Mild microwave ablation combined with HSP90 and TGF-β1 inhibitors enhances the therapeutic effect on osteosarcoma

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Abstract. Osteosarcoma is the most common malignant bone tumour and the second leading cause of cancer-related death in children and adolescents. Microwave ablation has an excellent therapeutic effect on bone tumours by instantaneously increasing the temperature in the tumour; however, there is a risk of damaging the surrounding healthy tissues by exposure to a high temperature when the treatment power is too large. In the present study, two anti-tumour reagents, a heat shock protein 90 (HSP90) inhibitor (PF-04929113) and a transforming growth factor-β1 (TGF-β1) inhibitor (SB-525334) were employed to enhance the therapeutic effect of mild-power microwave ablation. It was revealed that microwaving to 48°C combined with HSP90 and TGF-β1 inhibitors significantly increased the apoptotic rate of VX2 cells. The same results were observed during *in vivo* experiments using New Zealand rabbits to model osteosarcoma. In addition, the results indicated that the expression of cytochrome *c*, caspase-3 and caspase-9 were upregulated in response to the treatment, which indicated that the mitochondrial apoptotic signalling pathway had been activated. These findings may provide a novel strategy for the development of microwave ablation in osteosarcoma treatment, which could effectively kill tumour cells without damaging the surrounding normal tissues.

Introduction

Osteosarcoma is the most common malignant bone tumour in children and adolescents (1). Currently, the main treatment of osteosarcoma is surgical resection combined with neoadjuvant chemotherapy (2), but the long-term survival rate of primary osteosarcoma is still only ~67% (3). In recent years, microwave ablation has been successfully applied to the clinical treatment of bone tumours (3). To completely eliminate the tumour, microwave ablation currently requires ablation applied to the tumour safety boundary when treating para-articular tumours (4). However, this procedure has a risk of damaging the surrounding normal tissue when the temperature induced by microwave ablation is too high (5). Therefore, exploring a method that can kill tumour cells and reduce the temperature of the microwave ablation to protect surrounding normal tissues is of great importance for the clinical application of microwave ablation in osteosarcoma.

Tumours synthesize heat shock protein 90 (HSP90) in response to microwave stimulation (6). High expression of HSP90 increases the tolerance of tumour cells to heat and inhibits apoptotic signalling pathways (7). Furthermore, HSP90 is more abundant in tumour cells than in normal cells (8); it can inhibit the apoptosis of tumour cells by participating in various protective signalling pathways, including inhibiting the mitochondrial release of cytochrome *c* (cyto *c*), inhibiting the formation of apoptotic bodies, further enhancing the tolerance of tumour cells to heat and inhibiting tumour necrosis (9). Previous studies have revealed that HSP90 inhibitors are novel and effective anticancer drugs (10,11). Therefore, suppressing the expression of HSP90 in tumours under microwave ablation has the potential to enhance the therapeutic effect of microwave treatment.

Another key signalling molecule in tumour cells is transforming growth factor-β1 (TGF-β1), which can affect the growth, differentiation, metastasis and apoptosis of tumour cells (12). Animal experiments have demonstrated that TGF-β1

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is an effective target for tumour therapy (13,14). Previous studies have reported that small inhibitors of TGF-β1 can block SMAD phosphorylation and nuclear translocation, as well as inhibiting tumour morphological changes, cell proliferation, migration and angiogenesis (15,16). To reinforce the therapeutic effect of microwave ablation, TGF-β1 inhibitors can be combined with it in the treatment of osteosarcoma (17). It has been reported that both HSP90 and TGF-β1 can bind to the same receptor to have synergistic effects on tumour adhesion, migration and other functions (17). In addition, HSP90 inhibitors can suppress TGF-β1 signalling at the receptor level (17) (Fig. 1).

In the present study, microwave ablation was combined with HSP90 and TGF-β1 inhibitors to treat osteosarcoma (Fig. 2). To the best of our knowledge, this is a rare method of applying microwave ablation in the treatment of tumours. Based on previous findings concerning cell tolerance of hyperthermia (18), four microwave ablation temperatures (37, 41, 48 and 60˚C) were set in this study. It was discovered that the apoptotic rate of VX2 cells was significantly increased after microwaving at 48˚C combined with TGF-β1 and HSP90 inhibitors. The *in vivo* experiments also showed the same results. In addition, it was demonstrated that the expression of cytokines in the apoptotic signalling pathway were increased. Collectively, these findings may provide a mild microwave ablation method that can kill tumour cells while avoiding damage to the surrounding normal tissue.

Materials and methods

Cell culture. The rabbit squamous cell carcinoma VX2 cells (American Type Culture Collection) and the rabbit bone marrow mesenchymal stem cells (R-BMSCs; iCell Bioscience, Inc., cat. no. s018.) were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.), 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin solution. The cells were cultured in an incubator at 37˚C and 5% CO_2. When the cells occupied 90-95% of the of the bottom of the culture flask, they were digested and prepared in suspension with a density of $1x10^4$ cells.

After all cells were adhered to the plate for 20 h, fresh culture medium was added with one of the following conditions: 37 nM HSP90 inhibitor (PF-04929113; p) alone; 14.3 nM TGF-β1 inhibitor (SB-525334; s) alone; or both 37 nM PF-04929113 and 14.3 nM SB-525334 (p+s). The inhibitors were purchased from Gibco; Thermo Fisher Scientific, Inc. Culture medium with no inhibitors was regarded as the control group.

