

Capsaicin inhibits proliferation and induces apoptosis in osteosarcoma cell lines via the mitogen-activated protein kinase pathway

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Received March 19, 2017; Accepted August 29, 2017

DOI: 10.3892/or.2017.5960

Abstract. Capsaicin, a pungent molecular compound present in many hot peppers, exerts anticancer activities against various human cancer cell lines by inducing apoptosis. However, the effects of capsaicin on human osteosarcoma (OS) as well as the related mechanisms remain to be fully elucidated. In the present study, the anticancer effects of capsaicin on 3 human OS cell lines (MG63, 143B and HOS) were investigated. Various concentrations of capsaicin (50-300 μ M) effectively decreased cell viability in all 3 OS cell lines in a dose-dependent manner. Notably, capsaicin-induced apoptosis was observed when OS cells were treated with relatively high concentrations of capsaicin (starting at 250 μ M). In addition, the mitochondrial apoptotic pathway was involved in the capsaicin-induced apoptosis in the OS cells. Meanwhile, our results also indicated that at relatively low concentrations (e.g., 100 μ M), capsaicin could inhibit the proliferation, decrease the colony forming ability and induce G0/G1 phase cell cycle arrest of OS cells in a dose-dependent manner. Moreover, our results revealed that the anticancer effects induced by capsaicin on OS cell lines involved multiple MAPK signaling pathways as indicated by inactivation of the ERK1/2 and p38 pathways and activation of the JNK pathway. Furthermore, the results of animal experiments showed that capsaicin inhibited tumor growth in a xenograft model of human OS. In conclusion, these results indicate that capsaicin may exert therapeutic benefits as an adjunct to current cancer therapies but not as an independent anticancer agent.

Introduction

Osteosarcoma (OS) is the most frequent primary malignant bone tumor in children and young adults and has a high tendency for local invasion and early metastasis (1,2). Conventional treatments such as surgery, chemotherapy and radiotherapy still result in a poor prognosis for OS due to early lung metastasis. Recently, with the use of neoadjuvant chemotherapy and improvements in surgical techniques, the 5-year survival rate has improved for some patients with localized OS (3), but for patients with metastatic disease, the survival rate (~10-20%) remains unchanged (4,5). Furthermore, chemoresistance and adverse side-effects induced by chemotherapeutic agents cannot be neglected (6). Therefore, new effective therapeutic agents for OS are urgently needed.

Since plant-derived compounds promote apoptosis, autophagy, programmed cell death, mitotic catastrophe and senescence in cancer cells (7), many different natural compounds from various plants have been suggested to not only prevent, but also treat cancers. Several studies have reported that compounds extracted from natural plants exert inhibitory effects on OS (8,9). Capsaicin (*trans*-8-methyl-N-vanillyl-6-nonenamide), the most abundant and pungent component in a variety of hot peppers, has been used extensively as a food additive in countries worldwide (10). As a medicinal compound, capsaicin is currently used to treat pain and inflammation caused by various diseases, including rheumatoid arthritis, herpes zoster and cluster headaches (11). More recently, the anticancer effects of capsaicin have garnered increasing attention. The literature states that capsaicin suppresses several malignant human cell lines, including prostate cancer (12), leukemia (13), gastric cancer (14), hepatocarcinoma (15), glioma (16) and breast cancer (17). However, only a few studies have reported the effects of capsaicin on OS (18). Furthermore, since the molecular mechanisms involved in the anticancer effects of capsaicin are complicated, a lack of consensus regarding its mechanisms for inducing OS cell apoptosis exists in the literature. For instance, Ying *et al* (19) demonstrated that capsaicin possesses strong efficacy in inducing human OS cell apoptosis via activation of the AMPK signaling pathway

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Key words: capsaicin, osteosarcoma, apoptosis, proliferation, cell cycle, MAPK signaling pathway

and c-Jun NH₂-terminal kinases. Cho *et al* (20) found that capsaicin could induce apoptosis in the OS MG63 cell line and further demonstrated that the caspase cascade and antioxidant enzymes were the underlying regulatory signaling pathways involved in capsaicin-induced apoptosis. In addition, Jin *et al* revealed that capsaicin could induce immunogenic cell death in human OS MG63 cells *in vitro* (21). However, these results were predominantly obtained with relatively high concentrations of capsaicin. Other than apoptosis induction in OS cells, mechanisms that may explain the anti-OS activities at low concentrations of capsaicin remain unclear. Therefore, we evaluated the effects of capsaicin on proliferation, cell cycle arrest and apoptosis induction using 3 OS cell lines (MG63, 143B and HOS) and explored the underlying mechanisms with the goal of obtaining comprehensive results that describe the effect of capsaicin on OS cells.

Materials and methods

Reagents. Capsaicin (*trans*-8-methyl-N-vanillyl-6-nonenamide) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from HyClone (Logan, UT, USA). Cell viability and cytotoxicity test kit, Cell Counting Kit-8 (CCK-8), was purchased from Dojindo Molecular Technologies (Kimamoto, Japan). An Annexin V-FITC/propidium iodide (PI) double staining test kit was purchased from KeyGen Biotech (Nanjing, China). A 5-ethynyl-2'-deoxyuridine (EdU) cell proliferation assay kit was purchased from RiboBio (Guangzhou, China). RIPA lysis buffer, phenylmethanesulfonyl fluoride (PMSF), a bicinchoninic acid (BCA) protein assay kit, bovine serum albumin (BSA), a JC-1 mitochondrial membrane potential assay kit, a caspase-3 activity assay kit, and mouse anti-human actin (cat. no. AA128) and tubulin (cat. no. AT819) were purchased from Beyotime Biotech (Shanghai, China). Mouse anti-human proliferating cell nuclear antigen (PCNA) (cat. no. sc-53407) were purchased from Santa Cruz Biotechnology (San Francisco, CA, USA). Rabbit anti-human p21 (cat. YT3497) was purchased from ImmunoWay Biotechnology (Newark, DE, USA). Rabbit anti-human Bax (cat. no. 5023), Bcl-2 (cat. no. 4223), p-ERK1/2 (cat. no. 4370), ERK1/2 (cat. no. 4695), p-p38 (cat. no. 4511), p38 (cat. no. 8690), p-JNK (cat. no. 4668), JNK (cat. no. 9252) and Ki67 (cat. no. 9027) were purchased from Cell Signaling Technology (Boston, MA, USA). Goat anti-rabbit (cat. no. 111-035-003) and goat anti-mouse secondary antibodies (cat. no. 115-035-003) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Cell and cell culture. The OS cell lines MG63, 143B and HOS were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in DMEM containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in an atmosphere containing 5% CO₂.

Cell viability assay. Cells were seeded into 96-well plates at a density of 5,000 cells/well and incubated at 37°C for 24 h with 6 replicates. The cells were treated with various concentrations of capsaicin (0, 50, 100, 150, 200, 250 or 300 µM) for 24 h. Additionally, 2-wells containing capsaicin alone were

established to register the background absorbance at each concentration. Then, 10 µl of CCK-8 was added to each well, and the plates were incubated for an additional hour. The cells were subsequently placed in a microplate reader to detect absorbance at 450 nm. Cell viability was calculated using the following formula: Cell viability (%) = (test absorbance - background absorbance)/(control absorbance - background absorbance) x 100%.

Assessment of apoptosis using flow cytometry. Cellular apoptosis was detected via flow cytometry using an Annexin V-FITC/PI kit. Briefly, the cells were seeded into 10-cm dishes (10⁶ cells/dish) for 24 h and then treated with various concentrations of capsaicin (0, 100, 150, 200 or 250 µM) for 24 h. The cells were then collected and washed twice with ice-cold phosphate-buffered saline (PBS) and stained with Annexin V-FITC and PI according to the manufacturer's guidelines, after which the samples were read on a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The distribution of viable (FITC⁻/PI⁻), early apoptotic (FITC⁺/PI⁻), late apoptotic (FITC⁺/PI⁺) and necrotic (PI⁺/FITC⁻) cells was calculated and analyzed. Both early and late apoptotic cells were recorded as apoptotic cells, and the results were expressed as the percentage of total cells.

