Wnt/β-catenin signaling modulates piperine-mediated antitumor effects on human osteosarcoma cells

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Received May 16, 2019; Accepted January 14, 2020

DOI: 10.3892/mmr.2020.11000

Abstract. The plant extract piperine is used as a traditional Chinese medicine due to its anti-inflammatory effects and efficacy against numerous types of cancer. The aim of the present study was to investigate the antitumor mechanism of piperine in human osteosarcoma U2OS and 143B cell lines. The effects of piperine on cell apoptosis and invasion of human osteosarcoma cells were assessed using flow cytometry and Transwell assays. Moreover, western blotting was used to measure the effects of piperine on the protein expression levels of the metastasis markers matrix metalloproteinase-2 (MMP-2) and vascular endothelial growth factor (VEGF). In addition, the involvement of the Wnt/β-catenin signaling pathway in modulating the effects of piperine was examined via western blot analysis. The results of MTT and Transwell invasion assays indicated that piperine treatment dose-dependently reduced U2OS and 143B cell viability and invasion. Furthermore, a significant reduction was identified in MMP-2, VEGF, glycogen synthase kinase-3β and β-catenin protein expression levels, as well as the expression levels of their target proteins cyclooxygenase-2, cyclin D1 and c-myc, in U2OS cells after piperine treatment. In addition, similar results were observed in 143B cells. Therefore, the present study demonstrated the efficacy of piperine in osteosarcoma, and identified that the Wnt/β-catenin signaling pathway may modulate the antitumor effects of piperine on human U2OS and 143B cells.

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Abbreviations: COX-2, cyclooxygenase-2; GSK-3β, glycogen synthase kinase-3β; MMP-2, matrix metalloproteinase-2; VEGF, vascular endothelial growth factor

Key words: antitumor, piperine, osteosarcoma, Wnt/β-catenin signaling, cell viability, invasion

Introduction

Osteosarcoma has an annual incidence rate of 5/1,000,000 individuals and a mortality rate of >50% in patients <20 years old worldwide (1-4). Surgery combined with radiotherapy and chemotherapy is the primary treatment method for osteosarcoma (5). However, ≤80% of patients have poor prognosis due to metastatic lesions that are already present at the time of diagnosis, and the 5-year survival rate is ~30% (6). Given this poor disease prognosis, the development of effective therapeutic strategies and pharmaceuticals to reduce osteosarcoma malignancy are important.

Piperine is an alkaloid found in *Piper nigrum* Linn and *Piper longum* Linn (*Piper nigrum* L., family piperaceae). Piperine is used as a food flavoring and as a traditional Chinese medicine due to its pharmacological benefits (7,8). Moreover, piperine is used to treat gastrointestinal disorders such as constipation and diarrhea (9). Furthermore, it has well-characterized anti-inflammatory (10) and antitumor effects in numerous types of cancer, including breast, lung and liver cancer, and lymphoma (11-14). Piperine has been reported to dose-dependently (15-20) regulate cell growth and differentiation via the Akt/JNK/MAPK pathway (21), and can increase cytokine production via the mTOR signaling pathway (22).

Tumor metastasis is a complex process involving tumor cell dissociation, extracellular matrix degradation, infiltration and adhesion to vascular endothelial cells (23). Notably, matrix metalloproteinases (MMPs), such as MMP-2 and MMP-9, and collagen type IV are significantly upregulated in osteosarcoma and metastases, and are indices of poor prognosis (24). Moreover, MMP-2 downregulation can inhibit osteosarcoma metastasis and infiltration (21,25). Vascular endothelial growth factor (VEGF) is known to promote angiogenesis, and its upregulation is correlated with poor osteosarcoma prognosis (26,27). Furthermore, VEGF downregulation has been shown to reduce vascular density and inhibit metastases in osteosarcoma (28).

While the antitumor effect of piperine on U2OS cells has been reported (21), its underlying molecular mechanisms of action are not fully understood. As the Wnt/β-catenin signaling pathway is known to regulate cell proliferation and differentiation (29,30), the present study hypothesized that it may be involved in modulating the antitumor effects of

piperine. Therefore, the aim of the present study was to test this hypothesis; the results may provide a novel insight into the antitumor mechanism of piperine.

