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Original Article

Berberine suppresses osteoclastogenesis and osteolysis in a mouse model

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Abstract: Bone dissolution and aseptic loosening caused by wear particles are among the main causes of artificial joint replacement failure. The OPG/RANKL/RANK pathway has been found to play an important role in osteoclast biology in recent years, and the biological effects of wear particles induce imbalances in RANKL/OPG, stimulate osteoclast activity, and lead to the dissolution of bone around prostheses. Therefore, we can prevent bone dissolution induced by wear particles by blocking the activity of the osteoclast activating factor during osteolysis. To study the therapeutic effect and mechanism-of-action of berberine against wear particle-induced osteolysis, we established a model utilizing C57BL/6 mice experiencing polyethylene granule-induced bone dissolution. Specifically, 40 male, eight-week-old C57BL/6 mice were randomly divided into vehicle, sham, low-dose, and high-dose groups. After treatment with different interventions, the mice were sacrificed, and their serum analyzed by enzyme-linked immunosorbent assays, their skull cover bones micro-computed tomography-scanned and TRAP-stained, and their liver and small intestine pathology evaluated. In the mouse model, the inhibition of the signaling pathway of RANKL-induced osteoclast formation by berberine inhibited osteoclast formation and bone destruction in a dose-dependent manner, as well as reduced osteoclast-related receptor (OSCAR) and mouse type I collagen C-terminal peptide (CTX-1) expression. Berberine also promoted the formation of the bone-protecting element OPG, thus improving skull osteolysis induced by polyethylene granules. This study indicates berberine may be a new method of preventing the aseptic loosening of prostheses.

Keywords: Berberine, RANKL, OPG, osteoclast, osteolysis

Introduction

Arthroplasty is the most important 20th century surgical technical innovation [1]. At present, total hip arthroplasty has become the gold standard for treating severe hip trauma and bone disease [2]. Total hip arthroplasty is expected to increase 71%, to 635,000 procedures, by 2030 and primary total knee arthroplasty is expected to increase 85%, to 1.26 million procedures, by 2030 [3]. Total joint arthroplasty is a more and more frequent approach for the treatment of end-stage osteoarthritis in young and active adults; it successfully relieves joint pain and improves function, significantly enhancing the health-related quality of life [4, 5]. The main cause underlying postoperative revisions of prosthetic joint replacements is the aseptic loosening of prostheses [6, 7]. Recent studies have found that the use of prostheses results in the release of a large quantity of wear particles, which can cause macrophage-mediated osteoclast differentiation and the maturation of important associated factors, where osteoclast-induced osteolysis is the main underlying cause of late artificial joint loosening [8]. Therefore, the key avenue of preventing aseptic loosening of artificial joint replacements is restraining the bone resorption effect of local osteoclasts. In addition, wear particles have been experimentally shown to induce osteolysis in various animal models [9, 10], as well as induce an inflammatory response in macrophages [11, 12]. A recent hip arthroplasty retrospective study found that the polyethylene (PE)-associated loosening of prostheses is a result of periprosthetic osteolysis induced by metal and cement wear of particles, which is

the main cause for late joint replacement failure and two corrections [13-15]. When wear particles are released into the joint cavity, osteoclast differentiation and bone resorption increase, while osteoblast differentiation and mineralization are inhibited, causing prosthesis bone remodeling around the imbalance and leading to osteolysis and loosening [16].

The RANKL/RANK/osteoprotegerin (OPG) system is an important recent discovery in the osteoclast field. RANK combined with RANKL can activate signaling in precursor cells and differentiate precursor cells into mature osteoclasts, osteoclasts, and bone marrow stromal cells that secrete OPG and compete with RANK to prevent RANK and RANKL from interacting. RANKL, RANK, and OPG mRNA and protein expression can be detected in the peripheral membrane of the loosing artificial joint [17]. Wang found many tissues containing a large numbers of wear particles expressing RANKL, which may be involved with implant loosening. It has been suggested wear particles are associated with RANKL [18]. RANKL/RANK/OPG system activation is thought to be the cause of periprosthetic osteolysis and implant failure. Research shows periprosthetic osteolysis is due to the excessive activation of osteoclasts, which is initiated by the activation of osteoclast differentiation factors through RANK and RANKL signaling pathways [19, 20]. An important method of treating bone loss is to inhibit RANKL signaling in order to induce osteoclast differentiation [21]. Therefore, a potential means of preventing the loosening of prostheses is by interfering with RANKL/RANK/OPG signaling and preventing activation of these signaling pathways by various means.