Temperature rise curve. In each well of 48-well culture plates, 500 μl DMEM was added, after which a microwave needle fixed with a temperature measuring probe was inserted into the well; the temperature measuring probe was the same type of microwave temperature measuring probe used in the clinical treatment of bone tumours. The microwave therapy device was adjusted to 15 W under physiotherapy mode, and the starting microwave temperature of each well was 37˚C. The microwave time was set at 15, 20, 25, 30, 40, 50 and 60 sec. The microwave treatment was repeated three times for each treatment time, the temperature of the DMEM was recorded, and the time-temperature curve was plotted.

MTT assay. The VX2 cell suspension density was $7x10^4$ cells/ml and 500 μl suspension was added to each well of a 48-well plate. After incubation for 20 h in a 37˚C constant temperature incubator, fresh culture medium was added with the inhibitors (as aforementioned). After co-culture with inhibitors for 4 h, each group was heated by microwave to 37, 41, 48 and 60˚C. The R-BMSCs were used to repeat the aforementioned procedure and were microwaved to 48˚C. After 4 h, the cells were incubated with 5 mg/ml MTT solution in a 37˚C incubator for 4 h, and the crystal violet was dissolved with DMSO solution. The optical density values at 490 nm were measured using a microplate reader. The cell survival rates were calculated by reference to the control group at 37˚C. Each experimental condition was repeated three times.

Calcein/PI staining. After the cells were cultured with the inhibitors and heated, the supernatant was discarded, and 200 μl calcein/PI (Gibco; Thermo Fisher Scientific, Inc.) solution was added to each well. After 30 min incubation at 37˚C, the cell viability was observed under a fluorescence inverted microscope (Olympus Corporation) and images (magnification, x200) were captured.

Flow cytometry. Apoptosis of cells was tested in the following groups: Microwave alone (M); microwave + 37 nM PF-04929113 (M+p); microwave + 14.3 nM SB-525334 (M+s); and microwave + 37 nM PF-04929113 + 14.3 nM SB-525334 (M+p+s) groups. Following addition of the inhibitors, the groups received microwave ablation with a power of 15 W and a duration time of 40 sec. Subsequently, the cells were collected, washed twice with cold PBS, stained with Annexin V-FITC and PI (BD Biosciences, Inc.) for 30 min at 4˚C in the binding buffer and analysed by flow cytometry (FACSCalibur flow cytometer; BD Biosciences) and FlowJo software 10.6 (FlowJo LLC).

Cellular immunofluorescence assay. The cells in the control, M, M+p, M+s and M+p+s groups were fixed with 4% paraformaldehyde for 15 min at room temperature. After washing twice with PBS, cells were permeabilized using a 0.2% Triton X-100 solution and subsequently blocked with 3% BSA (neoFroxx GmbH) for 30 min at room temperature. Next, the cells were incubated for 8 h at 4˚C with primary antibodies against TGF-β1 (cat. no. sc-130348, 1:1,000), SMAD4 (cat. no. sc-73040, 1:1,500), HSP90 (cat. no. sc-101494, 1:1,000), caspase-3 (cat. no. sc-56046, 1:1,500), caspase-9 (cat. no. sc-56076, 1:1,000) and cyto *c* (cat. no. sc-75806, 1:1,000 all from Santa Cruz Biotechnology, Inc.), washed with PBS and incubated for 1 h at 37˚C with Alexa Fluor® 488 goat anti-rabbit IgG secondary antibody (1:1,000; cat. no. A32731, Invitrogen, Thermo Fisher Scientific, Inc.). Nuclei were stained using DAPI for 5 min at 37˚C. Images (magnification, x400) were acquired using a confocal laser-scanning microscope (Olympus Corporation).

Western blotting analysis. The cells in the control, M, M+p, M+s and M+p+s groups were lysed on ice with RIPA (Thermo Scientific, Inc.) for 30 min to extract the total protein. Protein concentration was assessed using a BCA protein assay kit. Protein (60 μg) from each sample were separated by
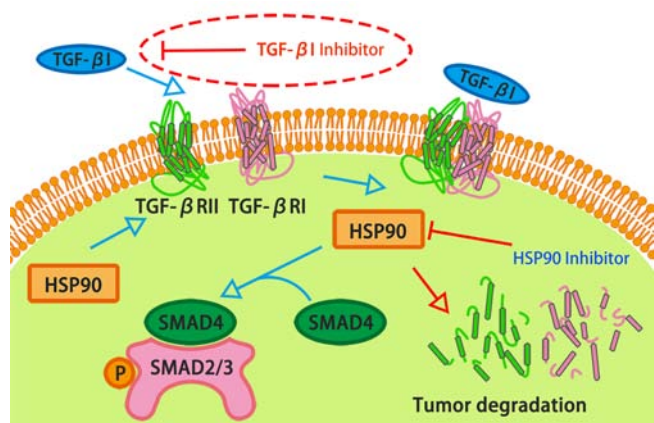
Figure 1. Schematic diagram of HSP90 inhibitor combined with TGF-β1 inhibitor promoting the tumour apoptosis-signalling pathway. HSP90, heat shock protein 90; TGF-β1, transforming growth factor-β1; SMAD, SMAD family member.
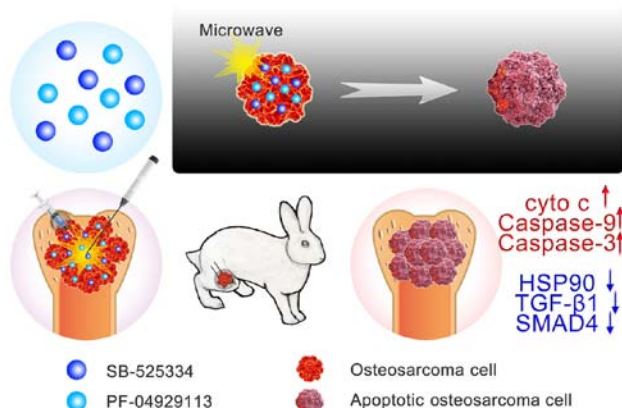

Figure 2. Schematic illustration of mild microwave ablation combined with HSP90 and TGF-β1 inhibitors. HSP90, heat shock protein 90; TGF-β1, transforming growth factor-β1; SMAD4, SMAD family member 4; cyto *c*, cytochrome *c*.

