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Original Article

Berberine inhibits the growth of osteosarcoma through modulating MMP/NM-23 and MAPK/JNK signal pathways

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Abstract: Objective: To investigate the effects and mechanisms of berberine (BBR) on the migration, invasion, proliferation and apoptosis of osteosarcoma cells in vitro. Methods: Proliferation of MG-63 and U2OS cells was measured by the CCK-8 assay. Cells migration was examined by wound-healing assay. The invasion and metastasis of cells were evaluated by transwell invasion assay. Cells apoptosis was determined by the flow cytometry. Caspase-3 activity in MG-63 and U2OS cells was measured, and Western blot was used to measure the levels of Bax, Bcl-2, MMP-2 and MMP-9 in cells. In addition, the osteosarcoma graft tumor model of mice was established. The tumorigenesis of MG-63 cells in nude mice was compared among three groups. Immunohistochemistry assay was used to measure the levels of MMP-2, MMP-9 and NM-23 in tumor tissue. Results: It was showed that BBR inhibited the proliferation of MG-63 and U2OS cells in vitro in time- and concentration-dependent manners. Moreover, BBR reduced the cells migration and invasion, also down-regulated the expressions of MMP-2 and MMP-9. BBR also inhibited the cells apoptosis by down-regulating the expression of Bcl-2 and up-regulating the expression of Bax. In nude mice, BBR obviously inhibited the tumorigenesis of MG-63 cells. Compared with the negative group, BBR decreased the levels of MMP-2 and MMP-9 and increased the level of NM-23. The molecular mechanism was associated with activation of the MAPK/JNK signal transduction pathway. Conclusions: BBR significantly regulates the biological behaviors of osteosarcoma cells and inhibits the growth of osteosarcoma. The molecular mechanism may be associated with the modulation of MMP/NM-23 and MAPK/JNK signals. BBR may be a potential drug for the treatment of osteosarcoma.

Keywords: Berberine (BBR), osteosarcoma, BALB/C-nu-nu mice, cell biological behavior, mechanism

Introduction

Osteosarcoma is a primary malignant tumor of the skeleton characterized by direct formation of immature bone or osteoid tissue by tumor cells. This tumor has a slight predilection for males, with a reported male-to-female ratio of about 1.5:1 to 2:1 [1]. In the long bones, osteosarcoma usually originate in the metaphysis, it is most common in the distal of femur and the proximal of tibia [2]. Micro-metastases exist in 80-90% of patients at onset, so osteosarcoma is a systemic disease with micro-metastasis [3]. Therapy options for osteosarcoma are comprehensive treatments based on the surgery. Before the advent of chemotherapy, the prognosis of osteosarcoma was poor, with 5-year disease-free survival rate ranging between 15% and 20% after surgical treatment [4]. Chemotherapy has improved the survival rate range to 75% to 80%. The likelihood of local recurrence is 5% to 7%, which is related to surgical resection margins and the responsiveness to chemotherapy [5].

Therapeutic research of osteosarcoma focuses on screening sensitive treatment methods and drugs [6-8]. Other treatment options or drugs should be considered in patients with drug-resistance or poor response to chemotherapy

drugs. Another research focus is to find new treatment targets or new anticancer drugs in order to improve the response of tissue to chemotherapeutic drugs, enhance the effect of chemotherapy, reduce the toxicity of chemotherapy drugs and reduce the side effects of chemotherapy [9, 10].

Berberine (BBR), known as huangliansu, is a common isoquinoline alkaloids, with molecular formula of $C_{20}H_{18}NO_4Cl$. It exists in many plants in the genus of Berberidaceae. Many studies have reported that BBR hydrochloride could inhibit the activities of Shigella dysenteriae, Streptococcus pneumoniae, Staphylococcus aureus, Streptococcus, Salmonella typhi and amoeba, and BBR has been widely used in treatment of intestinal infections and dysentery [11]. In recent years, some studies found that the BBR was cytotoxic and could inhibit the activities of liver cancer cells, leukemia cells and other malignant cells by inhibiting protein synthesis in cancer cells, blocking cell cycle and promoting apoptosis [12]. It was reported that BBR has a synergistic effect with hydrochloric acid cytarabine in vitro [13]. Another study also reported that BBR could induce the G1 arrest and apoptosis depended on the p53 gene in U2OS cells [14]. However, the data regarding the effect of BBR on the growth of osteosarcoma are still scarce. There are few reports on the role of BBR in the development of osteosarcoma.

Therefore, in this study, we focus on the effects of BBR on the proliferation, apoptosis, invasion and metastasis of osteosarcoma cells and its possible molecular mechanisms. We hope to explore if BBR would become the potential drug in the treatment of osteosarcoma and provide experimental evidence for the clinical treatment of osteosarcoma.

Materials and methods

Cell culture

Human osteosarcoma cell lines U2OS were purchased from the Wuhan Boster Biological Technology CO. LTD (Wuhan, China). Osteosarcoma MG-63 cell lines were supplied by Laboratory of Orthopaedic Surgery, Tongji Hospital Affiliated to Tongji Medical College, Huazhong University of Science and Technology. U2OS and MG-63 were cultured in DMEM high glucose medium and McCoy's 5α modified medium (Sigma, St. Louis, MO, USA). All media contained 10% FBS (Sigma, St. Louis, MO, USA), 100 μg/mL penicillin (Sigma, St. Louis, MO, USA) and 100 μg/mL streptomycin (Sigma, St. Louis, MO, USA). All cells were cultured in a humidified incubator of 5% CO_2 at 37°C. The medium was changed once every two days. Following treatment, the cells were harvested by trypsinization.

CCK-8 assay

Proliferation of cells was determined by the CCK-8 assay. MG-63 and U2OS cells were seeded in each well of 96-well plates. The cells were randomly divided into a control group, a vehicle group and a BBR group, with 5 duplicates in each group. After overnight incubation, the cells were treated with BBR (10 μg/mL, 50 μg/mL, 100 μg/mL) (Tianping Pharmaceutical Co., Shanghai, China) for 12, 24 and 48 h. Following BBR treatment, the medium was removed, and serum-free medium (100 μL) and CCK-8 solution (10 μL) (Dojindo, Kumamoto, Japan) were then added to each well and incubated for 2 h. The optical density was determined at 570 nm wavelength to determine whether the effect of BBR on the proliferation of MG-63 and U2OS cells is time-/concentration-dependent. Experiments in each group were repeated 3 times.

Wound-healing assay

Migration is one of the essential steps in the process of tumor cell metastasis. Cells were seeded in 6-well plates, and if the cells were confluence, wound-healing assay was performed with a 1000-μL sterile micropipette tip to scratch the cell monolayer. Then, the cells were randomly divided into Control group, DMSO group, 10 μg/mL BBR group, 50 μg/mL BBR group, and 100 μg/mL BBR group with 3 duplicates in each group, and the experiment was repeated 3 times. After incubated for 24 h, the plates were washed thrice with phosphate-buffered saline (PBS) and added with fresh serum-free medium at indicated time. Images of 5 randomly selected fields of the scratch area with or without treatment were photographed and measured at indicated times

under an inverted microscope. The cell migration capability was calculated based on the percentage (scratch width at indicated time/original scratch width).

Transwell invasion assay

The MG-63 and U2OS cells treated with DMSO, 10 μg/mL BBR, 50 μg/mL BBR, 100 μg/mL BBR alone for 24 h were resuspended to adjust the cell density to 3×10^5/mL. 100 μL suspension were seeded into the upper chamber precoated with 50 mg/L Matrigel™ (30 μL, 1:9) without FBS medium in triplicate, while the conditioned medium (500 μL) with 10% FBS was added to the lower chamber served as a chemo-attractant. Then, it was incubated for 24 h. Cells migrating to the lower surface of the membrane were fixed with 4% paraformaldehyde for 10 min, and stained with 0.1% crystal violet for 5 min. After washing, photomicrographs were taken in 8 randomly selected fields by microscopy (× 200). Finally, the number of cells were calculated.

