Effects of berberine on differentiation and bone resorption of osteoclasts derived from rat bone marrow cells

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Objective: To observe the effects of berberine on osteoclastic differentiation and bone resorption action in vitro, and to investigate the cellular mechanism of its inhibitory effects on bone resorption.

Methods: The multinucleated osteoclasts (MNCs) were derived by 1,25-dihydroxyvitamin D3 and dexamethasone from bone marrow cells in the coculture system with primary osteoblastic cells. The tartrate-resistant acid phosphatase (TRAP) staining and image analysis of bone resorption pit on dental slices were used to identify osteoclast. The activity of TRAP was measured by p-nitrophenyl sodium phosphate assay. The bone resorption pit area on the bone slices formed by osteoclasts was measured by computer image processing.

Results: At the concentrations of 0.1, 1.0 and 10.0 μmol/L, berberine dose-dependently suppressed the formation of TRAP-positive multinucleated cells, the TRAP activity and the osteoclastic bone resorption. The strongest inhibitory effect was exhibited at the concentration of 10.0 μmol/L, with the inhibiting rate of 60.45%, 42.12% and 72.69% respectively.

Conclusion: Berberine can decrease bone loss through inhibition of osteoclast formation, differentiation and bone resorption.

Keywords: berberine; osteoporosis; osteoclasts; bone resorption

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小檗碱对大鼠骨髓源性破骨细胞的分化及骨吸收功能的影响

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目的：研究小檗碱对破骨细胞分化及骨吸收功能的影响，探讨小檗碱抑制骨吸收的细胞学基础。

方法：采用原代培养的成骨细胞和骨髓单核细胞联合培养的方法，在1,25-(OH)2维生素D3和地塞米松作用下，使骨髓单核细胞分化形成破骨细胞。通过相差显微镜观察细胞形态，通过抗核蛋白酸性磷酸酶（tartrate-resistant acid phosphatase, TRAP）染色和观察骨片上骨吸收陷窝的形成鉴定破骨细胞。磷酸苯二钠法测定破骨细胞抗核蛋白酸性磷酸酶的活性，计算机图像分析技术测定骨片上破骨性骨吸收陷窝的面积。

结果：小檗碱在0.1～10 μmol/L范围内，浓度依序性地抑制TRAP阳性多核破骨细胞的形成和TRAP活性，减少破骨性骨吸收陷窝的面积；在10 μmol/L浓度下，对破骨细胞的抑制作用最强，对TRAP阳性多核破骨细胞的形成和TRAP活性的抑制率分别达到了60.45%和42.12%，骨吸收陷窝面积减少72.69%。

结论：小檗碱通过抑制破骨细胞的形成、分化和骨吸收功能减少骨质的丢失。

关键词：小檗碱；骨质疏松；破骨细胞；骨吸收

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Osteoporosis is a systemic skeletal disease characterized by low bone mineral density (BMD) and microarchitectural deterioration of bone tissue, leading to a consequent increase in bone fragility and fracture risk. Osteoporosis and concurrent bone fractures have been increasing remarkably along with the increase of life expectancy. It has ranked the seventh among all the common diseases, and become a major health hazard afflicting more than 200 million people worldwide\(^{[2]}\). At present, drugs used in clinical treatments of osteoporosis usually include estrogen, bisphosphonates and calcium. However, these drugs can produce undesirable side effects on some patients. Bisphosphonates can cause indisposition of gastrointestinal tract. Calcium is not easily absorbed. Estrogen can lead to a higher incidence of cardiovascular diseases, endometrial cancer and breast carcinoma, and has been abolished to treat osteoporosis in USA\(^{[2]}\). Thus, it is necessary to develop new substances with less undesirable side effects that can substitute or reduce the need for drugs used currently.

Berberine, an isoquinoline alkaloid that exists in a number of medicinal plants, such as Coptis chinensis (Huanglian), Phellodendron amurense (Huangbai), and so on, has been reported to possess various pharmacological actions including antibacterial, antinfection, anticancer, and apoptosis-induction\(^{[1-7]}\). In some traditional Chinese herbal formulas used for the treatment of osteoporosis, such as Er-Xian Decoction and Tsu-kan-gan, berberine has been verified to be the major active constituent to suppress bone resorption\(^{[8-10]}\). Our previous study demonstrated that berberine could increase BMD and decrease bone loss in ovariectomized (OVX) rats\(^{[11]}\). Berberine isolated from tsu-kan-gan could decrease the bone loss of OVX rats and inhibit formation of osteoclast induced by parathyroid hormone (PTH) and interleukin (IL)-1\(^\alpha\)\(^{[8,9,12]}\). Further studies investigated that berberine inhibited osteoclast formation and survival through suppression of NF-κB and Akt activation, while having no cytotoxic effects on bone marrow macrophages or osteoblastic cells\(^{[12]}\). However, the cytological mechanism for these inhibitory effects of berberine on bone resorption remains unknown.