SDS-PAGE using a 10% acrylamide gel and transferred to PVDF transfer membranes (EMD Millipore). Membranes were blocked for 1 h at room temperature with 5% non-fat dried milk in TBS containing 0.05% Tween-20 (TBST) buffer and incubated overnight at 4˚C with primary antibodies against TGF-β1 (cat. no. sc-130348, 1:2,000), SMAD4 (cat. no. sc-73040, 1:2,000), HSP90 (cat. no. sc-101494, 1:1,000), caspase-3 (cat. no. sc-56046, 1:1,100), caspase-9 (cat. no. sc-56076, 1:1,000) and cyto *c* (cat. no. sc-75806, 1:1,000 all from Santa Cruz Biotechnology, Inc.). After washing three times for 5 min in TBST buffer, the membranes were incubated with the HRP-Goat Anti-Mouse IgG (Jackson ImmunoResearch Laboratories, Inc., cat. no. 115-035-003) for 30 min at room temperature. Protein bands were detected with Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore) and analysed with the Bio-Image Analysis system: BOX F3 (Syngene) and ImageJ v1.8.0 (National Institutes of Health).

Establishment of an osteosarcoma model. All animal studies were approved by the Institutional Animal Care and Use Committee of Guangzhou General Hospital of Guangzhou Military Command of PLA (Guangzhou, China). Rabbits (n=31) were housed individually in stainless steel cages and maintained with free access to both food and water. The rabbits were housed in an environmentally controlled breeding room, with at least 10 air changes per hour. The animal feeding room was maintained between 18-26˚C and 30-70% relative humidity. Under the control of a timer, a12-h light/dark cycle was maintained. VX2 cells were suspended at a concentration of $1x10^7$ cells/ml. The tibial tuberosity of a New Zealand rabbit was exposed, 1 ml bone marrow was extracted and then 1 ml VX2 cell suspension was injected into the bone marrow cavity. After formation of the tumours, the tumour tissue was removed and cut into tumour tissue blocks (volume, 0.5 mm^3). Subsequently, the other 30 1.5 kg healthy male New Zealand rabbits were anaesthetized by injecting 30 mg/kg 3% pentobarbital into the ear vein. The tumour tissue blocks were transplanted into a femoral condyles bone defect (5 mm in diameter and 5 mm in depth) and the defect was closed with bone wax. The tumour model rabbits were obtained 1 week after implantation.

In vivo treatment. The tumour model rabbits were anaesthetized with 30 mg/kg 3% pentobarbital in the ear vein. Tumor volume was measured by Vernier calipers prior to surgery. The rabbits then received injections of the inhibitors followed by microwave treatment. The groups were: Control (no drugs or microwave treatment); M (microwave only); M+p (microwave plus PF-04929113, 55.5 nM, 100 μl); M+s (microwave plus SB-525334, 21.45 nM, 100 μl); and M+p+s (microwave plus the two inhibitors). Microwave ablation was conducted at a power of 15 W with a duration time of 40 sec under physiotherapy mode. The body weight and tumour sizes of the rabbits were measured every other day for a period of 21 days. Tumour size was measured with calipers and volume was calculated as follows: $V=ab^2/2$; where V (cm^3) is tumour volume, and a (cm) and b (cm) are tumour length and width, respectively. After all experimental data and samples were collected, the rabbits were euthanized by intravenous injection of 100 mg/kg pentobarbital sodium (19), the tumor tissues were <10% the weight of the rabbits at the time of sacrifice.

Haematoxylin and eosin (H&E) staining. The tumour tissues were fixed in 4% paraformaldehyde for >30 min at room temperature, embedded in paraffin and cut into 5-μm slices. The histological morphology of the tumours was acquired with H&E staining for 15 min at room temperature. All samples were observed under a fluorescence microscope (magnification, x400; BX51; Olympus Corporation).