Flow cytometry

Cells were treated with different concentrations of BBR and/or SB203580 (10 μmol/L) or JNK inhibitor SP600125 (10 μmol/L) for 24 h. The cells were washed twice with cold PBS and resuspended in 500 μL binding buffer at a concentration of 1×10^6 cells/mL. Then, 5 μL Annexin V-FITC solution and 5 μL PI (1 mg/mL) were added. The cells were incubated at 37°C for 30 min and analyzed by flow cytometry within 1 h. The number of apoptotic cells were counted and presented as a percentage of the total cell count.

Caspase-3 activity

MG-63 and U2OS cells were seeded in each well of 96-well plates. The cells were randomly divided into control group, vehicle group and BBR group, with 5 duplicates in each group. After overnight incubation, the cells were treated with BBR (10, 50, 100 μg/mL) for 24 h. Caspase-3 activity of MG-63 and U2OS cells was measured in accordance with the instructions of caspase-3 activity kit. The experiment was repeated 3 times.

Western-blotting assay

Total proteins from cells of different groups were separated by 12% SDS-PAGE, and the separated proteins were then electro-transferred onto nitrocellulose membranes using a transblot system (Bio-Rad, Hercules, CA, USA). The membranes were first blocked with 5% non-fat milk for 2 h at room temperature, followed by incubating with indicated primary Polyclonal rabbit antibody to Bax (Cell Signaling Technology, Beverly, MA, USA), Bcl-2 (Cell Signaling Technology, Beverly, MA, USA), MMP-2 (Cell Signaling Technology, Beverly, MA, USA), MMP-9 (Cell Signaling Technology, Beverly, MA, USA) and β-actin (Cell Signaling Technology, Beverly, MA, USA). Anti-rabbit antibody conjugated to HRP (Cell Signaling Technology, Beverly, MA, USA) was used as a secondary antibody. β-Actin was used as an intrinsic quality control. The bands were incubated in ECL-Plus reagent, and the chemiluminescence was measured on BioMax MR Film. The density of the bands was quantified using a Labworks image acquisition and analysis software (UVP, USA).

Animal experiments

Male BALB/c-nu/nu nude mice (4 weeks old, 22±4 g) were purchased from Shanghai Slac Laboratory Animal Co. Ltd. The mice were housed in laminar flow cabinets under specific pathogen free condition with food and water ad libitum in Huazhong University of Science and Technology, Tongji Medical College Experimental Animals Center (SPF grade). All the experiments were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and the Guidelines for Care and Use of Experimental Animals of Chinese Academy of Medical Sciences (Approval No. 2020-106). MG-63 cell line xenografts were established by subcutaneous injection (SC) of 1×10^7 cells at the dorsum of nude mice, and the treatment began when the tumors reached an average volume of 200-300 mm^3. Mice were randomized into 3 groups with 10 in each group and administrated: (a) Vehicle (Sterile saline, i.g.); (b) Doxorubicin (1 μg/g.d, i.p.); (c) BBR (50 μg/g.d, i.g.). The mice were checked daily for toxicity/mortality relevant to treatment, and the tumor volume was measured with a caliper 3

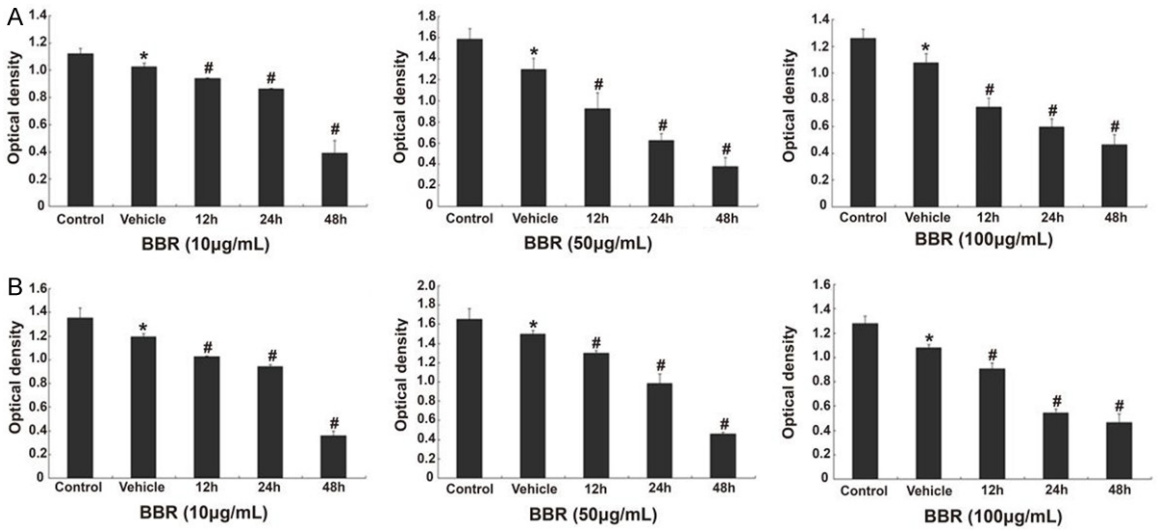


Figure 1. Berberine (BBR) inhibited the proliferation of cells in a time-dependent manner. A. The effects of BBR on MG-63 cell proliferation at different time points; B. The effects of BBR on U2OS cell proliferation at different time points. *P<0.05 compared with Control, #P<0.05 compared with Vehicle.

times a week, up to endpoint days. At the end of the treatment, mice were sacrificed through 150 mg/kg of 2% pentobarbital injection, and the tumor were peeled off and weighted. The growth curve of subcutaneous transplanted tumor was plotted with time as the horizontal axis and tumor volume as the vertical axis to evaluate the effect of BBR on the proliferation of MG-63 cells in *vivo*. To evaluate the anticancer activity of BBR, percentage of inhibition was calculated as following: V (cm^3) = L × W^2/2, where L represents the long axis length (cm) of the tumor, and W represents the short axis length (cm) of the tumor. Moreover, each tumor was partially cut and fixed with 4% paraformaldehyde, and the effect of BBR on the expressions of relevant proteins in tumor cells was analyzed by immunohistochemistry assays, which were conducted according to the immunohistochemistry kits (Abcam Company, USA).

Statistical analysis

All statistical data in this study were analyzed by SPSS 22.0 software (IBM company, Chicago, USA). The measurement data were presented as mean ± standard deviation (SD). The independent sample t-test was exploited for the comparison between two groups. One-way ANOVA with post hoc comparison following Bonferroni was performed among multiple groups, and one-way repeated measures ANOVA was conducted for data among different time points in one group. Statistical significance was accepted at the level of P<0.05.

Results

BBR inhibited proliferation in time- and dose-dependent manners

As seen in **Figure 1**, compared with control group, the proliferation ability of MG-63 cells and U2OS cells were significantly reduced in the vehicle group (all P<0.05). Compared with the vehicle group, the proliferation ability of MG-63 cells and U2OS cells treated with different concentration of BBR for 12 h were obviously reduced (all P<0.05). It was indicated that BBR induced a time-dependent decrease in the viability of MG63 and U2OS cells, and significant differences were observed (P<0.05). As shown in **Figure 2**, BBR induced a dose-dependent decrease in the viability of MG63 and U2OS cells, and statistical differences were found (P<0.05).