Berberine, i.e., berberine hydrochloride, is a natural isoquinoline alkaloid extracted from Chinese *Rhizoma coptidis* with extensive pharmacological properties. Berberine prevents glucocorticoid-induced loss of lumbar vertebrae cancellous bone by promoting bone formation and inhibiting bone absorption. Berberine activates the transcription factor protein Runx2 through the p38 mitogen-activated protein kinase pathway to promote the differentiation of osteoblasts [22, 23], inhibits acid phosphatase (TRAP) activity and the formation of trap-positive multi-nucleus osteoclasts, and reduces the area of bone sink resorption to prevent osteoporosis [24]. Further studies found berberine weakens osteoclast differentiation through RANKL induction of the nuclear factor-κB and nuclear factor of activated T-cells pathways and reduces bone loss, thus preventing aseptic loosening of prostheses [25]. However, to date, the effects of berberine have only been studied in cells with no studies on the effects of berberine in animal models. In the present study, we established an ultra-high-molecular-weight PE particle (UHMWPE)-induced osteolysis skull model in mice and characterized the effect of berberine on the size of the osteoclast population, skull bone resorption by osteoclasts and osteoblasts, and prevention of prosthesis wear particle-induced osteolysis and aseptic loosening.

Material and methods

Preparation of PE particles and reagents

Pure PE granules were purchased from Clariant (Gersthofen, Germany). The mean PE particle size was 1.84 ± 1.50 μm, where more than 32% of particles were less than 1 μm in size [26, 27].

The PE particles were soaked in 75% ethanol for 48 h and then sterilized with standard ethylene oxide [28, 29]. Finally, the PE particles were washed three times in phosphate buffered saline (PBS) and then suspended in sterile PBS at a concentration of 100 mg/ml. Berberine was purchased from the Chengdu Muenster Biotechnology Co. Ltd. (Sichuan, China).

Preparation of reagents and groups of mice

All experiments in this study were approved by the animal ethics committee of the Guangxi Medical University of China. Forty eight-week-old C57BL/6 male mice were provided by the laboratory animal center of Guangxi Medical University (mean weight 17.9 ± 0.7 g, range 17.2-18.6 g) and randomly divided into sham (only PBS), vehicle (PBS and PE particles), low-dose (2.5 mg/kg berberine and PE particles), and high-dose groups (5.0 mg/kg berberine and PE particles). The studies were completed at the Animal Laboratory of Guangxi Medical University. Ethical approval was ratified in accordance with the principles and procedures by the Animal Care Committee of Guangxi Medical University, China [No. 201707021].

Pattern of PE particle-induced bone destruction

The pattern of skull osteolysis due to PE particles in mice was established based on previous research [30, 31]. Mice were given intraperitoneal anesthesia of 2% pentobarbital sodium (0.01 ml/g), then the parietal hair was shorn, a cut was made along the parietal sagittal under aseptic conditions, subcutaneous tissue was separated to reveal the approximately 1 cm × 1 cm calvaria bone, and the periosteum was scratched. The sham group parietal sagittal line was injected with PBS (100 μl) and with PE particles (100 μl, 100 mg/ml) for the other groups and then the skin was sutured. Three days after the operation, berberine was administered every other day, where mice in the sham and vehicle groups were injected with 100 μl PBS. The low-dose group was injected with 2.5 mg/kg berberine and the high-dose group was injected with 5 mg/kg berberine. After 21 days, the mice were sacrificed by cervical dislocation and the skulls removed under aseptic conditions without tearing the seams of the skull. The intracranial brain tissue and skin were removed from the skull. The specimens were stored in 10% formaldehyde and examined by histology (hematoxylin and eosin and TRAP staining) and micro-computed tomography (micro-CT). Blood was collected from the femoral artery for analysis by enzyme-linked immunosorbent assay (ELISA). Livers and small intestines were collected for pathological analyses.

