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Combined application of Embelin and tumor necrosis factor-related apoptosis-inducing ligand inhibits proliferation and invasion in osteosarcoma cells via caspase-induced apoptosis

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Abstract. Embelin, as an inhibitor of the X-linked inhibitor of apoptosis protein (XIAP), may induce apoptosis in various types of cancer cells. The present study aimed to determine the effect of Embelin on the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis of osteosarcoma cells. Embelin and TRAIL were applied to U2OS and MG63 cells, respectively or in combination. MTT was initially used to detect the difference in survival rates between the group receiving combined application of 100 ng/ml TRAIL and 20 μmol/l Embelin and the individual application groups. Light microscopic quantification was used to detect the morphology of the osteosarcoma cells in each group. Determination of cell apoptosis was subsequently performed using flow cytometry. The invasive ability of the cells was detected by a Transwell assay, prior to relative protein expression being determined by western blot analysis. Based on all the test data, it was revealed that the survival rates and the invasive ability were significantly lower following the combined application of 100 ng/ml TRAIL and 20 μmol/l Embelin than following the individual application of either ($P<0.01$). Additionally, upregulating expression of caspases, as well as death receptor 5, and downregulating expression of XIAP and matrix metalloproteinase 9 (MMP-9), had more significant effects in the combined group compared with the individual group and the control group. All these results suggested that Embelin may enhance TRAIL-induced apoptosis and inhibit the invasion of human osteosarcoma cells.

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Introduction

Osteosarcoma is the most common type of bone cancer and it usually occurs in children and adolescents. It was also revealed that metastasis to the lung occurs quickly in patients with osteosarcoma (1). Although neoadjuvant chemotherapy in osteosarcoma has greatly enhanced the survival rate, numerous patients suffer from toxic side effects due to long-term application of chemotherapeutic drugs. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a biological agent that was identified in recent years, has a selective killing effect on tumor cells without causing damage to normal cells and has been considered to be a promising novel biological treatment in the near future (2,3). A large number of human tumor cells are sensitive to the apoptosis induced by TRAIL; however, osteosarcoma cells are not sensitive to TRAIL. Recent studies have suggested that X-linked inhibitor of apoptosis (XIAP) was frequently overexpressed in cancer cells and served an important role in the resistance to TRAIL in certain tumor cells (4-6). Embelin, identified from the fruit of the Embelia ribes BURM (Myrsinaceae), was originally discovered by screening a library of natural products derived from Oriental traditional medicine (7-9). Recently, scholars used Embelin as a small-molecule inhibitor of XIAP to induce the apoptosis of tumor cells and to restore the sensitivity of certain tumor cells to TRAIL (10). However, the therapeutic effect of Embelin on TRAIL-induced apoptosis in osteosarcoma cells has not been investigated. The present study examined whether Embelin may enhance the susceptibility of human osteosarcoma cells to TRAIL and investigated the associated mechanism.

Materials and methods

Reagents. The osteosarcoma MG-63 (code no: TCHu 124) and U2OS (code no: TCHu 88) cell lines were purchased from the Cell Research Center of the Chinese Academy of Sciences (Shanghai, China). RPMI-1640 medium, Dulbecco's modified Eagle's high glucose medium (DMEM) and trypsin were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Embelin was obtained from Abcam

(Cambridge, UK). Pan-caspase inhibitor z-VAD-fmk and caspase-8 inhibitor z-IETD-fmk were purchased from Abcam (Cambridge, UK). Rabbit antibodies against caspases 3 and 8, death receptor (DR) 5, XIAP and MMP-9 were purchased from Abcam.

Cell culture and research methods. The U2OS cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) and the MG63 cells were cultured in DMEM with 10% FBS. The two cell lines were cultured in an incubator in a humidified 5% CO_2 atmosphere. The cells entering the logarithmic growth phase were selected. In the pre-experiment, U2OS cells (0.8x105/ml) and MG63 cells (1x105/ml) were inoculated in 96-well plates with culture medium (Gibco; Thermo Fisher Scientific, Inc.) containing TRAIL with concentrations of 1, 10 and 100 ng/ml or containing Embelin with concentrations of 5, 10 and 20 μl/l or experimental control without drug. Following culture for 12, 24 and 48 h, 20 μl MTT was added and incubated at 37˚C for 4 h, prior to being dissolved in 150 μl dimethyl sulfoxide. The absorbance was subsequently measured at a wavelength of 570 nm. According to the pre-experiment on the concentration of TRAIL and Embelin using an MTT assay, concentrations of 1, 10 and 100 ng/ml were selected for the TRAIL group, concentrations of 5, 10 and 20 μl/l for the Embelin group, and 100 ng/ml TRAIL combined with 20 μl/ Embelin for the combined group. A concentration of 100 ng/ml TRAIL or 20 μmol/l Embelin did not reach the half maximal inhibitory concentration at 12, 24 and 48 h, according to the pre-experiment. The study was performed using the following time intervals: 12, 24 and 48 h.

MTT assay. U2OS cells ($0.8x10^5$/ml) and MG63 cells ($1x10^5$/ml) were inoculated in 96-well plates, and were added to the culture medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing reagents of different concentrations of 100 μl drug or experimental control without drug. Following culture for 12, 24 and 48 h, 20 μl MTT was added and incubated at 37˚C for 4 h, prior to being dissolved in 150 μl dimethyl sulfoxide. The absorbance was subsequently measured at a wavelength of 570 nm. The survival rate of tumor cells (%)=experimental group A value/control group A value x100 (11).

Detection of the morphology of apoptotic cells. The apoptotic cells were directly observed under an inverted phase contrast microscope at the magnification of x400. A cover slide was placed in the 6-well plate, and following apoptosis, cells were fixed using 99.5% absolute ethyl-alcohol at 37˚C for 10 min and stained with 0.5 ml Hoechst 33258 staining solution at 37˚C for 5 min. Images were subsequently captured using a fluorescence microscope at the magnification of x400 on the object slide covered by the cover slide and a drop of anti-fading solution from Hoechst Staining kit (Beyotime Institute of Biotechnology, Shanghai, China) was added.