BBR-treatment decreased cell motility

The results of wound healing assay showed that there was a statistical difference in the migration rate between the control group and the vehicle group (P<0.05). MG-63 cells and U2OS cells treated by different concentrations

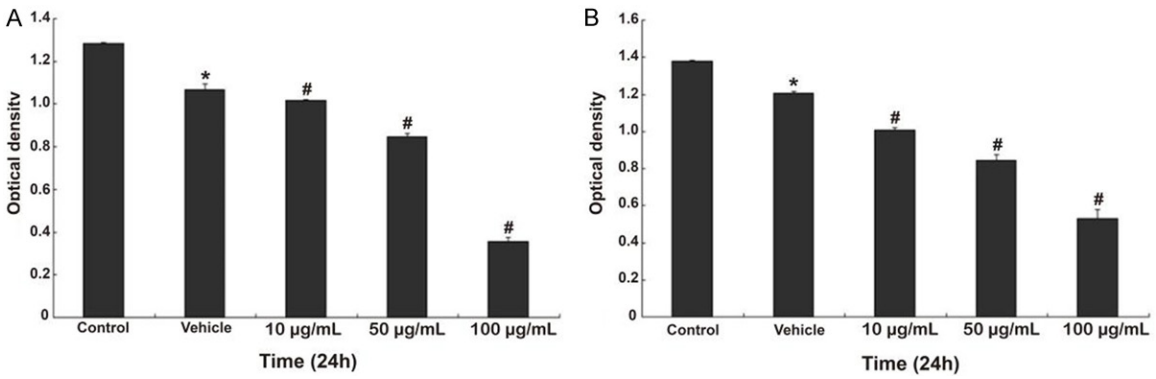


Figure 2. Berberine (BBR) inhibited the proliferation of cells in a dose-dependent manner. A. The effects of various concentrations of BBR on MG-63 cell proliferation; B. The effects of different concentrations of BBR on U2OS cell proliferation. **P<0.05* compared with Control, *#P<0.05* compared with Vehicle.

of BBR had a lower migration rate than the control group or vehicle group, as shown in **Figure 3** (*P<0.05*). Most importantly, BBR inhibited the cell migration in a dose-dependent manner. In addition, transwell assay results showed that compared with that in the control group or vehicle group, the invasion ability of MG-63 cells and U2OS cells treated with different concentrations of BBR was significantly reduced, and there were significantly differences for the numbers of migrated cells among cells treated with 10 μg/ml, 50 μg/ml, 100 μg/ml of BBR (all P<0.05). It is demonstrated that the effect of BBR on invasion ability of MG-63 cells and U2OS cells in *vitro* was performed in a dose-dependent manner.

BBR induced the apoptosis of MG-63 and U2OS cells

As shown in **Figure 4**, the results of flow cytometry showed that a dose-dependent increase was observed in the apoptotic rate of MG-63 and U2OS cells treated by BBR (P<0.05). Compared with that of the control group or vehicle group, the proportion of apoptotic cells in 10 μg/mL BBR group, 50 μg/mL BBR group and 100 μg/mL BBR group increased significantly (all P<0.05), indicating that BBR induced the apoptosis of MG-63 and U2OS cells, and the proportion of apoptotic cells increased significantly with the increase of BBR concentration.

As shown in **Figure 5**, compared with that of the control group, the proportion of apoptotic cells in the vehicle group was significantly increased (P<0.05), suggesting that DMSO induced the apoptosis of MG-63 and U2OS cells, further verifying the toxic effect of DMSO on MG-63 and U2OS cells. Compared with the 100 μg/mL BBR group, the 100 μg/mL BBR+SB203580 group and 100 μg/mL BBR+SP600125 group showed significantly lower apoptosis rate (all P<0.05), suggesting that p38 MAPK and JNK inhibitors significantly inhibited the apoptosis of MG-63 and U2OS cells induced by BBR. These results indicate that BBR induces the apoptosis of MG-63 and U2OS cells through p38 MAPK and JNK signal transduction pathways.

Effect of BBR on caspase-3 activity of MG-63 and U2OS cells

Caspase-3 activity of MG-63 and U2OS cells was measured at 8 h, 12 h and 24 h, respectively, after intervention with 100 μg/mL BBR. The results are shown in **Figure 6**. Compared with that in the control group or vehicle group, the intracellular caspase-3 activity increased in cells treated with 100 μg/mL BBR for 8 h. The activity continued to increase after 12 h, but it was decreased after 24 h. These results suggest that 100 μg/mL BBR can cause changes in caspase-3 activity in MG-63 and U2OS cells with the change form of “low-high-low” through time.

Effect of BBR on MMP-2 and MMP-9 expression in MG-63 and U2OS cells

As shown in **Figure 7**, Western-blotting analysis showed that BBR effectively restrained the expressions and activities of MMP-2 and

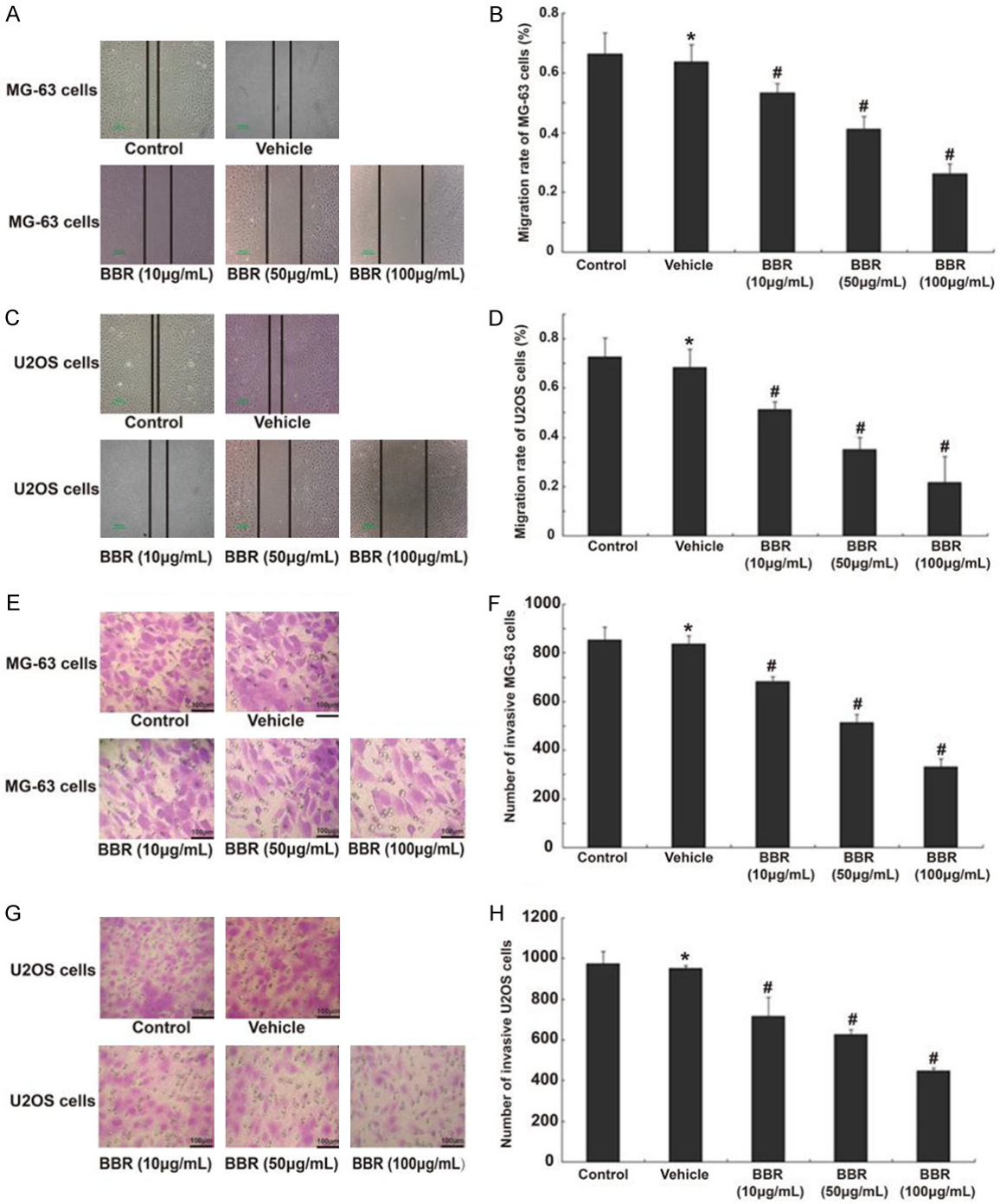


Figure 3. Effects of berberine (BBR) on migration and invasion ability of MG-63 and U2OS cells. A. Results of wound-healing assay of MG-63 cells. B. Migration rate of MG-63 cells. C. Results of wound-healing assay of U2OS cells. D. Migration rate of U2OS cells. E. Results of Transwell invasion experiment of MG-63 cells. F. The number of invasive MG-63 cells. G. Results of Transwell invasion experiment of U2OS cells. H. The number of invasive U2OS cells. *P<0.05 compared with Control. #*P*<0.05 compared with Vehicle.