Immunohistochemical staining. After deparaffinization and rehydration, the 5-μm tumor sections were treated with 3% hydrogen peroxide for 15 min at room temperature and blocked with 10% goat serum (Sigma-Aldrich; Merck KGaA) for 20 min at room temperature. Subsequently, they were incubated with primary antibodies in PBS for 8 h at 4˚C. After washing, secondary antibodies were added and incubated for 20 min at room temperature. The antibodies used were identical to the antibodies used in the cellular immunofluorescence assay. Subsequently, HRP-conjugated streptavidin and 3,3'-diaminobenzidine solution were added to the slides and developed for
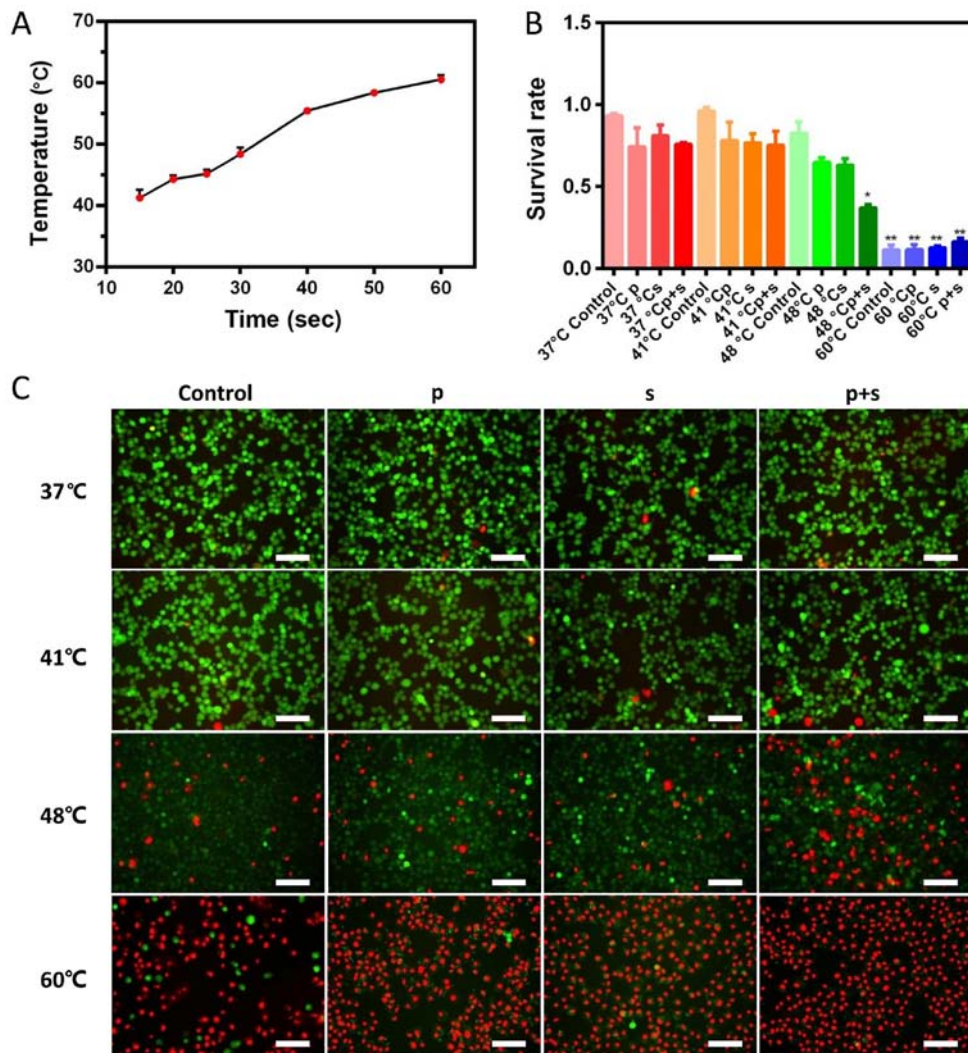
Figure 3. Fluorescent image of microwave ablation combined with inhibitors at the optimal ablation temperature. (A) Time-temperature curve under 15 W microwave ablation (n=3). (B) Effect of microwave ablation combined with inhibitors on the viability of tumour cells, compared with the 37°C control group (n=3). (C) Live VX2 cells stained with Calcein-AM are green and the dead cells are red; scale bar=100 μm. *P<0.05, **P<0.01 vs. 37°C control group. M, microwave; p, PF-04929113; s, SB-525334.

5 min. After rinsing with tap water for 10 min, they were counterstained with haematoxylin for 2 min at room temperature and differentiated with hydrochloric acid alcohol. All samples were observed under a fluorescence microscope (magnification, x400; BX51; Olympus Corporation). Image-Pro Plus 6.0 image analysis software (Media Cybernetics, Inc.) was used to analyse the integrated optical density (IOD) value of each image.

Statistical analysis. Statistical analysis was performed using SPSS version 23.0 (IBM Corp.). Data are expressed as the mean ± SD. Statistical comparisons among multiple treatment groups were measured using one-way ANOVA followed by a post-hoc multiple comparison Dunnett's test.

Results

Cell survival rate of VX2 cells decreases with microwave treatment combined with inhibitors. In the physiotherapy mode of the microwave therapy device, the temperature and time are linearly related when the power is 15 W (Fig. 3A). The times required to reach the temperatures of 41, 48 and 60°C were 15, 30 and 60 sec, respectively. MTT results showed that the survival rate of VX2 cells decreased with increasing temperature in all groups. The addition of inhibitors at 37 and 41°C had no significant effect on the survival rate of VX2 cells compared with the 37°C control group. When the temperature was 48°C, the survival rate of VX2 cells was 82.75% in the control group, 64.81% in the p group, 63.18% in the s group and 37.18% in the p+s group (Fig. 3B). The cell survival rate of the 48°C M+p+s group was significantly lower compared with the 48°C control, M, M+s and M+p groups (P<0.05). The cell survival rate in the 60°C group was significantly lower compared with the other temperature groups (P<0.05; Fig. 3B).