Micro-CT

PE particles were cleared away with 10% formaldehyde 24 hours before the scan to eliminate interference. The calvaria of the mice were scanned using a high-resolution micro-CT system (Skyscan 1076; Skyscan, Kontich, Belgium) with the following parameters: resolution of 18 mm, X-ray energy of 80 kV and 100 mA, and rotation of 180° at a 0.9° angle. Three-dimensional images, which were input in the computer, were generated for each mouse calvaria bone scan. For the rebuilt micro-CT images, a 6 mm × 6 mm region of interest (ROI) with PE particles was chosen for further exhaustive analysis. The ratios of bone mass to tissue volume (BV/TV) and bone mineral density (BMD) of all the specimens were measured and the bone resorption pits were calculated and the amount of porosity calculated using the software Image J (NIH, Bethesda, Maryland, USA).

Histological analysis

For decalcification, the mouse calvaria were stored for one month in 10% EDTA (pH 7.4) at 4°C, embedded in paraffin, and then sliced into 5 μm tissue sections with the center of the cross-section at the midline of skull. High-power microscopy was used to visualize and take photos of samples for quantitative analysis. Using image analysis software (NIH, Bethesda, Maryland, USA), the number of multinucleated osteoclasts positive for TRAP staining was counted and the area of bone erosion measured.

Enzyme-linked immunosorbent assay (ELISA) evaluation

Blood was collected from the celiac artery of the mice. Then, serum was prepared via centrifugation (350 × g for 10 min at 4°C). Using an ELISA kit (Wuhan High-Tech Medicine, Hubei, China) we analyzed the level of RANKL (receptor activator of nuclear factor κB ligand), OSCAR (osteoclast-associated receptor), CTX-1 (cross linked C-telopeptide of type I collagen), and OPG (osteoprotegerin).

Statistical analysis

Values are presented as the means ± standard deviation (SD). One-way analysis of variance (ANOVA) was carried out using SPSS statistical software version 22.0, followed by the Student's T-test. $P < 0.05$ was considered statistically significant.

Results

Berberine suppresses PE particle-induced osteolysis

The evaluation of the mouse skulls was performed using micro-CT and three-dimensional reconstruction. **Figure 1A** displays microsatellite images 2 weeks after bone destruction, which revealed no obvious bone resorption pits on the surface of the sham group skulls. Compared with the sham group, the skull surface of the vehicle group displayed obvious osteolysis with extensive bone resorption pits in the soluble bone region. Compared to the vehicle group, the damaged areas in the drug