MMP-9 in MG-63 and U2OS cells compared with the control group or vehicle group (all P<0.05). Among MG-63 cells and U2OS cells treated with the different concentration of BBR, there were significant differences in the expression levels of MMP-2 and MMP-9. These

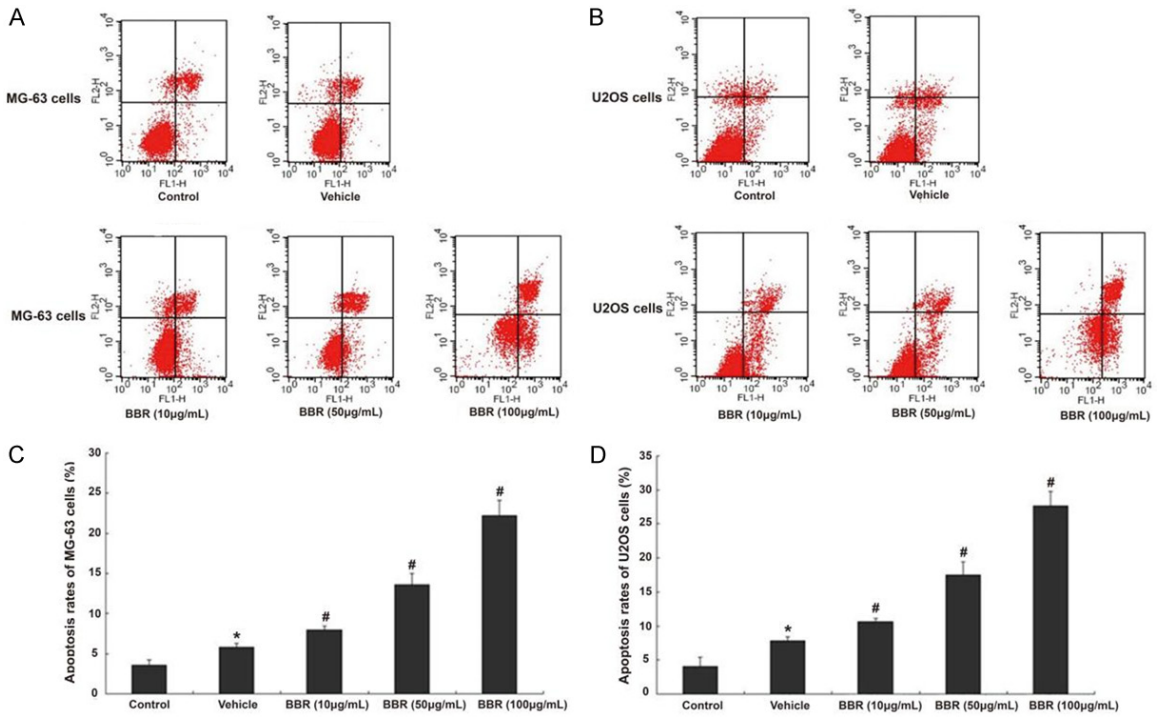


Figure 4. Effects of berberine (BBR) on apoptosis of MG-63 and U2OS cells. A. The flow cytometry results of MG-63 cells. B. The flow cytometry results of U2OS cells. C. Quantitative analysis of apoptosis rate in MG-63 cells. D. Quantitative analysis of apoptosis rate in U2OS cells. *P<0.05 compared with Control. #P<0.05 compared with Vehicle.

results indicate that BBR reduce the invasion ability of MG-63 and U2OS cells by down-regulating the expressions of MMP-2 and MMP-9.

Effects of BBR on the expressions of Bcl-2 and Bax in MG-63 and U2OS cells

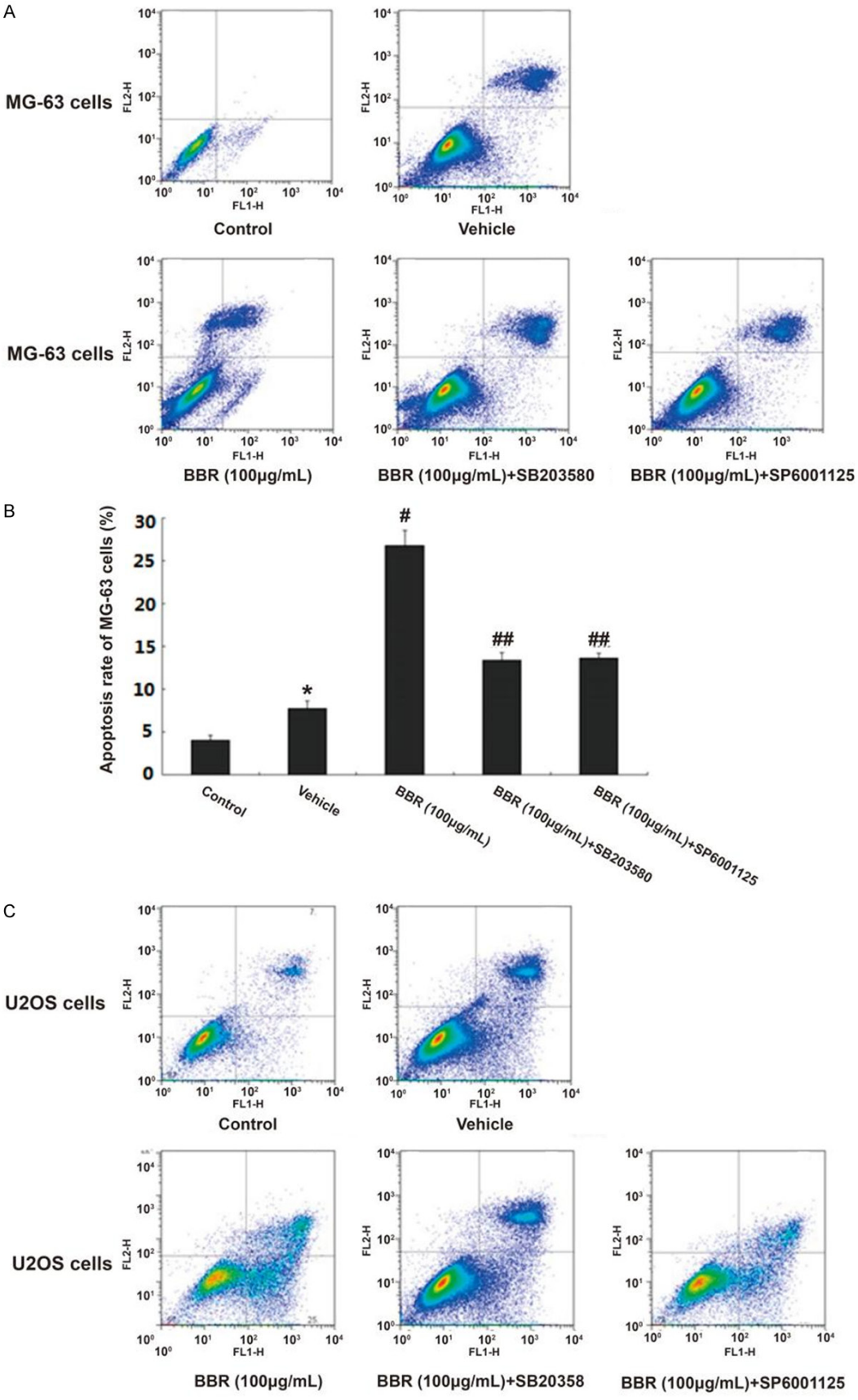
As shown in **Figure 8**, Western blot results showed that after 24-h stimulation, the expression levels of Bcl-2 in 10 μg/mL BBR group, 50 μg/mL BBR group and 100 μg/mL BBR group were significantly decreased (all P<0.05), while the expression levels of Bax in the BBR groups were significantly increased (all P<0.05), as compared with those in the vehicle group. It is suggested that BBR can induce apoptosis of MG-63 and U2OS cells by down-regulating the expression of Bcl-2 and up-regulating the expression of Bax.