Determination of cell apoptosis by flow cytometry. Following culture for 12, 24 or 48 h, apoptosis was detected using the Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA). Cells in 6-well plates ($5x10^5$/well) were detached by trypsinization and washed three times in phosphate-buffered saline, centrifuged at 4˚C at 1,000 x g for 5 min and resuspended in 195 μl Annexin V-FITC binding buffer (BD Biosciences). Annexin V-FITC (5 μl) was added and mixed. Following this, the U2OS and MG63 cells were stained by Annexin V-FITC in binding buffer in the dark for 10 min at room temperature. The cells were subsequently centrifuged at 4˚C at 1,000 x g for 5 min and were resuspended in 190 μl Annexin V-FITC binding buffer. Finally, 10 μl propidium iodide (PI) staining solution was added and mixed at 4˚C for 15 min. The U2OS and MG63 cells were maintained on ice in the dark and immediately subjected to flow cytometric analysis (12). The data were analyzed using the Cell Quest software (version 7.5.3; BD Biosciences, San Jose, CA, USA).

Determination of the invasion ability. The invasive ability of U2OS and MG63 cells was calculated by the number of cells that passed through a polycarbonate membrane (8 μmol/l pore). The chamber was washed with serum-free medium (Gibco; Thermo Fisher Scientific, Inc.), and then 20 μl Matrigel (dilution, 1:8; BD Biosciences) was added to evenly cover the surface of the polycarbonate membrane. Pre-processed DMEM (200 μl) containing $2x10^5$ cells with 10% FBS was placed into the upper Transwell chamber while 600 μl DMEM with 10% FBS was placed into the lower chamber. The Transwell invasion system was added to the cell incubator for 24 h. The upper chamber was removed and the cells were stained with 1% crystal violet for 15 min at room temperature (13). Invading cells that attached to the surface of the membrane were observed under an inverted phase contrast microscope at a magnification of x400.

Western blot analysis. Since MG-63 cells exhibit certain characteristics of osteoblasts and U2OS cells are more malignant and stable than MG-63 cells, U2-OS cells were selected for western blot analysis (14). A total Protein Extraction kit (Beyotime Institute of Biotechnology, Haimen, China) was used to extract the total protein from U2OS cells, according to the manufacturer's protocol. Protein concentration was then determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol. Protein (40 μg/lane) was separated by 10% SDS-PAGE using an SDS-PAGE Gel kit (Beyotime Institute of Biotechnology), according to the manufacturer's protocol, prior to being transferred onto 0.2-μm pore polyvinylidene fluoride membranes (Beyotime Institute of Biotechnology). The membrane was blocked with 5% skimmed milk in TBST at room temperature for 2 h, prior to being incubated with antibodies against the following: rabbit anti-caspase-3 (dilution, 1:5,000; cat. no. ab32351), rabbit anti-caspase-8 (dilution, 1:5,000; cat. no. ab25901), rabbit anti-DR-5 (dilution, 1:1,000; cat. no. ab199357), rabbit anti-XIAP (dilution, 1:1,000; cat. no. ab21278; all Abcam) and rabbit anti-MMP-9 (dilution, 1:1,000; cat. no. 10375-2-AP; ProteinTech Group, Inc., Chicago, IL, USA) in TBST containing 1% bovine serum albumin overnight at 4˚C. A rabbit anti-β-actin monoclonal antibody (dilution, 1:5,000; cat. no. ab8227; Abcam) was used as a control for caspase-3 and caspase-8 while a rabbit anti-GAPDH

