ORIGINAL ARTICLES

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The effect of biochanin A on cell growth, apoptosis, and migration in osteosarcoma cells

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Biochanin A has been reported to be associated to tumour cell proliferation, apoptosis and drug resistance in hepatocellular carcinoma, prostate cancer, and colorectal cancer, etc, while the relation of biochanin A on osteosarcoma is not clear. Hence, in this study, we examined the effects of biochanin A treatment on osteosarcoma cell lines MG63 and U2OS on proliferation, apoptosis, invasion and migration. We then investigated the involved molecular mechanism and found a time- and dose-dependent inhibition of cell viability in MG63 and U2OS cells with biochanin A treatment. Under the same circumstances, an increased ratio of cells in G0/G1 phase but a decreased ratio of cells in G2/M phase was observed. In addition, after biochanin A treatment, apoptotic rates clearly increased and decreased migration and invasion ability were observed in MG63 and O2OS cells. Meanwhile, relevant genes involved in cell proliferation, apoptosis, invation and migration demonstrated altered expressions in MG63 and U2OS cells. The present study supports the assumption that biochanin A has suppressive effects on osteosarcoma through regulating cell proliferation, apoptosis, invasion, and migration.

1. Introduction

Osteosarcoma is one of the primary malignant bone tumors exhibiting a high incidence in adolescence (Eleutério et al. 2015; Janeway et al. 2012; Hu et al. 2016). The standard treatments for osteosarcoma comprised a combination of surgery, drug therapy and radiotherapy (Cates 2017; Li et al. 2017; Alessio et al. 2017). Chemotherapy is one of the main pharmacological options. Despite of advances in chemotherapeutic strategy for osteosarcoma that have improved clinical outcomes (Kubo et al. 2017; Senerchia et al. 2017; Asling et al. 2016), the total effect of chemotherapy remains unsatisfactory. The main issue that lead to the failure of chemotherapy is the development of drug resistance in osteosarcomatous cells (Song et al. 2017; Wang et al. 2017). Hence, exploring new drug options is imperative for improving treatment outcomes of patient with osteosarcoma.

Phytochemicals, due to their gentle effects and less adverse reactions, are now in the focus of research for treatment of osteosarcoma (Hu et al. 2015; Chang et al. 2015). Biochanin A is an active constituent of *Trifolium pratense* L. (Renda et al. 2013) that has multiple effects like antitumorigenesis, reduction of oxidative stress, treatment of osteoporosis, anti-inflammation, regulation of blood glucose levels and treatment of allergies (Chen et al. 2015; Jalaludeen et al. 2016; Wu et al. 2015; Azizi et al. 2014). These results indicate that biochanin A has high potentials in treating different diseases. However, the efficacy of biochanin A in osteosarcoma has not been reported. Therefore, in this study, the functions and mechanisms of biochanin A in osteosarcoma treatment were evaluated *in vitro*.

The present study used MG63 and U2OS human osteosarcoma cell lines as targets of biochanin A interference treatment, Therefore, the effect of biochanin A inhibition on MG63 and U2OS cell viability, cell cycles, apoptotic rate and capacity of invasion and migration was examined while the alterations of associated genes were detected, which may provide evidence of biochanin A as a treatment option for osteosarcoma.

2. Investigations and results

2.1. Effect of biochanin A on MG63, U2OS cell viability

The molecular structure of biochanin A is shown in Fig. 1A. To realise the effect of Biochanin A on cell viability of human osteosarcoma cell line MG63 and U2OS, we treated them with various doses of biochanin A and detected the cell activity using MTT technique. The MTT results indicate a biochanin A dose- (Fig. 1B) and schedule-dependent (Fig. 1C) inhibition of the cell viability in both MG63 and U2OS cells. According to this result, the dose of biochanin A 40 μM and the length of treatment period (48 h) were determined for the subsequent experiment.

2.2. Effect of biochanin A on MG63 and U2OS cell cycles

We used 40 μM of biochanin A treating MG63 and U2OS cells. After 48 h of treatment, the alteration in cell cycle was examined using a flow cytometer. The results are shown in Fig. 2A and Fig. 2B. In comparison to control groups, with biochanin A treatment, more MG63 and U2OS cells remained in the G0/G1 phase, a decreased number was in the G2/M phase ($P<0.05$). These results suggest that biochanin A is associated with proliferation of osteosarcoma cell through regulating cell cycles.

2.3. Effect of biochanin A on cell apoptosis of MG63 and U2OS cells

We used 40 μM of biochanin A to treat MG63 and U2OS cells. After 48 h of treatment, the alteration in cell apoptosis was examined using a flow cytometer (FCM), as shown in Fig. 3. Compared with control groups, after treatment with salidroside, MG63 (Fig. 3A) and U2OS cells (Fig. 3B) showed increased apoptotic rates ($P<0.05$). These findings suggest that biochanin A has an effect of apoptotic induction in osteosarcoma cells.