In bone microenvironment, osteoblast and osteoclast are involved in bone remodeling. In the process of bone remodeling, osteoblast deals with bone formation, and osteoclast deals with bone resorption. These two kinds of cells maintain a well-coordinated balance\(^{[12]}\). The excess of osteoclastic bone resorption over osteoblastic bone formation results in the osteoporosis and other bone metabolic diseases\(^{[14,15]}\). In order to verify the inhibitory effects of berberine on bone resorption and understand the cytological mechanism, we investigated its influences on the formation and differentiation of osteoclasts derived from rat bone marrow cells, as well as the osteoclastic pit area formed on bone slices. This study will provide basis for extending the clinical application of berberine.

1 Materials and methods

1.1 Materials

1.1.1 Materials and reagents  Sprague-Dawley rats (3 to 4 days old, weighed 7 to 8 g) were purchased from the Experimental Animal Center of the Second Military Medical University [animal license number is SCXK (Hu) 2007-0003]. Berberine was isolated from Cortex Phellodendri Amurenensis, and identified by spectrum analysis. Data of proton nuclear magnetic resonance spectroscopy (\(^{1}H\) NMR) and mass spectrometry (MS) were coincidence with previous studies\(^{[16]}\). The purity of berberine is up to 99% according to high performance liquid chromatography (HPLC) analysis.

Trypsin, collagenase II, fetal calf serum (FCS) and α-modified minimum essential medium (α-MEM) were purchased from Gibco (USA). 1, 25-dihydroxyvitamin D3, dexamethasone, naphthol AS-BI phosphate and pararosaniline were purchased from Sigma (USA). Triton X-100 and toluidine blue were obtained from Shanghai Sangon Biological Engineering Technology & Service Co., Ltd. Dimethyl sulfoxide (DMSO), disodium  p-nitrophenyl-phosphate, 4-nitrophenol, diolamine, ethylene glycol methyl ether and potassium sodium tartrate were domestic analytical reagent grade.

1.1.2 Apparatus  The purification workbench (SW-CJ-2FD) was purchased from Suzhou Purification Equipment Co., Ltd (China). CO\(_2\) incubator (MCO-15AC) was from SANYO Electric Co., Ltd (Japan). Inverted microscope (CKX41) was from Olympus Corporation (Japan). ELx 800 absorbance microplate reader was purchased from Bio-Tek Instruments (USA). 96-well culture plates were purchased from Costar (USA).

1.1.3 Preparation and treatment of cover slips and bone slices  Cover slips were cut into 0.5 cm \(\times\) 0.5 cm slices, and soaked in chronic acid mixture overnight, rinsed thoroughly with tap water and distilled water, then dried in oven, sterilized before use.

Ivory bones were cut into 40 \(\mu\)m thick bone slices, and trimmed into 0.5 cm \(\times\) 0.5 cm, and preserved in 75% ethanol at 4 °C. The bone slices were ultrasonically treated for 30 min, thoroughly rinsed with distilled water, soaked in 75% ethanol for 24 h and sterilized by ultraviolet.

1.2 Methods

1.2.1 Isolation and culture of primary rat osteoblasts  Primary osteoblastic cells were prepared according to Liu et al\(^{[17]}\). Osteoblastic cells were isolated from the calvarias of 3 to 4 days new born rats. Five calvarias were collected and digested by using a solution of phosphate-buffered saline (PBS) containing 0.25% trypsin at 37 °C for 30 min. The digestive solution was discarded, and the rat calvarias were rinsed with D-Hank’s solution for three
times and subjected to a solution of PBS containing 0.05% trypsin and 1 g/L collagenase at 37 °C for 1 h. The cells of the second digestion were collected and then cultured in α-MEM containing 10% FCS. These cells had typical properties of osteoblast such as alkaline phosphatase activity. All cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. The cells were passaged and used at the 4th passage for experiments.

