

Strontium ranelate reduces the progression of titanium particle-induced osteolysis by increasing the ratio of osteoprotegerin to receptor activator of nuclear factor- κ B ligand *in vivo*

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Received June 22, 2017; Accepted November 2, 2017

DOI: 10.3892/mmr.2017.8292

Abstract. The present study aimed to investigate the effects of strontium ranelate (SR), an anti-osteoporotic drug, on osteolysis in an experimental mouse model of aseptic loosening. A total of 45 female C57BL/6J mice each received implantation of one titanium alloy pin into the tibia, followed by intraarticular injection of titanium particles. One week following surgery, mice were randomly divided into three groups: Control group (no additional treatment), SR625 group (treated with SR at a dose of 625 mg/kg/day), and SR1800 group (treated with SR at a dose of 1,800 mg/kg/day). SR was administered via oral gavage once every day for 12 weeks. Micro-computed tomography scanning and hematoxylin/eosin staining were used to assess osteolysis around the prosthesis. Immunohistochemistry and reverse transcription-quantitative polymerase chain reaction analysis were used to measure the expression of receptor activator of nuclear factor- κ B ligand (RANKL) and osteoprotegerin (OPG). Compared with the control, the SR625 and SR1800 groups exhibited a significantly increased pulling force of the titanium alloy pin. Bone volume and the bone surface/volume ratio in the periprosthetic tissue were significantly increased in the SR-treated groups. Significant differences were

observed between the SR1800 group and control group with respect to trabecular thickness and trabecular number. Mechanistically, SR downregulated the expression of RANKL and upregulated the expression of OPG in the periprosthetic tissue. In addition, SR was observed to inhibit wear particle-associated osteolysis in a dose-dependent manner. In conclusion, the present data illustrated that SR inhibited titanium particle-induced osteolysis *in vivo*.

Introduction

Aseptic loosening induced by wear particles has become one of the most critical contributors to arthroplasty failure (1). Wear particles are debris from joint replacement implants that are able to induce inflammation and bone resorption at the interface between the prosthesis and its adjoining bone (2,3). Various measures have been used for the prevention and treatment of aseptic loosening. Strontium ranelate (SR) is an anti-osteoporotic drug, and has the potential to reduce the risk of spinal and hip fractures in postmenopausal women (4). SR is able to promote the proliferation of pre-osteoblasts, suppress the production and activity of osteoclasts, and increase osteoclast apoptosis (5,6). Therefore, SR may be considered to be a potential treatment for aseptic loosening.

Receptor activator of nuclear factor- κ B ligand (RANKL) is secreted by osteoblasts and other cell types, including endothelial and active T cells (7,8), and various inflammatory factors may stimulate its secretion (9-11). Upon binding to its membrane receptor (RANK), RANKL activates the nuclear factor (NF)- κ B signaling pathway and induces osteoclast differentiation, inhibits osteoclast apoptosis, and promotes osteoclast adhesion to the bone surface (12-14). Osteoprotegerin (OPG), a soluble competitive decoy receptor for RANK, is able to inhibit the NF- κ B signaling pathway by interfering with the RANKL-RANK interaction (11,15). OPG is secreted by a number of types of cells, including osteoblasts and mesenchymal stem cells (16). The interaction between OPG, RANKL and RANK, therefore, may serve an essential role in the regulation of bone metabolism (17-19).

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Abbreviations: BV, bone volume; BV/TV, bone volume fraction; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor- κ B ligand; SR, strontium ranelate; Tb.N, trabecular number; Tb.Th, trabecular thickness; Ti, titanium; μ CT, micro-computed tomography

Key words: aseptic loosening, titanium particles, strontium ranelate, receptor activator of nuclear factor- κ B ligand, osteoprotegerin

The present study aimed to investigate whether treatment with SR may inhibit aseptic loosening in an experimental mouse model that simulates artificial joint replacement, and reflects the interaction between wear particles and periprosthetic tissues (20), and to examine the potential biochemical mechanisms of action of SR.

Materials and methods

Preparation of wear particles. Unmixed titanium (Ti) particles (Zimmer Biomet, Warsaw, IN, USA) with an average size of 5 μm were used. Prior to injection, the particles were rinsed in 70% ethanol for 48 h at room temperature, washed twice in PBS, and autoclaved at 180°C for 6 h to remove endotoxins. A commercial detection kit (E-Toxate; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to test whether the treated wear debris contained endotoxins or not (21).