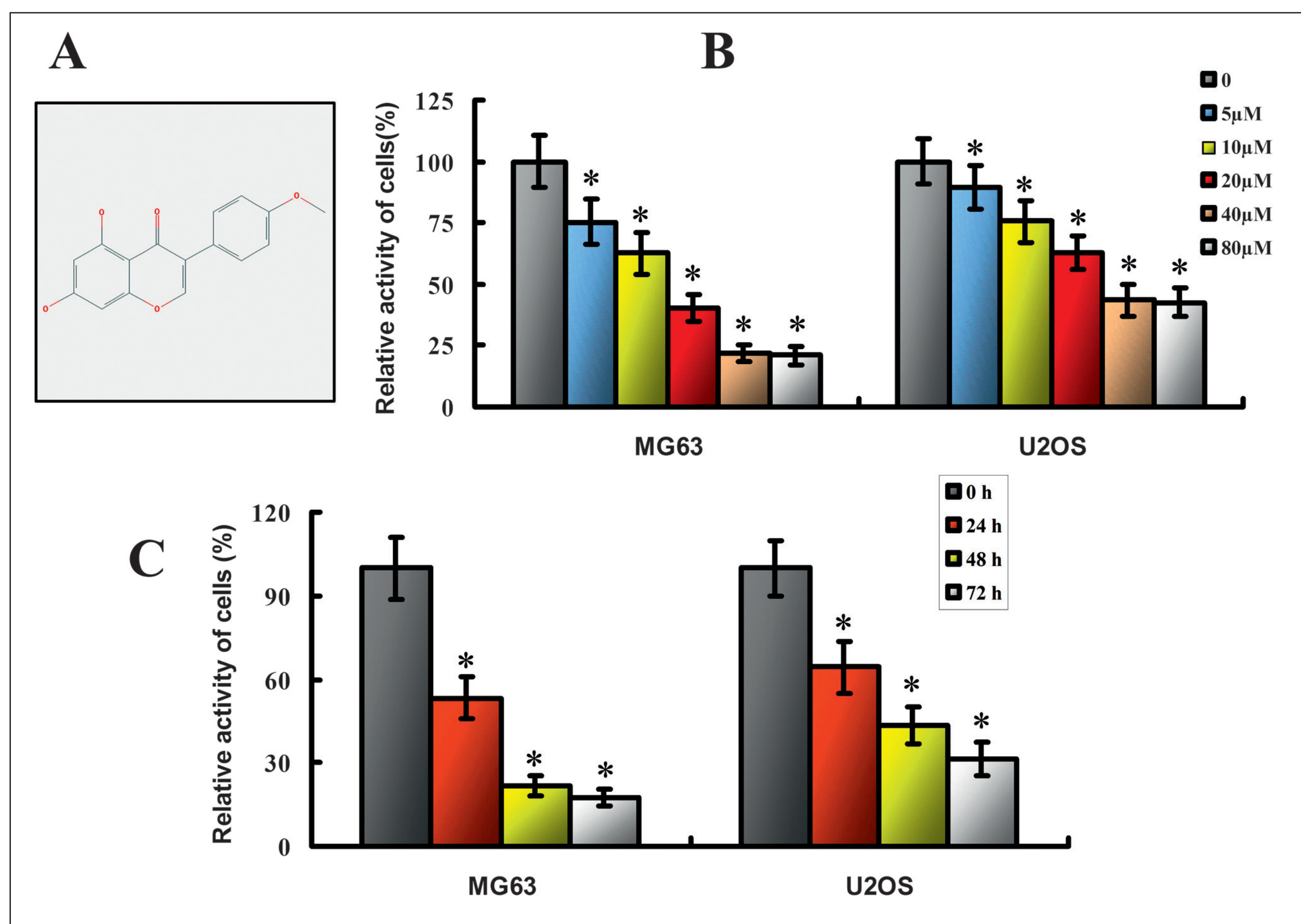

Fig. 1: Effect of biochanin A on the activity of osteocarcinomatous cell line MG63 and U2OS
Molecular structure of biochanin A was exhibited as A. Effects of biochanin A on MG63 and U2OS cells with different concentrations were illustrated as B, and effects of biochanin A on cells with different time were illustrated as C. Results revealed that biochanin A could inhibit the activity of MG63 and U2OS cells with dose- and time-dependent manner.
Note: ①*$P < 0.05$ *vs* 0μM in B; ②*$P < 0.05$ *vs* 0 h in C

2.4. Effect of biochanin A on MG63 and U2OS cell capacity of invasion and migration

After treatment of MG63 and U2OS cells with 40 μM biochanin A for 48 h, we used a wound assay to observe changes in the cell ability of migration and a transwell assay to examine changes in the cell ability of invasion. The results are shown in Fig. 4. Compared to control groups, treatment of biochanin A induced inhibition of cell ability of migration and invasion in both MG63 (Fig. 4A) and U2OS cells (Fig. 4B). According to the results, biochanin A may inhibit osteosarcoma cell invasion and migration.