Effect of BBR on tumorigenesis of MG-63 cells in nude mice

The results of tumor volume and the nude weight of transplanted tumor in various groups at different time points are shown in **Figure 9**. Compared with the negative control group, the volume and naked weight of nude mice in the positive control group were significantly different (all *P<0.05*), suggesting that intraperitoneal injection of Adriamycin (1 μg/g.d) significantly affected the growth of transplanted tumor in nude mice. Compared with the negative control group, there were significant differences in the volume and naked weight of nude mice in the BBR group (all *P<0.05*), suggesting that gavage of BBR (50 μg/g.d) significantly affected the growth and proliferation of transplanted tumor in nude mice. Compared with the positive control group, there was no significant difference in the volume and naked weight of nude mice in the BBR group (*P>0.05*), suggesting that the peritoneal injection of BBR (50 μg/g.d) was less effective than intraperitoneal injection of Adriamycin (1 μg/g.d) in the term of the growth of transplanted tumor in nude mice.

Effect of BBR on the expression of MMP-2, MMP-9 and NM-23

As shown in **Figure 10**, the expression levels of MMP-2 and MMP-9 were statistically different in positive control group or BBR group as com-


A
MG-63 cells
FL2-H
FL1-H
Control
Vehicle
MG-63 cells
BBR (100μg/mL)
BBR (100μg/mL)+SB203580
BBR (100μg/mL)+SP6001125
B
Apoptosis rate of MG-63 cells (%)
30
25
20
15
10
5
0
#
##
##
*
Control
Vehicle
BBR (100μg/mL)
BBR (100μg/mL)+SB203580
BBR (100μg/mL)+SP6001125
C
U2OS cells
Control
Vehicle
U2OS cells
BBR (100μg/mL)
BBR (100μg/mL)+SB20358
BBR (100μg/mL)+SP6001125

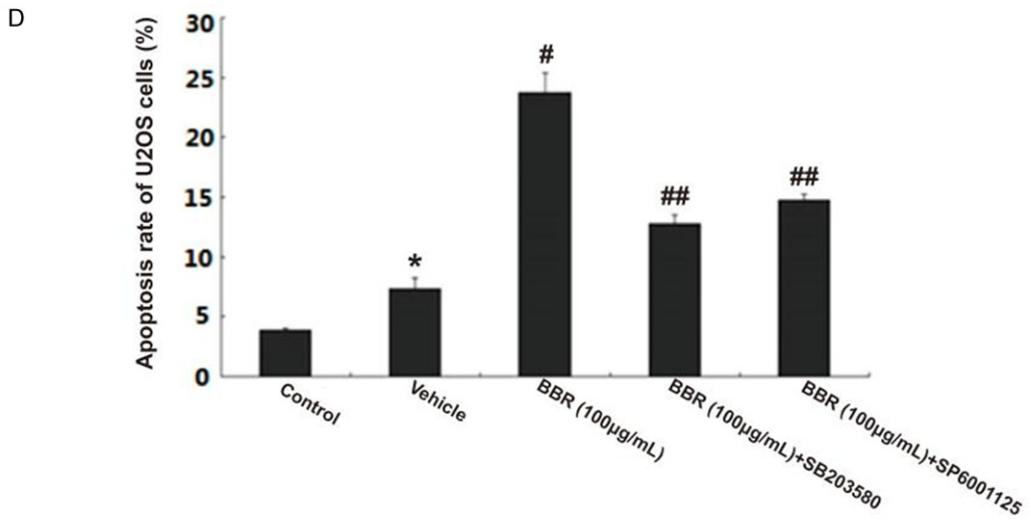


Figure 5. Berberine (BBR) activates apoptosis of MG-63 and U2OS cells through p38MAPK and JNK signaling pathways. A. P38 MAPK and JNK inhibitors inhibited BBR induced apoptosis of MG-63 cells indicated by the flow cytometry assay. B. Histogram of the quantitative analysis of MG-63 cells apoptosis. C. P38 MAPK and JNK inhibitors inhibited BBR induced apoptosis of U2OS cells indicated by the flow cytometry assay. D. Histogram of the quantitative analysis of U2OS cells apoptosis. *P<0.05 compared with Control. #P<0.05 compared with Vehicle. ##P<0.05 compared with 100 μg/mL BBR.

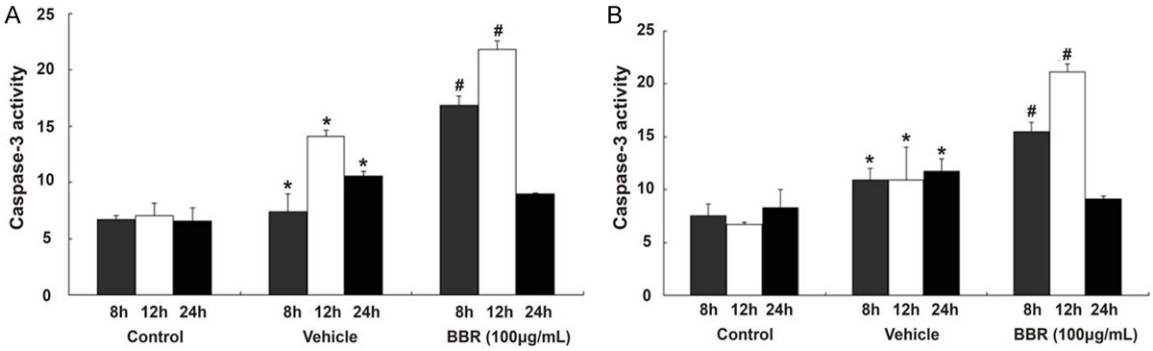


Figure 6. Effect of berberine (BBR) on caspase-3 activity of MG-63 and U2OS cells. A. Effect of BBR on caspase-3 activity in MG-63 cells. B. Effect of BBR on caspase-3 activity in U2OS cells. **P*<0.05 compared with Control. *#P*<0.05 compared with Vehicle.

pared to those in the negative control group (P<0.05), suggesting that the positive control agent Adriamycin and BBR hydrochloride affected the expression levels of MMP-2 and MMP-9. Compared with the positive control agent Adriamycin, BBR hydrochloride showed no statistical difference in the expression of MMP-2 (P>0.05), while there was a statistical difference in the expression of MMP-9 between the positive control and BBR groups (P<0.05). Compared with negative control group, the expression level of NM-23 was statistically different in the positive group or BBR group (P<0.05), suggesting that the positive contrast agent Adriamycin and BBR hydrochloride could influence the expression of NM-23. There was no statistical difference in the level of NM-23 between the control group and BBR group (P>0.05). The above results indicate that BBR can reduce the expression levels of MMP-2 and MMP-9 and increase the expression of NM-23.