Animal experiment. A total of 45 10-week-old female C57BL/6J mice, each weighing 20 \pm 2 g, were used in the present study. All mice were maintained with pressure-controlled ventilation at a constant temperature of 25°C and a relative humidity of 40-70% in a 12/12-h light/dark cycle, and were given lab chow and water *ad libitum*. The study protocol was approved by the Animal Ethics Committee of Ningxia Medical University (Yinchuan, China).

Animal experiments were performed as previously described (20). In all mice, an intraperitoneal injection of Nembutal (0.6% pentobarbital sodium) was given to induce general anesthesia, and the murine joint prosthesis model was established in the right lower extremities. Under sterile conditions, the tibial plateau was exposed through the medial parapatellar approach and one Ti pin was gently implanted into the proximal tibia, with the pin head being maintained in the same plane as the tibial plateau surface. The skin incision was washed with normal saline containing 100 U/ml penicillin and 100 mg/ml streptomycin, and each layer was separately closed with absorbable sutures (20). Prior to surgically inserting the Ti pin, the mouse tibial canal was injected with 10 μl Ti suspension (4 \times 10⁴ particles of Ti in normal saline). Subsequently, every 2 weeks following surgery, 20 μl Ti particles were injected into the joint capsule at week 2, 4, 6, 8, 10 and 12. Mice were randomly divided into three groups for treatment with SR (S12911-2; PROTELOS[®]; Servier, Stoke Poges, UK): Control group (joint prosthesis only), SR625 group (joint prosthesis and SR at a dose of 625 mg/kg/day), and SR1800 group (joint prosthesis and SR at a dose of 1,800 mg/kg/day). A total of 7 days post-surgery, mice were given SR via intragastric gavage. Animals were treated consecutively for 12 weeks and were sacrificed for histological analysis, immunohistochemical (IHC) analysis, Ti prosthesis steadiness examination and micro-computed tomography (μCT) analysis.

Pullout test to assess Ti prosthesis steadiness. Following sacrifice, the tibia containing the Ti pin was removed (20). To expose the Ti pin head, all muscles and tissues around the bone were carefully removed. Each bone was fixed to a special clamp using dental cement, which was designed to align the long axis of the implant with the long axis of the HP-100 Control Electronic Universal Testing Machine (Yueqing

Zhejiang Instrument Scientific Co., Ltd). With the position of the mouse limb and the custom fixture controlled, the pin was pulled out of the tibial canal at a rate of 2.0 mm/min. Load data were recorded using automatic software (Edburg version 1.0; Yueqing Instrument Co., Ltd., Yueqing, China).

μCT scans. Following removal of all soft tissues, tibias from four mice per group were fixed in 4% paraformaldehyde, at 4°C for 4 weeks. The fixed shin bones were scanned by μCT (SkyScan 1176; Bruker microCT, Kontich, Belgium) at a resolution of 9 μm . The μCT scans were acquired at a 900-ms exposure time, 45-kV voltage and 550-mA current. Automatic data analysis software (NRecon version 1.1.11; Bruker microCT) was used to reconstruct and acquire images based on the μCT analyses, and to determine the bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), bone volume (BV), and specific bone surface (BS/BV) of the shin bone surrounding the Ti pin. All horizontal cutting images were captured at two-fifths of the titanium nail, which was 2 mm from the lower edge of the top hat.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. The 260/280 absorbance ratio was measured to verify RNA purity (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). First strand cDNA was synthesized with 1 μg total RNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). A total of 2 μl cDNA was used for each PCR mixture, containing SYBR[®] Premix Ex Taq[™] II (Tli RNaseH Plus; Takara Biotechnology Co., Ltd., Dalian, China). The reaction was subjected to a 40-cycle amplification of 95°C for 30 sec, 95°C for 5 sec, and 60°C for 30 sec. The relative mRNA expression of selected genes was normalized to GAPDH and quantified using the 2^{- $\Delta\Delta\text{C}_q$} method (22).

PCR primers used in the present study were: RANKL forward, 5'-TCCTGAGCCTCCATGAAAACG-3' and reverse, 5'-CCCACACTGTGTTGCAGTTC-3'; OPG forward, 5'-TGAAGTACCGGAGCTGTCCCC-3' and reverse, 5'-AGGCCA TATGTGCTGCAGTTTCG-3'; and GAPDH forward, 5'-TTG TCAAGCTCATTGGGCTCATT-3' and reverse, 5'-GCC ATGTAGGTCCACCCATG-3'.