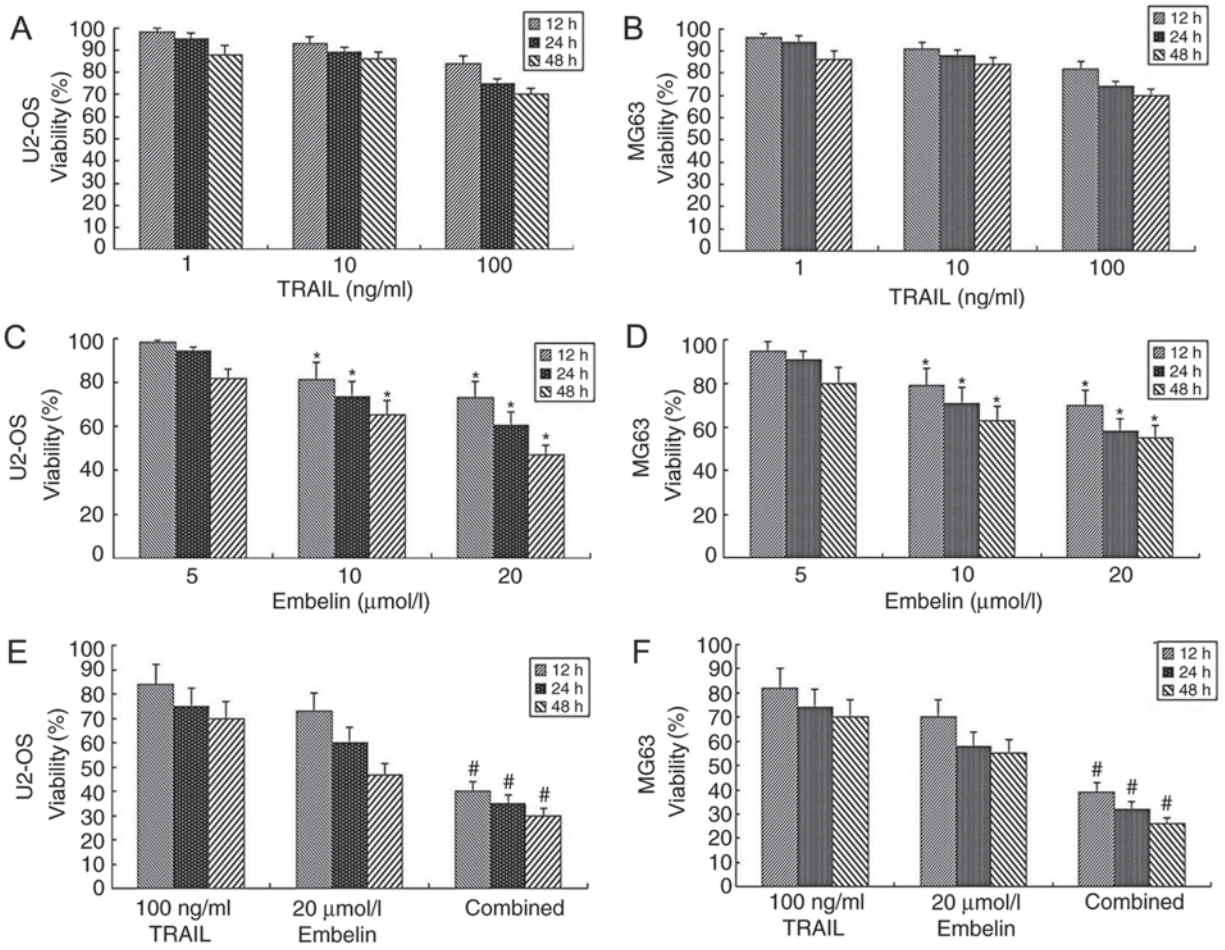

Figure 1. Viability of U2OS and MG63 cells, as measured by MTT after 12, 24 or 48. (A) The viability rate of U2OS cells treated with different concentrations of TRAIL. (B) The viability rate of MG63 cells treated with different concentrations of TRAIL. (C) The viability rate of U2OS cells treated with different concentration of Embelin. (D) The viability rate of MG63 cells treated with different concentration of Embelin. (E) The viability rate of U2OS cells treated with a combination of 100 ng/ml TRAIL and 20 μmol/l Embelin. (F) The viability rate of MG63 cells treated with a combination of 100 ng/ml TRAIL and 20 μmol/l Embelin. The data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. the control group; #P<0.01 vs. the TRAIL group or Embelin group. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

monoclonal antibody (dilution, 1:5,000; cat. no. ab181602; Abcam) was used as a control for DR-5, XIAP and MMP-9. The membranes were subsequently washed three times for 30 min in Tris-buffered saline containing Tween 20 (1xTBST) and were incubated with the horseradish peroxidase-conjugated AffiniPure goat anti-rabbit IgG secondary antibody (dilution, 1:50,000; cat. no. ab182016; Abcam) diluted with TBST for 2 h at room temperature, prior to being washed again in TBST three times for 30 min. A BeyoECL Plus kit (Beyotime Institute of Biotechnology) was used to detect protein bands, according to the manufacturer's protocol. MF-Chemisis 2.0 (DNR Bio-Imaging Systems, Ltd., Neve Yamin, Israel) and GelCapture software (version 2.24, DNR Bio-Imaging Systems, Ltd.) were used to obverse and analyze the protein bands (15,16).

Statistical analysis. Statistical analysis was performed using Windows SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation of three independent experiments. Data were compared using one-way analysis of variance, followed by the Student-Newman-Keuls test. P<0.05 was considered to indicate a statistically significant difference.

Results

Viability of tumor cells in the presence of combined TRAIL and Embelin. While concentrations of 1, 10 or 100 ng/ml TRAIL were used in U2OS or MG63 cells, no significant difference in the dose-inhibiting effect was observed between different groups (P>0.05; Fig. 1A and B). This observation was consistent with the pre-experimental results. In the pre-experiment, it was revealed that the use of TRAIL alone with concentrations ranging between 0.1 and 1,000 ng/ml has little effect on U2OS or MG63 cells. As the purpose of the present study was to examine and verify the effect of combined application of Embelin and TRAIL to osteosarcoma cells, appropriate concentrations were selected. When concentrations of 5 μmol/l Embelin were applied to U2OS or MG63 cells at 12, 24 or 48 h, no significant difference in the dose-inhibiting effect was observed compared with at 0 h (P>0.05; Fig. 1C and D). However, when concentrations of 10 or 20 μmol/l Embelin were applied to U2OS or MG63 cells, cell viability was inhibited (P<0.05; Fig. 1C and D). When a combination of 100 ng/ml TRAIL and 20 μmol/l Embelin was applied to U2OS or MG63 cells, the viability rate was