2.5. Effect of biochanin A on proliferation, apoptosis, invasion and migration associated genes of MG63 and U2OS cells

As shown in Fig. 5, proliferation, apoptosis, invasion and migration associated genes varied after biochanin A treated in MG63 (Fig. 5A) and U2OS (Fig. 5B) cells. From the qPCR and Western blot data, we observed significant downregulation of PCNA, cyclinD1, Bcl-2, MMP-9 gene mRNA and protein expression in both MG63 and U2OS cell along with upregulation of Bax and E-cadherin expression ($P<0.05$). On the other hand, after treatment with biochanin A, MG63 cell has decreased expression of N-cadherin gene mRNA and protein ($P<0.05$), while U2OS cell did not shows a significant difference in treatment of biochanin A in comparison to control groups ($P>0.05$). Results revealed that biochanin A might be involved in proliferation, apoptosis, invasion and migration of osteosarcoma cells by regulating associated genes.

2.6. Effect of biochanin A on caspase-3 of MG63 and U2OS cells

In addition to that, the results demonstrate increased caspase-3, cleaved caspase-3 protein and caspase-3 activity in MG63 and U2OS cells treated with biochanin A, compared to control groups ($P<0.05$) (Fig. 6). Results revealed that biochanin A could inhibit cell activity by regulating caspase-3.

3. Discussion

Osteosarcoma is a common malignancy with depressing therapeutic outcomes and poor prognosis due to high morbidity and mortality (Archer et al. 2016). Pharmacological therapy plays a critical role in the treatment of osteosarcoma. In particular, chemotherapy remains an indispensable therapeutic option (Chen et al. 2017). Methotrexate (MTX), cisplatin (CDP), doxorubicin (ADM) and ifosfamide (IFO) are common drugs that have been effective in combination treatment for osteosarcoma (Zhang et al. 2016; Aznab et al. 2017; Huang et al. 2015). However, resistance to chemotherapy is one of the limitations resulting in recurrence or metastasis in osteosarcoma (Zhang et al. 2017). Hence, exploring novel drug therapies is of great clinical significance in treating osteosarcoma.

Phytochemicals have recently achieved some success in treating cancers (Tariq et al. 2017; Li et al. 2017; Han et al. 2015). In osteosarcoma treatment, herbal compounds have also been proved effective. So resveratrol and *Artemisia annua* extract have shown immunosuppressive effects on osteosarcoma cells (Zou et al. 2015; Tang et al. 2015). *Trifolium pratense* L. is another widely