Histopathological and IHC analysis. The tibia was fixed in 4% paraformaldehyde for 24 h at 4°C, and immersed in EDTA solution for decalcification. The samples were dehydrated in a graded series of ethanol followed by xylene, prior to being embedded in paraffin at 60°C. Sections (5 μm) were cut perpendicular to the long axis of the tibia using an RM2235 Rotary Microtome-Basic Instrument (Leica Microsystems, Inc., Buffalo Grove, IL, USA). Sections were stained with hematoxylin and eosin (H&E) for histomorphometric analysis: 0.5% water-soluble Eosin for 5 min at 23°C and Hematoxylin for 3 min at 23°C. IHC staining for OPG and RANKL was implemented to assess the activity of osteoclastogenesis. EDTA was preheated to 60°C in a pressure cooker, then glass slides added to the autoclave for 2 min, then allowed to cool for 20 min. Following washing with PBS, the slides were incubated

at 23°C for 10 min with 3% hydrogen peroxide, washed again with PBS and incubated with primary antibodies overnight at 4°C. The primary antibodies used were: Rabbit polyclonal anti-OPG (cat. no. ab183910; 1:300; Abcam, Cambridge, UK); and rabbit polyclonal anti-RANKL (cat. no. ab9957; 1:300; Abcam). To exclude the possibility of nonspecific staining, negative controls were performed with PBS instead of primary antibodies. Then the slides were incubated with secondary antibodies (Enzyme-labeled goat anti-rabbit IgG polymer) part of the PV-9001 kit (Sino Biological, Beijing, China) at 23°C for 40 min. Standardized IHC images were obtained with a microscopic imaging system (DM2000 LED; Leica Microsystems, Inc.), and positive expression was calculated using Image-Pro Plus version 6.0 software (Media Cybernetics Inc., Rockville, MD, USA).

Statistical analysis. Data are presented as the mean \pm standard deviation. Results were analyzed by one-way analysis of variance, among the three groups. The least significant difference post-hoc test was performed for the distinction of means between different groups. $P < 0.05$ was considered to indicate a statistically significant difference. SPSS 19.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis.

Results

Treatment with SR increases the pulling force of the Ti pin. The average pulling load was 1.21 ± 0.61 N for the control group. Compared with the control group, significant increases in pulling force were detected in the SR625 group (8.51 ± 0.52 N, $P < 0.01$) and SR1800 group (13.42 ± 0.13 N, $P < 0.01$). A significant difference in pulling load was additionally observed between the SR625 and SR1800 groups ($P < 0.01$; Fig. 1).

Treatment with SR improves bone microstructure around the prosthesis. μ CT scanning demonstrated differences in the bone microstructure among the three groups. Osteolysis around the control group pin was most marked (Fig. 2). Tb.Th, Tb.N, BS/BV, BV and BV/TV data were obtained from μ CT analysis of the region of interest. Compared with the control group ($11.709 \pm 0.720\%$), BV/TV was significantly increased in the SR625 group ($13.390 \pm 0.628\%$, $P = 0.048$) and SR1800 group ($15.288 \pm 0.184\%$, $P = 0.002$) in a dose-dependent manner ($P = 0.031$). Similarly, compared with the control group (0.109 ± 0.006 mm³), BV was significantly increased in the SR625 group (0.125 ± 0.004 mm³, $P = 0.048$) and SR1800 group (0.142 ± 0.002 mm³, $P = 0.002$) in a dose-dependent manner ($P = 0.031$). Conversely, compared with the control group (75.89 ± 1.82 1/mm), a significant decline in BS/BV was observed in the SR625 group (64.98 ± 1.77 1/mm, $P = 0.005$) and SR1800 group (60.36 ± 1.06 1/mm, $P = 0.001$), although without a dose-dependent effect ($P = 0.123$).

Additionally, compared with the control group (0.041 ± 0.001 mm), Tb.Th was increased in the SR625 group (0.043 ± 0.001 mm, $P = 0.175$) and significantly increased in the SR1800 group (0.047 ± 0.001 mm, $P = 0.011$; Fig. 3D). Furthermore, compared with the control group (2.841 ± 0.218 1/mm), Tb.N was increased in the SR625 group (3.050 ± 0.157 1/mm, $P = 0.154$) and significantly increased in the SR1800 group (3.219 ± 0.027 1/mm, $P = 0.028$; Fig. 3E).