Caspase-3 activity assessment. The activity levels of caspase-3 were detected using a caspase-3 assay kit according to the manufacturer's instructions. In brief, after cells were treated with various concentrations of capsaicin (0, 100, 150, 200 or 250 µM), they were collected, lysed and centrifuged at 16,000 x g for 20 min at 4°C. The supernatant containing protein was collected, and the protein concentrations were measured using BCA methods. Then, ~50 µg of protein was incubated with buffer containing Ac-DEVD-pNA (2 mM) at 37°C overnight, and the absorbance of yellow pNA (the cleavage product) was measured using a microplate reader at a wavelength of 405 nm. In addition, caspase-3 activity was calculated as a fold of the optical density (OD) of the different capsaicin concentrations relative to the OD of the control group.

Mitochondrial membrane potential assessment. The mitochondrial membrane potential (ΔΨ_m) was detected using a JC-1 mitochondrial membrane potential assay kit. Cells were seeded in 10-cm dishes at a density of 10⁶ cells/dish for 24 h, and then treated with various concentrations of capsaicin for an additional 24 h. Cells were collected and suspended in 0.5 ml of medium and incubated with 0.5 ml of JC-1 at 37°C for 20 min, after which the samples were washed 2 times with ice-cold JC-1 buffer and measured using flow cytometry.

EdU proliferation assay. The effects of capsaicin on OS cell proliferation were determined using an EdU cell proliferation assay kit according to the manufacturer's protocol. Briefly, cells were seeded into 24-well plates at an initial density of 5x10⁴ cells/well and incubated for 12 h to allow adherence. Afterwards, they were treated with various concentrations of capsaicin (0, 100, 150, 200 or 250 µM) for another 24 h. The cells were then incubated with 50 µM EdU for 2 h before they were fixed with 4% paraformaldehyde. After the cells were

permeabilized with 0.3% Triton X-100, the incorporated EdU was stained with Apollo 488 (green fluorescence), and the nuclei were stained with Hoechst 33342 (blue fluorescence). Finally, the percentage of EdU-positive cells was determined using fluorescence microscopy.

Colony formation assay. Cells were seeded into 6-well plates at a density of 500 cells/well and incubated for 12 h in medium supplemented with 10% FBS. Next, the cells were treated with various concentrations of capsaicin (0, 100, 150, 200 or 250 μM) for 24 h, after which they were incubated with complete medium for another 10 days until colonies began to form. The medium was discarded, and the cells were washed twice with ice-cold PBS. After the cells were fixed with 4% paraformaldehyde for 20 min, they were stained with 0.1% crystal violet for 10 min. The number of clones containing at least 50 cells in 4 different visual fields was counted under a microscope.

Cell cycle analysis. Cells were seeded in 10-cm dishes at a density of 10^6 cells/dish and treated with various concentrations of capsaicin (0, 100, 150, 200 or 250 μM) for 24 h. Cells were collected and fixed in 70% ice-cold ethanol at -20°C overnight. Then, the cells were incubated with 10 mg/ml RNase and 50 $\mu\text{g/ml}$ PI for 30 min. The cell cycle distribution was assessed using flow cytometry.

Western blot analysis. After cells were treated with capsaicin at the indicated concentrations (0, 100, 150, 200 or 250 μM) for 24 h, they were lysed in RIPA lysis buffer containing PMSF and phosphatase inhibitors to extract the total intracellular proteins. Protein samples (30-50 $\mu\text{g/lane}$) were separated on a 8-12% gel using SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes, which were blocked with either 5% skim milk or 5% BSA at room temperature for 1 h and then incubated with the corresponding primary antibodies (1:800) overnight at 4°C . After the membranes were washed with Tris-buffered saline with Tween-20 (TBST), they were incubated with secondary antibody for 1 h at 37°C . The membranes were washed, and the reactive protein bands were detected with an enhanced chemiluminescence (ECL) detection system and developed on film.

Xenograft tumor model. Male nude mice (4 weeks old) were supplied by the Experimental Animal Center of Chongqing Medical University. All animal studies were approved by the Ethics Committee at Chongqing Medical University. The mice were housed with free access to a commercial diet and water under specific pathogen-free conditions. After the mice were acclimated for one week prior to study initiation, they were then subcutaneously injected with 100 μl of an HOS cell suspension in sterile PBS at a density of 2×10^6 cells/ml. After the tumor volume reached 100 mm^3 , capsaicin treatment was initiated. Ten mice were randomized into 2 groups (5/group). The capsaicin group was administered capsaicin 50 mg/kg body weight in 100 μl of PBS containing 0.2% ethanol, and the control group received 100 of PBS containing 0.2% ethanol; the treatments were administered via oral gavage. The groups received their respective treatments every 3 days for a total of 4 weeks until the mice were sacrificed. The tumor volume

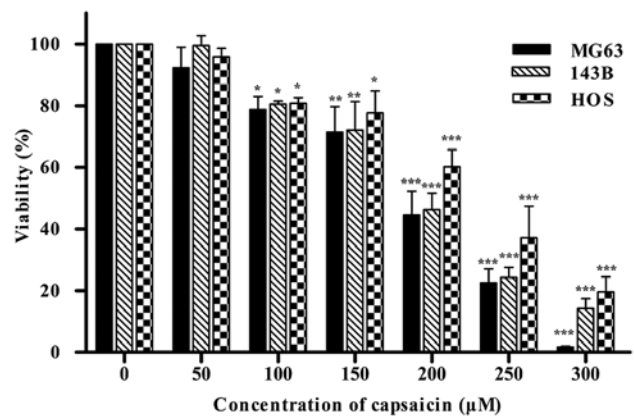


Figure 1. Effect of capsaicin on OS cell viability. Three different human OS cell lines (MG63, 143B and HOS) were treated with various concentrations of capsaicin (0, 50, 100, 150, 200, 250 or 300 μM) for 24 h. Cell viability was measured using a CCK-8 assay. The experiment was performed in triplicate. The results of the data from 3 independent experiments are expressed as the percentage of capsaicin-treated cells vs. untreated cells (0 μM). The data are presented as the mean \pm SD; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with untreated cells (0 μM).

was measured every 3 days after treatment according to the following formula: $1/2 \times a^2b$ (a is the short axis and b is the long axis of the tumor). Mice were sacrificed under anesthesia on day 28, and the xenograft tumors from each animal were removed, measured, and then embedded in paraffin for immunohistochemistry (IHC).

Proliferation index in the xenograft tumor. IHC was performed to evaluate PCNA and Ki67 expression in xenograft tumor tissues. Briefly, paraffin-embedded xenograft tumor tissue sections were dewaxed in xylene and subsequently rehydrated in a graded series of ethanol. Antigen retrieval on the deparaffinized sections was performed by immersing the samples in 0.1 M citrate buffer (pH 6.0), boiling the sections in the microwave for 10 min, and then cooling the sections to room temperature. Endogenous peroxidase activity was then blocked by immersing the sections in methanol containing 3% hydrogen peroxide for 10 min. After the sections were blocked in goat serum for 10 min at room temperature, they were incubated with the antibodies targeting Ki67 (1:200) and PCNA (1:200) overnight at 4°C . Then, the sections were incubated with the secondary antibody at 37°C for 30 min. Streptavidin-conjugated peroxidase was added to the sections, which were incubated for another 10 min at room temperature. Diaminobenzidine (DAB) substrate was added for 5 min to visualize the bound antibodies. PCNA and Ki67 expression was evaluated by counting the number of positive cells from 5 randomly fields under a light microscope at a magnification of $\times 400$. Data are presented as the percentage of the positive cells.

Statistical analysis. All the data are presented as the mean \pm standard deviation (SD) of 3 independent experiments. Analysis of variance (ANOVA) and Student's t-test were used to determine significant differences between groups. A two-tailed P-value < 0.05 was considered to indicate a significant difference.