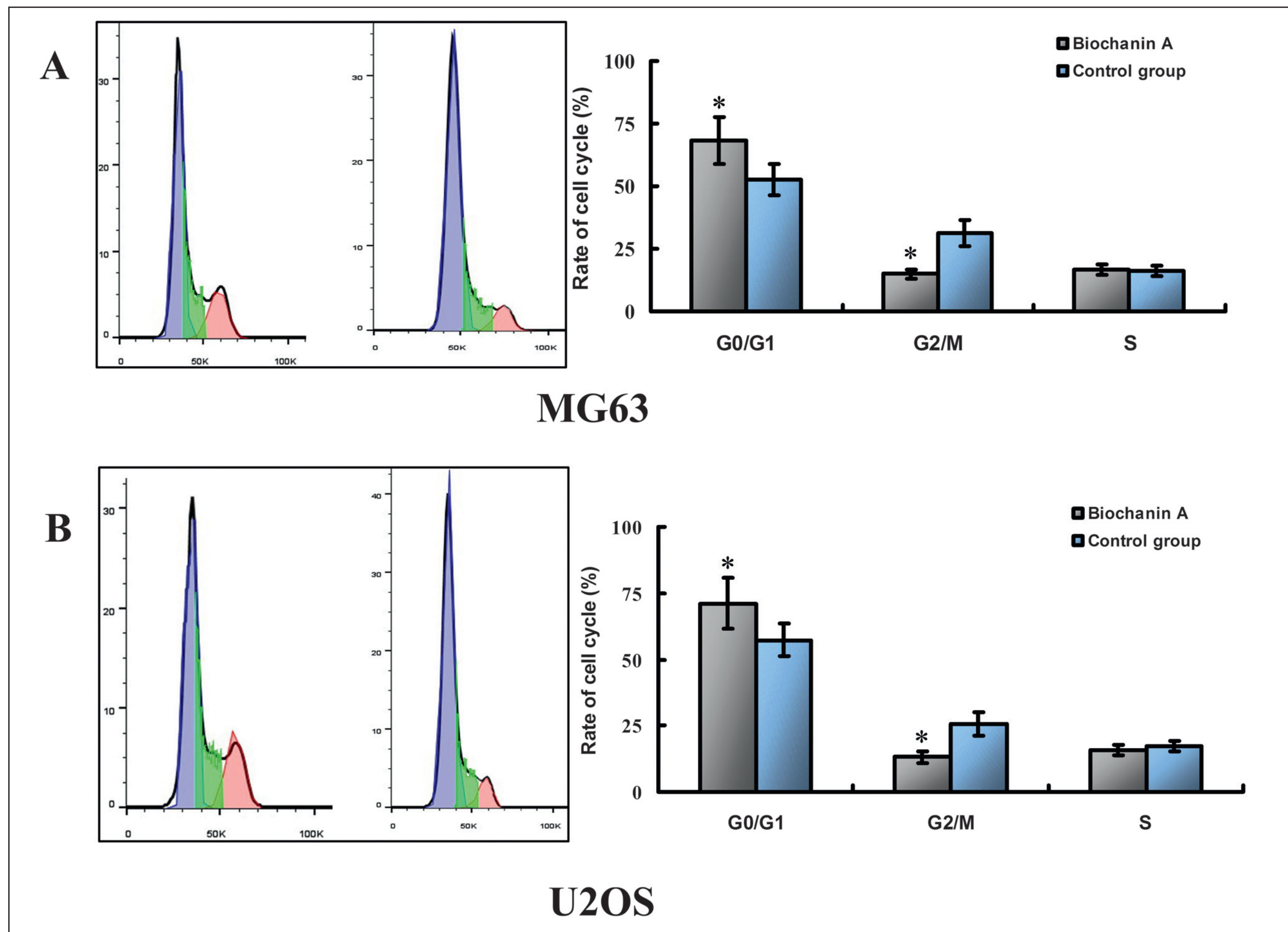

Fig. 2: Effect of biochanin A on cell cycle of MG63 and U2OS cells. Results of flow cytometry (FCM) showed that after treated with biochanin A, cycle of MG63 and U2OS cells varied significantly. Compared with control group, the ratio of G0/G1 stage cells increased in biochanin A group, and decreased ratio in G2/M stage (*P*<0.01), whereas no significant difference in S stage (*P*>0.05), as shown in A and B.
Note: $^*P < 0.01$ *vs* control group

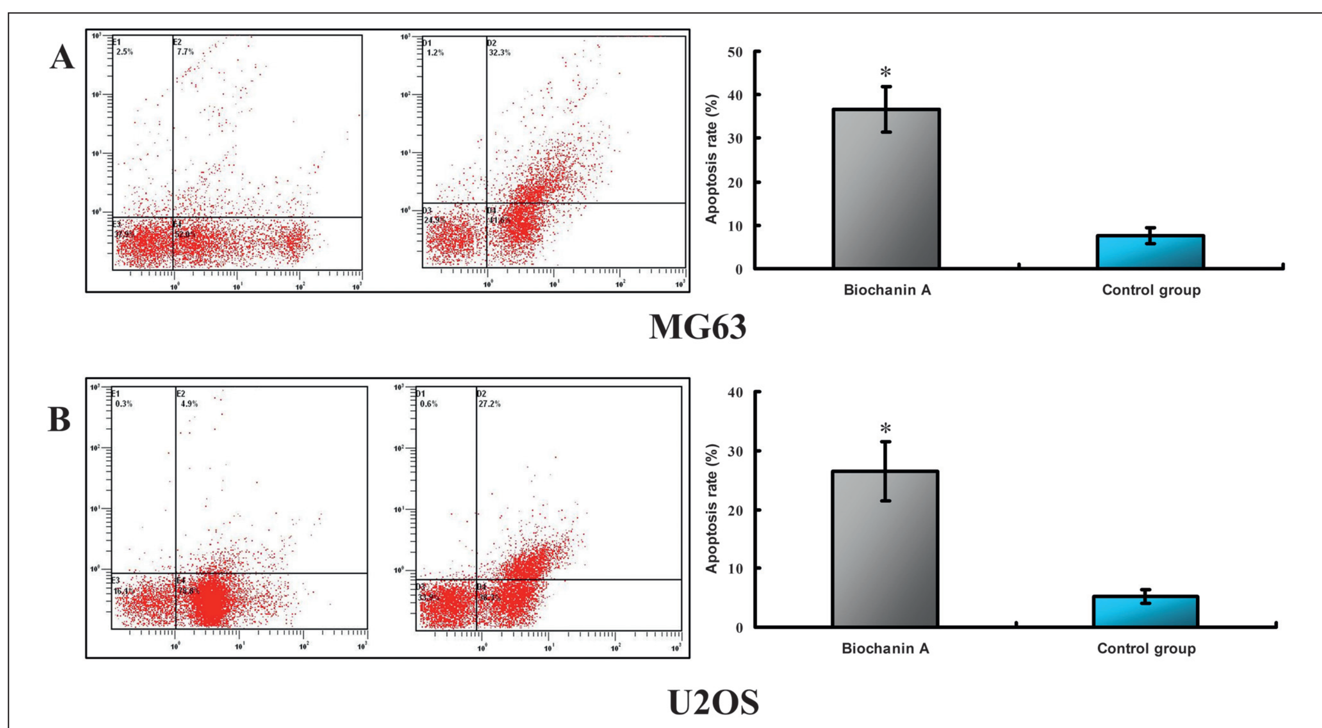

Fig. 3: Effect of biochanin A on apoptosis rate of MG63 and U2OS cells. MG63 and U2OS cells treated with biochanin A showed variation on apoptosis rate with FCM test. Compared with control group, the apoptosis rate in biochanin A group increased (*P*<0.01), and results of MG63 cells (A) was in accordance with U2OS cells (B).
Note: $^*P < 0.01$ *vs* control group