1.2 Induction of osteoclasts from rat bone marrow Briefly, 5 SD rats which were 3 to 4 d old were sacrificed by cervical dislocation. After 5-minute sterilization with 75% alcohol, the femurs were disarticulated, with the ends removed, and the bone marrow cells were flushed out by using a 1-mL syringe and washed in PBS buffer twice. Single cell suspension was prepared by repeated pipetting with a 5-mL pipette (10^-5 M). Primary osteoclastic cells of passage 4 (1 × 10^4/mL) and bone marrow cells (1 × 10^6/mL) were cocultured in inducing medium (α-MEM medium with 10% FCS, 1 × 10^{-8} mol/L 1, 25-(OH)₂-VD₃, and 1 × 10^{-7} mol/L dexamethasone) at 37 °C in a humidified atmosphere of 5% CO₂ in 96-well plates (100 µL per well). After 8 days, the bone marrow cells differentiated into mature multinucleated osteoclasts (MNCs).

1.2.3 Determination of biological activity

1.2.3.1 Identification and counting of positive-tartrate-resistant acid phosphatase MNCs Primary osteoblasts (1 × 10^5 cells/mL) and bone marrow cells (1 × 10^6 cells/mL) were placed on 96-well plates containing cover glasses and cultured in α-MEM at 37 °C in a humidified atmosphere of 5% CO₂ for 24 h. Then the cells were treated with berberine of 0.1, 1, 10 µmol/L, while blank wells and negative controls were still treated with α-MEM for 10 days. Then, osteoclasts were identified by tartrate-resistant acid phosphatase (TRAP) staining. The cells were fixed for 30 min with 2.5% glutaraldehyde in PBS at 4 °C, washed thrice with distilled water, and then incubated in TRAP staining solution for 60 min at 37 °C (0.2 mol/L acetic acid buffer 18 mL, pH 5.0, 62 mmol/L azaparosanilime 1 mL, naphthol AS-BI phosphatase 1 mL, potassium sodium tartrate 282.22 mg). The glass cover slips were washed in distilled water for three times, dehydrated with gradient ethanol, and sealed with glycerol-gelatin. Cells possessing three or more nuclei were counted as multinucleated osteoclasts under microscope examination.

1.2.3.2 Assay for osteoclast TRAP activity Primary osteoblasts (1 × 10^5 cells/mL) and bone marrow cells (1 × 10^6 cells/mL) in α-MEM were seeded into 96-well culture plates and cultured for 8 days until mature. Then the cells were treated with berberine at concentrations of 0.1, 1, 10 µmol/L for 48 h, respectively. Meanwhile, blank wells and negative controls were treated with α-MEM. After the cells were washed twice with PBS buffer, 20 μL of 0.1% Triton X-100 was added to lyse the cells at room temperature for 15 min, and 100 μL of reaction solution (0.4 g sodium p-nitrophenolphosphate and 2.0 g potassium tartrate, dissolved in 200 mL of deionized water, adjusting pH to 3.5 with 1 mol/L HCl) was added and the cells were incubated at 37 °C for 30 min. To stop the reaction, 100 μL of 1 mol/L NaOH was added to each well. The ultraviolet absorbance was measured at 405 nm. At the same time, the positive cells for TRAP were counted. The activity of TRAP was expressed as moles p-nitrophenol per minute per 100 osteoclasts.

1.2.3.3 Determination of bone resorption pit

100 µL suspension of primary osteoblasts (1 × 10^5 cells/mL) and bone marrow cells (1 × 10^6 cells/mL) in α-MEM were seeded into 96-well culture plates and cultured for 24 h with a sterilized dental slice in each well. The cells were treated with berberine at concentrations of 0.1, 1, 10 µmol/L for 12 days. Meanwhile, blank wells and negative controls were treated with α-MEM. At the end of incubation, dental slices were fixed in 2.5% glutaraldehyde, treated in 0.25 mol/L NH₄OH to remove adherent cells, and then stained with 1% toluidine blue solution for 10 min at room temperature. After that, the bone slices were sealed in neutral gum. Resorption pits on bone slice were observed and numbered in 20 random fields of vision under a microscope. The images of pit area were collected and quantified with image analysis software (Leica Q550IW, Germany).

1.3 Statistical analysis All experiments were repeated at least three times. Results were evaluated for statistical significance on a minimum of 15 replicates. All data were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was performed using the SPSS 13.0 software. The significance level was 0.05.

2 Results

2.1 Identification of osteoclast Primary osteoblasts and bone marrow cells were adhered in normal α-MEM within 24 h. Osteoblasts were in close contact with each other to form a compact layer at the bottom of the plate, while the bone marrow cells were much smaller. As time went on, the bone marrow cells became bigger round cells with prominance and pseudopodia. After 8-day culture, bone marrow cells differentiated into mature osteoclasts characterized with numerous nucleus and vacuole in cytoplasm. There existed more osteoclasts near the mineralized bone nodules.