As shown in Fig. 3C, most of the VX2 cells were viable at 37 and 41°C. A small number of dead cells was observed in the groups treated with inhibitors. When the temperature reached 48°C, an increased number of dead cells was found and the number of dead cells in the p+s group was greater than in the control group. At 60°C, only a few live cells were observed in the control group. These results were in accordance with the MTT results.
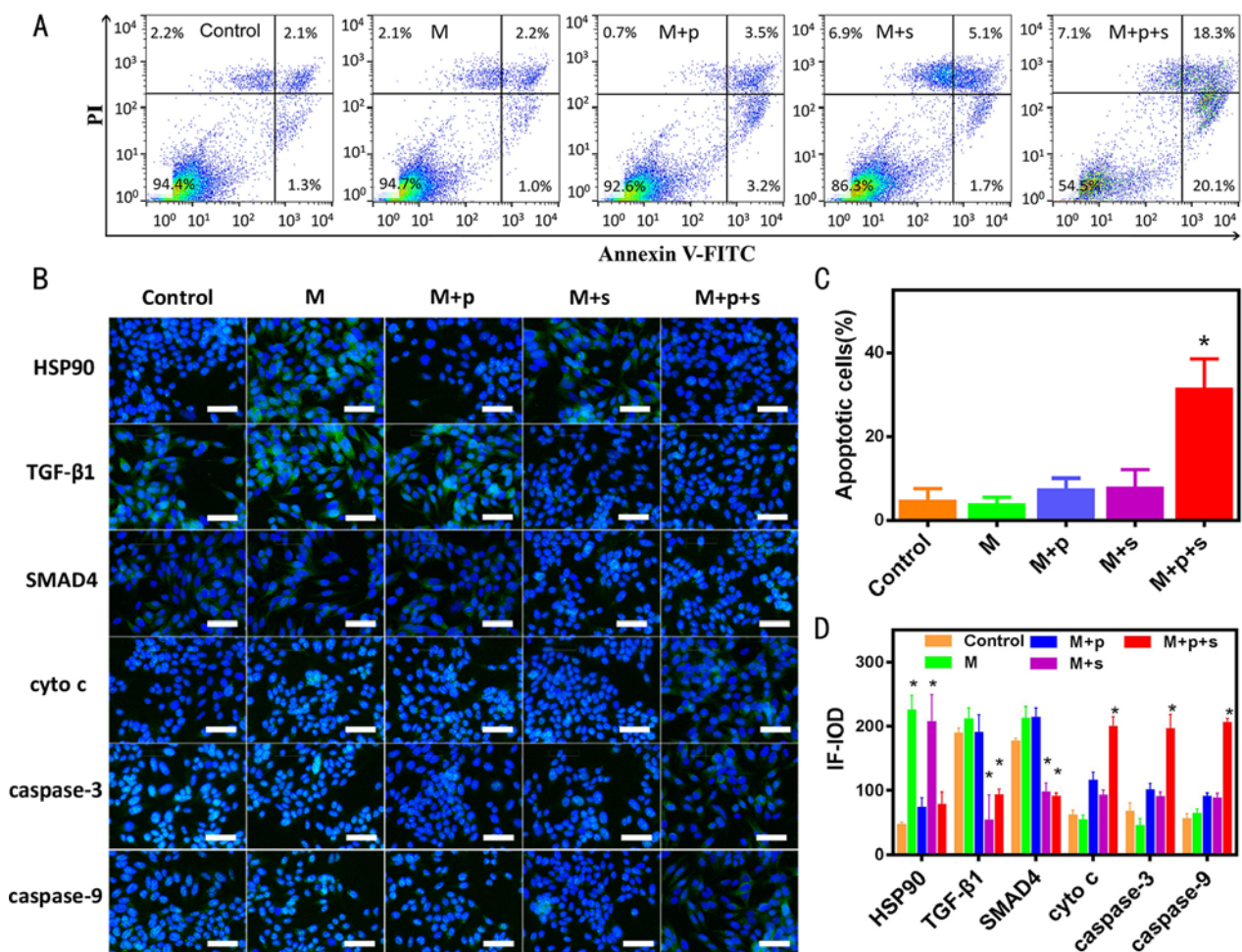
Figure 4. Microwave to 48˚C combined with HSP90 and TGF-β1 inhibitors promotes the apoptosis of VX2 cells. (A) Proportion of apoptosis after microwave ablation combined with inhibitors in VX2 cells. (B) Apoptosis-related signalling proteins detected by immunofluorescence staining; blue, DAPI; green, Alexa Fluor showing positively expressed protein; scale bar=50 μm. (C) Corresponding statistical results of flow cytometry (n=3). (D) Corresponding statistical results of cellular immunofluorescence images (n=3). *P<0.05 vs. control group. cyto *c*, cytochrome *c*; HSP90, heat shock protein 90; IF-IOD, immunofluorescence-integrated optical density; M, microwave; p, PF-04929113; PI, propidium iodide; s, SB-525334; TGF-β1, transforming growth factor-β1.

Treatment with inhibitors followed by microwave ablation increases cell apoptotic rate. As shown in Fig. 4A, when cells were subjected to microwaving at 48˚C, the apoptotic rate was 3.4% in the control group, 3.2% in the M group, 6.7% in the M+p group, 6.8% in the M+s group and 38.4% in the M+p+s group. The quantitative data revealed that the M+p+s group had a significantly higher apoptotic rate compared with the control group (Fig. 4C).

As determined by immunofluorescence, HSP90, TGF-β1 and SMAD4 expression was increased in the M group, whereas cyto *c*, caspase-3 and caspase-9 expression was not altered in the M group when compared with the control group (Fig. 4B and D). In the M+s group, the expression of apoptotic downstream signalling pathway-related proteins were similar to that of the M group. The expression of cyto *c*, caspase-3 and caspase-9 was significantly increased in the M+p+s group compared with the M group (Fig. 4B and D), indicating that the TGF-β1 inhibitor could promote the signalling pathway of apoptosis. In addition, the IOD values of HSP90 in the M and M+s groups were significantly increased, whereas those in the M+p and M+p+s groups were similar to those in the control group. The IOD values of TGF-β1

in the M+p and M+p+s groups were significantly decreased when compared with the control group (Fig. 4B and D).

Western blotting analysis. Fig. 5A and B shows the expression levels of proteins associated with the HSP90 and TGF-β1/SMAD4 signalling pathways. In the M+p+s groups, the expression levels of TGF-β1 and SMAD4 were significantly decreased. HSP90 in the VX2 cells was significantly upregulated by microwaving to 48˚C, whereas the expression levels of the other proteins in the M group were similar to that of the control group. When combined with the HSP90 inhibitor (M+p group), the expression of HSP90 in VX2 cells was reduced. The expression levels of cyto *c*, caspase-3 and caspase-9 were markedly increased in the M+p+s group when compared with the control group.