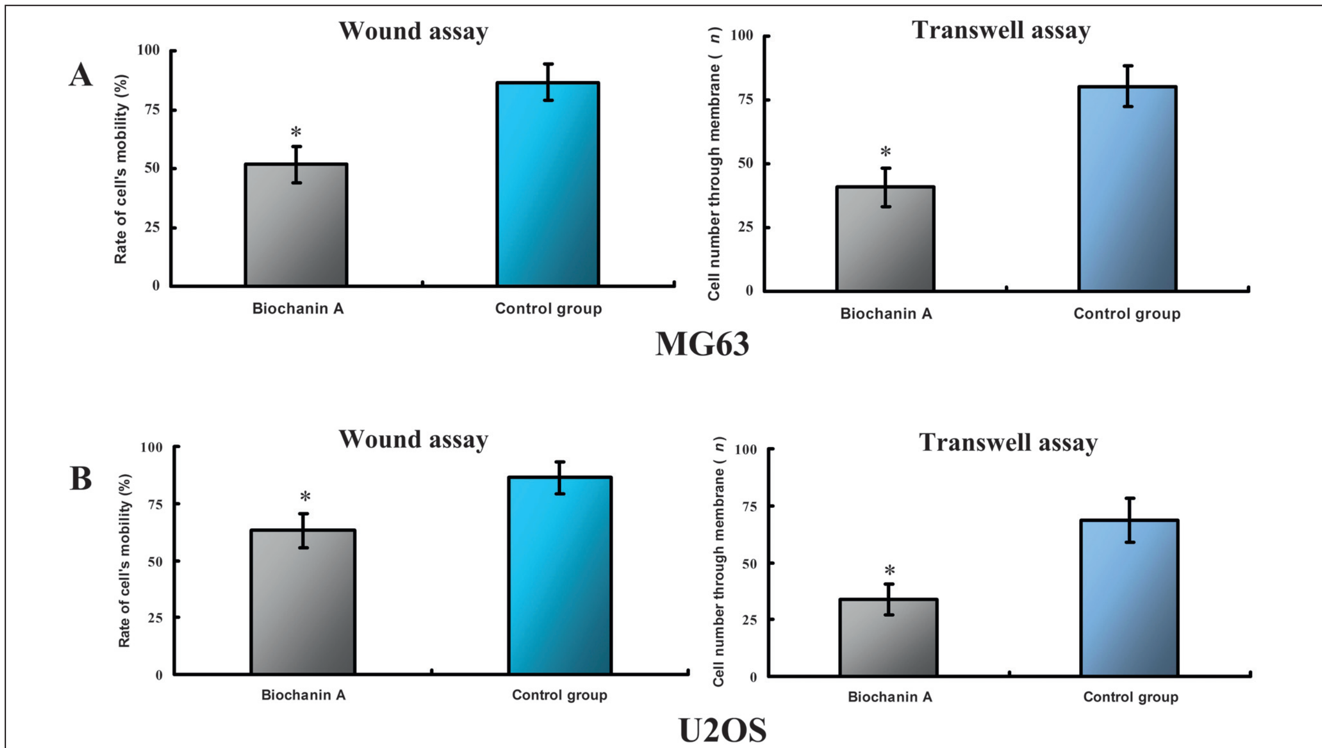

Fig. 4: Effects of biochanin A on migration and invasion of MG63 and U2OS cells. After treated with Biochanin A, MG63 and U2OS cells were subjected to Wound assay to detect cell migrating activity, and Transwell chamber assay to detect cell invasion activity. Results of MG63 cells (A) was in accordance with U2OS cells (B). Note: *P<0.01 *vs* control group.

