins and aggrecan [6,25,26,31].

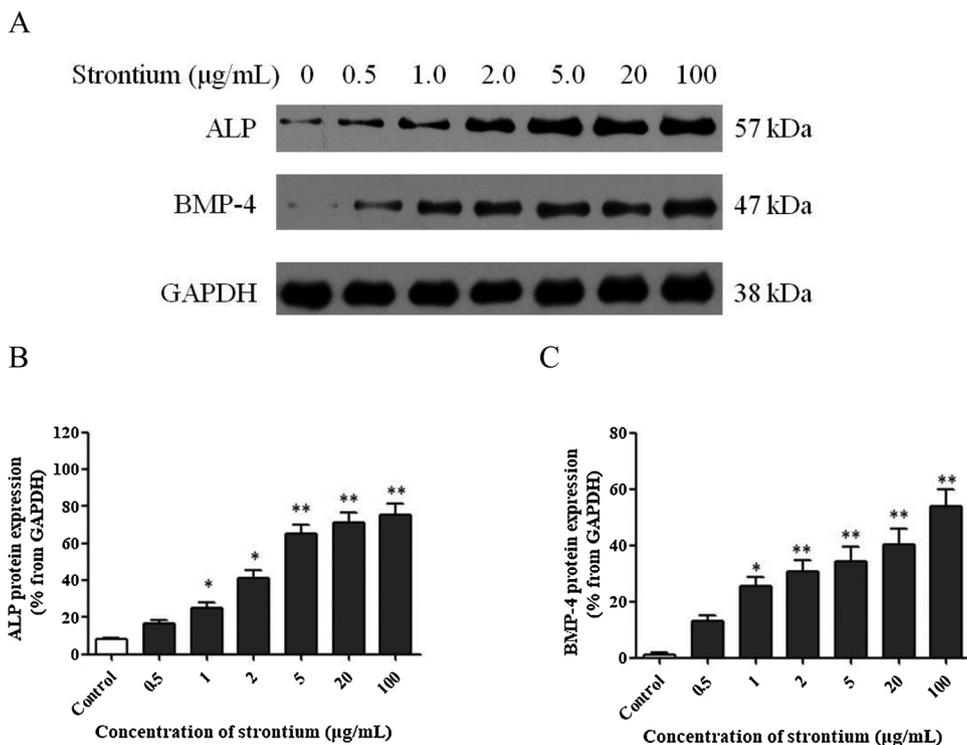


Fig. 2. Effect of strontium on ALP and BMP-4 protein expression in rat chondrocytes. A: Western blotting results of ALP and BMP-4; B: protein expression level of ALP; C: protein expression level of BMP-4. \*  $P < 0.05$ ; \*\*  $P < 0.01$  versus the control group.

Some cytokines have also drawn attention. These include insulin-like growth factors (IGFs), transforming growth factors (TGFs), and BMPs. BMPs, a multifunctional family, are famous for the ability to induce bone formation [18]. *In vitro*, studies have demonstrated that BMP-4 can induce chondrogenesis under serum-free culture condition. This occurred at sites where expression of Sox9, a marker of chondrogenesis, was high [32]. Since BMP-4 could also promote cartilage differentiation, it is possible that strontium affects the expression level of BMP-4? No comparable study has yet been performed on chondrocytes cultured *in vitro*. In the present study, results showed that the administration of strontium *in vitro* increased BMP-4 expression in chondrocytes. BMP-4 increases cartilage matrix production by irritating the synthesis of type II collagen and inhibiting the production of type X collagen, in this way maintaining chondrogenic phenotype [33–35]. Hence, strontium may stimulate cartilage matrix formation by inducing the expression of BMP-4. Further studies should be performed to establish the specific mechanism.

ALP is ubiquitously distributed, and it has been extensively investigated. Chondrocytes can secrete ALP, which becomes activated in hypertrophic chondrocytes and cartilage matrix in the hypertrophic zone [36,37]. Studies have shown that ALP levels are higher during the early stages of the osteoblast differentiation [38]. We here hypothesized that strontium could increase the activity of ALP in chondrocytes. Results proved that ALP activity increased with exogenous strontium in a concentration-dependent manner. Zeitouni et al. found that ALP was activated by treatment with medium supplemented with osteogenic agents [39]. ALP plays a central role in bone metabolism and strontium stimulates chondrocyte anabolism without affecting chondroresorption [25]. It can induce bone formation through its positive effects on osteoblastic differentiation and by increasing ALP activity in murine preosteoblasts and osteoblasts *in vitro* and *in vivo* [40–42]. In this way, the effect of strontium on chondrocytes was indicated by the level of ALP expression level. Likewise, we found strontium could promote BMP-4 expression, which may reveal the mechanisms underlying the action of strontium on cartilage formation. Further research is warranted to carry on.

## 5. Conclusion

The current study indicates that a certain concentration of strontium (0.5–100 µg/mL) can promote ALP and BMP-4 expression in a dose-dependent manner.

## Compliance with ethical standards

In the present study, all experimental procedures were conducted with the approval of the Institutional Animal Care and Use Committee of Northwest A&F University, Yangling, China. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

## Conflict of interest

The authors declare that they have no conflicts of interest.

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