After primary osteoblasts and bone marrow cells were cultured in α-MEM for 8 d, bone marrow cells were differentiated into mature osteoclasts. The cytochemical staining for TRAP showed that some round and bigger osteoclasts were visible under a inverted phase contrast microscope, and some red
sediment was found in their cytoplasm but not in nucleus, while the osteoblast-like cells were not stained (Figure 1).

2.2 Effects of berberine on osteoclast differentiation in morphology. After being continuously treated with berberine at 0.1, 1 and 10 μmol/L for 8 days, the osteoclasts derived from bone marrow cells were not clear in shape and morphology; the edge of membrane was shrunk, and nucleus, vacuoles, prominence and pseudopodia were not distinct; some cells were degraded into fragments and in the status of apoptosis. Berberine at the concentration of 10 μmol/L significantly inhibited maturation and differentiation of osteoclasts, and induced the apoptosis of osteoclasts (Figure 3).

![Figure 1: Osteoclasts stained by TRAP (Inverted phase contrast microscopy, ×400)](image1)

After the primary osteoblasts and marrow cells were co-cultured for 12 d on the bone slices in the presence of 1,25-(OH)2-VD3 and dexamethone, numerous resorptive pits appeared on the surface of bone slices. After being stained with toluidine blue, the resorption pits could be identified easily by their blue color. The resorption pits were roundish and elliptical, with irregular shape; their margins were rodent, and bottoms were rough under microscope. The untreated dental slices possess a very homogeneous surface (Figure 2).

![Figure 2: Bone resorption pits formed by osteoclasts (Inverted phase contrast microscopy, ×1000)](image2)

A$_1$: Control; B$_1$: Dental slices cocultured with osteoclasts treated with 10 μmol/L berberine.

2.3 Effects of berberine on TRAP-positive multinucleated osteoclast formation. As shown in Figure 4, after being treated with berberine at concentration of 0.1, 1 and 10 μmol/L for 10 d, the numbers of TRAP-positive multinucleated osteoclasts were significantly reduced compared to the negative control. These results indicated that berberine could inhibit the formation and differentiation of osteoclasts induced by 1,25-(OH)2-VD3 and dexamethone. At concentration of 10 μmol/L, the inhibitory effect of berberine was stronger with its inhibitory rate up to (60.45±2.67) %.

2.4 Effects of berberine on osteoclastic TRAP activity. After the osteoclasts were treated with berberine for 48 h, the TRAP activity was dose-dependently suppressed at concentrations of 0.1, 1 and 10 μmol/L, and respectively decreased by (20.20±4.14) %, (29.1±4.95) % and (42.12±2.73) % as compared to the negative control (Figure 5).
2.5 Effects of berberine on the formation of osteoclastic bone resorption pit As shown in Figure 6, the bone marrow cells were induced to differentiate into osteoclasts by 1,25-(OH)\textsubscript{2}-VD\textsubscript{3} and dexamethasone in the coculture system with osteoblasts; the mature osteoclasts resorbed the bone matrix and formed bone resorption pits on ivory bone dental slices. After osteoclasts were treated with berberine at 0.1, 1 and 10 \(\mu\text{mol/L}\) for 12 d, the bone resorption pits formed on the surface of bone slices were (47.55 \pm 5.62) %, (40.40 \pm 4.74) % and (27.31 \pm 4.91) % respectively as compared to the negative control. These results indicated that berberine performed a drastic and significant dose-dependent decrease in bone resorption pit at the concentrations of 0.1, 1 and 10 \(\mu\text{mol/L}\).

3 Discussion

Osteoclasts are MNCs that mediate bone resorption in bone remodeling. Some investigations suggested that in physical conditions, osteoblasts and osteoclasts are mutual dependent, and osteoclasts are involved in osteoclast formation and differentiation by producing some local factors to promote mature and differentiation of osteoclasts\textsuperscript{10}. Therefore, it is more significant to study the effects of drug on osteoclasts derived from bone marrow cells by coculture with osteoblasts.