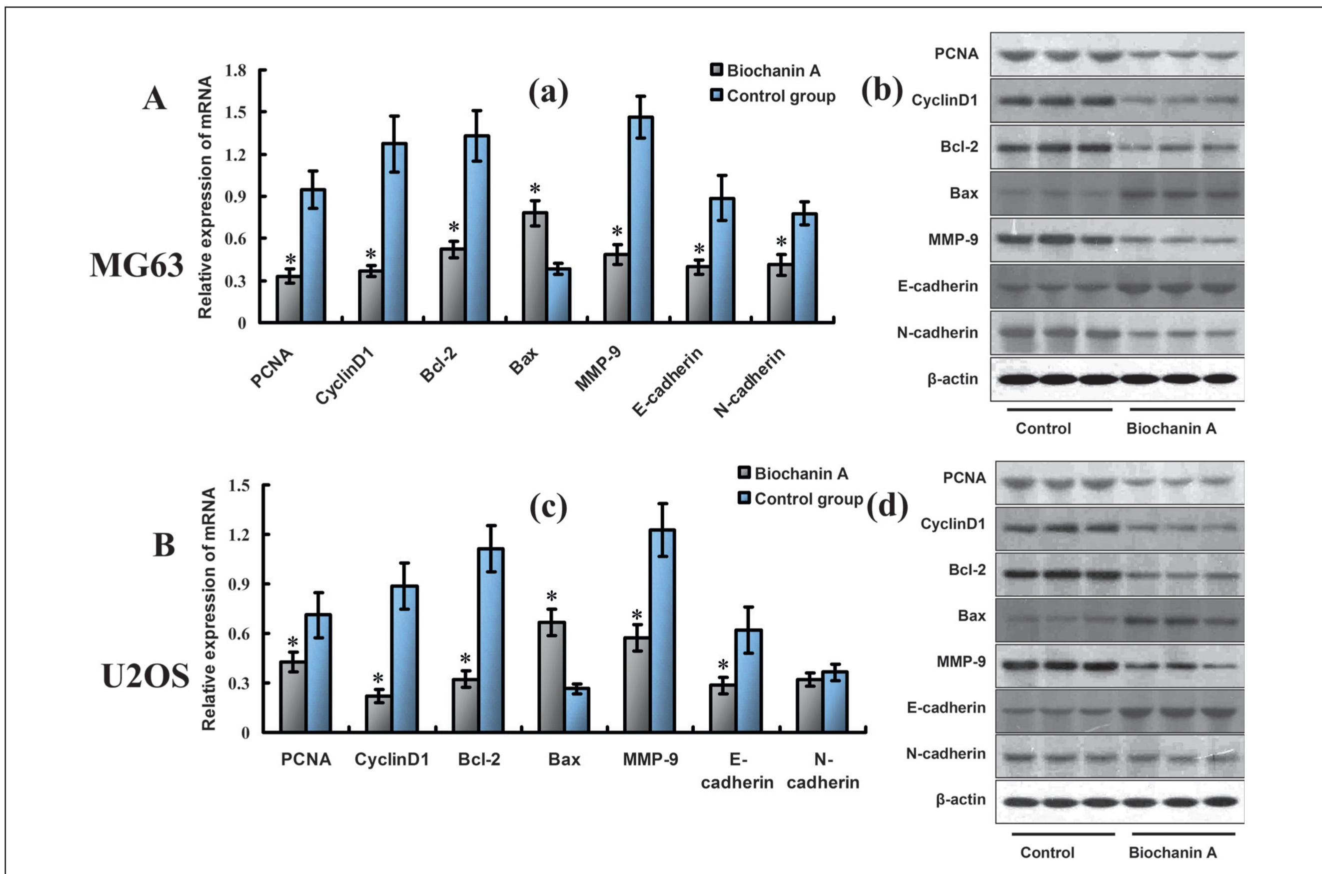

Fig. 5: Effect of biochanin A on proliferation, apoptosis, migration and invasion associated genes of MG63 and U2OS cells. MG63 and U2OS cells were treated with biochanin A, and then were subjected to qPCR [(a) and (c)] and Western blotting assays [(b) and (d)], to detect the mRNA or protein expression levels of PCNA, cyclinD1, Bcl-2, Bax, MMP-9, E-cadherin, N-cadherin genes. Results showed that expressions of PCNA, cyclinD1, Bcl-2, MMP-9, N-cadherin decreased but Bax, E-cadherin increased in MG63 cells, which was illustrated in A. As to U2OS cells, there was no significant difference for N-cadherin gene between biochanin A group and control group, and results of MG63 cells (A) was in accordance with U2OS cells (B) about other genes.
Note: *P<0.01 *vs* control group

