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Effect of strontium ranelate on rabbits with steroid-induced osteonecrosis of femoral head through TGF-β1/BMP2 pathway

F.-Y. PAN[1], Z.-M. LI[2], X.-W. LIU[1], Y. LUO[1], Z. MA[1], S.-X. FENG[1], N. XU[1]

[1]Joint Department of Orthopedics, 970 Hospital of the PLA JLSF, Yantai, China
[2]Department of Medical Service, 970 Hospital of the PLA JLSF, Yantai, China

Fengyu Pan and *Zhenming Li* contributed equally to this work

Abstract. – OBJECTIVE: To study the effect of strontium ranelate (SR) on steroid-induced osteonecrosis of the femoral head (SIONFH) in rabbits and its regulatory mechanism.

MATERIALS AND METHODS: The ONFH model was established in 30 rabbits using steroid and they were randomly divided into Control group, Model group, and SR group. After SR intervention, the rabbits were sacrificed and sampled. The pathological injury of the femoral head in each group was detected *via* hematoxylin-eosin (HE) staining, the level of vascular endothelial growth factor (VEGF) in the femoral head in each group was detected *via* enzyme-linked immunosorbent assay (ELISA). The messenger ribonucleic acid (mRNA) and protein expression levels of transforming growth factor-β1 (TGF-β1), as well as the bone morphogenetic protein 2 (BMP2) in the femoral head in each group, were determined using Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Western blotting.

RESULTS: The rabbit model of SIONFH was successfully established. Compared with Control group, the Model group had a severer pathological injury of the femoral head, a lower level of VEGF in the femoral head, significantly decreased mRNA and protein levels of TGF-β1 and BMP2. Compared with Model group, the SR group had markedly improved pathological injury of the femoral head, a higher level of VEGF in the femoral head, significantly increased mRNA and protein levels of TGF-β1, as well as BMP2.

CONCLUSIONS: SR can remarkably improve the pathological injury of the femoral head and increase the expression of VEGF in SIONFH rabbits, whose potential mechanism may be related to the activation of the TGF-β1/BMP2 signaling pathway.

Key Words:
Strontium ranelate, TGF-β1/BMP2 signaling pathway, Osteonecrosis of femoral head, VEGF.

Introduction

Osteonecrosis of the femoral head (ONFH), also known as ischemic necrosis of femoral head, is a very common and refractory disease in orthopedics, including trauma-induced, alcohol-induced, and steroid-induced ONFH (SIONFH)[1-3]. In the early stage of the disease, its pathological manifestations are dull pain and hidden pain of the hip joint, accompanied by the aggravation of hip pain after exercise, as well as limitation of motion and claudication in severe cases. If there is no timely treatment, the ischemia and necrosis of hip femoral head may occur, with the collapse in the necrotic area, seriously affecting the quality life of patients, and posing a heavy burden on society and families[4,5]. Therefore, searching for safe and effective drugs or methods is the emphasis of research on SIONFH.

The pathological manifestation of SIONFH is the vascular damage of femoral head. With the development of the disease, the residual blood volume in the femoral head is insufficient to exert the normal function of osteocytes at the injury site, thus leading to necrosis and apoptosis of osteocytes[6]. Several studies have demonstrated that transforming growth factor-β1 (TGF-β1) plays an important role in ONFH and it can be seen from the clinical specimen data that the expression level of TGF-β1 in bone tissues in SIONFH declines. Therefore, its expression level is positively correlated with the degree of ONFH[7,8], thus further suppressing the expression of the downstream target bone morphogenetic protein 2 (BMP2). BMP2 exists in the bone matrix, which can induce the differentiation of stromal cells into chondrocytes and osteocytes, promoting the expression of vascular endothelial growth factor

Corresponding Author: Fengyu Pan, MM; e-mail: panfengyu107@outlook.com

(VEGF), creating a microenvironment benefitting vascularization for osteocytes[9,10]. The above findings suggest that if the TGF-β1/BMP2 signaling pathway is activated in time, the occurrence or development of SIONFH can be inhibited to some extent.