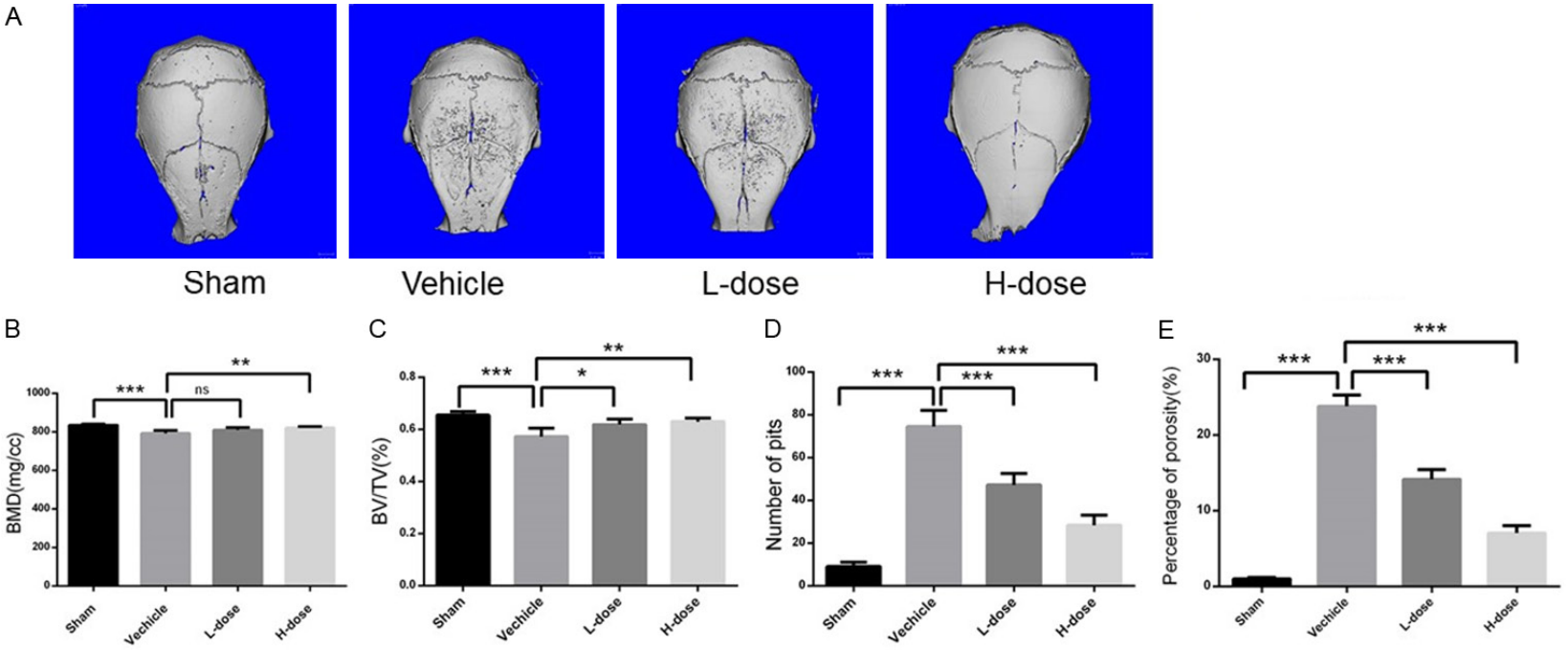

Figure 1. The effect of berberine on polyethylene particle-induced calvarial osteolysis in mice. A. Typical micro-computed tomography scan images of polyethylene (PE)-induced osteolysis in skulls from each group. B. Bone mineral density (BMD). C. The ratio of bone volume to tissue volume (BV/TV). D. Number of bone resorption pits. E. Porosity of specimens in regions of interest (6 mm × 6 mm). N = 6. Outcomes are expressed as the mean ± SD. ns: p value > 0.05, *p value < 0.05, **p value < 0.01, and ***p value < 0.001.

intervention groups were smaller, and the degree and number of the bone absorption pits were significantly reduced. The effect of berberine on bone destruction was most obvious in the high-dose group, where the bone structure was closest to the sham group. Compared with the vehicle group, there was no significant difference in bone mineral density (BMD) in the low-dose group ($P > 0.05$), but the high-dose group was significantly increased ($P < 0.01$), which was closest to the sham group (**Figure 1B**). The ratio of bone volume to tissue volume (BV/TV) values were ordered this way: sham group ($P < 0.001$) > high-dose group ($P < 0.01$) > low-dose group ($P < 0.05$) > vehicle group, indicating berberine increased the bone mass of the skulls in a dose-dependent manner (**Figure 1C**). The amount of bone resorption pits in the vehicle group was more than in the Berberine treatment groups (2.5 and 5 mg/kg/day) (**Figure 1D**). In addition, berberine was found to reduce porosity in skull ROIs ($P < 0.001$) and effectively improve the PE granule-induced bone dissolution of the skull cover bones (**Figure 1E**).