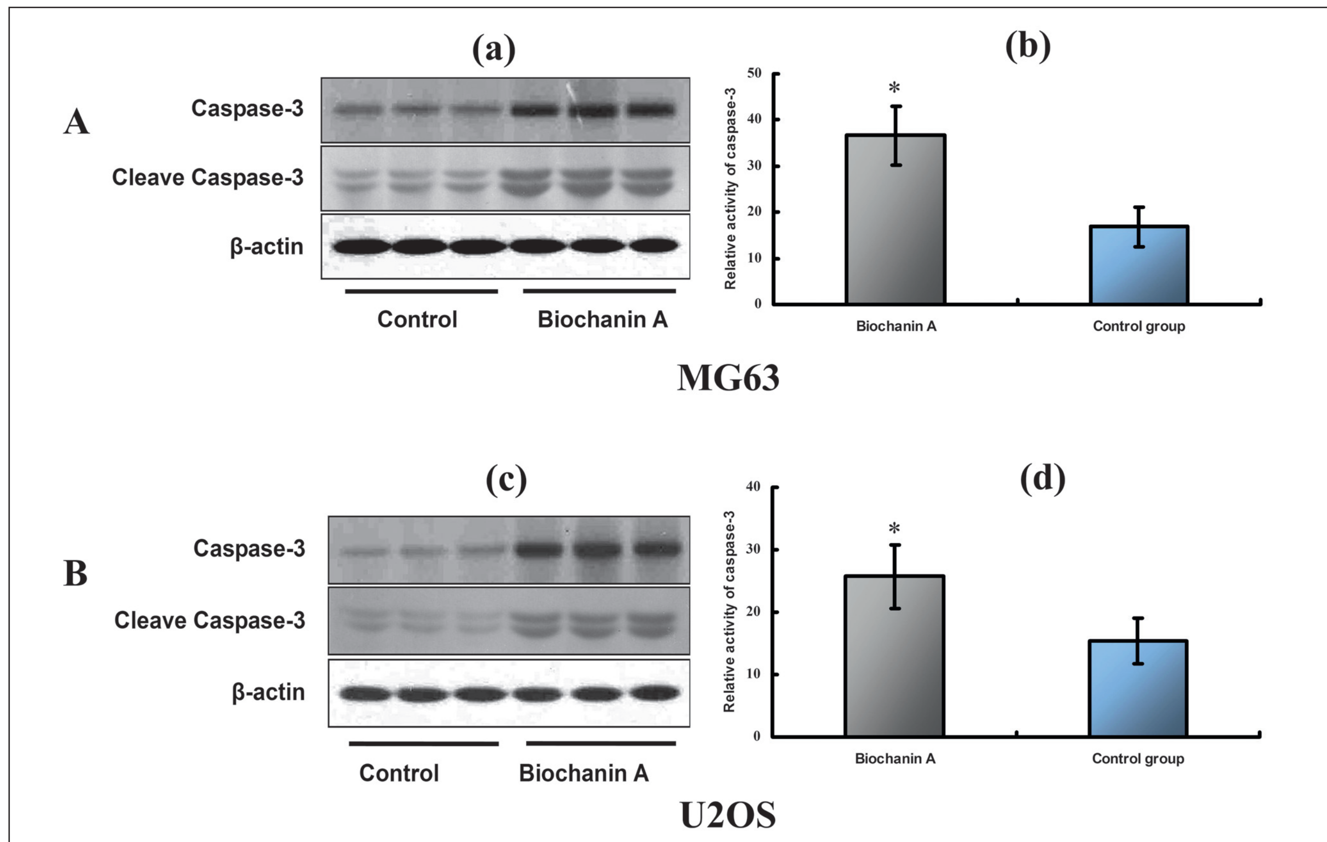

Fig. 6: Effect of biochanin A on the expressions of caspase-3, cleave caspase-3 and caspase-3 activity in MG63 and U2OS cells. After treated with biochanin A, expressions of caspase-3, cleaved caspase-3 increased significantly, and activity of caspase-3 also increased, as shown in A and B. Results of MG63 cells (A) was in accordance with U2OS cells (B).
Note: **P*<0.01 *vs* control group

employed herbal drug that has effects of immunoregulation, cough relief, anti-inflammation and treating ulcers (Ligacheva et al. 2016; Vlaisavljevic et al. 2014). Biochanin A is one of the active ingredients extracted form *Trifolium pratense* L (Zgórka 2011). It can affect cell capability of proliferation, apoptosis and drug resistance in liver, prostate and colorectal cancer (Youssef et al. 2016; Ahmad et al. 2013; Fokialakis et al. 2012). Yet, effects of biochanin A on osteosarcoma have not been reported. In this study, we found that biochanin A was effective in suppressing the cell activity of MG63 and U2OS. The result indicates that biochanin A may not only serve as an inhibitor of tumour cell proliferation, invasion and migration but can also act as a promoter of apoptosis in osteosarcoma. Thus, biochanin A may be a potential new pharmacological option in treating osteosarcoma, although more research is required.

Uncontrolled growth is one main feature of tumor cells (Wang et al. 2017). Inhibition of tumour growth can be achieved by suppression of cancer cell proliferation. A high rate of tumour cell proliferation is associated with cell cycle disorders because among cancer cells, the number of cells in the G0/G1 phase is smaller than that in the G2/M or S phase. Therefore, regulating cell cycles is a pivotal pathway in tumour treatment (Li et al. 2017). In the present study, biochanin A was found to increase the percentage of cells in the G0/G1 phase while decreased the number of cells in the G2/M phase. It was also found to downregulate PCNA and cyclinD1 gene expression in both MG63 and U2OS osteosarcoma cells. The PCNA is an important marker of cell proliferation. It is closely associated with cell DNA synthesis and considered a factor in activation of cell proliferation (Wang et al. 2016). CyclinD1 is a cell proliferation promoter. It activates G1 phase-specific cyclin-dependent kinases 4 (CDK4) thereby initiating cell cycle progression from resting to mitosis through regulating a series of downstream genes (Zhu et al. 2017). Therefore, biochanin A may affect osteosarcoma tumour growth through inhibition of PCNA and CyclinD1 expression.