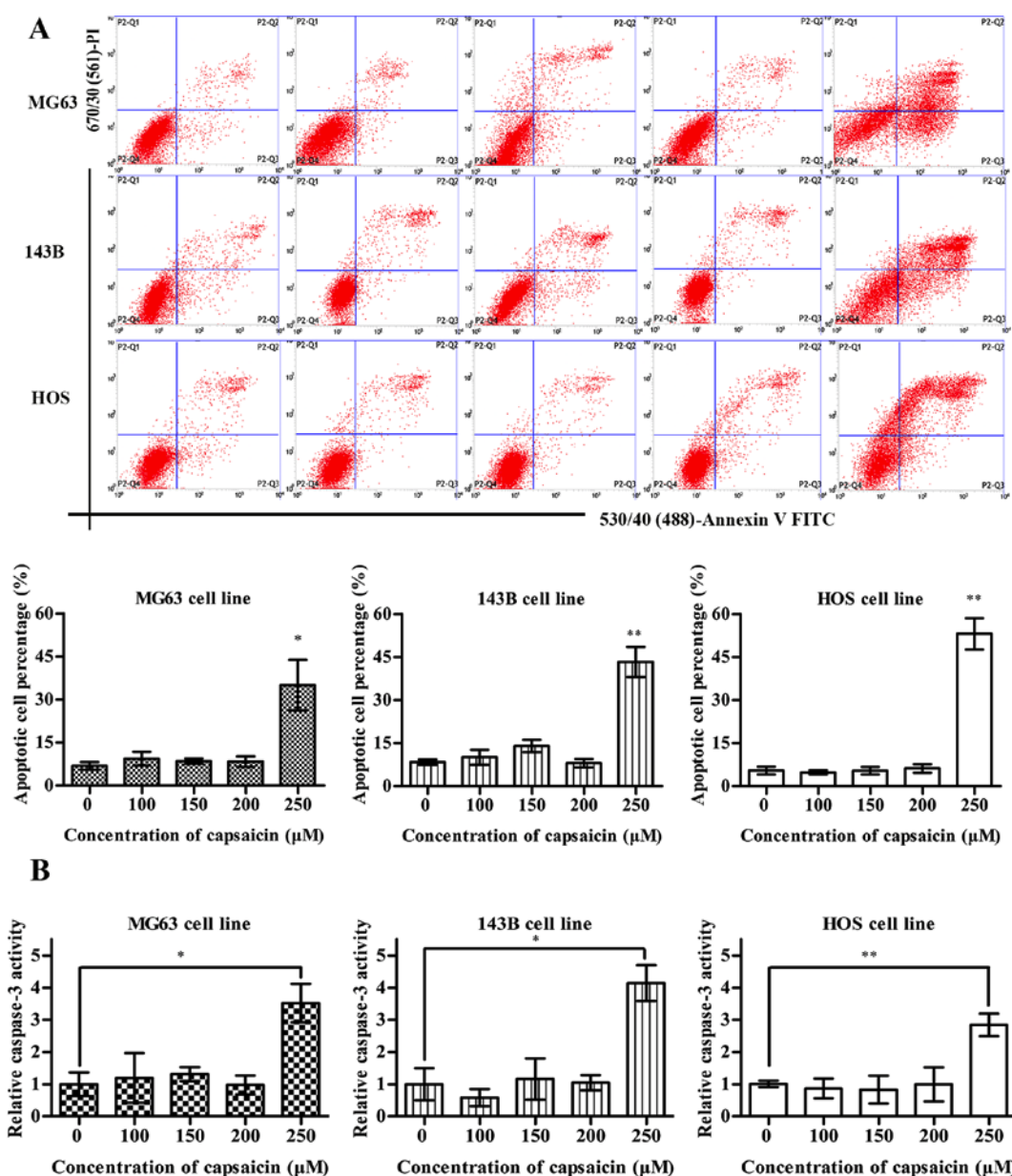


Figure 2. Apoptotic effect of capsaicin on OS cells. (A) Three OS cell lines (MG63, 143B and HOS) were treated with various concentrations of capsaicin (0, 100, 150, 200 or 250 μM) for 24 h and then stained with Annexin V-FITC and PI prior to analysis via flow cytometry. The percentage of apoptotic cells is presented as the mean \pm SD of 3 independent experiments; * $p < 0.05$ and ** $p < 0.01$ vs. the control. (B) Effects of capsaicin on caspase-3 activity in MG63, 143B and HOS cells were determined using a caspase-3 activity assay kit, and the results are expressed as fold changes relative to untreated cells. The data are presented as the mean \pm SD from 3 independent experiments; * $p < 0.05$ and ** $p < 0.01$ vs. the control (0 μM).

Results

Capsaicin decreases the viability of OS cells in a dose-dependent manner. The CCK-8 assay was used to examine the effects of capsaicin on the viability of OS cells *in vitro*. Three OS cell lines (MG63, 143B and HOS) were treated with a wide range of concentrations of capsaicin (0, 50, 100, 150, 200, 250 or 300 μM) for 24 h. As shown in Fig. 1, starting at 100 μM capsaicin, the cell viability progressively decreased with elevated concentrations of capsaicin in all the 3 OS cell lines with a IC_{50} value of ~ 200 μM . These results indicated that capsaicin could decrease the viability of OS cells in a dose-dependent manner, with 100 μM determined as the minimal effective concentration in all 3 OS cell lines.

Capsaicin induces apoptosis in OS cells at a relatively high concentration. To elucidate whether the decrease in OS cell viability caused by capsaicin was associated with apoptosis induction, the number of apoptotic OS cells that were treated with various concentrations of capsaicin (0, 100, 150, 200 or 250 μM) was determined via flow cytometry using Annexin V-FITC/PI double staining. Notably, OS cell apoptosis was only observed at relatively high concentrations of capsaicin (starting at 250 μM) in all 3 OS cell lines. In addition, no apoptotic effects were observed at capsaicin concentrations between 0 and 200 μM (Fig. 2A). As caspases are proteolytic enzymes that critically mediate apoptosis, the increased caspase activity results in cell apoptosis, with caspase-3 activation as an ultimate executioner of apoptotic