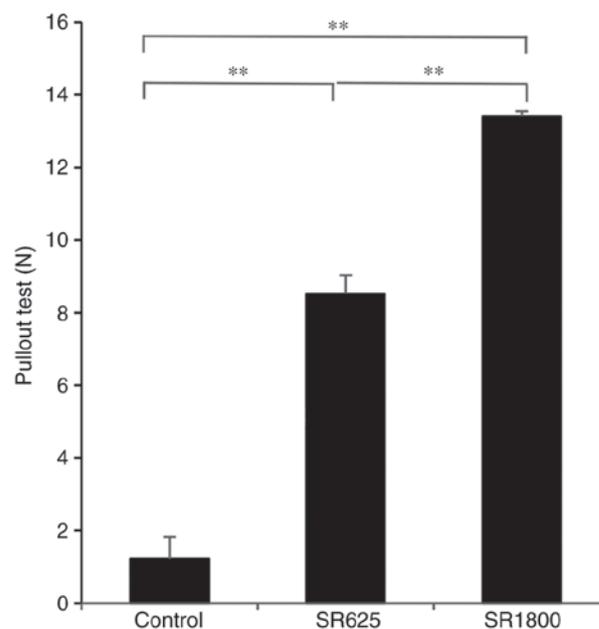


Figure 1. Pulling force required to remove the titanium pin implant in the three groups ** $P < 0.01$. SR, strontium ranelate.

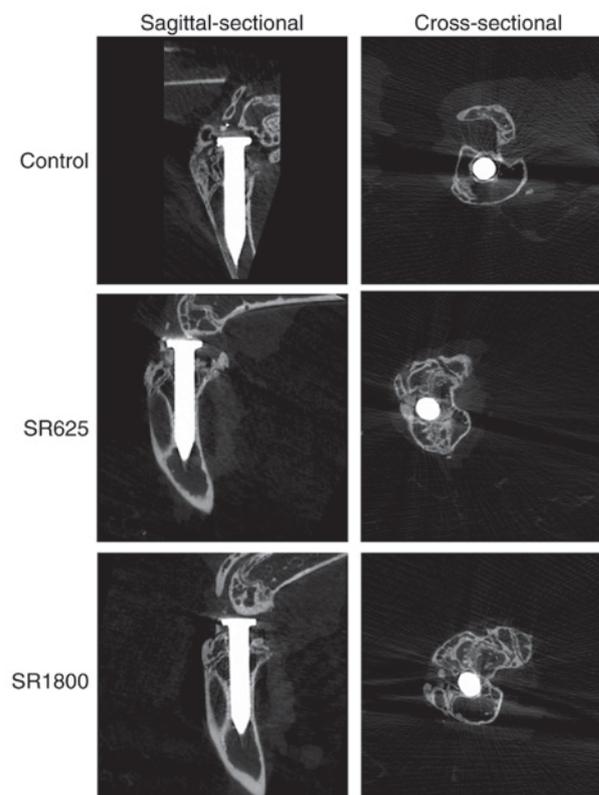


Figure 2. Sagittal-sectional and cross-sectional micro-computed tomography scans of titanium implants. SR, strontium ranelate.

Treatment with SR increases OPG expression and decreases RANKL expression in the periprosthetic tissue. H&E staining indicated areas of bone resorption (Fig. 4). IHC was used to detect the expression of OPG and RANKL in all groups. Fig. 5 illustrates the expression of OPG and RANKL in the bone around prosthesis. Compared with the control group,