Berberine attenuates the formation of osteoclasts and inhibits PE particle-induced calvarial inflammation

In order to explore whether berberine can inhibit the formation of osteoclasts and alleviate inflammation, we evaluated cranial pathological changes in mice by hematoxylin and eosin (10 ×, **Figure 2A**) and TRAP staining (20 ×, **Figure 2B**). Bone destruction was found to occur in areas in which PE particles had been injected. Inflammatory cells, such as lymphocytes, macrophages, and osteoclasts in particular, aggregated on the surface of the eroded bone. The number of osteoclasts increased after the injection of PE granules. By contrast, the number of osteoclasts and the amount of skull erosion (**Figure 2C**) clearly decreased in the drug intervention groups in an inverse manner ($P < 0.001$ vs. vehicle group). Therefore, berberine was confirmed to prevent the formation of osteoclasts.

The inhibitory effect of berberine on RANKL and OSCAR expression

In order to confirm the main mechanism of berberine inhibition of RANKL-induced osteoclast formation involved RANKL-dominated downstream signaling and feedback reductions in RANKL expression, RANKL and OSCAR expression was quantified using ELISA. We found that the levels of RANKL and OSCAR in the vehicle group were obviously higher than of the levels in the sham group ($P < 0.001$). Furthermore, the expressions of serum RANKL and OSCAR in the animals treated with berberine were observably lower, and their concentrations were lower in the high-dose group ($P < 0.001$) than they were in low-dose group ($P < 0.05$) (**Figure 3A**, **3B**). Therefore, the serum levels of RANKL and OSCAR decreased after treatment with berberine, which is related to osteoclast differentiation.

Berberine inhibits bone dissolution

ELISA was used to measure the concentration of CTX-1 for the purpose of exploring whether berberine is effective in treating the bone damage induced by PE particles and evaluating whether the osteolysis was improved after treatment with berberine. The concentration of CTX-1 was higher in the vehicle group than it was in the other three groups, and the level in the high-dose group ($P < 0.001$) was lower than the level in the low-dose group ($P < 0.01$) (**Figure 3C**). Therefore, berberine significantly reduced the levels of CTX-1 concentrations in a dose-dependent manner. These results indicate that berberine could inhibit bone dissolution.

Berberine promotes OPG expression

The expression of OPG by osteoblasts is protective in bone remodeling. In order to investigate whether berberine affects OPG expression in osteoblasts, ELISA was used to measure OPG. The vehicle group was found to have lower levels than the sham group ($P < 0.001$). The drug intervention group had high levels of OPG compared to the vehicle group, where the high-dose group ($P < 0.01$) was higher than the low-dose group ($P < 0.05$) (**Figure 3D**). In conclusion, berberine regulated the balance between osteoclast and osteoblast differentiation during osteolysis.

Discussion

At present, more than 1 million sets of joint prostheses are implanted every year in China. Due to an aging society and improvement in liv-