Strontium ranelate (SR) (5-[bis(carboxymethyl)amino]-2-carboxy-4-cyano-3-thiopheneacetic acid strontium, molecular weight: 513.49, molecular formula: $C_{12}H_6N_2O_8SSR_{28}H_2O$)[11,12] is composed of two stable strontium atoms and one molecule of ranelate. Strontium atoms can participate in the process of bone calcification and aggregate in osteoblasts to increase collagen synthesis. Moreover, they can inhibit osteoclast-mediated bone resorption. Clinically, SR is mainly used to prevent and treat osteoporosis in postmenopausal women and reduce the risk of fracture. Its efficacy in osteoporosis has been greatly considered by researchers, but its effect on SIONFH is rarely reported[13]. In this paper, therefore, the rabbit model of SIONFH was established *via* injection of dexamethasone sodium phosphate to observe the effect of SR on ONFH in rabbits and explore its mechanism of action to provide an experimental basis for the application of SR in the treatment of SIONFH.

Materials and Methods

Reagents

Dexamethasone sodium phosphate injection was purchased from CISEN Pharmaceutical, TRIzol from Thermo Fisher Scientific (Waltham, MA, USA), enzyme-linked immunosorbent assay (ELISA) kit of VEGF from R&D System (Minneapolis, MN, USA), hematoxylin-eosin (HE) staining fluid from Solarbio (Beijing, China), first-strand complementary deoxyribonucleic acids (cDNAs) synthesis kit, p38 MAPK and TNF-α primers from Invitrogen (Carlsbad, CA, USA), rabbit anti-TGF-β1 and BMP2 primary antibodies, as well as horseradish peroxidase (HRP)-labeled secondary antibodies from Bioss (Beijing, China).

Instruments

The optical microscope was purchased from Olympus (Tokyo, Japan), electrophoresis apparatus and semi-dry membrane transfer machine from Bio-Rad (Hercules, CA, USA), thermostat water bath kettle from Changzhou Aohua Instrument Co., Ltd., Reverse Transcription-Polymerase Chain Reaction (RT-PCR) instrument from Thermo Fisher Scientific (Waltham, MA, USA), microtome from Leika (Wetzlar, Germany), spectrophotometer from Shanghai Huguang Scientific Instrument Co., Ltd. (Shanghai, China), and analytical balance from Tianjin Jingtuo Instrument Technology Co., Ltd. (Tianjin, China).

Animals

A total of 30 clean-grade rabbits were purchased from Jiangsu HFQ Biotechnology Co., Ltd. [license No.: SYXK (Jiangsu, China) 2014-0018] and fed in separate cages in a quiet environment. They had free access to food and water. The fodder was regularly replaced, with cleaning and the removal of feces every day to avoid contaminating it. This research was approved by the Animal Ethics Committee of 70 Hospital of the PLA JLSF Animal Center.

Establishment of Rabbit Model of SIONFH

The above 30 rabbits were randomly divided into Control group, Model group, and SR group. The rabbits in Model group were injected intraperitoneally with dexamethasone sodium phosphate (20 mg/kg) every 3 days for 8 weeks, while those in SR group were gavaged with SR (800 mg/kg) every day for 8 weeks based on the treatment in Model group. The gentamicin solution was injected to prevent infection in Model group and SR group. Besides, the rabbits in Control group were gavaged with an equal dose of normal saline as controls.

Detection of Pathological Injury of Femoral Head in Each Group Via HE Staining

HE staining is the most commonly used staining method for detecting pathological injury. Hematoxylin is alkaline and it can stain the chromatin in the nucleus blue-violet, while eosin is acidic and it can stain the cytoplasm and matrix red. Once the rabbits were sacrificed, the femoral head was fixed with 4% paraformaldehyde and decalcified with ethylenediaminetetraacetic acid (EDTA)-Na_2 solution for 4 weeks. Then, the tissues were embedded in paraffin, sliced into 8 μm-thick sections, and deparaffinized and hydrated with xylene and ethanol solution, followed by the HE staining. After re-dehydration, transparentization, and sealing, the staining was observed under the optical microscope.