Materials and methods

Chemical reagents. DMSO and MTT were purchased from Sigma-Aldrich (Merck KGaA). Piperine (molecular weight, 285.35 kDa; National Institutes for Food and Drug Control) was dissolved in DMSO at the concentration of 150 μM and stored at -20˚C. An Annexin V-FITC/PI double staining cell apoptosis detection kit was obtained from Nanjing KeyGen Biotech Co., Ltd. NQBB FBS was obtained from Wuhan ChunDuBio Co., Ltd. Anti-MMP-2 (cat. no. 10373-2-AP; 1:1,000) was purchased from ProteinTech Group, Inc. Anti-VEGF (cat. no. GB11034; 1:3,000), anti-c-Myc (cat. no. GB13076; 1:500), anti-cyclin D1 (cat. no. GB11079; 1:1,000), anti-cyclooxygenase-2 (COX2; cat. no. GB11072; 1:500), anti-β-catenin (cat. no. GB11015; 1:500) and anti-glycogen synthase kinase-3β (GSK-3β; cat. no. GB11099; 1:1,000) were purchased from Wuhan Servicebio Technology Co., Ltd.

Cell culture. Human osteosarcoma U2OS and 143B cells were provided by Cheeloo College of Medicine, Shandong University. 143B cells were identified by STR from Shanghai Cinoasia Institute, and the results showed that the cells were not contaminated, had homology with HOS/KHOS-240s cells and were human osteosarcoma cells. The cells were cultured in McCoy's 5A medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS at 37˚C in a 5% CO_2 humidified incubator until cells reached the logarithmic growth phase; cells were then harvested for subsequent experiments.

MTT cell viability assay. U2OS cells ($4x10^3$ cells/well) and 143B cells ($1x10^3$ cells/well) were seeded in 96-well plates and incubated at 37˚C in a 5% CO_2 humidified incubator with different piperine concentrations (0, 50, 100 and 150 μM) for 24, 48 and 72 h. Subsequently, cell viability was determined using an MTT kit (Cell Titer 96AQ; Promega Corporation). The supernatant was aspirated and 0.05% DMSO (150 μl) was added to each well, and then shake at a low speed (3.2 g) for 10 min to fully dissolve the formazan. The optical density (OD) values of piperine-treated cells were measured at 490 nm using an ELISA microplate reader (Rt2100c; Rayto Life and Analytical Sciences Co., Ltd.). Inhibition rate %=(1-OD value of experimental group/OD value of 0 μM group) x 100%.

Flow cytometry. U2OS cells ($5.0x10^4$ cells/well) and 143B cells ($1.0x10^4$ cells/well) were seeded in 6-well plates and incubated at 37˚C in 5% CO_2 with different concentrations of piperine (0, 50, 100 and 150 μM) for 48 h. Cells were then harvested and 3 ml pre-chilled PBS was added at 4˚C, which were centrifugated at 337 x g for 5 min at room temperature and 200 μl binding buffer was then used to suspend the supernatant. Next, double fluorescence staining was performed with 20 μg/ml PI and 5 μl Annexin V-FITC for 15 min at room temperature before analysis with a flow cytometer (Beckman CytoFLEX; Beckman Coulter, Inc.) and FlowJo software version 10.0.7 (Stanford University). Annexin V-FITC (green) and PI (red) fluorescence intensities were detected using the FITC channel (FL1) and PI channel (FL2), respectively. Total apoptotic rate was calculated as follows: Total apoptotic rate=early apoptotic rate + late apoptotic rate.

Transwell invasion assay. A Matrigel Transwell 24 holes with an aperture of 3.0 μm (Corning, Inc.) assay was performed to assess cell invasion. Matrigel matrix (50 mg/l; BD Biosciences) was diluted in serum-free medium at a 1:3 ratio to reconstitute the basement membrane. U2OS and 143B cells ($5x10^4$ cells/ml) were separately added to the upper Transwell chamber, and 10% FBS-containing culture medium containing different concentrations (50-150 μM) of piperine was added to the lower chamber. After incubation for 48 h at 37˚C in 5% CO_2, the cells in the upper chamber were fixed with 75% ethanol at room temperature for 10 min, stained with 0.1% crystal violet for 5 min at 37˚C and examined using light microscopy (Olympus Corporation) at x400 magnification. In total, five random visual fields were used to count the total number of migrating cells, and the images were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.).