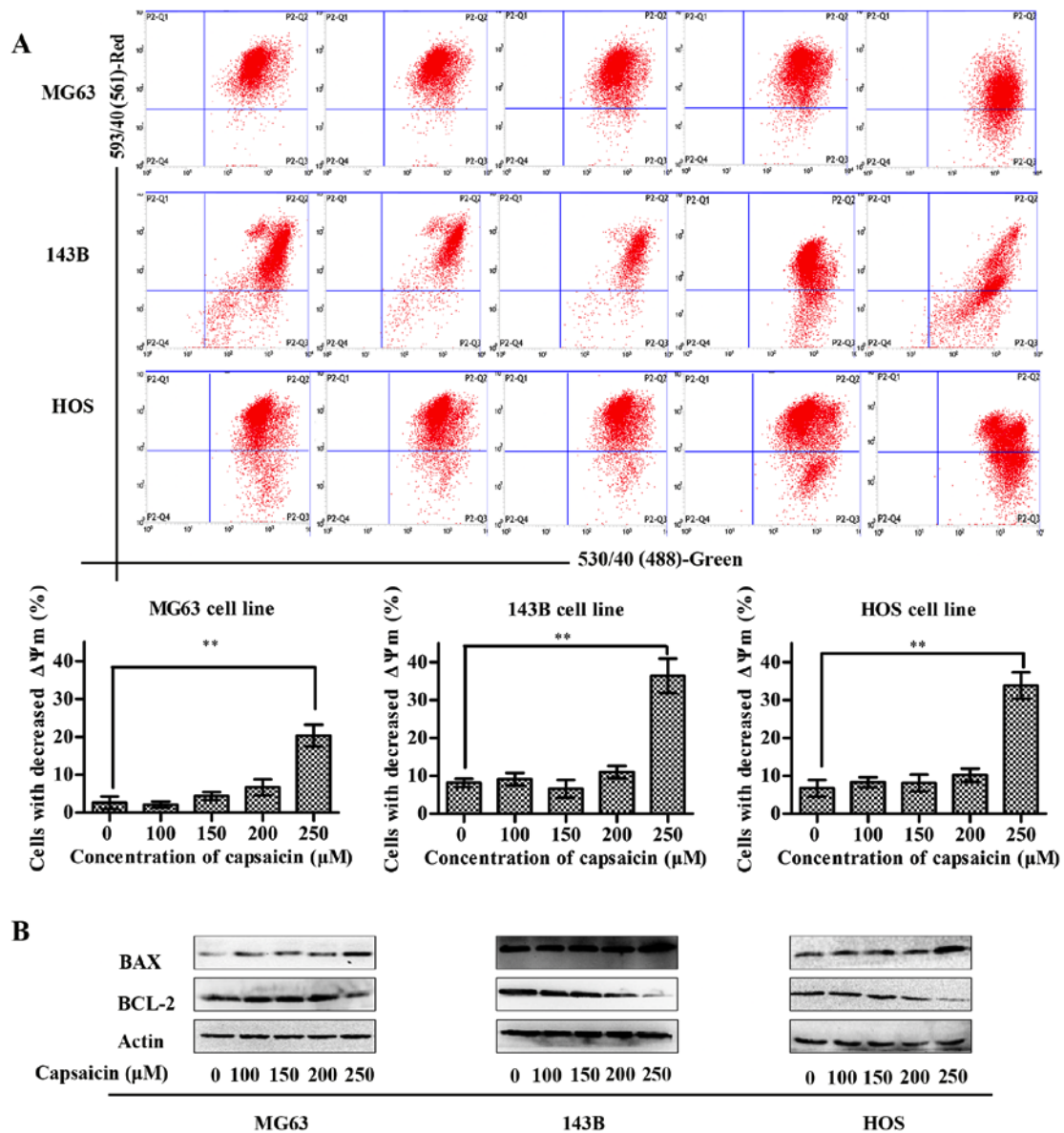


Figure 3. The mitochondrial apoptosis pathway is involved in capsaicin-induced apoptosis in OS cells. (A) Three OS cell lines (MG63, 143B and HOS) were treated with various concentrations of capsaicin (0, 100, 150, 200 or 250 μM) for 24 h. The $\Delta\Psi\text{m}$ was measured using the JC-1 fluorescent probe and assessed via flow cytometry. The chart illustrates the percentage of all 3 cell lines presenting a decrease in the $\Delta\Psi\text{m}$. The data are presented as the mean \pm SD of 3 independent experiments; ** $p < 0.01$ vs. the control. (B) OS cells were treated with various concentrations of capsaicin (0, 100, 150, 200 or 250 μM) for 24 h. Cell lysates were prepared to assess Bax and Bcl-2 protein expression via western blotting. The data are representative of 3 independent experiments.

pathways. As shown in Fig. 2B, only treatment with the highest tested concentration of capsaicin (250 μM) for 24 h resulted in elevated caspase-3 activities in the OS cell lines, which were 3.5-, 4- and 3-fold higher compared to the control treatment (0 μM) in MG63, 143B and HOS cells, respectively. Meanwhile, when cells were treated with low concentrations of capsaicin (between 100 and 200 μM) for 24 h, no obvious increase in activated caspase-3 was observed in all 3 OS cell lines. In conclusion, these results suggested that high concentrations of capsaicin could modulate the activation of caspase-dependent apoptotic signaling pathways in OS cells.

The mitochondrial apoptosis pathway is involved in capsaicin-induced apoptosis in OS cells. Caspase-3 can be activated in apoptotic cells both by death receptor-mediated (extrinsic)

and mitochondrial-mediated (intrinsic) pathways (22). To further determine whether capsaicin-induced apoptosis in OS cells is mediated by mitochondrial dysfunction, we measured the mitochondrial membrane potential ($\Delta\Psi\text{m}$) by adding JC-1 dye to the cells and measuring the fluorescence using flow cytometry. An intact mitochondrial membrane allows for JC-1 accumulation in the mitochondria, which may emit red fluorescence. Conversely, the loss of the $\Delta\Psi\text{m}$ prevents this accumulation, and JC-1 may remain as a monomer in the cytosol and emit green fluorescence (23). Thus, an increase in green fluorescence is indicative of a decrease in $\Delta\Psi\text{m}$. As shown in Fig. 3A, when cells were treated with low concentrations of capsaicin (between 100 and 200 μM) for 24 h, no obvious variations in $\Delta\Psi\text{m}$ were observed compared to the control cells (0 μM capsaicin) in all 3 OS cell lines. However,

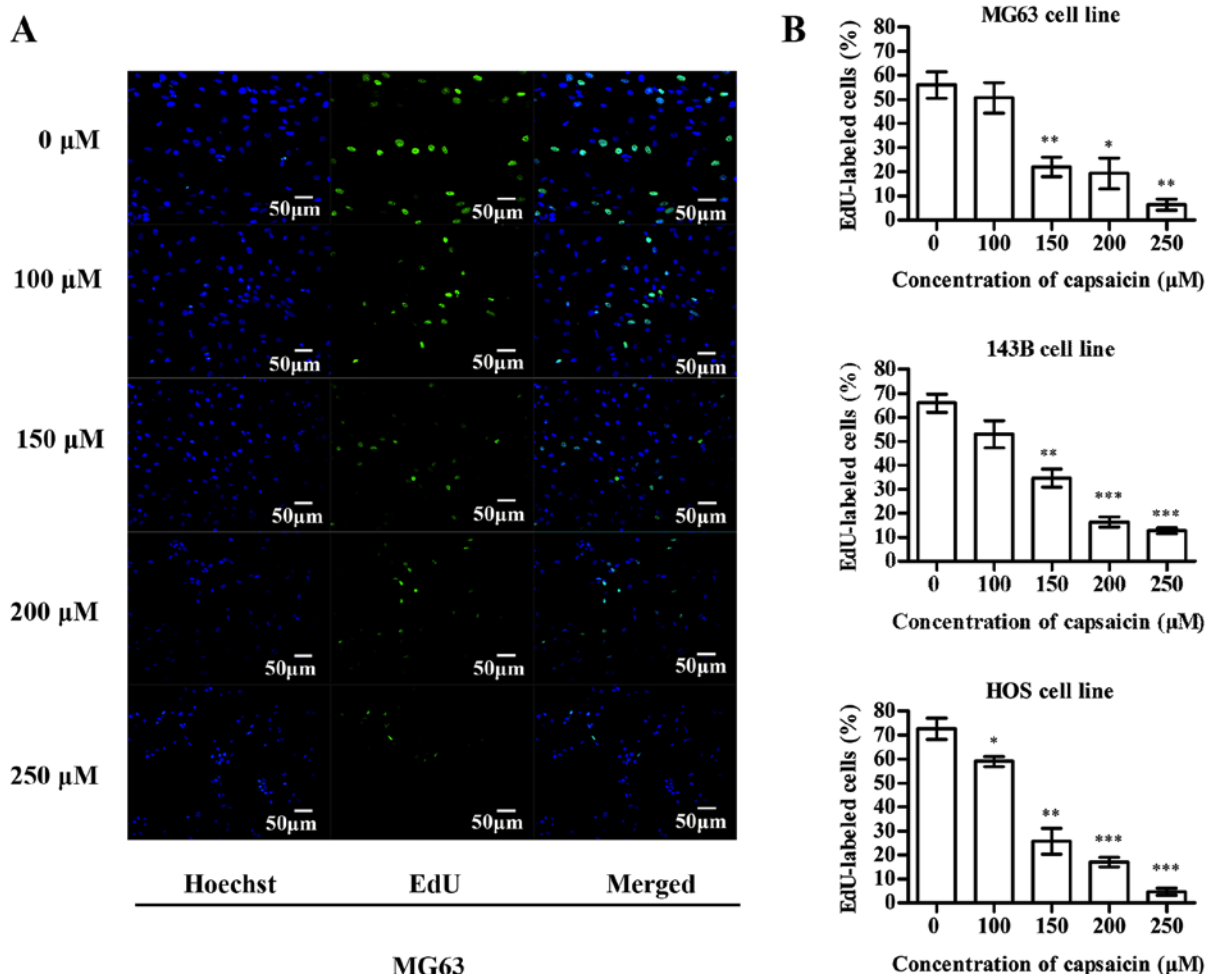


Figure 4. Inhibitory effects of capsaicin on proliferation. (A) The inhibitory effects of capsaicin on MG63 cells were assessed using the EdU proliferation assay. EdU-labeled cells were examined under a fluorescence microscope (MG63). (B) The ratios of EdU-positive cells of all 3 OS cell lines are presented as the mean \pm SD of 3 independent experiments; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. the control (0 μM).

compared to control cells, all 3 OS cell lines treated with 250 μM capsaicin exhibited significant decreases in the $\Delta\Psi\text{m}$. Since the $\Delta\Psi\text{m}$ was reduced in capsaicin-treated cells, we further assessed the Bax and Bcl-2 protein expression, both of which are involved in the mitochondrial apoptotic pathway. As shown in Fig. 3B, compared to the control (0 μM capsaicin), treatment with 250 μM capsaicin for 24 h induced the upregulation of Bax protein and downregulation of Bcl-2 protein in all 3 OS cell lines. However, there were no differences in Bax and Bcl-2 protein expression among the OS cell lines treated with capsaicin at relative low concentrations which were from 0 to 200 μM . Taken together, these data indicated that capsaicin induced OS cell apoptosis starting at the concentration of 250 μM via the mitochondrial apoptotic pathway.