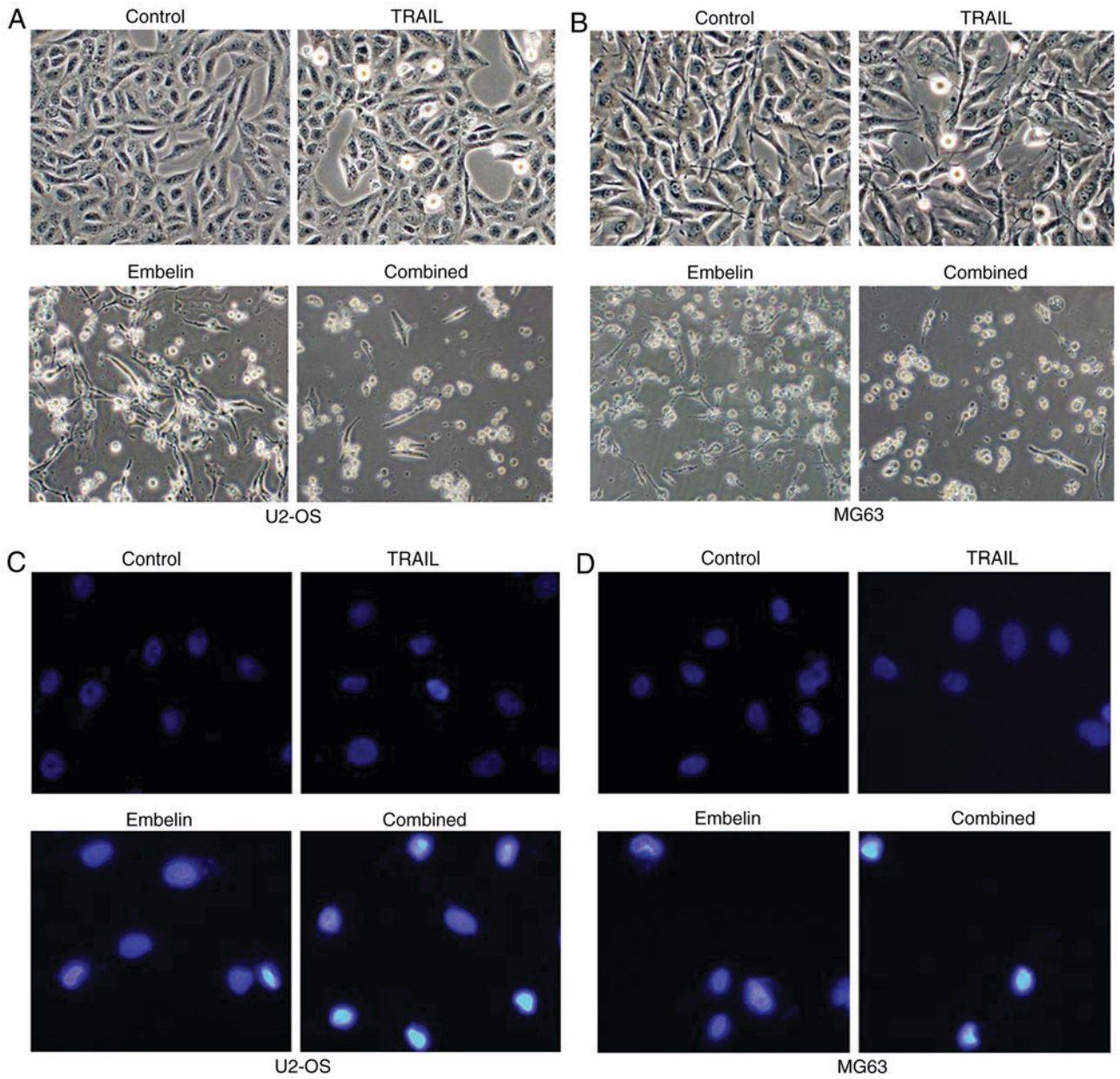

Figure 2. Morphological changes and fluorescent staining of U2OS and MG63 cells treated with different drugs after 48 h. (A) The morphological appearance of U2OS cells (control, treated with 100 ng/ml TRAIL, treated with 20 μmol/l Embelin or treated with a combination of the two) under an inverted phase contrast microscope. (B) The morphological appearance of MG63 cells (control, treated with 100 ng/ml TRAIL, treated with 20 μmol/l Embelin or treated with a combination of the two) under an inverted phase contrast microscope. (C) Fluorescent staining of U2OS cells (control, treated with 100 ng/ml TRAIL, treated with 20 μmol/l Embelin or a combination of the two) under a fluorescence microscope. (D) Fluorescent staining of MG63 cells (control, treated with 100 ng/ml TRAIL, treated with 20 μmol/l Embelin or a combination of the two) under a fluorescence microscope. Magnification, x400. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

significantly lower (P<0.01), compared with the individual treatment groups (Fig. 1E and F). The results of the present study demonstrated that the combined application of TRAIL and Embelin had a strong inhibitory effect on the viability rates of U2OS and MG63 cells.

Changes in the apoptosis rate of U2OS and MG63 cells. U2OS and MG63 cells were added to the surface of a dish in an angular position (Fig. 2A and B). Following the application of 100 ng/ml TRAIL or 20 μmol/l Embelin, few of the osteosarcoma cells were small and round (Fig. 2A and B). However, with the combined application of 100 ng/ml TRAIL and 20 μmol/l Embelin, chromosomes were condensed, and cells became non-adherent and suspended in the DMEM (Fig. 2A and B). Cell death was blocked by joint application of TRAIL and Embelin following the use of 150 μM pan-caspase inhibitor z-VAD-fmk or 150 μM caspase-8 inhibitor z-IETD-fmk (Fig. 3A and B), which indicated that the process of cell death requires caspases activation. In a field of fluorescence microscope, the majority of U2OS and MG63 cells in the control group were lightly-stained and the morphology of the cells were condensed and exhibited fluorescence (Fig. 2C and D), while in the cells treated with the individual application of 100 ng/ml TRAIL or 20 μmol/l Embelin, only a small number of cells were lightly-stained and condensed (Fig. 2C and D). With the combined application of