Microwave ablation has no significant effects on the survival rate of R-BMSC cells. As shown in Fig. 5C there was no significant difference in the survival rate of the R-BMSCs after microwave treatment at 48˚C compared with the control group, even when inhibitors were used in combination.
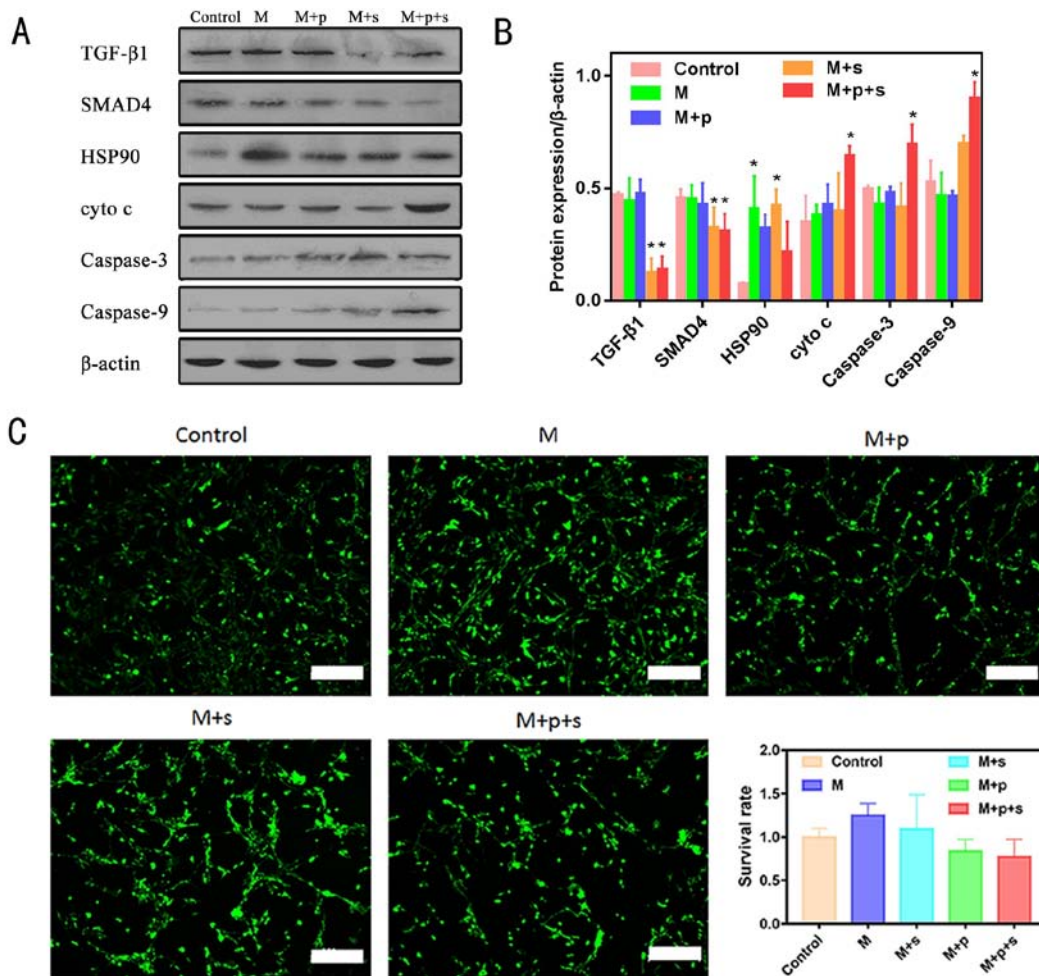
Figure 5. Microwaving to 48°C combined with HSP90 and TGF-β1 inhibitors promotes the apoptosis of VX2 cells. (A) Expression of typical proteins involved in the HSP90 and TGF-β1/SMAD4 signalling pathway in VX2 cells after microwave ablation combined with inhibitors. (B) Corresponding statistical results of western blotting (n=3). (C) Cell staining and MTT assay of the VX2 cells after microwave ablation combined with inhibitors in the R-BMSC cells; live R-BMSC cells stained with Calcein-AM are green and dead cells are red; scale bar=100 μm. *P<0.05 vs. control group. cyto *c*, cytochrome *c*; HSP90, heat shock protein 90; M, microwave; p, PF-04929113; s, SB-525334; TGF-β1, transforming growth factor-β1.

Changes in tumour volume and body weight. The weight of the rabbits in each group increased over 0-21 days, the rabbits were ~1.5 kg before surgery and ~2.0 kg 21 post-surgery (Fig. 6A). However, there were no significant differences in body weight among the groups at each time point. No rabbit was found to have multiple tumours. As shown in Fig. 6B, there was an increase in tumour volume in all groups over the 0-21 days after microwave ablation. The M+p+s group had the smallest volume at 21 days post-surgery, the volume was ~58.05% of the control group and was lower than that of the other groups. In addition, there were a large number of tumour cells observed in the control group, which decreased in the groups subjected to microwave treatment; the M+p+s group had the lowest number of tumour cells among these groups (Fig. 6E).

Immunohistochemistry. As shown in Fig. 6C and F, HSP90 expression in VX2 tumour tissue was significantly increased after microwaving at 48°C, whereas the expression levels of the other proteins were similar to that of the control group. When combined with the HSP90 inhibitor (M+p group), the expression of HSP90 in VX2 tumour tissues decreased to close to that of the control group. When combined with the TGF-β1

inhibitor (M+s and M+p+s groups), the expression levels of TGF-β1 and SMAD4 were significantly decreased compared with the control group. The expression of cyto *c*, caspase-3 and caspase-9 markedly increased in the M+p+s group when compared with the control group.