Capsaicin inhibits the proliferation and decreases the colony formation ability of OS cells. The inhibitory effects of capsaicin on OS cell proliferation was detected using the EdU incorporation assay. In MG63 cells (Fig. 4A), compared to the untreated groups (0 μM), the percentage of EdU-labeled cells decreased after treatment with multiple concentrations of capsaicin (between 100 and 250 μM) for 24 h. Similarly, the EdU-labeled 143B and HOS cells showed a dose-dependent decrease after a 24-h treatment with capsaicin (Fig. 4B). These observations

indicated that capsaicin exerted significant inhibitory effects on OS cell proliferation. Furthermore, we determined the tumorigenicity of OS cells using the colony formation assay. After a 24-h treatment with various concentrations of capsaicin, OS cells were cultured for another 10 days, and the number of colonies was counted. As shown in Fig. 5A and B, capsaicin decreased the number of colonies in a dose-dependent manner starting at 100 μM in all the 3 OS cell lines.

Capsaicin induces cell cycle arrest at the G0/G1 phase in OS cells. Cell proliferation is a complicated process closely related to cell cycle progression. To further investigate whether modulating the cell cycle is responsible for capsaicin-mediated cell growth inhibition, we analyzed the number of cells at the G0/G1, S and G2/M phases using flow cytometry. As shown in Fig. 6A and B, after treatment with various concentrations of capsaicin for 24 h, the number of cells in the G0/G1 phase was increased in accordance with a decrease in cell number in the S phase in all 3 OS cell lines. In MG63 cells, the percentage of control cells (0 μM) in G0/G1 was $38.32 \pm 2.03\%$, which increased to 44.9 ± 2.16 , 54.12 ± 0.90 , 59.93 ± 2.52 and $68.56 \pm 2.30\%$ after treatment with 100, 150, 200 and 250 μM capsaicin, respectively, for 24 h. Meanwhile, the percentage of cells in the S phase showed a corresponding decrease

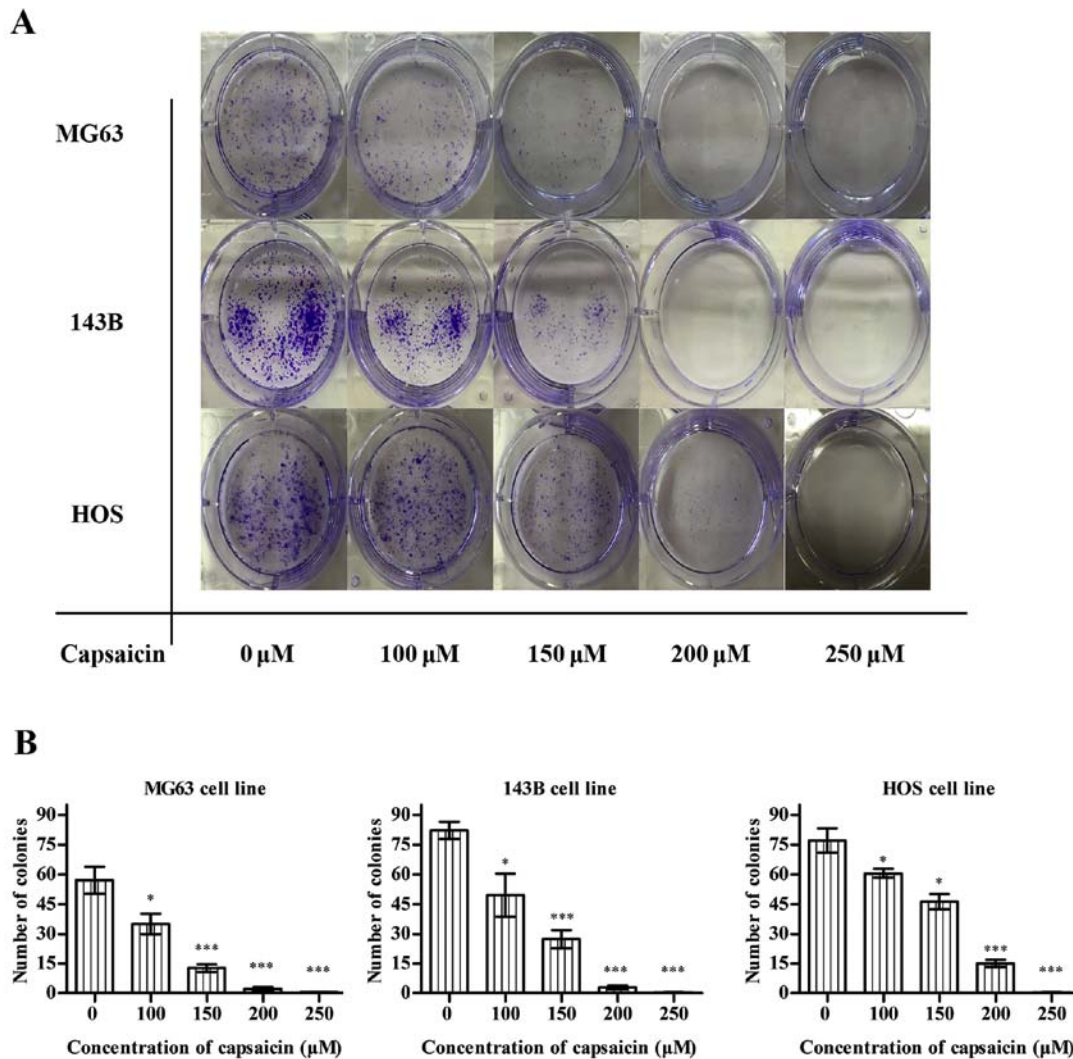


Figure 5. Capsaicin decreases the colony formation ability of OS cells. (A) The colony formation assay was performed in all 3 OS cell lines (MG63, 143B and HOS). (B) The numbers of colonies were counted under a microscope. The data are presented as the mean \pm SD of 3 independent experiments. * $p < 0.05$ and *** $p < 0.001$ vs. the control (0 μ M).

from 52.38 ± 2.01 to 47.95 ± 1.21 , 33.29 ± 0.99 , 32.81 ± 1.80 and $27.44 \pm 2.32\%$ after treatment with 100, 150, 200 and 250 μ M capsaicin, respectively. Capsaicin treatments in the other 2 OS cell lines (143B and HOS) showed similar outcomes. PCNA and P21 have been demonstrated as important cell cycle regulatory factors. P21 is a cofactor that interacts with PCNA to influence cell cycle regulation (24). To investigate whether the observed capsaicin-induced G0/G1 arrest was caused by any interaction with PCNA and/or P21, western blotting was performed to detect changes in PCNA and P21 expression. In the OS cell lines, the expression of PCNA was decreased with the increasing capsaicin concentrations compared to the control cells. Conversely, P21 expression was slightly increased in cells treated with 100 μ M capsaicin and showed further significant increases with higher capsaicin concentrations. Furthermore, similar changes in P21 and PCNA expression were also observed in the other 2 OS cell lines (Fig. 6C and D). Taken together, these data indicated that capsaicin inhibited OS cell proliferation by inducing cycle arrest at the G0/G1 phase, which may involve the regulation of P21 and PCNA.