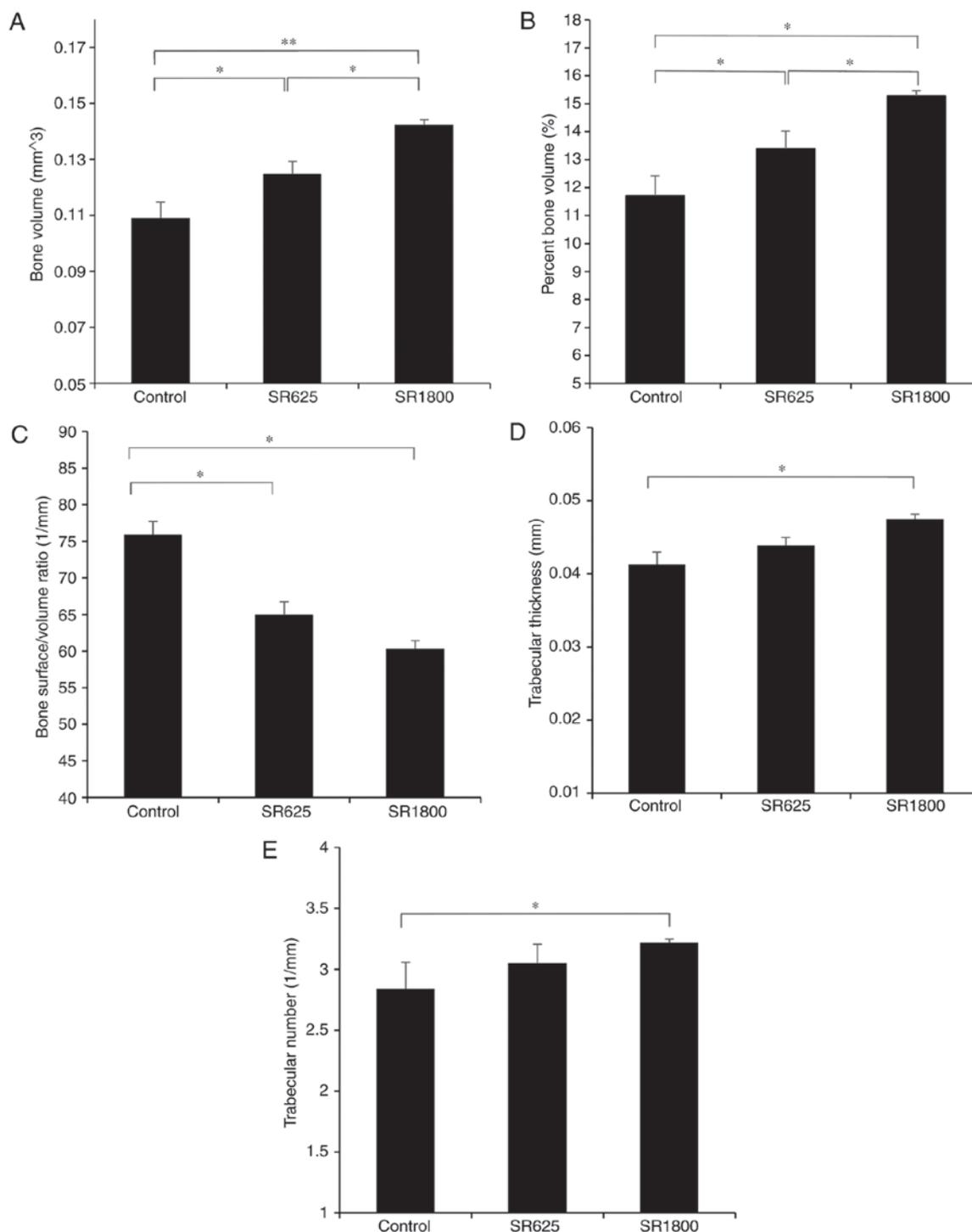


Figure 3. Micro-computed tomography analysis of bone microstructure. (A) Bone volume, (B) percentage bone volume, (C) bone surface density, (D) trabecular thickness and (E) trabecular number were measured. * $P < 0.05$; ** $P < 0.01$. SR, strontium ranelate.

the expression level of OPG was significantly increased in the SR1800 group (0.422 ± 0.010 vs. 0.770 ± 0.012 , respectively; $P < 0.001$; Fig. 6A) and the expression levels of RANKL were significantly decreased (0.723 ± 0.011 vs. 0.221 ± 0.009 , respectively; $P < 0.01$; Fig. 6B). Similarly, compared with the control group, expression levels of OPG were significantly increased in the SR625 group (0.422 ± 0.010 vs. 0.590 ± 0.007 , respectively; $P < 0.01$; Fig. 6A) and levels of RANKL were significantly decreased (0.723 ± 0.011 vs. 0.400 ± 0.018 , respectively; $P < 0.01$; Fig. 6B). In addition, the expression levels of

OPG and RANKL were significantly increased and decreased, respectively, to a greater extent in the SR1800 group compared with the SR625 group ($P < 0.01$).