Detection of VEGF Content in Femoral Head in Each Group Via ELISA

After the last treatment with SR, the rabbits were anesthetized with 10% chloral hydrate, and the femoral head tissues were extracted. According to the instructions of the ELISA kit, standards and samples with gradient dilution were added and incubated after calcification, the plate was washed and horseradish peroxidase (HRP)-labeled streptavidin-biotin was added, both of which would strongly bind to each other. After the plate was washed again, the blue developing solution was added. Also, the stop buffer was added to terminate the reaction and at this moment the solution turned yellow. The absorbance was measured using the microplate reader (λ=450 nm), based on which the VEGF concentration was calculated.

Detection of Messenger Ribonucleic Acid (mRNA) Levels of TGF-β1 and BMP2 in Femoral Head in Each Group Via RT-PCR

The femoral head tissues (100 mg) were fully lysed with 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) lysis buffer and the total RNA was extracted using chloroform and isopropanol. According to the instructions of the first-strand complementary deoxyribose nucleic acid (cDNA) kit, RNA was reversely transcribed into cDNA, followed by PCR amplification using 25 μL of reaction system: denaturation at 95°C, annealing at 65°C and extension at 72°C for a total of 30 cycles. 1 μL of forward primers and 1 μL of reverse primers were added (Table I). After gel electrophoresis for reaction products, the optical density value was observed.

Detection of Protein Levels of TGF-β1 and BMP2 in Femoral Head in Each Group Via Western Blotting

The tissues were lysed using radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) and the proteins were collected. The dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) gel was prepared, and the loading buffer was added into proteins, followed by a boiling water bath for 5 min for protein denaturation. Then, the proteins were added into the gel pores for electrophoresis. The proteins on the gel were transferred onto the polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland), sealed with blocking buffer, incubated with TGF-β1 (1:1000) and BMP2 (1:1000) primary antibodies overnight, and incubated again with secondary antibodies. Finally, the color was developed in bands using diaminobenzidine (DAB) and the optical density value of bands was analyzed using the ImageJ software (Media Cybernetics, Silver Springs, MD, USA).

Table I. Primer sequences of TGF-β1 and BMP2.

Gene	Sequences
TGF-β1	CTAATGGTGGAAACCCACAACG TATCGCCAGGAATTGTTGCTG
BMP2	ACCCGCTGTCTTCTAGCGT TTTCAGGCCGAACATGCTGAG
GAPDH	ACAACTTTGGTATCGTGGAAGG GCCATCACGCCACAGTTTC

Statistical Analysis

The data were imported into Excel to establish the database and processed using Statistical Product and Service Solutions (SPSS) 17.0 (SPSS, Chicago, IL, USA) software. The measurement data were expressed as mean ± standard deviation. The *t*-test was used for analyzing measurement data. The differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ suggested statistically significant differences.

Results

SIONFH Model

In Model group, the rabbits had low spirit and hair loss at 2 days after the injection of dexamethasone sodium phosphate. With the prolongation of time, the food intake was reduced. After 4 weeks, the rabbits gradually suffered from difficulty in standing and claudication, and two of them died. In Control group, the rabbits had normal spirit, activity and food intake. In SR group, the low spirit and difficulty in standing were improved compared to those in Model group. The bone mineral density (BMD) of the femoral head detected indicated that the rabbit model of SIONFH was successfully established (Figure 1).

SR Could Improve Pathological Injury of SIONFH Rabbits

According to the HE staining (Figure 2), the cell membrane was disrupted and the nuclei