MAPK signaling pathways are involved in the capsaicin-induced toxic effect on OS cells. MAPK signaling pathways are important for tumor development, and regulating these pathways has been previously demonstrated to affect capsaicin-induced anticancer activity (25). Thus, we determined whether MAPKs were implicated in the inhibitory effects of capsaicin in OS cells (Fig. 7A and B). In the 3 OS cell lines, the levels of total ERK1/2, p38 and JNK were not altered in cells subjected to capsaicin treatment compared to untreated cells. However, treatment with various concentrations of capsaicin resulted in significant decrease in p-ERK1/2 and p-p38 in a dose-dependent manner, which corresponded to the inhibitory effects of capsaicin on proliferation observed in the present study. In contrast, the p-JNK levels remained at basal levels in the presence of up to 200 μ M capsaicin, but these levels significantly increased at a concentration of 250 μ M in all 3 OS cell lines, which corresponded to the capsaicin-induced apoptotic effects observed in the present study. Taken together, these results demonstrated that the capsaicin-induced effects on OS cells may involve the regulation of MAPK pathways.

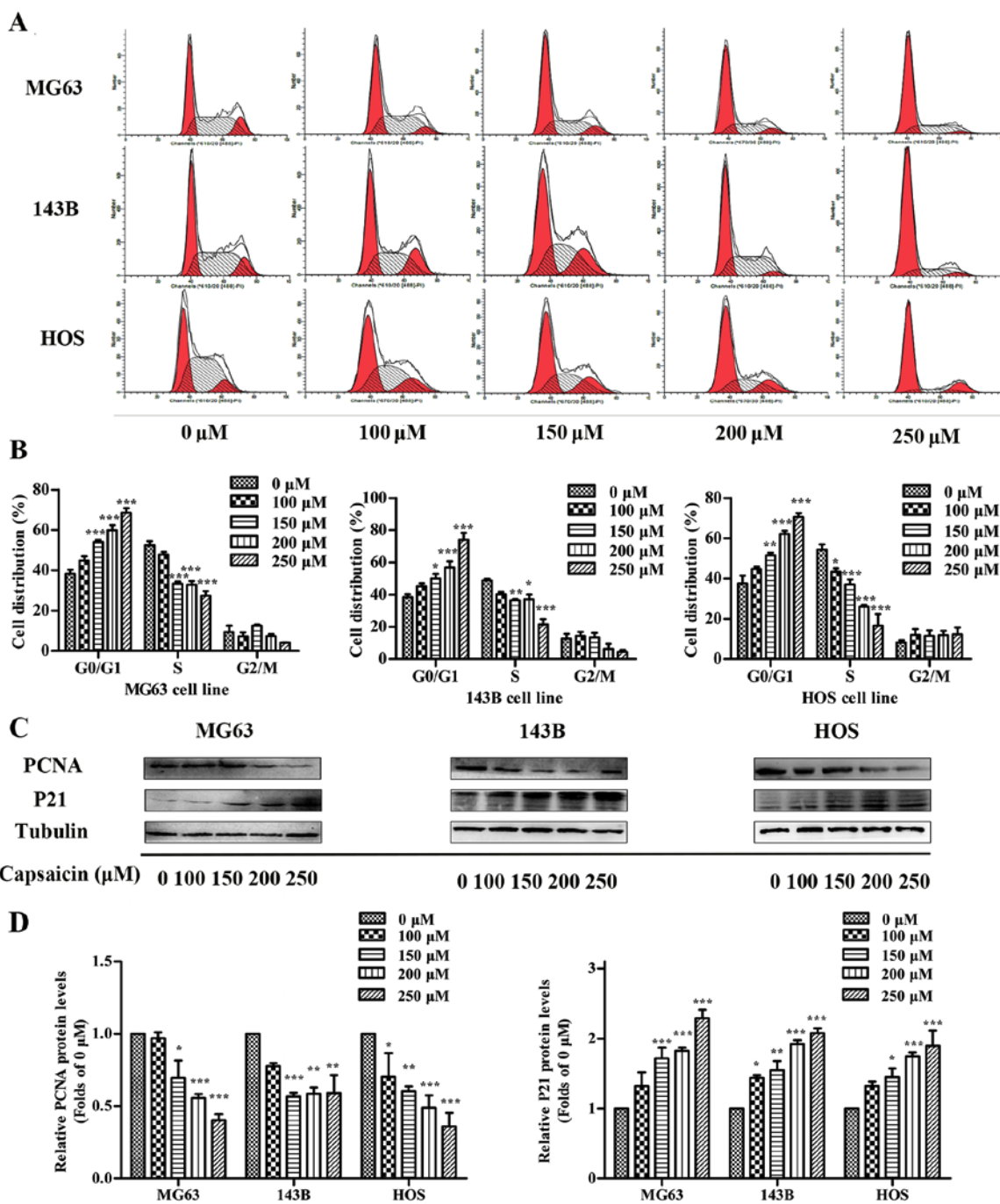


Figure 6. Effects of capsaicin on cell cycle distribution in OS cells. (A) Three OS cell lines (MG63, 143B and HOS) were treated with various concentrations of capsaicin (0, 100, 150, 200 or 250 μM) for 24 h, stained with PI and analyzed using flow cytometry. (B) The percentage of cells in each phase is presented as the mean \pm SD of 3 independent experiments; * p <0.05, ** p <0.01 and *** p <0.001 vs. the control (0 μM). (C) OS cells were treated with various concentrations of capsaicin (0, 100, 150, 200 or 250 μM) for 24 h. Cell lysates were prepared to assess PCNA, P21 and tubulin (loading control) protein expression levels via western blotting. (D) The results are shown as the fold changes in the levels of PCNA and P21 normalized to the level of actin. The data are presented as the mean \pm SD of 3 independent experiments; * p <0.05, ** p <0.01 and *** p <0.001 vs. the control (0 μM).

Capsaicin inhibits tumor growth in a xenograft model of human OS. We evaluated the *in vivo* antitumor potential of capsaicin using an OS xenograft model. HOS cells were subcutaneously implanted in nude mice, and the tumor volumes were measured every 3 days. As shown in Fig. 8A, the capsaicin-treated group exhibited significantly smaller OS tumors than the control group. No significant difference in body weight was observed during the experimental period

between the control and capsaicin-treated groups (Fig. 8B). At the end of the experiment, the tumor weight measurements indicated that capsaicin significantly decreased the tumor weight compared to that in tumors from the control group (Fig. 8C). Furthermore, the proliferation indices (as indicated by PCNA and Ki67 expression) were lower in tumor specimens from the capsaicin-treated group than those from the control group (Fig. 8D). These findings