Discussion

BBR is known to have a wide range of biological and pharmacological activities, such as

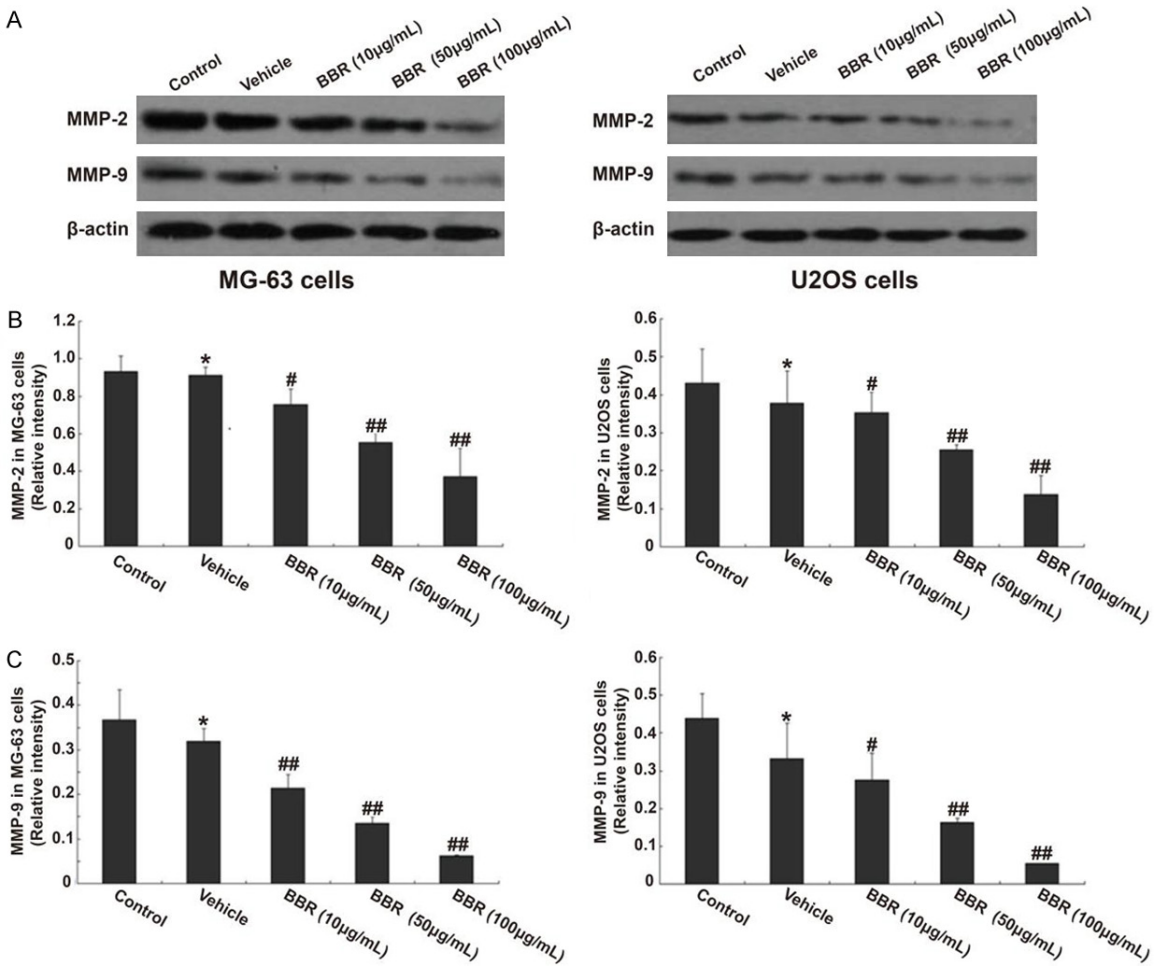


Figure 7. Effects of berberine (BBR) on MMP-2 and MMP-9 expressions in MG-63 and U2OS cells. A. Western blot results of MMP-2 and MMP-9 in MG-63 and U2OS cells. B. The results of gray scale scanning of MMP-2 expression in MG-63 and U2OS cells. C. The results of gray scale scanning of MMP-9 expression in MG-63 and U2OS cells. *P<0.05 compared with Control; #P<0.05 compared with Vehicle. ##P<0.05 compared with Vehicle.

anti-diarrhea, anti-arrhythmia and anti-inflammation [15, 16], and is therefore widely used in the treatment of systemic diseases including cardiovascular, endocrine and gastrointestinal diseases [17]. BBR hydrochloride also possesses anti-tumor activity [18, 19] and has shown the ability to inhibit human colon cancer cell growth by inhibiting COX-2 transcription activity, aromatic amine N-acetyltransferase activity and DNA topoisomerase I and II [20-24]. In this study, we mainly discussed whether BBR hydrochloride has an effect on the proliferation, apoptosis, migration and invasion of osteosarcoma cell lines MG-63 and U2OS. Our results showed that BBR inhibited the proliferation of MG-63 and U2OS cells in time- and concentration-dependent manners in vitro. BBR could also reduce the migration and invasion capacity of MG-63 and U2OS cells. The possible molecular mechanism could be that BBR downregulates the expressions of MMP-2 and MMP-9. In in vivo experiments, it was found that the xenograft tumor was completely located in the capsule, without obvious invasion to surround tissues, lung metastasis and other distant metastases. Immunohistochemistry in the biopsy revealed that the expressions of MMP-2 and MMP-9 were significantly decreased. All the above results confirmed that BBR effectively reduced the migration and invasion ability of osteosarcoma cells. Meanwhile, it was found that BBR could successfully induce the apoptosis of MG-63 and U2OS cells and lead to changes in caspase-3 activity of MG-63 and U2OS