Western blot analysis. U2OS and 143B cells were treated with different concentrations of piperine (0, 50, 100 and 150 μM) for 48 h prior to protein extraction at 37˚C. After the cells were harvested and washed with RIPA buffer (Wuhan Servicebio Technology Co., Ltd.; cat. no. G2002) total protein from cells was extracted using a protein extraction kit (cat. no. KGBSP002; Nanjing KeyGen Biotech Co., Ltd.). Protein concentration was measured by bicinchonic acid protein kit (Wuhan Servicebio Technology Co., Ltd.) Proteins (20 μg) were then separated by SDS-PAGE with 10% gels. The separated protein bands were transferred onto nitrocellulose membranes blocked with 5% skimmed milk/TBST(0.1% Tween-20) at 37˚C for 60 min, which were probed with the respective primary antibodies [Anti-MMP-2 (1:1,000; ProteinTech Group, Inc.) Anti-VEGF (1:3,000), anti-c-Myc (1:500), anti-cyclin D1 (1:1,000), anti-cyclooxygenase-2 (COX2; 1:500), anti-β-catenin (1:500) and anti-glycogen synthase kinase-3β (GSK-3β; 1:1,000; Wuhan Servicebio Technology Co., Ltd.)] overnight at 4˚C. Subsequently, the membranes were incubated with a corresponding horseradish peroxidase-labeled secondary antibody (1:3,000; cat. no. 23301; Wuhan Servicebio Technology Co., Ltd.) for 30 min at room temperature, before being developed with an enhanced chemiluminescence reagent (Wuhan Servicebio Technology Co., Ltd.). Protein bands were analyzed using AlphaEaseFC software (ver 4.0.0; Alpha Innotech).

Statistical analysis. Statistical analyses were performed using SPSS version 16.0 software (SPSS, Inc.). Data from ≤3 independent experiments are presented as the mean ± SD. A one-way ANOVA followed by Bonferroni post-hoc analysis was performed to compare groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Piperine inhibits cell proliferation and induces apoptosis. MTT assay results indicated that piperine significantly inhibited U2OS and 143B cell proliferation ($P<0.05$; Fig. 1). It was revealed that cell density was reduced in the presence