RT-qPCR analysis of OPG and RANKL in periprosthetic tissues demonstrated that, compared with the control group, the expression of OPG was significantly upregulated and the expression of RANKL was significantly decreased in response to treatment with SR ($P < 0.01$). Additionally, this effect was significantly enhanced in the SR1800 group compared with the SR625 group ($P < 0.05$; Fig. 7).

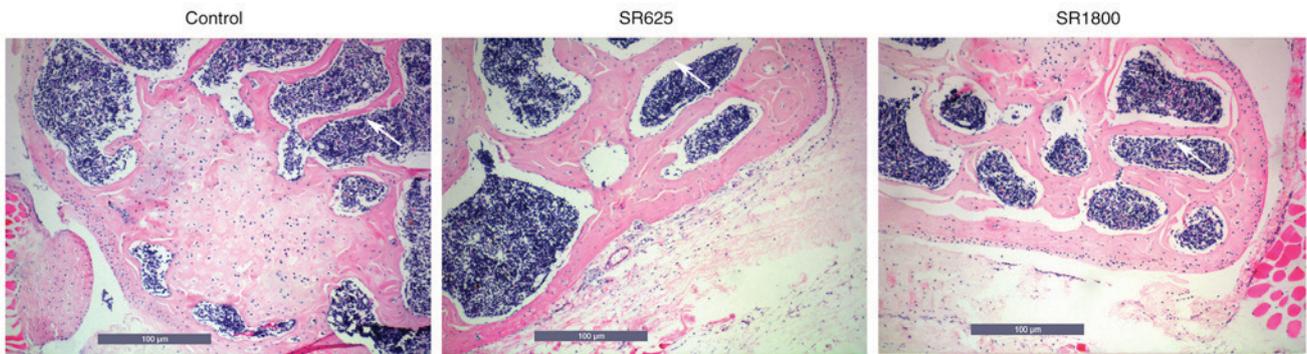


Figure 4. Histological assessment of periprosthetic tissue. Scale bar=100 μ m. SR, strontium ranelate.