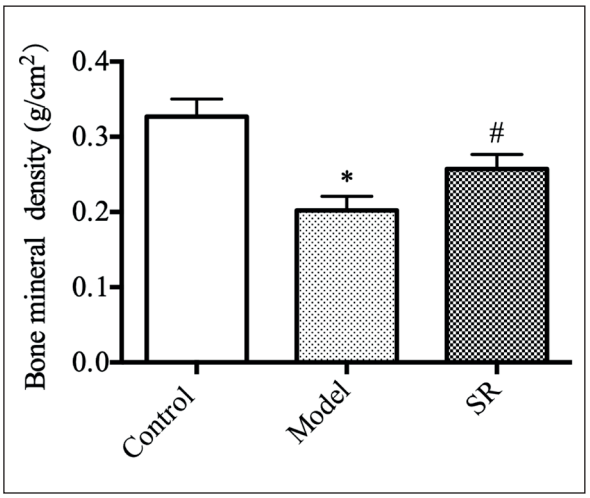

Figure 1. Comparison of BMD of femoral head among groups (*p<0.05: Control group *vs*. Model group, #p<0.05: Model group *vs*. SR group).

were arranged disorderly, with congestion in the femoral head tissues in Model group at 8 weeks after modeling compared to those in Control group. In SR group, the congestion was alleviated; the adipocytes and empty lacuna were reduced compared to those in Model group.

SR Could Increase VEGF Content in Femoral Head of SIONFH Rabbits

ELISA results and statistical analysis showed that the VEGF level in the femoral head tissues declined in Model group compared with that in Control group (*p<0.05), while it was increased in SR group compared with that in Model group (#p<0.05) (Figure 3).

SR Could Raise mRNA Levels of TGF-β1 and BMP2 in Femoral Head of SIONFH Rabbits

The RT-PCR bands (Figure 4) showed that the mRNA levels of TGF-β1 and BMP2 in the femoral head tissues declined in Model group compared with those in Control group (*p<0.05, *p<0.05), while they were increased in SR group compared to those in Model group (#p<0.05, #p<0.05). The statistical results are shown in Figure 5.

SR Could Raise Protein Levels of TGF-β1 and BMP2 in Femoral Head of SIONFH Rabbits

The Western blotting bands (Figure 6) revealed that the protein levels of TGF-β1 and BMP2 in femoral head tissues declined in Model group compared to those in Control group (*p<0.05, *p<0.05), while they were increased in SR group compared to those in Model group (#p<0.05, #p<0.05). The statistical results are shown in Figure 7.

Discussion

In recent years, reports have demonstrated that the morbidity rate of ONFH shows an increasing trend year by year. In particular, SIONFH accounts for approximately 30-50%, but there are no effective treatment means except for hip replacement surgery in the late stage of ONFH[14,15]. SIONFH is a metabolic lesion caused by ischemia and hypoxia of the femoral head due to long-term administration of excessive hormone drugs. The therapeutic effects of modern medicine are not

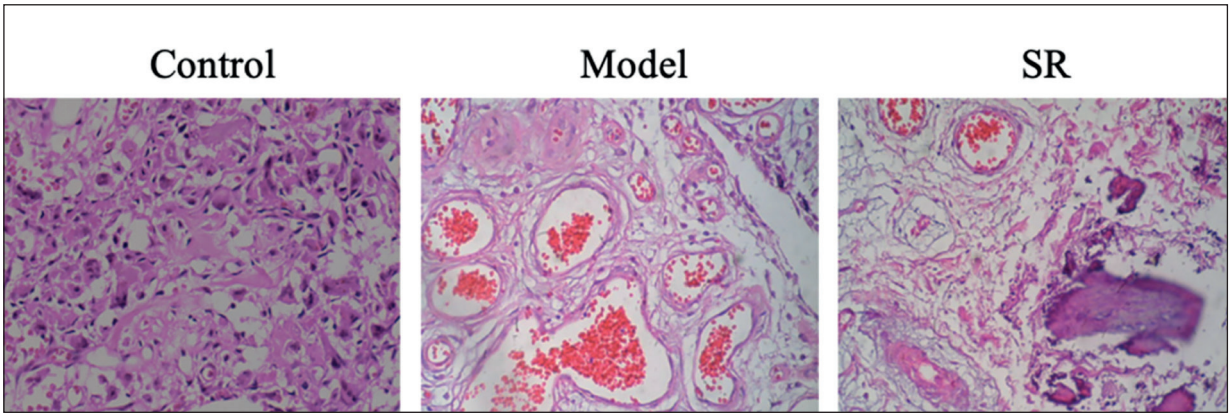

Figure 2. Pathological morphology of femoral head in each group (magnification 20×).