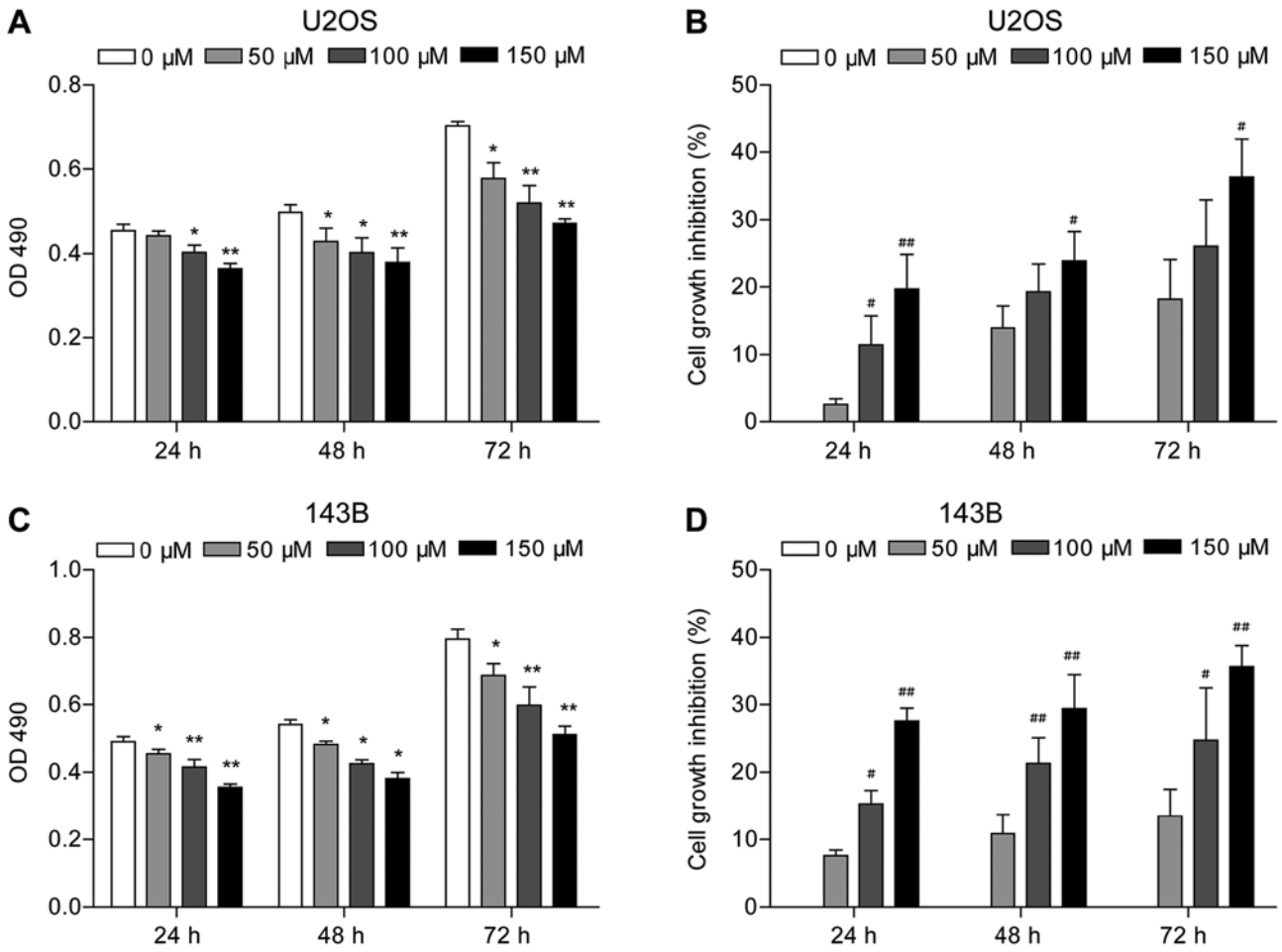

Figure 1. Piperine inhibits U2OS and 143B cell proliferation, as determined by MTT assay. (A) U2OS and (C) 143B cell density OD_{490} profile of the control group (0 μM) and the piperine experimental groups at 24, 48 and 72 h after treatment. Inhibition rate %=(1-OD value of experimental group/OD value of 0 μM group) x 100%. Effect of piperine on percentage of (B) U2OS and (D) 143B cell growth inhibition at 24, 48, and 72 h after treatment. *P<0.05, **P<0.01 vs. Control (0 μM). $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. Control (25 μM). OD, optical density.

of piperine compared with untreated cells, with the lowest OD_{490} obtained for cells treated for 24 h with 150 μM piperine (P<0.01; Fig. 1A and C). Moreover, the strongest growth inhibition was observed in response to 150 μM piperine for 72 h (35% for U2OS and 35.7% for 143B; P<0.01; Fig. 1B and D). In U2OS cells, the apoptotic rate was positively associated with piperine concentration (Fig. 2). The apoptotic rate in the absence of piperine was 1.89%, whereas the apoptotic rates increased to 2.12, 4.63 and 19.1%, in the presence of 50, 100 and 150 μM piperine, respectively (P<0.05; Fig. 2A). For 143B cell the apoptotic rates was 10.29, 13.28, 24.85 and 36.7% in the presence of 0, 50, 100 and 150 μM piperine, respectively (P<0.05; Fig. 2B)

Table I. Data from the Transwell assay

	Invaded cells	
Treatment group, μM	U2OS	143B
0	365.6±23.8	320.3±30.0
50	266.2±39.7	246.9±48.3
100	167.2±20.6	128.5±37.7
150	20.4±4.8	45.8±10.2

Data are presented as the mean ± SD.

Piperine inhibits cell invasion by downregulating MMP-2 and VEGF protein expression. The present results suggested that osteosarcoma cells treated with 50, 100 and 150 μM piperine exhibited reduced cell(U2OS: Number of invading cells 266, 167 and 20 per field; 143B: 246, 128 and 45 per field, respectively; P<0.01; Fig. 3; Table I). Furthermore, western blotting results demonstrated a dose-dependent decrease in MMP-2 and VEGF protein expression levels in the presence of piperine, with the most significant reduction observed at 150 μM piperine for both proteins. However, with increasing concentration of piperine, U2OS had a more significant effect on MMP-2 and VEGF compared with 143B cells (Fig. 4).

Piperine suppresses cell proliferation and invasion via the Wnt/β-catenin signaling pathway. Western blot analysis results revealed that the protein expression levels of GSK-3β and β-catenin were significantly reduced in U2OS cells after piperine treatment, and downregulation of these proteins was inversely associated with piperine concentration. Notably,