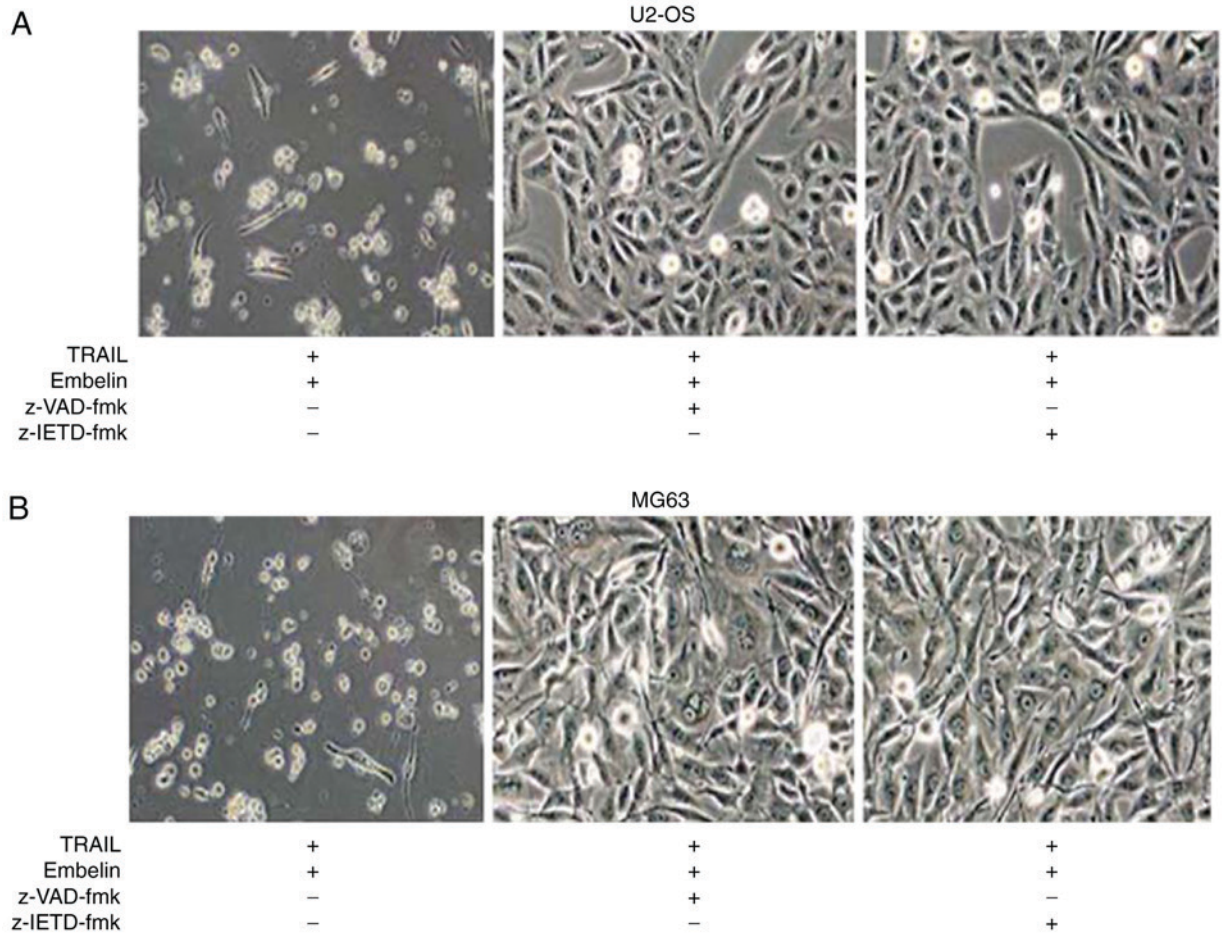

Figure 3. Morphological changes of U2OS and MG63 cells under the effects of chemotherapeutic agents after 48 h. (A) U2OS cells was co-treated with 100 ng/ml TRAIL and 20 μmol/l Embelin in the presence of 150 μM z-VAD-fmk or 150 μM z-IETD-fmk and images were captured under an inverted phase contrast microscope. (B) MG63 cells were co-treated with 100 ng/ml TRAIL and 20 μmol/l Embelin in the presence of 150 μM z-VAD-fmk or 150 μM z-IETD-fmk and images were captured under an inverted phase contrast microscope. Magnification, x400. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

100 ng/ml TRAIL and 20 μmol/l Embelin, a large number of cells were condensed and exhibited fluorescence, indicating the apoptosis of numerous cells (P<0.05) (Fig. 2C and D).

Effect of Embelin on the invasive ability of U2OS and MG63 cells. The Transwell experiments indicated that the number of U2OS and MG63 cells in the combined treatment group passing through the polycarbonate membrane was significantly less than that in the individual treatment groups and the control group (Fig. 4A and B). Cells that were able to invade to the lower side of the membrane in the Transwell assays after 24-h incubation were quantified (Fig. 4C and D). These data indicated that the combined application of TRAIL and Embelin may diminish the invasion of U2OS and MG63 cell lines more significantly than the individual application of either treatment.

Effect of Embelin on the apoptosis of U2OS and MG63 cells. Annexin V and PI staining experiment results demonstrated that, with the combined application of 100 ng/ml TRAIL and 20 μmol/l Embelin, the number of apoptotic cells was more than that in the control group and the individual treatment groups (P<0.01; Fig. 5). As Annexin V and PI staining results demonstrated in Fig. 5A, the early apoptotic rates of the control group, the TRAIL-only group, the Embelin-only group and the combined treatment group in U2OS cells were 0.73, 3.54, 12.33 and 20.71%, respectively. The late apoptotic rates in U2OS cells were 1.87, 6.12, 6.82 and 10.84%, respectively, and the total apoptotic rates in U2OS cells were 2.60, 9.66, 19.15 and 31.55%, respectively. A similar trend was also observed in the MG63 cells (Fig. 5B). The early apoptotic rates of the control group, the TRAIL-only group, the Embelin-only group and the combined treatment group in MG63 cells were 0.94, 3.51, 5.76 and 10.17%, respectively, the late apoptotic rates in MG63 cells were 2.43, 4.18, 10.64 and 15.14%, respectively, and the total apoptotic rates in MG63 cells were 3.37, 7.33, 16.40 and 25.31%, respectively. These data indicated that the combined treatment may increase the apoptosis rate of U2OS and MG63 cells, compared with individual treatment with either drug (Fig. 5C and D).

The expression of caspase-3, caspase-8, cleaved caspase-3, DR5, XIAP and MMP-9 in U2OS cells. Following exposure to 100 ng/ml TRAIL, 20 μmol/l Embelin or the combined application of the two for 48 h, expression levels of caspase-3, caspase-8, cleaved caspase 3, DR5, XIAP and MMP-9 in U2OS cells were