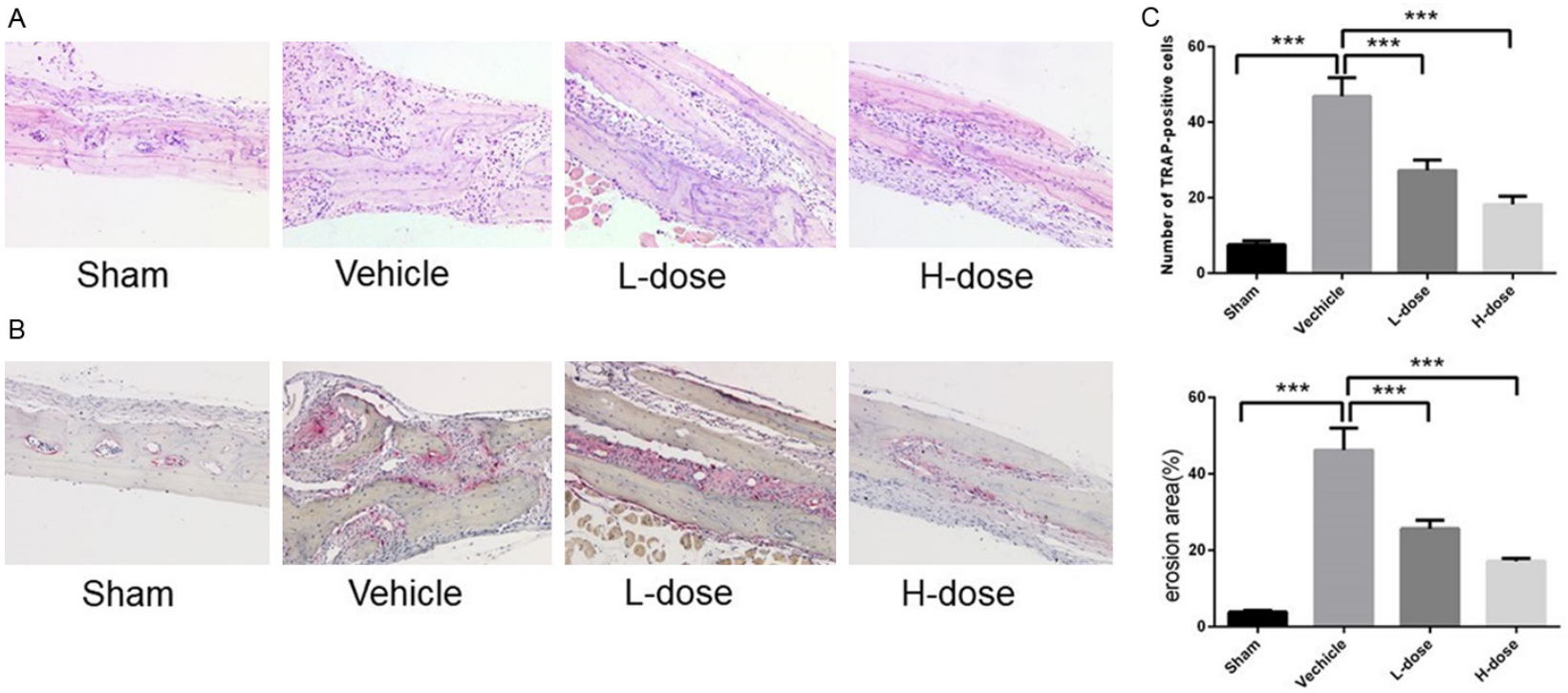

Figure 2. Histological staining of skull cover bone slices from each group. A. Hematoxylin and eosin staining (10 ×). B. TRAP staining (20 ×). C. TRAP-positive cell number/wide-field-vision and area of erosion area in skull cover bone in a fixed-size area (n = 6). The results are expressed as the mean ± SD. **p* value < 0.05, ***p* value < 0.01, and ****p* value < 0.001.

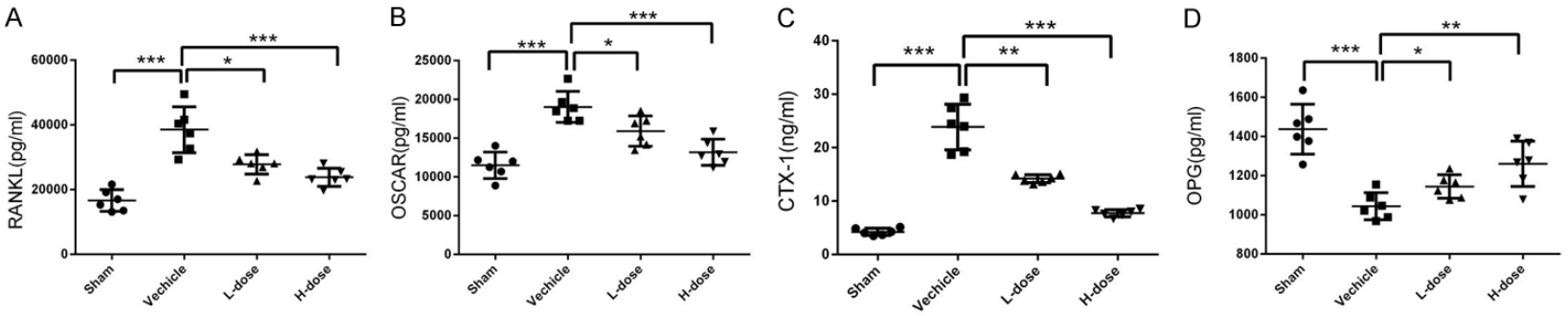

Figure 3. ELISA quantifications of the RANKL, OSCAR, CTX-1, and OPG expression levels. (A) RANKL, (B) OSCAR, (C) CTX-1, and (D) OPG concentrations in a model of PE particle-induced osteolysis (n = 6). Results are expressed as the mean ± SD. **p* value < 0.05, ***p* value < 0.01, and ****p* value < 0.001.