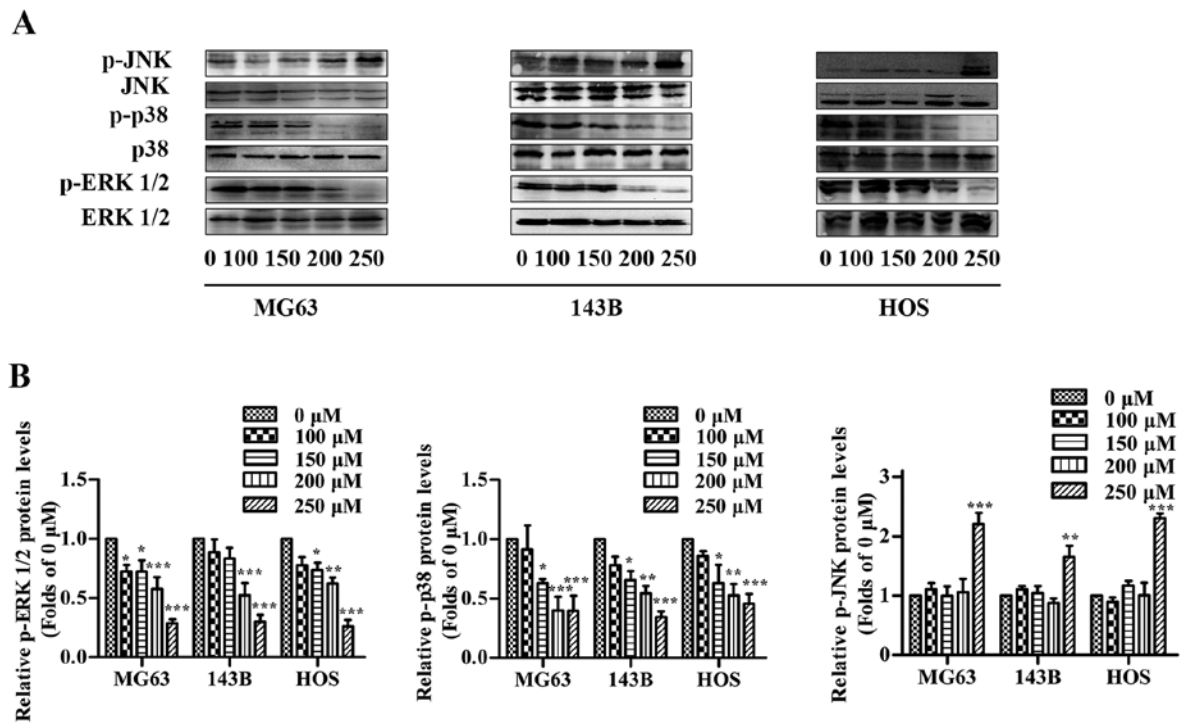


Figure 7. Effects of capsaicin on MAPK signaling pathways in OS cells. OS cells were treated with various concentrations of capsaicin (0, 100, 150, 200 or 250 μM) for 24 h. (A) Cell lysates were prepared to assess the expression levels of p-ERK1/2, ERK1/2, p-p38, p38, p-JNK and JNK using western blotting. (B) The results are shown as the relative levels of p-ERK1/2, p-p38 and p-JNK to ERK1/2, p38 and JNK, respectively. The data are presented as the mean \pm SD of 3 independent experiments; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs. the control (0 μM).

indicated that capsaicin efficiently suppressed OS tumor growth *in vivo*.

Discussion

It is widely accepted that eliminating cancer cells by inducing cell apoptosis is a crucial element in chemotherapy (26,27). Capsaicin is an active molecular component of hot chili peppers, and significant evidence has shown that capsaicin can trigger apoptosis in numerous cancer cell lines. In addition, the significant concentration of capsaicin that could trigger apoptotic cell death for most cancer cells varied between 200 and 300 μM (28). Lee *et al* (29,30) reported that the prominent apoptotic effect of capsaicin on A172 human glioblastoma cells and HepG2 human hepatoma cells were initially observed at concentrations of 200 and 250 μM , respectively. In the present study, we investigated capsaicin-induced apoptosis in osteosarcoma (OS) cells using 2 independent methods: detection of phosphatidylserine (PS) translocation through Annexin V/PI double staining and measurement of caspase-3 activation. Our results showed that capsaicin-induced apoptosis was observed at a concentration of 250 μM in all 3 tested OS cell lines; these data were in accordance with previous results in other human cancer cells. Furthermore, the $\Delta\Psi\text{m}$ of OS cells was decreased after treatment with 250 μM capsaicin, which were coincident with the apoptosis results. Together with the observed upregulation of Bax and simultaneous downregulation of Bcl-2 in OS cells after treatment with 250 μM capsaicin, our results indicated that capsaicin could induce apoptosis in OS cells through the intrinsic pathway starting at a concentration of 250 μM .

Most studies exploring the toxicity of capsaicin in OS cells have focused on the mechanisms underlying capsaicin-induced apoptosis (18,20). In addition, numerous studies have reported that the capsaicin-induced anticancer effects are primarily dependent on apoptotic machinery. Nevertheless, apoptosis induction by capsaicin cannot be considered as a default pathway, particularly since defective apoptosis is considered a major hallmark of cancer cells (31). Moreover, the apoptotic effects induced by capsaicin were usually observed at high concentrations. Thus, it is likely that capsaicin may work through other pathways to exert its anticancer effects on cancerous cells. Based on our results, capsaicin-associated toxicity in OS cells was not completely coincident with apoptotic effects, which began to manifest at a concentration of 250 μM . Indeed, the results of the CCK assay indicated that capsaicin decreased the viability of OS cells in a dose-dependent manner from 0 to 300 μM with an IC_{50} value of ~ 200 μM in all the 3 OS cell lines. Specifically, the ability of capsaicin to reduce the CCK-8 value in the cells may merely reflect its negative effects on mitochondrial metabolic activity in the cell, which can be influenced by many factors such as cell number, viability, proliferation and apoptosis. Therefore, the decrease in OS cell viability in the absence of apoptosis induction upon treatment with relatively low concentrations of capsaicin may involve some other mechanisms.

Along with the extensive research available on the anticancer effects of capsaicin, recent literature has indicated that cell survival mechanisms may be activated in cancer cell lines subject to capsaicin treatment. Lewinska *et al* (32) reported that capsaicin did not trigger apoptosis in prostate carcinoma cells (DU145) and lung carcinoma cells (A549) at doses up to

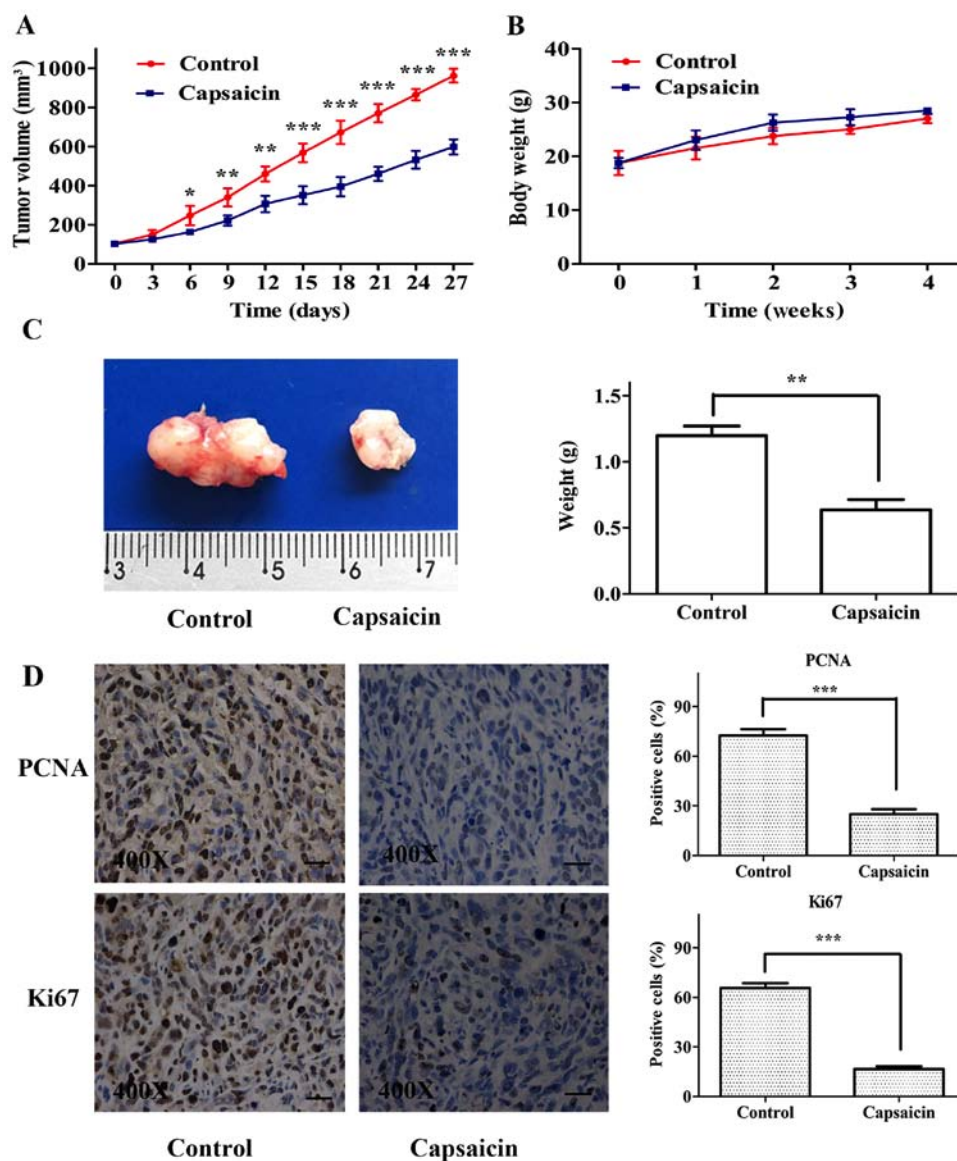


Figure 8. Capsaicin suppresses the growth of human OS xenograft tumors *in vivo*. Nude mice bearing HOS human OS xenograft tumors were treated with either vehicle (control group) or capsaicin (capsaicin group). (A) The volumes of the xenograft tumors were measured and compared at the indicated time points. (B) The weight of each nude mice was measured and compared at the indicated time points. (C) At week 4 after treatment initiation, the xenograft tumors were retrieved from the mice, and the tumor weight was measured and compared. (D) Xenograft tumors were stained with PCNA and Ki67 antibodies using IHC (original magnification, x400). The data are presented as the mean \pm SD of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs. control.