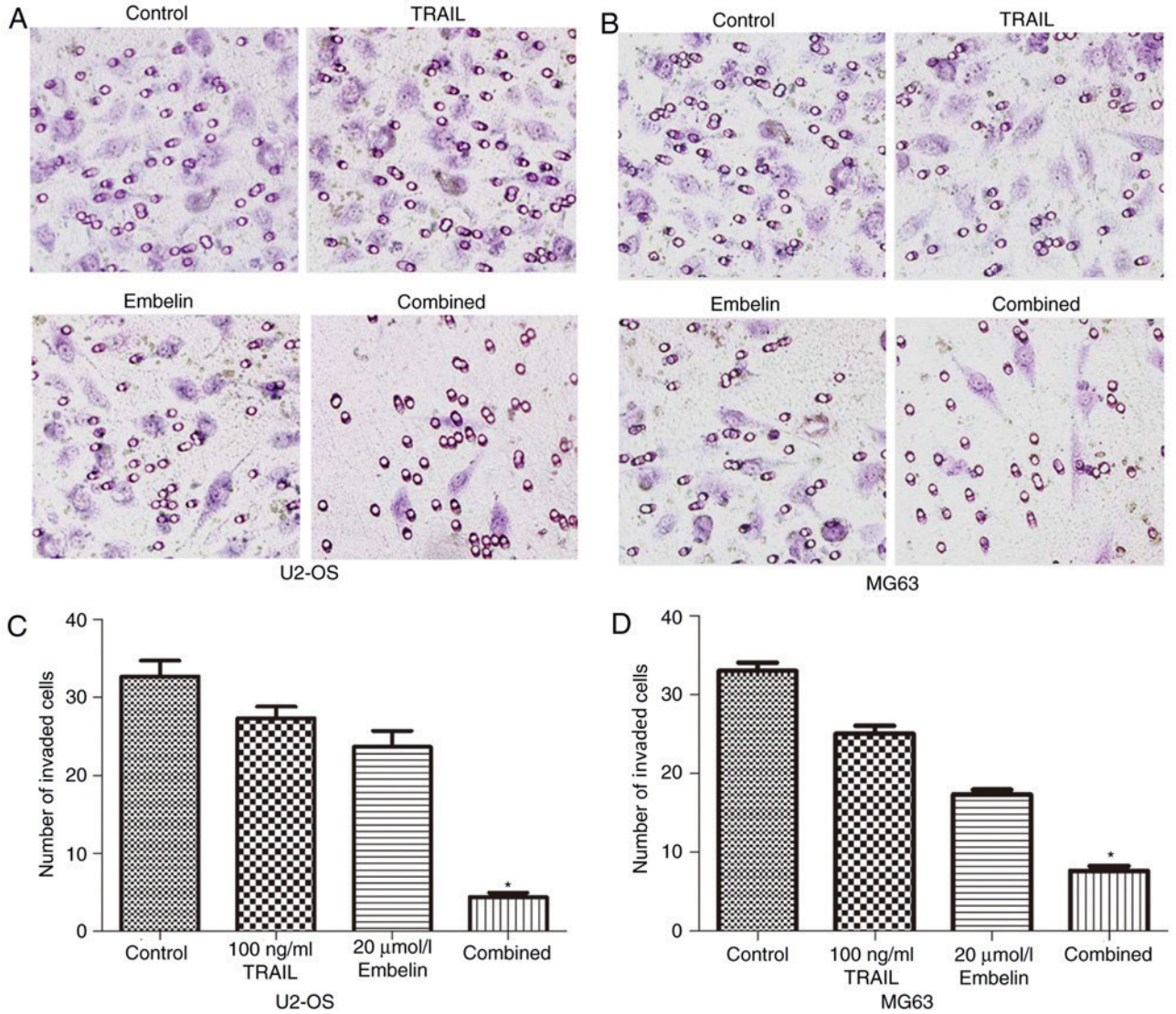

Figure 4. Invasion in U2OS and MG63 cells treated with different drugs after 24 h. (A) Crystal violet staining of the U2OS cells (control, treated with 100 ng/ml TRAIL, treated with 20 μmol/l Embelin or treated with a combination of the two) that passed through the polycarbonate membrane under an inverted phase contrast microscope. (B) The crystal violet staining of the MG63 cells (control, treated with 100 ng/ml TRAIL, treated with 20 μmol/l Embelin or treated with a combination of the two) that passed through the polycarbonate membrane under an inverted phase contrast microscope. (C) Quantification of U2OS cells that had attached to the basal side of the membrane. (D) Quantification of MG63 cells that had attached to the basal side of the membrane. Data are presented as the mean ± standard deviation of three independent experiments. Magnification, x400. *P<0.05 vs. the control group. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

measured using western blot analysis in the present study. The levels of caspase-3, caspase-8, cleaved caspase 3 and DR5 in the combined treatment group were significantly higher than that of the individual treatment groups and the control group (P<0.05). By contrast, levels of XIAP and MMP-9 in the combined treatment group were significantly lower than in the individual treatments groups and the control group (P<0.05) (Fig. 6).

Discussion

TRAIL is a member of the TNF super family of cytokines (17,18). TRAIL is a potentially important anticancer agent, since it selectively kills malignant cells while leaving normal cells unaffected (19,20). TRAIL induces apoptosis through interactions with its death domain containing receptors DR4 and DR5 (21,22). Once activated, TRAIL receptors recruit Fas-associated protein with death domain as a major cellular adaptor protein, which triggers the auto-activation of caspase-8 and subsequently leads to the activation of downstream caspases, including caspases-3, -7 and -9. This resulted in the cleavage of cellular substrates and ultimately cell death. However, certain tumor cells, including gastric, hepatic, lung and osteosarcoma cells are not sensitive to TRAIL (23-26). The present study also demonstrated that there was no significant difference in the viability rate of U2OS and MG63 cells between the TRAIL group and the control group, which confirmed the tolerance of U2OS and MG63 cells to TRAIL (Fig. 1A). Although previous studies have revealed that the combination of TRAIL and certain chemotherapy drugs may improve the sensitivity of osteosarcoma cells to TRAIL (27,28), to a certain extent, such treatment regimens continue to depend upon chemotherapy drugs and side effects to healthy cells are inevitable. Therefore, identifying a treatment