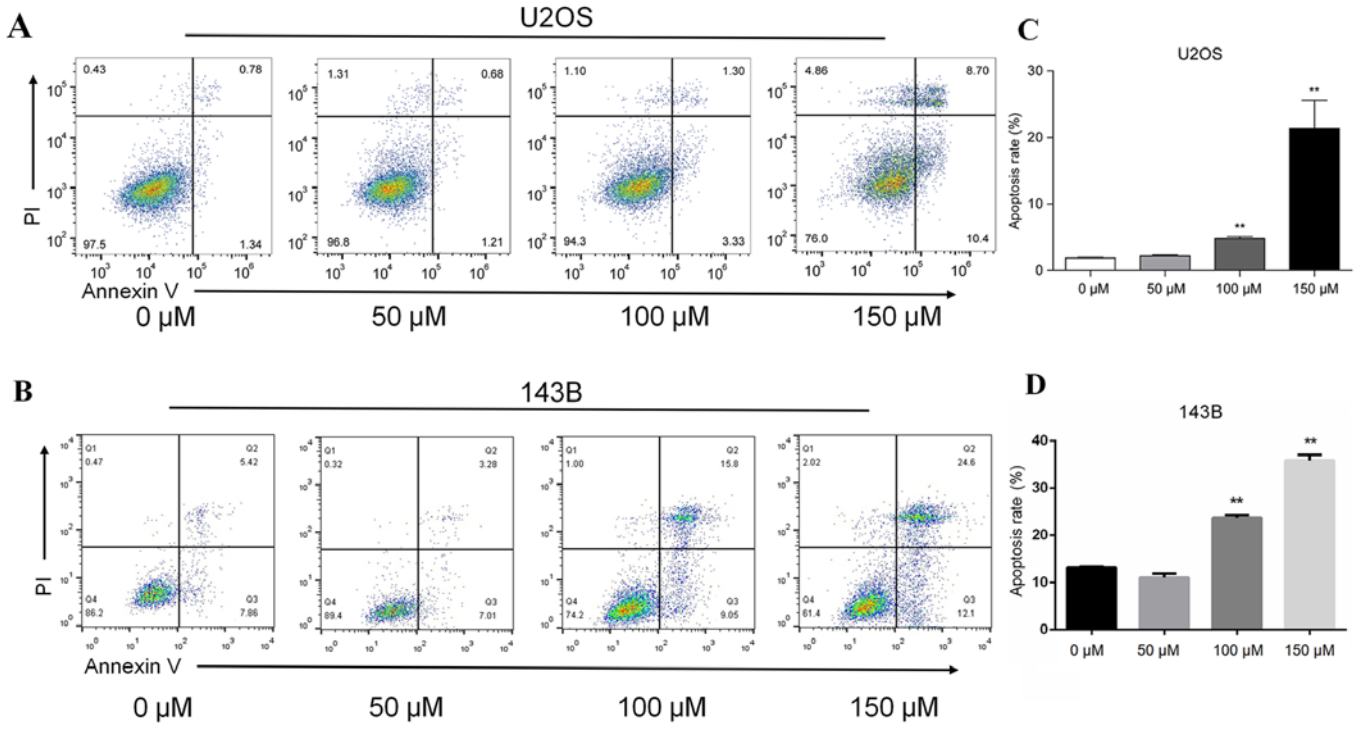

Figure 2. Piperine induces U2OS and 143B cells apoptosis. Flow cytometry profiles of Annexin V FITC/PI double stained (A) U2OS and (B) 143B cells after 48 h piperine treatment at 0μM, 50μM,100μM and 150 μM. C and D represent the apoptosis rate of U2OS and 143B cells after treatment with different concentrations of piperine for 48 h. **P<0.01 vs. Control (0 μM).