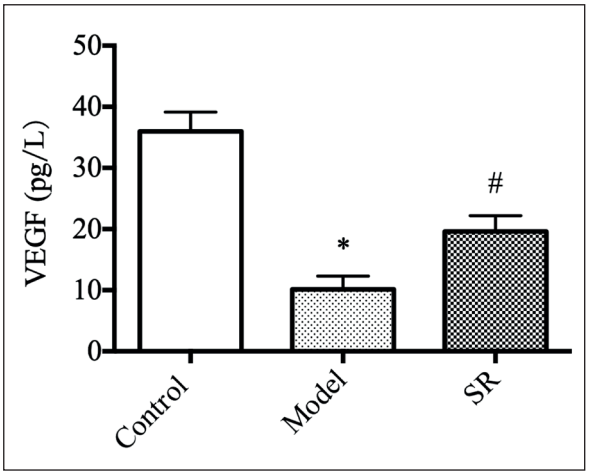

Figure 3. Comparison of VEGF level (*p<0.05: Control group *vs*. Model group, #p<0.05: Model group *vs*. SR group).

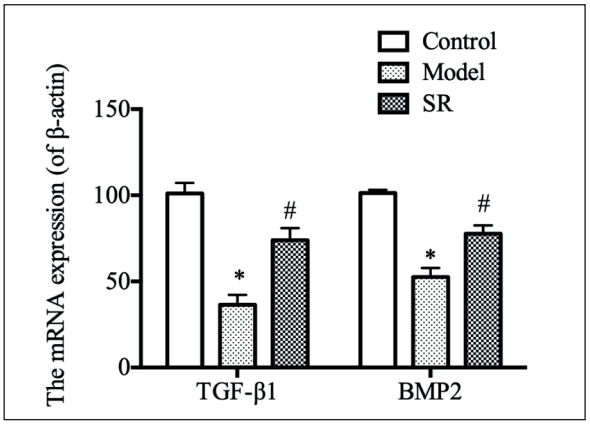

Figure 5. Comparison of mRNA levels of TGF-β1 and BMP2 in femoral head tissues among groups (*p<0.05: Control group *vs*. Model group, #p<0.05: Model group *vs*. SR group).

satisfactory[16]. The clinical effect of SR, as a safe and effective first-line drug for osteoporosis, has been proven, and it has dual effects on bone metabolism, which can inhibit bone resorption and promote bone formation. However, the application of SR in the treatment of SIONFH is rarely reported.

The pathogenesis of SIONFH is complex and diverse, mainly including lipid metabolism disorder theory, osteoporosis theory, intraosseous hypertension theory, apoptosis theory, and vascular damage theory. Xu et al[17] have found that vascular damage theory occupies a very important position, and researchers have confirmed that ONFH is closely associated with insufficient blood supply to the femoral head. VEGF is a kind of glycoprotein secreted by platelets, macrophages, and osteoblasts, which, as a vascular growth factor, plays a key role in angiogenesis. Weinstein et al[18] found that in the mouse model of ONFH, the lower expression of VEGF corresponds to the severer ONFH. VEGF is regulated by BMP2 *in vivo*. BMP2 is the most important signaling protein regulating bone formation and the results of *in vivo* and *in vitro* experiments manifested that BMP2 possesses a strong osteogenesis effect. Moreover, BMP2 is also an important regulator in the TGF-β1/BMP2 signaling pathway, which is regulated by TGF-β1. Zhang et al[19] found that PRP can significantly improve the hemorheological indexes in New Zealand white rabbits after tibial fractures, increase the VEGF level, and repair the local damaged vessels, whose mechanism may be related to the regulation of the TGF-β1/BMP2 signaling pathway.