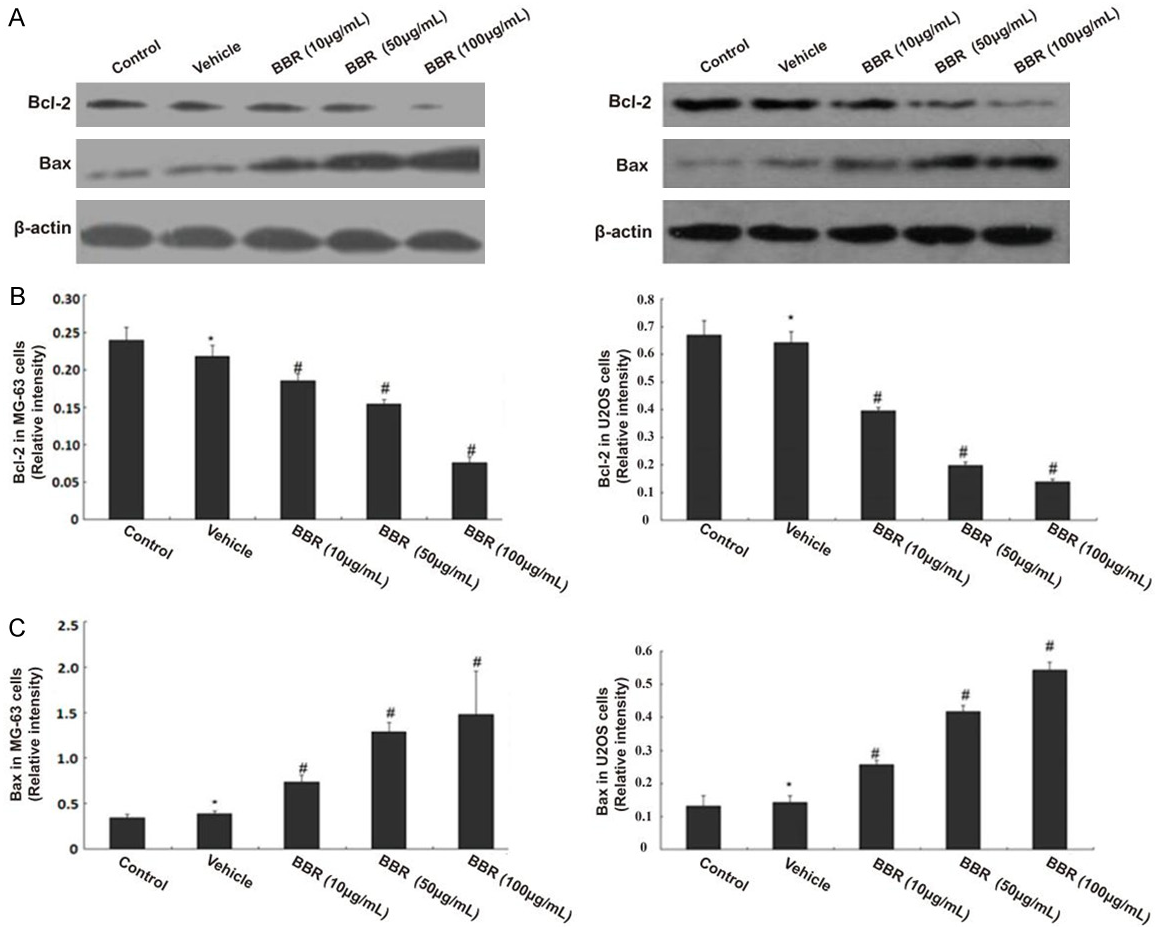


Figure 8. Effects of berberine (BBR) on the expressions of Bcl-2 and Bax in MG-63 and U2OS cells. A. Western blot results of Bcl-2 and Bax in MG-63 and U2OS cells. B. The results of gray scale scanning of Bcl-2 expression in MG-63 and U2OS cells. C. The results of gray scale scanning of Bax expression in MG-63 and U2OS cells. **P*<0.05 compared with Control; #*P*<0.05 compared with Vehicle.

cells. The possible molecular mechanisms are that BBR down-regulates the expression of Bcl-2 and increases the expression of Bax. Also, the apoptosis of MG-63 and U2OS cells was dependent on the activation of MAPK and JNK signal transduction pathways by BBR.

Clonal proliferation is one of the basic characteristics of tumor, and inhibition of clonal proliferation is one of the main objectives of tumor therapy. The anti-tumor proliferation effect of BBR hydrochloride has been widely investigated in various cell lines. It was found that BBR could induce G1-stage arrest of human epidermoid cancer cells, human oral cancer cells [25], Caucasian human glioblastoma cells and mouse leukemia cancer cell lines. BBR can also induce embryonic fibroblasts (Balb/c3T3 cells), gastric cancer (snu-5) cell lines and leukemia cells in non-tumor Balb/c mice to cell growth arrest at G2 stage [26, 27]. The inhibition effect of BBR on tumor cells was more apparent and showed less toxicity to normal cells. The concentration of BBR at less than the median lethal dose (50 μmol/L) can significantly inhibit HepG2 proliferation. Moreover, BBR, even in a high concentration levels (1 mmol/L), showed no or only small cytotoxicity in the liver cells separated from SD rats [28, 29]. This indicates that the anti-proliferation effect of BBR on different cell lines was also different. In our study, BBR could inhibit the proliferation of MG-63 and U2OS cells in time- and concentration-dependent manners and inhibit the growth of osteosarcoma xenograft tumor in BALB/c-nuo-nu mice compared with the negative control group, but the effect was far less than that of Adriamycin. To some extent, these results

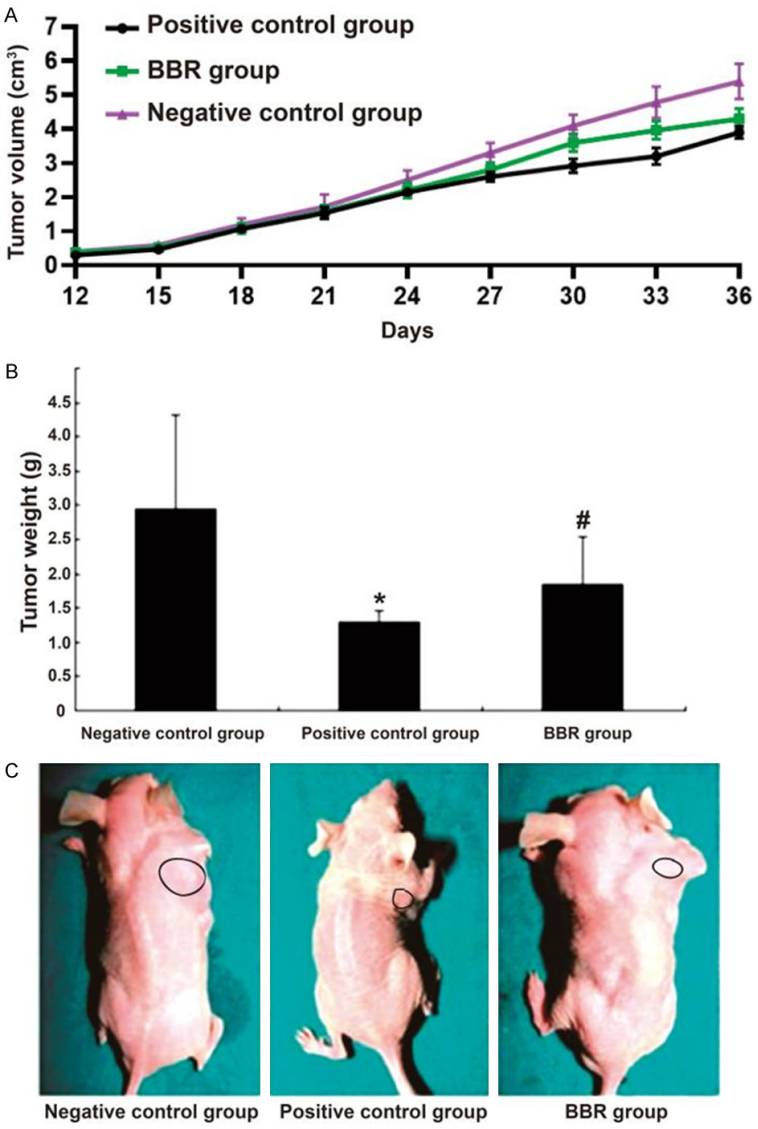


Figure 9. Effect of berberine (BBR) on tumorigenesis of MG-63 cells in nude mice. A. Growth curves of subcutaneous tumors in nude mice among negative control group (N=10), positive control group (N=10) and BBR group (N=10). B. Histogram of subcutaneous tumor naked weight. C. Subcutaneous tumors in nude mice of different groups. Arrows indicated the subcutaneous tumors. #P<0.05 compared with positive control group. *P<0.05 compared with negative control group.

indicate that BBR inhibits the proliferation of osteosarcoma cells. The potential mechanism of the anti-proliferation of BBR hydrochloride may be associated with the regulation of cell cycle arrest [30-33].

From the perspective of cell apoptosis, the failure of normal apoptosis of tumor cells leads to the occurrence of tumors. Therefore, Inducing apoptosis of tumor cells is also an approach of tumor therapy and the mechanism of many potential cancer protectors, blockers or inhibitory drugs in tumor treatments. Previous studies have demonstrated that BBR promotes apoptosis in many cancer cell lines and non-tumor cells, such as HL 60, BALB/c 3T3, KB cells, SNU-5 and oral epithelial cells [29, 34-36]. Our results support that BBR successfully induced apoptosis of MG-63 and U2OS cells. BBR promoted apoptosis of MG-63 and U2OS cells by down-regulating the expression of Bcl-2 and up-regulating the expression of Bax. After the addition of SB203580 and SP600125 inhibitors of p38MAPK and JNN, the apoptosis of BBR-induced MG-63 and U2OS cells were significantly inhibited. Therefore, BBR possibly mediates the apoptosis of MG-63 and U2OS cells by activating p38 MAPK and JNK signaling pathways. In the experiments, we also observed that changes in caspase-3 activity in MG-63 and U2OS cells treated by BBR, indicating that BBR intervention in MG-63 and U2OS cells eventually led to caspase-3 activation and self-apoptosis.