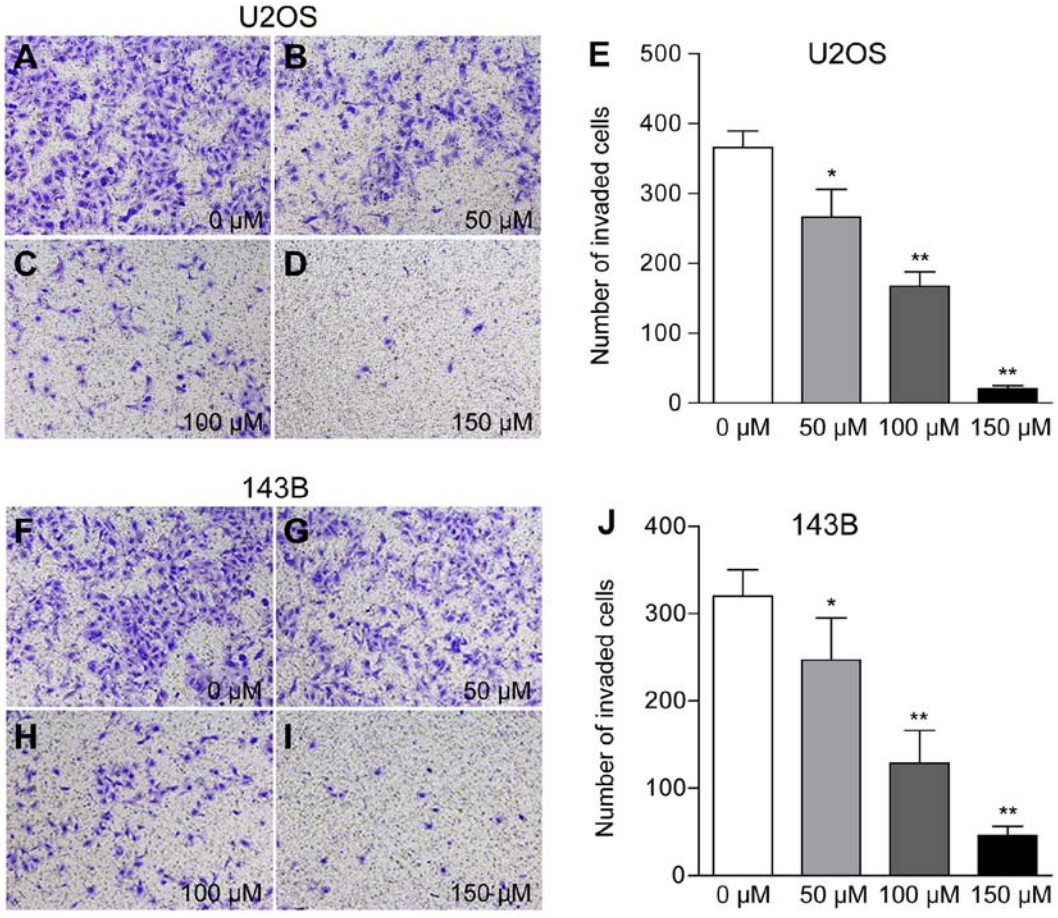

Figure 3. Transwell assay results showing piperine-mediated reduction in cell invasion. Images of (A-D) U2OS and (F-I) 143B cells treated with (A and F) 0, (B and G) 50, (C and H) 100 and (D and I) 150 μM piperine for 48 h. Magnification, x100. (E and J) Semi-quantitative profile of microscopy images of piperine-treated U2OS cells. *P<0.05, **P<0.01 vs. 0 μM.