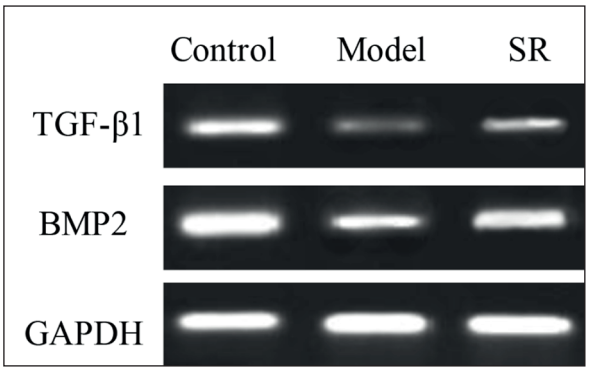

Figure 4. RT-PCR bands. TGF-β1 and BMP2 in the femoral head tissues declined in Model group compared with those in Control group.

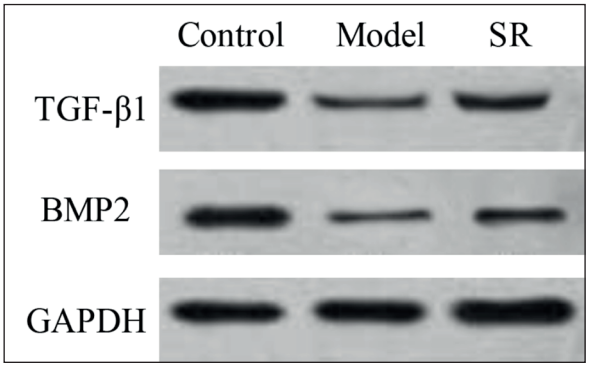

Figure 6. Western blotting bands. protein levels of TGF-β1 and BMP2 in femoral head tissues declined in Model group compared to those in Control group.

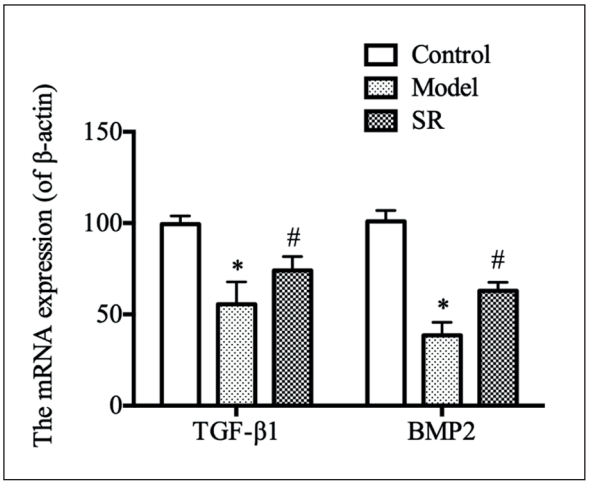

Figure 7. Comparison of protein levels of TGF-β1 and BMP2 in femoral head tissues among groups (*p<0.05: Control group *vs.* Model group, #p<0.05: Model group *vs.* SR group).

In this paper, therefore, the rabbit model of SIONFH was established *via* the injection of dexamethasone sodium phosphate, and the BMD was evaluated. The results showed that the BMD was significantly decreased in Model group but increased after SR treatment. Next, the pathological changes in the femoral head tissues were detected *via* HE staining, and it was found that SR could significantly improve the pathological injury of femoral head in ONFH rabbits. According to the detection results of VEGF content in femoral head tissues, SR could remarkably raise the level of VEGF in femoral head tissues of ONFH rabbits, indicating that the improvement of femoral head function in ONFH rabbits by SR may be associated with the increased levels of angiogenic factors in femoral head tissues. Wang et al[20] also found that early SIONFH is closely related to the protein expression of VEGF and the decreased expression level of VEGF will lead to significantly reduced blood supply to the femoral head, demonstrating that the VEGF expression plays an important role in the pathogenesis of ONFH. To further explore the regulatory mechanism of SR in ONFH rabbits, the expression levels of TGF-β1 and BMP2 in the femoral head tissues of rabbits were determined through RT-PCR and Western blotting.

Conclusions

We revealed that SR could exert a protective effect on bones in SIONFH rabbits by evidently activating the TGF-β1/BMP2 signaling pathway. The research results provide new experimental data for the application of SR in the treatment of SIONFH from the basic experimental level.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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