Discussion

The use of microwave therapy on tumours is associated with the risk of causing thermal damage to the surrounding normal tissues due to the high temperatures generated by the microwave thermal effect. A previous study reported the effect of hyperthermic temperatures on cells, the findings were as follows: Between 37 and 41°C, cells maintained a dynamic balance; between 41 and 48°C for >60 min triggered irreversible damage to the cells; between 48 and 60°C for only 4-6 min led to irreversible damage to proteins, DNA and cells; >60°C caused the protein to immediately coagulate, and the cells were immediately and irreversibly damaged (18).

Based on the aforementioned studies, four microwave ablation temperatures (37, 41, 48 and 60°C) were set in this experiment, and the effect of microwave ablation combined
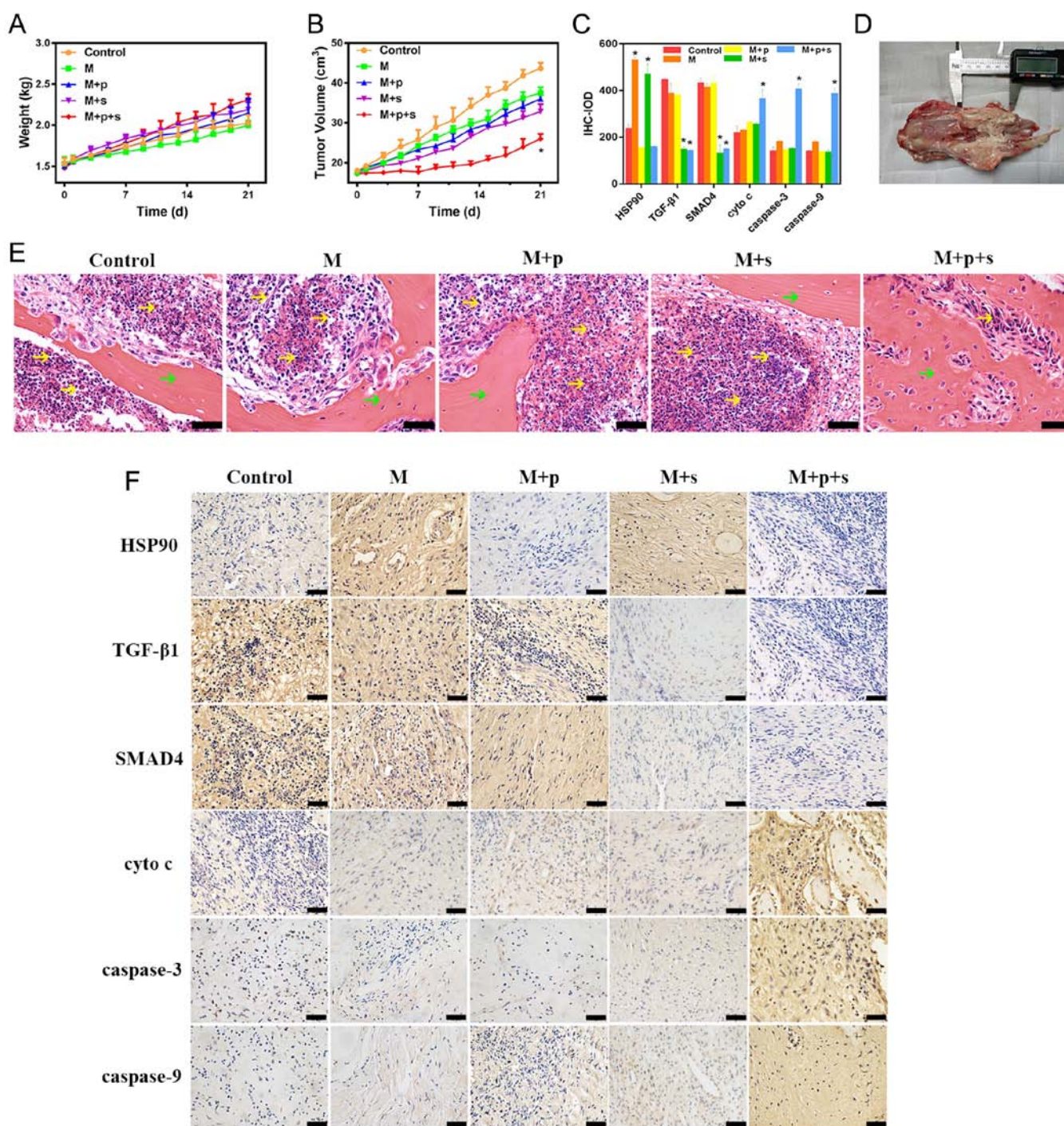
Figure 6. *In vivo* experiment results. (A) Changes in body weight of the New Zealand rabbits; data are expressed as the mean ± SD (n=6). (B) Changes in tumour volume after osteosarcoma treatment with mild microwave ablation combined with TGF-β1 and HSP90 inhibitors. (C) Corresponding statistical results of immunohistochemical images (n=6). (D) Representative tumour tissue. (E) H&E staining was used to detect the changes of tumour tissue after tumour treatment with microwave ablation combined with inhibitors; yellow arrows indicate tumour tissue and green arrows indicate bone tissue; scale bar=50 µm. (F) Immunohistochemical detection of the signalling pathway-related proteins after mild microwave ablation combined with inhibitors for the treatment of osteosarcoma; scale bar=50 µm. *P<0.05 vs. control group. cyto *c*, cytochrome *c*; HSP90, heat shock protein 90; IHC-IOD, immunohistochemistry-integrated optical density; M, microwave; p, PF-04929113; s, SB-525334; TGF-β1, transforming growth factor-β1.

with the TGF-β1 and HSP90 inhibitors on the survival rate of osteosarcoma tumours was investigated. The present study revealed that the survival rate of VX2 cells after microwaving to 48°C combined with TGF-β1 and HSP90 inhibitors, the rate of apoptosis was significantly higher than the microwave or inhibitor treatment groups alone. The results of the *in vivo* experiments also demonstrated the same synergistic

tumour treatment effect, without any systemic effect on the experimental rabbits, as during the experiment, there was no significant difference in the weight of the rabbits in each group.