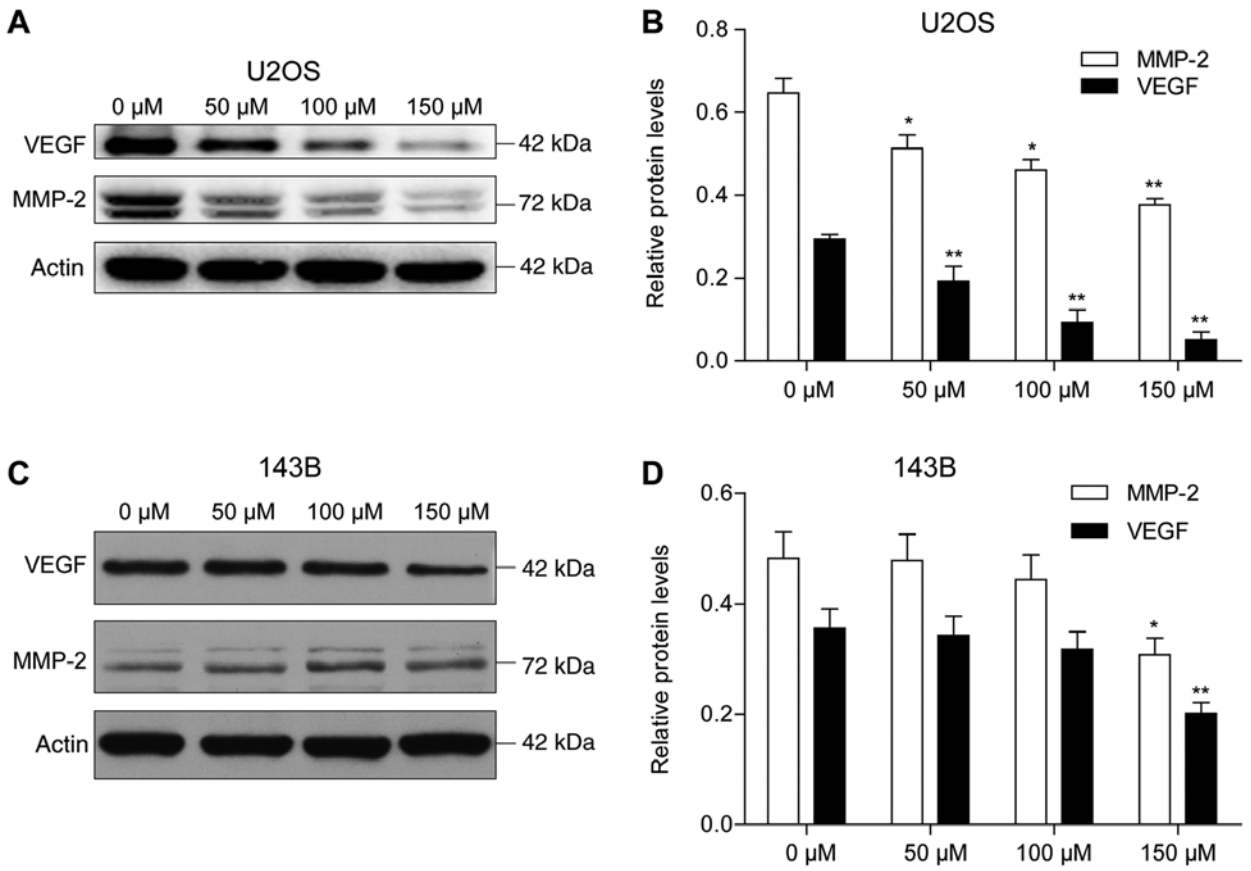

Figure 4. Piperine downregulates VEGF and MMP-2 protein expression levels in U2OS and 143B cells. Western blot analysis of VEGF and MMP-2 protein expression levels in (A) U2OS and (C) 143B cells treated with 0, 50, 100 and 150 μM piperine. Actin was used as the loading control. (B and D) Semi-quantitative profiles of western blotting data of VEGF and MMP-2 at the four tested piperine concentrations. *P<0.05, **P<0.01 vs. 0 μM. MMP, matrix metalloproteinases; VEGF, vascular endothelial growth factor.

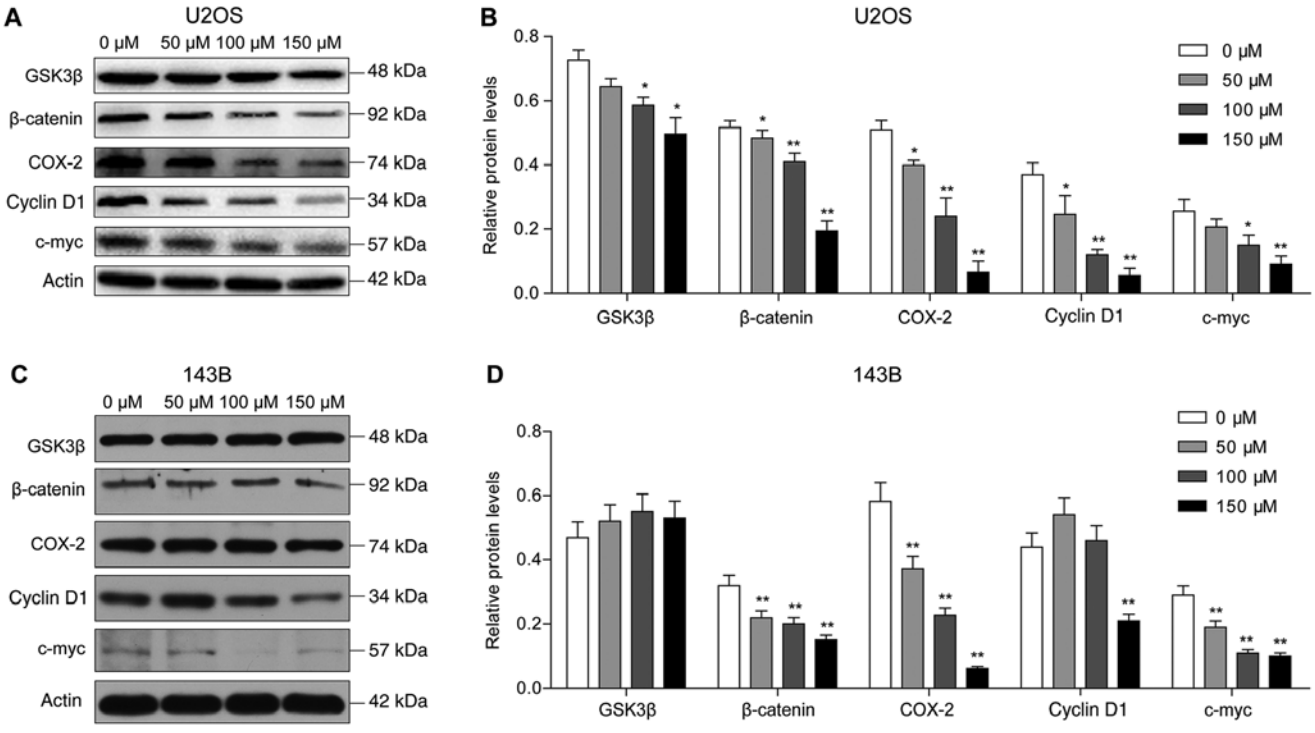

Figure 5. Effect of piperine treatment on the Wnt/β-catenin signaling pathway in U2OS and 143B cells. Western blot analysis of GSK-3β and β-catenin expression levels, and their target genes COX-2, cyclin D1 and c-myc after 48 h piperine treatment in (A) U2OS and (C) 143B cells. Actin was used as the loading control. (B and D) Semi-quantitative profile of the western blotting data. *P<0.05, **P<0.01 vs. 0 μM. COX-2, cyclooxygenase-2; GSK-3β, glycogen synthase kinase 3β.

the lowest protein expression levels were observed following treatment with 150 μM piperine ($P<0.05$; Fig. 5). In addition, reduced expression levels of the target proteins COX-2, cyclin D1 and c-myc were observed with increasing piperine concentrations ($P<0.01$; Fig. 5). In 143B cells, it was demonstrated that β-catenin protein expression was significantly reduced, and the expression levels of COX-2 and c-myc were decreased with increasing piperine concentrations.