Invasion and distant metastasis of tumor to surrounding tissues are one of the biological characteristics of tumor, which is also one of the main causes for death of patients. Therefore, inhibition or elimination of invasion and distant metastasis of tumor to surrounding tissues is one of the main goals in tumor treatments, as well as one of the means to improve the prognosis and prolong survival time of patients. Tumor invasion and metastasis is a very complicated pathological process. The degradation and invasion of histological barriers such as basement membrane and extracellular matrix are the key links

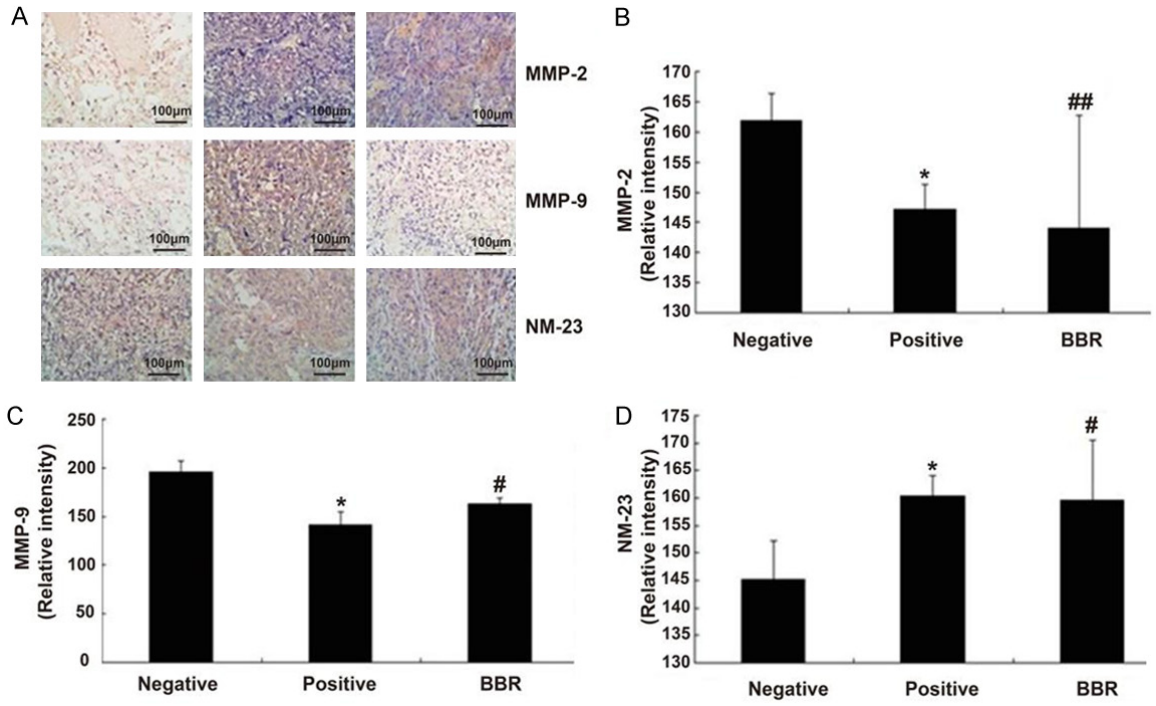


Figure 10. Effect of BBR on the expressions of MMP-2, MMP-9 and NM-23 in BALB/c-nu-nu mouse osteosarcoma transplanted tumor tissue. A. Immunohistochemistry of MMP-2, MMP-9 and NM-23 in BALB/c-nu-nu mouse osteosarcoma transplanted tumor tissue. B. The expression of MMP-2 in transplanted tumor tissues. C. The expression of MMP-9 in transplanted tumor tissues. D. The expression of NM-23 in transplanted tumor tissues. *P<0.05 compared with negative control group. #P<0.05 compared with positive control group. ##P<0.01 compared with positive control group.

of tumor invasion and distant metastasis. Many studies have found that matrix metalloproteinases can disrupt the degradation balance of the matrix, promote cancer cells to break through the histological barriers, and result in adjacent tissue invasion and distant tissue metastasis [37, 38]. Gelatinases, as one of important enzymes, is considered to be a major proteolytic enzyme in the process of tumor invasion and metastasis. It has been found that the increased activity and expression of MMP-2 are closely related to the invasion/metastasis potential and prognosis of various human malignant tumors [39]. In our study, the results showed that BBR reduced the migration and invasion ability of MG-63 and U2OS cells and down-regulated the expressions of MMP-2 and MMP-9 in MG-63 and U2OS cells. Immunohistochemistry also indicated that the expressions of MMP-2 and MMP-9 in the tissue was decreased significantly, while the expression of NM-23 was increased. Similarly, BBR significantly inhibited the expressions of MMP-2 and MMP-9 in SNU-5, HL-60 and WEHI-3 [40, 41]. In the study regarding migration and invasion of 5-8F cells and leukemia stem cells, it was found that BBR reduced the cells migration and invasion ability by up-regulating the expression of NM-23 [42]. The inhibitory mechanism of metalloproteinases is partially regulated by the decrease of intracellular oxygen free radicals such as free oxygen free radical scavengers and vitamin E [43]. In addition, the balance between MMP and TIMP is also an important aspect. The above studies in vivo/vitro studies confirmed that BBR may reduce the migration and invasion ability of osteosarcoma cells by down-regulating the expressions of MMP-2 and MMP-9 and up-regulating the expression of NM-23.

Interestingly, we found that the transplanted tumor was completely located in the capsule, with no obvious invasion to the surrounding tissues. Lung tissue sections showed no metastatic lesions in the lung. In this study, the weight loss of tumor-bearing mice in the group with BBR hydrochloride peritoneal injection

was much smaller than that of the tumor-bearing mice in the Adriamycin group, and the skin of the tumor-bearing mice in the BBR hydrochloride group was ruddy and smooth without wrinkles, while the skin of the tumor-bearing mice in the Adriamycin group was rough and more wrinkles. This indirectly indicates that BBR may have the effect of improving the malignant fluid quality caused by tumor. The specific mechanism is unknown, but it has been reported that Rhizoma Coptidis can improve the effects of AOM-induced lipid peroxidation, protein-bound carbohydrates and antioxidant status in rats [44]. In addition, Iizuka reported that, supplementation of coptis chinensis significantly reduced weight loss in human esophageal cancer cell line YES-2 nude mouse model, and BBR, as the main component, inhibited the secretion of IL-6 in a dose-dependent manner in YES-2 cells [45]. Similar results have been reported in colon-loaded 26/20 cell line mice [46]. The above findings all support the results in this study and make effective explanations. In addition, BBR also enhances the anticancer activity of carmustine, cyclophosphamide or lampstand extract in animal studies [47]. This synergistic effect was also observed when applying coptis chinensis with radiation in lung cancer models in vivo and in vitro [48]. This indirectly suggests that BBR may also be a sensitizer for some tumor therapy drugs.

This study has some limitations. First, our study could not fully replicate the development of osteosarcoma in humans. Second, the mechanisms of signal transduction system for BBR intervention were not further elucidated. Additional long-term and in vivo human studies are needed to confirm our findings and to further support BBR as a therapeutic drug for osteosarcoma treatment.

In conclusion, this study not only explored the role of BBR in the treatment of osteosarcoma and its molecular mechanism at a cellular level, but also further clarifies the possible molecular mechanism of BBR's role and the signal transduction mechanism involved in the activation of BBR at the whole animal level. Our findings provide new research direction, ideas and theoretical basis for future studies on prevention and treatment of osteosarcoma.

Disclosure of conflict of interest

None.

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