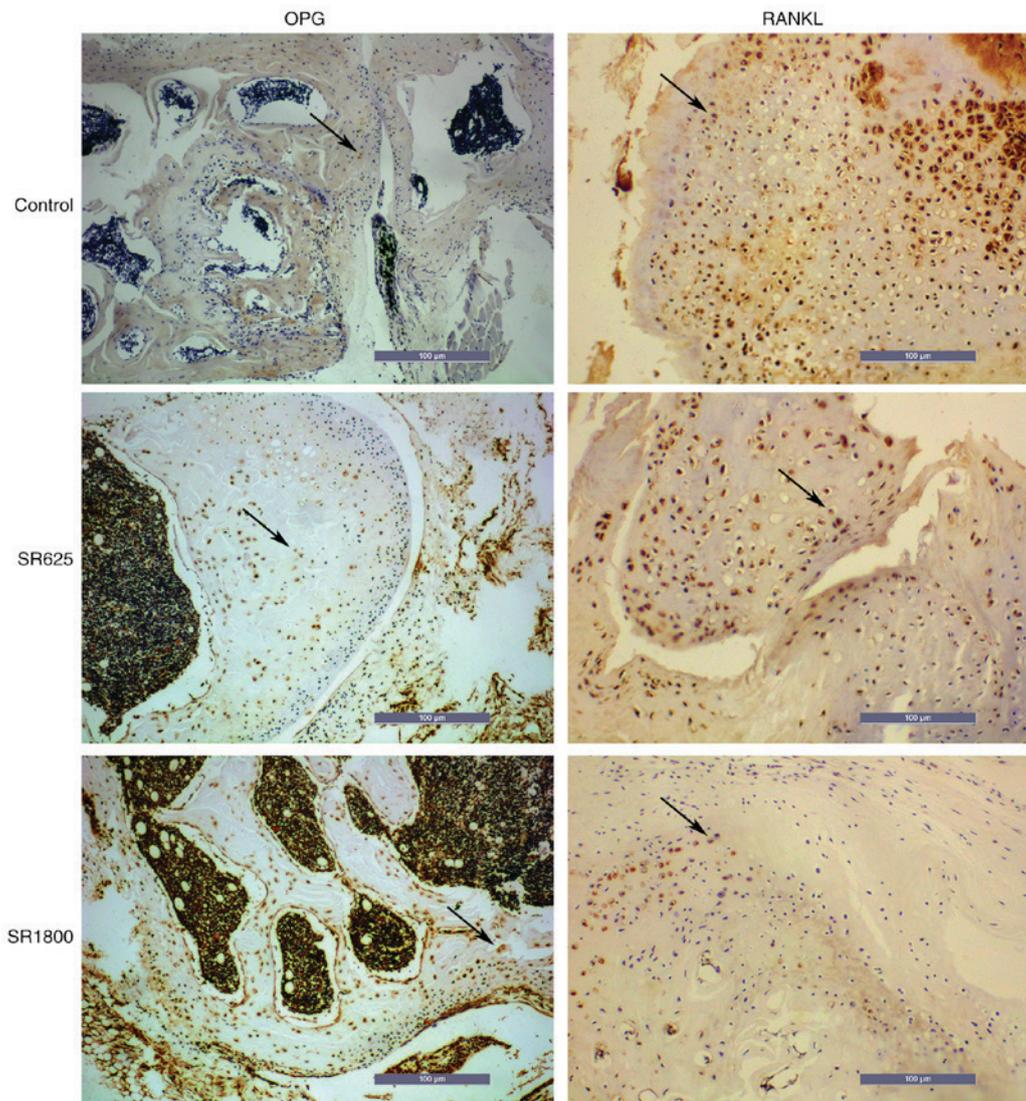


Figure 5. Immunohistochemical staining of OPG and RANKL. Scale bar=100 μ m. OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor- κ B ligand; SR, strontium ranelate.

Discussion

Total knee arthroplasty is an effective and reliable treatment for the terminal stage of knee arthritis. Following surgery, symptoms may effectively be controlled and joint function restored (23-25). Aseptic loosening is one of the

long-term complications of total joint replacement and is an important factor affecting the success rate of joint replacement. The pathogenesis of aseptic loosening is not clear, although previous studies indicated that an imbalance of osteogenesis and osteolysis around the prosthesis is the root cause (5,6).

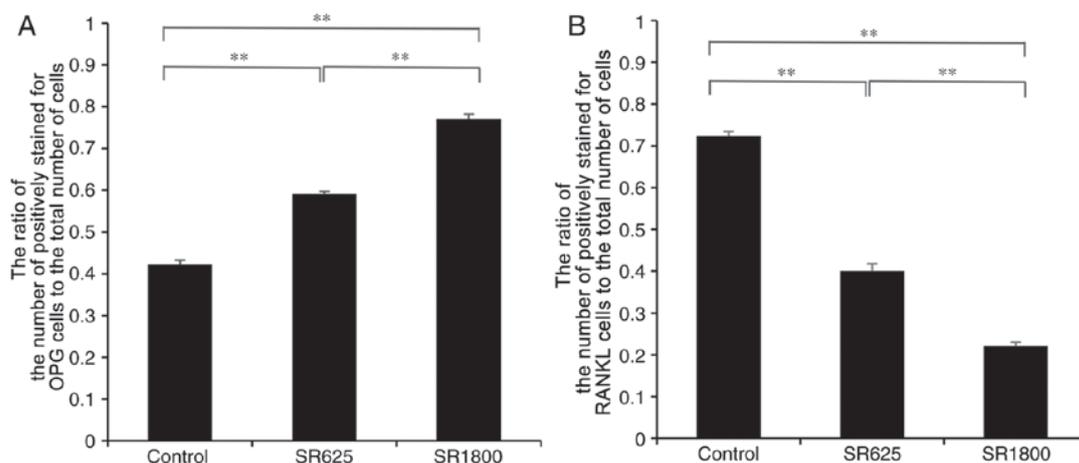


Figure 6. Immunohistochemical staining of OPG and RANKL. Semi-quantification of (A) OPG and (B) RANKL protein expression levels in periprosthetic tissues. The ratios of the number of positively stained for OPG and RANKL cells to the total number of cells were plotted. ** $P < 0.01$. OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor- κ B ligand; SR, strontium ranelate.