The effect of this combination therapy could be attributed to the combination of two inhibitors blocking the synergistic activity of TGF-β1 and HSP90. Although HSP90 is essential for the survival of healthy cells, cancer cells have been shown to

be more dependent on HSP90, and therefore require increased active HSP90 compared with normal tissues (20), which may provide a novel target for tumour therapy. In the present study, live/dead staining and MTT assays were used to verify that microwave ablation at 48˚C combined with inhibitors had no significant effect on the survival rate of R-BMSCs. This may be because normal cells are less sensitive to thermal stimulation than tumour cells (21). A previous study reported that the anti-tumour effect of the HSP90 inhibitor may be mediated by a significant decrease in TGF-β1 at the post-translational level rather than the transcript level, followed by a decrease in SMAD2/3 (22). Moreover, TGF-β1 and HSP90 can bind to the same receptor (23), and previous studies have investigated their synergistic effects on tumour cell adhesion, migration and immunoglobulins (24,25). TGF-β1 has no effect on the adhesion and migration levels of tumour cells, but the addition of HSP90 can increase the adhesion of metastatic tumour cells without affecting the migration of primary tumour cells (26,27).

In the present study, the results of cellular immuno-fluorescence and Immunohistochemistry demonstrated that SB-525334 blocked the expression of TGF-β1 and SMAD4, and PF-04929113 blocked the expression of HSP90 but significantly increased the expression of apoptosis-related proteins cyto *c*, caspase-3 and caspase-9. In Fig. 4D and B, HSP90 in the M+p group decreased, but cyto c and caspases were also affected by other signaling pathways, thus there was no significant decrease overall. When an injury signal is transmitted to the mitochondria, mitochondrial outer membrane permeabilization leads to the release of cyto *c* into the cytoplasm (28), where it binds and activates apoptotic peptidase activating factor 1 (APAF1) (29). APAF1 has a caspase activation and recruitment binding domain (CARD), which is homologous to the initiating apoptotic protease caspase-9, so that activated APAF1 can aggregate and activate caspase-9 in a CARD-CARD manner, forming a cytochrome apoptotic body composed of cyto *c*, APAF1 and caspase-9, which activates caspase-3 and ultimately induces apoptosis (30).

In another signalling pathway associated with tumour proliferation/apoptosis, TGF-β1 phosphorylates and activates TGF-β1 type receptors (31). Phosphorylation of receptor-specific SMAD2/3 allows them to oligomerize with the common mediator SMAD4, thereby mediating the proliferation of tumour cells (32). Since TGF-β1 and HSP90 are able to bind to the same receptors, HSP90 inhibitors can target TGF-β1 signalling at the receptor level (23). HSP90 inhibitors can induce caspase activation by proteolysis (33). Caspase activation is mainly carried out by hydrolysis (34), and thus an immunoblot analysis was performed using an anti-lytic caspase-3/8/9 antibody. It was found that the caspase-9 content of the M+p+s group cells was significantly higher than that of untreated cells (33). The cleaved form of caspase-3 was detected in untreated cells, and the caspase-3 content of the M+p+s group cells was significantly increased compared with untreated cells (33).

When tumour cells are damaged, hypoxia triggers an increase in mitochondrial membrane permeability and cyto *c* translocates from the mitochondrial matrix to the cytoplasmic matrix (35). The resulting apoptotic body, a complex composed of cyto *c*, APAF1 and caspase-9, then activates the apoptosis-executing caspase-3 to induce apoptosis (36). These reports are consistent with the results of the present study, which suggested that the combination treatment activated tumour cell apoptosis by activating the mitochondrial apoptotic pathway. The immunohistochemical results also revealed that the expression of cyto *c*, caspase-3 and caspase-9 were enhanced when microwave ablation was combined with TGF-β1 and HSP90 inhibitors, which promoted osteosarcoma apoptosis. A previous study reported that the HSP90 inhibitor 17-DMAG could enhance the apoptotic effect of hyperthermic conditions on melanoma cells via melanoma-induced apoptosis (37), which differs from the inhibitor used of the present study. HSP90 can also inhibit exogenous apoptotic signalling pathways (38); its related anti-tumor effects require further study.

In summary, this study investigated the use of microwaving to 48˚C combined with HSP90 and TGF-β1 inhibitors. It was demonstrated that this combination could decrease cell survival rate and promote apoptosis in osteosarcoma tumours, providing a novel strategy for osteosarcoma treatment by microwave ablation while also offering a solution for the protection of surrounding normal tissues.

Availability of data and materials

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

Authors' contributions

QY, YZ and LC conceived and designed the study. LC, MW, ZL, MY, SC, WW and BL performed the experiments and collected the data. LC, WW and MW were major contributors in drafting and revising the manuscript. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

All animal studies were approved by the Institutional Animal Care and Use Committee of Guangzhou General Hospital of Guangzhou Military Command of PLA.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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