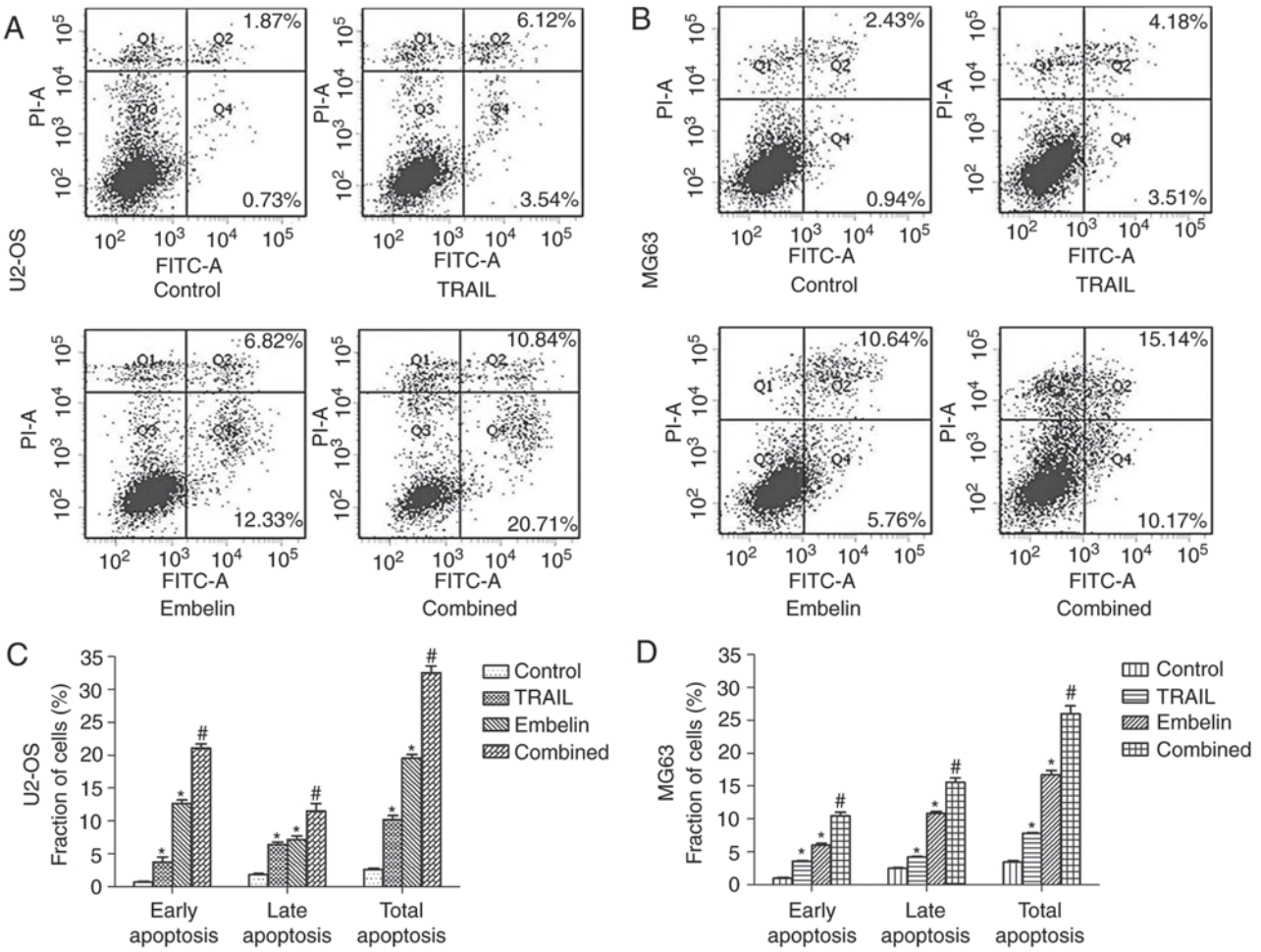

Figure 5. Apoptosis in U2OS and MG63 cells treated with different drugs after 24 h. The four groups of (A) U2OS and (B) MG63 cells (control, treated with 100 ng/ml TRAIL, treated with 20 μmol/l Embelin or treated with a combination of the two) were incubated for 24 h. Cells stained with FITC-conjugated Annexin V and PI were analyzed by flow cytometric analysis. In the lower right quadrant, early apoptotic cells were observed, and the necrotic or late apoptotic cells were located in the upper right quadrant. The percentage of early apoptotic, late apoptotic and total apoptotic (C) U2OS and (D) MG63 cells (control, treated with 100 ng/ml TRAIL, treated with 20 μmol/l Embelin or treated with a combination of the two) for 24 h. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.01 vs. control group; $^{\#}$P<0.01 vs. TRAIL group or Embelin group. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; FITC, fluorescein isothiocyanate; PI, propidium iodide.

plan regarding TRAIL that has fewer side effects has become an aim for numerous researchers worldwide (29). Recent studies regarding biologically targeted therapy have revealed that the function of TRAIL in conducting the death signal into cells may be doubled by the high expression of DR4 or DR5 (30,31). Such studies may speed up the advancement in application of TRAIL in the treatment of osteosarcoma and have become a hot topic. Therefore, the aim of the present study was to identify a drug that may be used in combination with TRAIL to reduce the resistance of osteosarcoma cells to TRAIL by activating DR4 or DR5.

XIAP is one of the best characterized and most potent endogenous inhibitors of the caspases and is therefore considered a key physiological regulator of cell death (5,6). XIAP inhibits the upstream caspase-9 by binding it to its BIR3 domain, and the downstream caspase-3 and caspase-7 by binding them to its BIR2 domain. XIAP expression is elevated in numerous types of cancer including lung, ovarian, colon and kidney cancer, and myeloid leukemia, which was also responsible for resistance to chemotherapy (32). Embelin was identified ~50 years ago as an active component of the Embelia ribes BURM (33). Myrsinaceae, which has been used as traditional medicine for thousands of years to treat a diverse range of illnesses, including fever, inflammatory diseases and various gastrointestinal ailments and therefore, must pose a low toxicity threat (34). Until recently, Embelin has been regarded as an inhibitor of XIAP to induce the proliferation suppression and apoptosis of human cancer cells in various organs, including pancreatic, colon and prostate cancer, as well as leukemia (35-37). At, present, to the best of our knowledge, no reports have stated that Embelin inhibited the proliferation and invasion of osteosarcoma cells. Furthermore, a recent study demonstrated that Embelin may upregulate the expression of DR4 or DR5, and increase the susceptibility of tumor cells to TRAIL (38). In our pre-experiment, it was revealed that Embelin posed a killing effect on osteosarcoma cells and, based on this, further research was performed to investigate whether or not Embelin may enhance the TRAIL-DR5 pathway, thereby increasing the sensitivity of osteosarcoma cells in order to identify a novel strategy for the use of TRAIL.

The results of the present study suggested that the combined application of TRAIL and Embelin had a synergistic apoptotic