250 μ M. In addition, they demonstrated that the DNA damage response may be involved in the resistance of DU145 and A549 cancer cells to apoptosis. Furthermore, Choi *et al* (17) reported that capsaicin did not induce apoptosis in the breast cancer cell lines MCF7 and MDA-MB-231, and they also confirmed that capsaicin-induced autophagy associated with endoplasmic reticulum stress may play a critical role in impeding cell death. Chien *et al* (18) suggested that an anti-apoptotic pathway was activated in parallel with pro-apoptotic pathway and was mediated by autophagy in G292 OS cells. In conclusion, capsaicin may exert dual effects on cancer cells in a dose-dependent manner. The explicit protective mechanisms involved in promoting OS cell survival in response to low concentrations of capsaicin need to be elucidated in future studies. In addition to these above mentioned protective mechanisms (which have yet to be proven), the results from the present study showed that the decrease in OS cell viability at low concentrations

of capsaicin was not the result of apoptotic cell death. Thus, we speculated that a capsaicin-mediated decrease in metabolic activity at lower concentrations (as estimated using the CCK-8 assay) may reflect a cytostatic function of capsaicin rather than its ability to stimulate apoptosis. We further investigated whether capsaicin could inhibit the growth of OS cells. Since inhibition of DNA synthesis in cancer cells can be quantified by EdU incorporation into cancer cells, which correlates with cell proliferation (33), we conducted the EdU label assay to determine the proliferation status of OS cells. After treatment with 100, 150, 200 or 250 μ M of capsaicin for 24 h, the number of EdU-labeled cells in the treated groups decreased significantly in a dose-dependent manner. Unlike the capsaicin-induced apoptotic effects in OS cells, which only occurred at high concentrations of capsaicin, these results indicated that the capsaicin-induced loss of OS cell viability was primarily due to the inhibition of proliferation rather than

cell apoptosis, particularly in cells treated with a relatively low concentration of capsaicin.

The ability of cancer cells to form colonies is essential for the metastasis of a malignant tumor to distant organs (34). Therefore, we investigated the colony forming ability of the 3 OS cells and observed that capsaicin treatment significantly reduced the number of colonies in a dose-dependent manner compared with the untreated cells; these data highlight the great antiproliferative effects of capsaicin on OS cells.

Cells proliferate through the cell cycle, which is divided into the G0/G1, S and G2/M phases. Throughout the cell cycle, DNA checkpoints ensure the integrity of DNA replication. Increasing evidence has indicated that capsaicin leads to DNA damage, resulting in cell cycle modulation (35). To the best of our knowledge, there are few studies concerning capsaicin-induced cell cycle arrest in OS cells. Jin *et al* (36) reported that capsaicin exerted G0/G1 cell cycle arrest in human colon cancer cells. In contrast, Lin *et al* (37) observed that capsaicin increased the number of human KB cancer cells in G2/M phase. These inconsistent findings on capsaicin-induced cell cycle arrest in cancer cells suggested that the mode of this activity depends on the phenotypic and genotypic diversity of different cancer cells. To address the OS cell cycle arrest that may account for capsaicin-induced proliferation inhibition, we investigated the cell cycle distribution of OS cells treated with capsaicin. Our results demonstrated for the first time that capsaicin induced G0/G1 arrest in 3 human OS cell lines in a dose-dependent manner, which was consistent with the inhibitory effects on proliferation. Furthermore, previous studies have also shown that capsaicin-induced G0/G1 arrest in other cancer cells occurs via induction of the cyclin-dependent kinase inhibitor P21 (38,39). Tong *et al* (40) reported that transfection of an antisense RNA targeting PCNA resulted in growth inhibitory effects and G0/G1 phase arrest in bladder cancer. Next, we performed western blotting to determine whether capsaicin modulated the OS cell cycle by regulating P21 and PCNA. Congruously, our results showed that P21 expression was increased and associated with a decrease in PCNA expression in all 3 OS cell lines in a dose-dependent manner, which may be related to capsaicin-induced cell cycle arrest.

The MAPK family plays a critical role in OS cell survival, proliferation and angiogenesis. In addition, some other studies showed that capsaicin could alter the MAPK pathways in cancer cells (25,41). Based on previous studies, we hypothesized that capsaicin may mediate its anti-OS effects by regulating MAPK signaling pathways. In the present study, all 3 cell lines exhibited p-ERK1/2 and p-p38 downregulation after capsaicin treatment in a dose-dependent manner, whereas p-JNK was increased significantly only at a concentration of 250 μ M. The ERK1/2 signaling pathway is known to contribute to cell proliferation, which is also partially involved in G0/G1 cell cycle arrest (42,43). Previous studies have reported that capsaicin induced anticancer effects on fibrosarcoma cells partially by suppressing ERK1/2 phosphorylation in human fibrosarcoma cells (44). Consistent with previous findings, our results indicated that capsaicin significantly inhibited ERK1/2 phosphorylation, which was correlated with a decrease in cell viability, inhibition of proliferation and induction of G0/G1 cycle arrest. In contrast, regulation of p38 phosphorylation was

diverse among different cancer cell lines treated with capsaicin. Park *et al* (25) reported that capsaicin suppressed the phosphorylation of p38 in human AGS gastric cancer cells. However, Liu *et al* (41) demonstrated that capsaicin mediated its anticancer effects by activating p38 in human renal carcinoma. In the present study, capsaicin significantly inhibited the phosphorylation of p38 in all 3 OS cell lines, which was also correlated with the capsaicin-induced antiproliferative effects on OS cells. Furthermore, the JNK signaling pathway has been associated with apoptosis in tumor cells, including OS (45,46). In the present study, the p-JNK levels were significantly elevated after treatment with 250 μ M capsaicin, which was correlated with the capsaicin-induced apoptotic effects in all 3 OS cell lines; these data suggest that capsaicin-induced OS cell apoptosis is at least partially involved in activating the JNK signaling pathway. Our results are consistent with those of previous findings that capsaicin could increase JNK phosphorylation and thus promote apoptosis in cancer cells (41,47). Taken together, the findings in the present study strongly suggest that inactivation of ERK1/2 and p38 plays an important role in capsaicin-induced inhibition of proliferation and cell cycle arrest in OS cells, whereas JNK activation participated in capsaicin-induced apoptosis.

Finally, the xenograft tumor model was used to investigate the inhibitory effects of capsaicin on OS cells *in vivo*. Our results demonstrated that capsaicin possessed potent antiproliferative effects on HOS xenograft tumors. Moreover, capsaicin administration did not significantly affect the body weight of mice, which indicated that capsaicin may be a relatively effective and safe agent against OS.

In conclusion, our results showed that capsaicin has profound *in vitro* and *in vivo* antiproliferative effects against human OS cells. Moreover, MAPK signaling pathways were involved in the anti-OS effects induced by capsaicin. The inactivation of ERK1/2 and p38 may participate in the capsaicin-induced inhibition of proliferation in OS cells starting at a concentration of 100 μ M. However, capsaicin-induced apoptosis in OS cells, which was observed at a dose of 250 μ M, may be involved in JNK activation. These results may enrich our understanding of the mechanisms that mediate the capsaicin-induced anti-OS effects. In addition, the capsaicin-induced apoptotic effects in OS cells were only observed upon treatment with a relatively high concentration of capsaicin. Nevertheless, the inhibition of proliferation and cell cycle arrest induced by capsaicin could be observed at lower concentrations. Thus, these results strongly indicate that capsaicin may exert more therapeutic benefits as an adjunct to current cancer therapies rather than as an independent anticancer agent.

Acknowledgements

This study was supported by the Chongqing Science and Technology Committee foundation (cstc2017jcyjB0312).

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