Bone resorption is the process during which lyso-somal enzyme in osteoclasts produces a local acid microenvironment, and various acid hydrolase lyses the inorganic salt and organic substances of bone. These lysates are released into the extracellular fluid, and resorption pits are formed on the bone surface. In the process of coculture of osteoclasts with bone slices, osteoclasts broke down bone and released the minerals to form resorption pit on bone slices. Berberine decreased the area of osteoclastic bone resorption pit on bone slices, and at the concentration of 10 \(\mu\text{mol/L}\), the inhibitory rate reached 72.69%. These results indicated that berberine possesses significant inhibitory effects on bone resorption. Osteoclastic bone resorption is mediated by the formation of new osteoclasts and the resorption activity of osteoclasts. In our experiment, berberine inhibited the formation and differentiation of osteoclasts derived from bone marrow cells by 1,25-(OH)\textsubscript{2}-VD\textsubscript{3} and dexamethasone with 60.45% inhibitory rate. The activity of TRAP is directly related with osteoclastic bone resorption. Berberine significantly decreased the TRAP activity of mature osteoclasts induced for 8 days. These results indicated that berberine inhibited bone resorption and decreased bone loss by dual effects — decreasing the number of osteoclasts and inhibiting the TRAP activity of osteoclasts.

Previous animal experiment found that berberine could increase the bone density and trabecular thickness, and decrease the number of osteoclasts in ovariectomized rats, and these inhibitory effects on bone loss were mainly related with bone resorption activity of osteoclasts\textsuperscript{9,10}. Our results further verified the inhibitory effects of berberine on osteoclasts, and supported the Hu et al\textsuperscript{12} that berberine inhibited formation and differentiation of osteoclast induced by RANKL. In our experimental system, osteoclasts were derived in coculture of osteoblasts and bone marrow cells, and pre-experimental results showed that berberine did not promote or inhibit proliferation and ALP activity of osteoclasts in calvaria culture of new born rats. These results indicated that berberine did not inhibit osteoclast formation and differentiation through producing cytotoxic effects on osteoclasts.

Berberine has various pharmacological effects including antimicrobial activity against a variety of
organism such as bacteria, viruses, fungi, helminthes and chlamydia. Berberine has been reported to block the induction of edema on mouse ear by 12-O-tetradecanoylphorbol-13-acetate (TPA)\(^{(39)}\) and inhibit cyclooxygenase-2 (COX-2) transcriptional activity through the regulation of activator protein 1 (AP-1) transcription factor. Pretreatment of berberine inhibited the production of exudates and prostaglandin E\(_2\) (PGE\(_2\)) in carrageenan induced air pouch of Wistar rats\(^{[50]}\). COX-2 and PGE\(_2\), a major eicosanoid product of the COX-2-catalyzed pathway, play key roles in normal bone tissue remodeling. COX-2 and PGE\(_2\) stimulated osteoclastogenesis through inhibition of osteoprotegerin (OPG) secretion, stimulation of RANKL production by osteoblasts, and up-regulation of RANK expression in osteoclasts\(^{[2]}\). COX-2 has been demonstrated to be a critical regulator of mesenchymal cell differentiation into osteoblasts and an essential element in bone repair\(^{[52]}\). It has also been reported that COX-2 and PGE\(_2\) play important roles in osteoclast formation \emph{in vitro}\(^{[39]}\), and are required for debris-induced osteoclastogenesis and osteolysis in an \emph{in vitro} mouse calvaria model\(^{[34]}\). These findings demonstrated that the inhibitory effects on bone resorption of berberine are related with inhibitory effects on the production of COX-2 and PGE\(_2\).

\textit{Rhizoma Coptidis} and \textit{Cortex Phellodendri Amurensis} contain plenty of berberine and similar chemical constituents. Although it has not been reported that these crude herbs are used individually to treat osteoporosis in clinical practice, it was recorded in ancient book \textit{Yao Pin Hua Yi} that \textit{Cortex Phellodendri Amurensis} prepared with wine, and being taken together with Siwutang, had the action of strengthening bone, and could treat pains of the lions and knees, and weakness of lower limbs. Our previous study also found that \textit{Cortex Phellodendri Amurensis} could decrease bone loss in ovariec- tomized rats, and had the action on prevention and treatment of osteoporosis\(^{[7]}\). However, the clinical application of \textit{Cortex Phellodendri Amurensis} in prevention and treatment of osteoporosis need further studies.

Natural medicines, which are suitable for long time use with fewer side effects, have become the focus of antiosteoporotic medicine. In the present, most antiosteoporotic constituents from natural medicines are phytoestrogens, such as isoflavones, lignans, coumarins, and so on. Berberine is not in the category of phytoestrogen-like substance, and cannot produce stimulant action on the uterus and mammary glands of postmenopausal women, so the treatment with berberine can avoid the adverse effects of estrogen-like substance. Berberine, which is widely distributed in more than 100 kinds of plants from 14 genuses of 7 families, has abundant plant resources. Berberine has been clinically applied to treat intestinal infection for a long time, and has not been found clear adverse effects. Therefore, berberine can be investigated and developed into a new clinical antosteoporotic potential.

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