ing standards, social demand for artificial joint replacement is increasing. However, 10 years after the replacement operations, the rate of cumulative overhaul of joint failure caused by aseptic loosening is over 12% [32]. The main cause of joint loosening and osteolysis is biological reactions to abrasive particles at the bone implant interface [33]. Many studies have considered the release of wear particles around the prosthesis, which can be inferred to be a key factor in aseptic loosening [34, 35]. After joint prosthesis replacement, wear particles can stimulate RANKL expression in cells around the prosthesis and inhibit OPG expression, triggering an increase in the RANKL/OPG ratio, the induction of osteoclast differentiation, and an increase in the number of osteoclasts and resulting in osteolysis and aseptic loosening of the prosthesis [36, 37]. Some studies found RANKL, RANK, and OPG are expressed in the pseudomembranous area around the joint replacement. In addition, wear particles were present in RANKL-positive cells.

To date, wear particle-induced cell signaling involved in osteolysis at the molecular level has still not been completely delineated. In vivo and in vitro experiments have revealed a role for cellular signal transduction pathways. The RANK/RANKL/OPG axis is an important pathway in the promotion of osteoclast differentiation from osteoblasts into osteoclasts. The RANK/RANKL/OPG axis regulates osteoblast and osteoclast activity, thereby regulating the balance between osteogenesis and bone absorption in the human body [36].

Von Knoch [38] found exogenous OPG can inhibit osteoclasts, effectively preventing UHMWPE-induced osteolysis in the rat skull dissolution experimental model. This is strong evidence for a role for the RANK/RANKL/OPG system in wear particle-induced osteolysis. Veigl [39] suggested the RANK/RANKL/OPG system has an unshakable position in osteolysis of prostheses and found RANKL was expressed in patients with artificial joint revision and bone dissolution only in tissues containing large amounts of debris. Itonaga [40] showed that pseudomembranous macrophages contain osteoclast precursor cells. These cells are not only phenotypically similar to macrophages, but RANK-expressing pseudomembranous macrophages also produce various bone resorption-stimulating factors in the presence of wear particles, which may be through the effect of RANKL and OPG production stimulation of osteoclast differentiation and activation. Nagai [41] considered differences in the effect of wear particles on osteoclast formation to be mainly due to different levels of RANKL and RANK induced by different wear particles.

In this study, a mouse model of wear particle-induced skull dissolution was established, similar to other studies of titanium particle-induced osteolysis [42, 43]. The effect of berberine on PE particle-induced bone dissolution was evaluated by ELISA, TRAP staining, and micro-CT scans. The number of TRAP-positive cells decreased significantly following berberine treatment, indicating berberine could inhibit the formation of osteoclasts. An ELISA analysis showed RANKL expression by osteoclasts was decreased and OPG expression increased, suggesting berberine may prevent wear particle-induced osteolysis by inhibiting RANKL and promoting OPG expression. Micro-CT revealed an observable decrease in bone absorption pits, especially in the high-dose group. This demonstrates berberine inhibited PE particle-induced bone dissolution in a dose-dependent manner.

In conclusion, we established a model of PE-particle mouse skull dissolution and studied the effect and mechanisms of berberine on bone dissolution at the animal level. PE particles can induce osteolysis, and berberine can reduce osteoclastogenesis and bone dissolution by regulating RANKL in a dose-dependent manner. In view of the improvement in PE granule-induced bone dissolution in mice, berberine is a possible new drug for preventing and treating bone dissolution and the aseptic loosening of prostheses.

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Disclosure of conflict of interest

None.

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