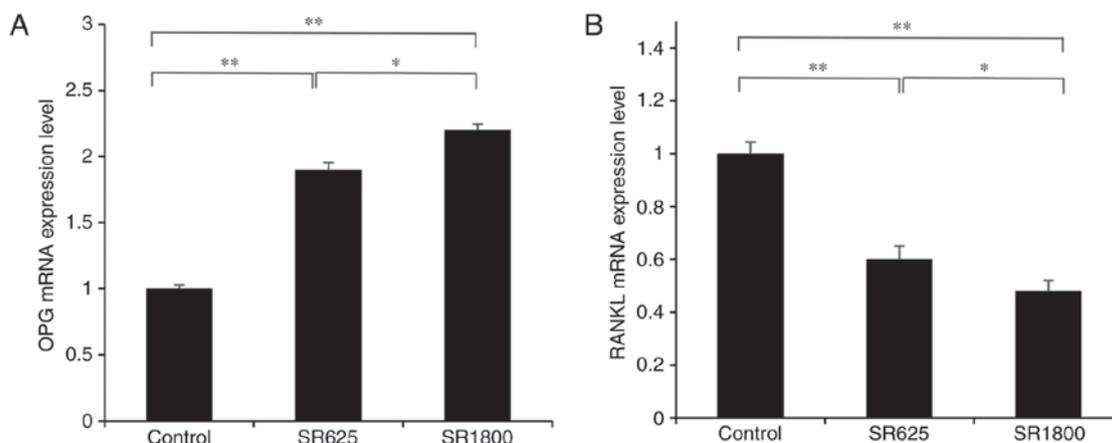


Figure 7. RT-qPCR analysis of OPG and RANKL. Relative mRNA expression levels of (A) OPG and (B) RANKL in periprosthetic tissue, as assessed by RT-qPCR analysis. * $P < 0.05$; ** $P < 0.01$. OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor- κ B ligand; SR, strontium ranelate; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

In the present study, the pulling force to remove the Ti implant from the bone was enhanced following treatment with SR in a dose-dependent manner. This finding supports the idea that SR may be potentially effective against bone resorption. In accordance with the above, Liu *et al* (26) demonstrated that BV and BV/TV were significantly increased following treatment with SR. In another study by Lu *et al* (27), following treatment with SR, Tb.Th, bone density and BV/TV were significantly enhanced compared with the control. However, no significant differences in bone mineral density were noted between the treatment groups and the control. In the present study, BV and BV/TV around the periprosthetic tissue were significantly different between the control group and treatment groups. In addition, BV/TV was observed to be negatively associated with the dose of SR. SR dose did not significantly affect BS/BV in the present study.

An aim of the present study was to determine the effect of SR on Tb.Th and Tb.N in mice with periprosthetic osteolysis. There were no statistically significant differences in Tb.Th or Tb.N between the SR625 group and the control group. However, significant differences in these parameters were observed between the SR1800 group and the control group. These results

indicated that SR was able to increase BV, BS/BV (though not significantly), BV/TV, Tb.N and Tb.Th following aseptic loosening induced by wear particles, suggesting that SR may inhibit the development of aseptic loosening. μ CT and H&E staining indicated that SR significantly reduced bone osteolysis compared with the control group. In this experiment, the bone formation rate was not measured, which is a limitation of the present study and requires investigation in the future.

In agreement with previous studies (3,28), it was demonstrated that SR significantly decreased the level of RANKL and increased the secretion of OPG. The ratio of OPG to RANKL serves an important role in the balance of bone mass and bone metabolism (29-32). The homeostasis between bone formation and resorption is essential for the regulation of bone mass (33-36). Osteoclasts are responsible for dynamic bone resorption, and their differentiation and apoptosis are regulated by the ratio of OPG to RANKL (37,38). The binding of RANKL to RANK may be prevented by OPG, therefore the concentration of OPG and RANKL has an important influence on bone resorption (39,40). The present study demonstrated that OPG and RANKL were significantly upregulated and downregulated,

respectively, in the SR groups compared with the control group, at the mRNA and protein level. These findings support a key role of SR in inhibiting the differentiation of osteoclasts by regulating the ratio of RANKL/OPG in the aseptic loosening model.

It may be noted that previous studies have reported serious side effects with SR, such as Stevens-Johnson syndrome and toxic epidermal necrolysis (41,42) although these were not observed in the present study. Topical application of SR is a promising method (43). Prostheses coated with SR may be able to inhibit aseptic loosening (44,45).

In conclusion, SR inhibited wear particle-associated osteolysis effectively, in a dose-dependent manner. SR additionally downregulated the RANKL/OPG ratio, implying that SR may be a potential therapy for aseptic loosening.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81460333/H0606).

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