Discussion

The present results suggested that piperine treatment increased apoptosis, and reduced cell invasion and proliferation of U2OS and 143B cells. Notably, cell proliferation was most significantly inhibited in response to treatment with the highest concentration of piperine (150 μM) for the longest treatment duration (72 h). Furthermore, the dose-dependent decrease observed in the protein expression levels of metastatic markers in the presence of piperine were in line with previous studies on the roles of these proteins in tumor metastasis and heterotopic angiogenesis (8,14,18). Therefore, the present results provided evidence on the role of piperine in inhibiting osteosarcoma cell proliferation and metastasis (21). In addition, the present western blotting results identified that the Wnt/β-catenin signaling pathway, which has a role in regulating osteosarcoma cell proliferation and apoptosis (31-34), may regulate piperine-mediated osteosarcoma apoptosis.

The core mediator of the Wnt/β-catenin signaling pathway, β-catenin, is regulated by phosphorylation, which is promoted by GSK-3β, and degradation via the ubiquitin/proteasome pathway (26). β-catenin mutations can cause abnormal activation of Wnt target genes, thus leading to cancer development (35). In addition, dysregulation of the Wnt signaling pathway can affect downstream gene expression (36). The present results suggested that piperine dose-dependently downregulated the protein expression levels of GSK-3β and β-catenin, as well as those of the downstream oncogenic proteins cyclin D1, c-Myc and COX-2. Therefore, piperine may effectively inhibit U2OS and 143B cell proliferation and metastasis, and ectopic angiogenesis via regulation of the Wnt/β-catenin signaling pathway. The PI3K/Akt pathway is frequently hyperactivated in osteosarcoma, and contributes to disease initiation and development (37). Moreover, inhibition of this pathway via small compounds is a potential therapeutic approach for osteosarcoma (38). Previous studies have shown that Wilms' tumor 1 knockdown in MG-63 cells results in deregulation of cell cycle proteins and downregulation of the PI3K/AKT pathway (39,40). Piperine has previously been reported to inhibit cell proliferation and metastasis of U2OS cells (21), and affect several signaling pathways, such as the Akt/MAPK and mTOR pathways (21,22,41), in several cancer cell types. However, to the best of our knowledge, the present study is the first to demonstrate that the Wnt/β-catenin signaling pathway may mediate the antitumor effects of piperine.

In the present study, it was demonstrated that piperine exerted a lower inhibitory effect on the 143B cell line compared with the U2OS cell line, and therefore, it was difficult to select a cell line to be used as control cells. Thus, the present study did not include a negative control. Furthermore, the cytotoxicity of piperine on healthy cells has not been thoroughly investigated in previous studies despite the well-characterized anticancer effects of piperine. However, it has been previously reported that piperine administration does not increase hepatorenal toxicity in nude mice (42,43). Thus, piperine may not cause osteosarcoma-specific cytotoxic effects.

In relation to the modest antitumor efficacy of piperine shown in the present study, further *in vivo* and clinical experiments are required to evaluate its efficacy on osteosarcoma proliferation, and to clarify the underlying molecular mechanisms. In addition, future studies will investigate whether combination drug treatment with cisplatin increases antitumor potency on osteosarcoma growth. The present study did not use agonists or antagonists to confirm the involvement of the Wnt/β-catenin pathway. However, the present study proposed that the expression levels of upstream signaling molecules in the Wnt/β-catenin pathway, such as β-catenin and GSK-3β, as well as the downstream target proteins COX-2, cyclin D1 and c-myc, could demonstrate the involvement of the Wnt/β-catenin pathway in piperine-mediated osteosarcoma inhibition. Furthermore, future *in vivo* experiments will use specific agonists and inhibitors of the Wnt/β-catenin pathway.

In conclusion, the present results suggested that the Wnt/β-catenin signaling pathway underlies the antitumor effects of piperine on U2OS and 143B cell proliferation, apoptosis and invasion. However, further research is needed to investigate the anti-osteosarcoma mechanism of piperine.

Acknowledgements

The authors would like to thank Professor Ming Zhang and Assistant Researcher Wenxia Li of Key Laboratory of Cardiovascular Remolding and Function Research Chinese Ministry of Health for their help in the present study.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LN contributed to the conception of the study. YBQ performed the experiments, data analysis and wrote the manuscript. WY and MS contributed to data interpretation and analyses. All authors have read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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