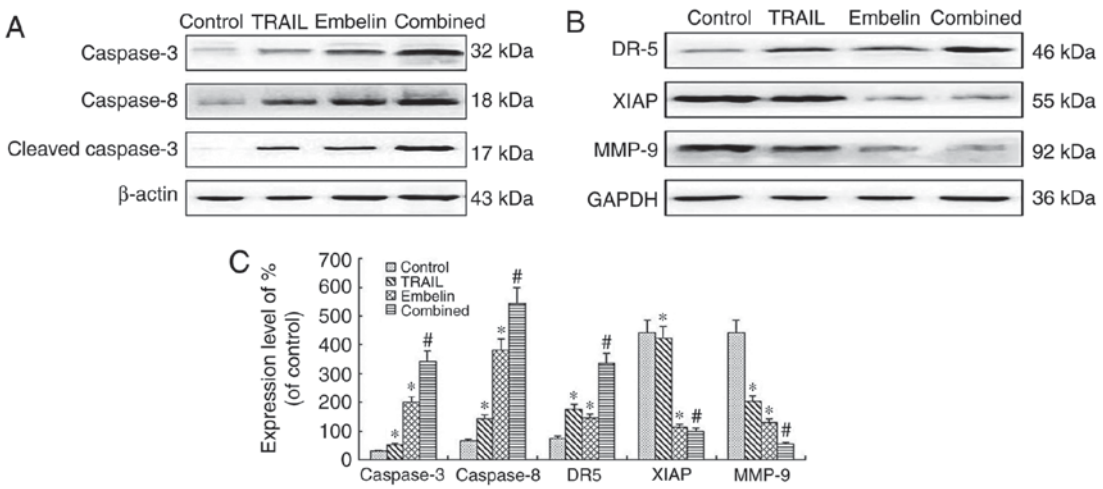

Figure 6. Western blot analysis of the expression of proteins after U2OS cells were cultured for 48 h. (A) The levels of caspase-3 (32 kDa), caspase-8 (18 kDa) and cleaved caspase-3 (17 kDa) were analyzed by western blot analysis. There was a marked increase in the expression of caspase-3, cleaved caspase-3, caspase-8 in the combined treatment group, P<0.01. (B) The levels of XIAP (55 kDa), MMP-9 (92 kDa) and DR5 (46 KDa) were analyzed by western blot analysis. There was a marked downregulation in the expression of XIAP and MMP-9 in the combined treatment group, P<0.01. However, there was a marked upregulation in the expression of DR5 in the combination group, P<0.01. (C) Quantification of relative protein expression. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.01 vs. control group; #P<0.01 vs. TRAIL group or Embelin group. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; XIAP, X-linked inhibitor of apoptosis protein; MMP-9, matrix metalloproteinase 9; DR5, death receptor 5.

effect compared with that of the individual application of either treatment. The results of the MTT assay demonstrated that combined treatment had a stronger inhibitory effect on the viability rate of U2OS and MG63 cells compared with the individual application of either agent (Fig. 1). Furthermore, combined treatment caused more distinct morphological changes compared with the application of each agent individually (Fig. 2). The present study also demonstrated that the killing effect of the combined treatment was achieved by inducing the apoptosis of osteosarcoma cells, as revealed by Hoechst staining (Fig. 2C and D). Furthermore, Annexin V and PI staining results indicated that the combined application of TRAIL and Embelin also served an important role in the apoptotic effect on osteosarcoma cells (Fig. 5). The apoptosis-induced effects on the combined treatment group were much stronger than that of the individual treatment groups and the control group. These results are consistent with those of previous studies on other types of tumor cell (39). The results of the Transwell invasion chamber experiments revealed that the inhibition of invasion in the combined treatment group was more significant than that in the control group and the individual treatment groups (Fig. 4A and B). Furthermore, the expression of MMP-9, which is well known to be the most important indicator of invasion ability (40), was significantly lower in the combined treatment group than in the control group and the individual treatment groups (Fig. 6B). All these results revealed that the combination of Embelin and TRAIL may inhibit the invasion of osteosarcoma cells. Similarly to the change in MMP-9 expression, the expression of XIAP was also downregulated due to the inhibitory effects of the treatment with Embelin (Fig. 6B). In addition, the combination of the two treatments upregulated the expression of DR5, cleaved-caspase-3 and caspase-8 (Fig. 6A and B). The total caspase-3 expression and the cleaved-caspase-3 expression were also increased. Based on the aforementioned results, the mechanism through which Embelin enhances TRAIL-induced apoptosis of osteosarcoma may be that Embelin upregulates the expression of DR5, thereby increasing the susceptibility of osteosarcoma cells to TRAIL, and that Embelin inhibits the expression of XIAP, which may upregulate the expression of caspases. These two pathways eventually result in the apoptosis of osteosarcoma cells.

Despite the fact that a number of studies have reported that the combination of Embelin and TRAIL induced the apoptosis of cancer cells, the results of the present study exhibit certain differences. Yang *et al* (41) demonstrated that Embelin inhibited the LMP1-mediated upregulation of XIAP and TRAIL resistance in nasopharyngeal carcinoma cells. The combined application of Embelin and TRAIL are limited to nasopharyngeal carcinoma cells overexpressing LMP1 proteins. In the present study, the combined application of Embelin and TRAIL were not limited to any over-expressing protein. The study of Yang *et al* (42) revealed that the underlying mechanism through which combined application of Embelin and TRAIL suppresses the NF-κB-dependent survival pathway in human acute myeloid leukemia cells. By contrast, the present study suggested that Embelin enhanced the TRAIL-induced apoptosis of osteosarcoma cells via the activation of the TRAIL-DR5-caspase signal pathway. By contrast, the indicators and methods used in the present study are similar to those used in a previously study that employed Embelin as a small-molecule inhibitor of XIAP to induce the apoptosis of tumor cells and restore the sensitivity of non-small cell lung cancer cells to TRAIL (38). However, in the present study, the therapeutic effect of Embelin on the TRAIL-induced apoptosis of osteosarcoma cells was investigated.

In conclusion, the results of the present study suggested that the XIAP inhibitor Embelin enhanced the TRAIL-induced apoptosis and inhibited the invasion of osteosarcoma cells, which may be related to the activation of the TRAIL-DR5-caspase signal pathway. Therefore, Embelin may sensitize osteosarcoma cells to TRAIL and alleviate the toxic side effects of the chemotherapy drug on the human body and therefore, the

combination of TRAIL and Embelin may be a promising treatment for patients with osteosarcoma. However, XIAP siRNA and *in vivo* experiments are required in order to further validate the results of the present study. Since B cell lymphoma 2 (Bcl-2) family proteins serve an important role in Embelin-enhanced TRAIL-induced cell apoptosis, whether or not caspase-induced apoptosis depends on Bcl-2 requires further investigation.

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Availability of data and materials

All data and materials described in the manuscript are available upon reasonable request from the corresponding author.

Authors' contributions

TH conceived and designed the experiments, HQ and YC performed the experiments; HQ, XL and WZ wrote the paper; TL and GJ performed the western blot analysis, SC and PL helped perform the analysis. All authors read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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