One of the hallmarks of tumour cell is the resistance to apoptosis (Zhang et al. 2017). Therefore, promoting programmed cell death in tumour cells is a vital aspect in curing cancer. This study demonstrated that treatment of MG63 and U2OS cells with biochanin A significantly increased the apoptotic rate. This result indicates that the promotion of cell apoptosis is likely one reason why biochanin A may be applied for osteosarcoma treatment. Bcl-2 enhances apoptotic resistance in cancerous cells through the mitochondrial pathway and therefore increases drug resistance in tumour cells. Bax on the other hand promote cell apoptosis in cancerous cells. Bcl-2 and Bax have a synergistic effect on regulation of cell apoptosis with different Bcl-2/Bax ratio (Golestani et al. 2014; Wu et al. 2011). Our research hightlighted that biochanin A treatment contributed to decreased Bcl-2 expression but increased Bax expressions in osteosarcoma cells. Biochanin A may participate in osteosarcoma cell death through mediating mitochondrial pathway, although a further understanding of the molecular mechanisms is needed. Caspase-3 is one of the essential genes in apoptotic promotion, which directly induces cell death in tumour cells (Yang et al. 2017). The activated caspase-3 is one of the direct factors affecting apoptosis. Therefore, this study detected levels of caspase-3, activated caspase-3, and cleaved caspase-3 in MG63 and U2OS cells under biochanin A treatment. We found that after biochanin A treatment, the expression of caspase-3 and cleaved caspase-3 significantly increased along with increased activity of casepase-3, compared to the control groups. This finding indicates that regulation of cell apoptosis is another important mechanism of biochanin A in treating osteosarcoma.

Inhibition of cell invasion and migration of cancer cells is beneficial to control of tumour progression and improvement of therapeutic outcomes (Zhuo et al. 2017). During the process of

invasion and migration, tumour cells undergoing epithelial-mesenchymal transition (EMT) plays an essential role. Therefore, we carried out an experiment to develop understanding for the relation between biochanin A treatment and the cell capacity to invade and migrate in MG63 and U2OS cells. The results demonstrate that under treatment of biochanin A, the cell capacity of invasion and migration of MG63 and U2OS cells significantly decreased. This indicates that biochanin A may play an inhibitive role in osteosarcoma invasion and metastasis. We then took a further step in investigating the association of biochanin A with EMT by examining the expression of MMP-9, E-cadherin and N-cadherin. MMP-9 is an important member of MMPs, which degrades extracellular matrix (ECM) and therefore contributes to cell migration (Li et al. 2015). E-cadherin is an important factor of cell-cell adhesion. It is classified as a cancer depressor because the loss of E-cadherin may lead to destruction of cytoskeleton promoting cells invasion and migration (Iseki et al. 2017). The expression of N-cadherin in epithelial tumour characterizes tumorigenesis as it may induce EMT, reinforce tumour cell activeity and promote the interaction between tumour and neighbouring cells (Labernadie et al. 2017). We observed that the osteosarcoma cells treated with biochanin A demonstrated underexpression of MMP-9 and N-cadherin but overexpression of E-cadherin. This result suggests that biochanin A may have suppressive funtion in cancer invasion and migration through mediation of tumour EMT.

In conclusion, in the current study, biochanin A showed a significant suppressive effect on osteosarcoma cells due to its ability to mediate tumour cell proliferation, apoptosis, invasion and migration. This study supports the assumption that biochanin A use may be beneficial in clinical therapy. More detailed understanding requires more experiments *in vivo* and clinical trials in a larger scale.

4. Experimental

4.1. Cell line and reagents

Human osteosarcoma cell lines MG63, U2OS were purchased from the Shanghai Institute of Biochemistry and Cell Biology, and cultured and regenerated in Research Center of our hospital. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was from Sigma Co. USA. DMEM culture media and trypsin solutions were produced by Gibco USA. Trizol reagents were all supplied by Invitrogen,, USA. Antibodies of PCNA, cyclinD1, Bcl-2, Bax, caspase-3, cleaved caspase-3, MMP-9, E-cadherin, N-cadherin, and β-actin were obtained from Sigma USA. Related PCR primers were synthesized by Sangon Biotech (Shanghai) Co., China. Biochanin A was offered by Sigma Co., USA.

4.2. Cell culture

Human osteosarcoma cell lines MG63 and U2OS were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100mg/ml streptomycin at 5 % CO_2, 37 °C for incubation. Cells lines were lysed using 0.25% trypsin solutions in combination with 0.02% EDTA and applied for studies when growing to 60-70%.

4.3. MTT Assay

Each group of cells was prepared as monolayer culture and seeded in 96-well plates at 5×10^4 cells/ml. 20 µl of MTT reagent (5 mg/ml) was subsequently added into each well for 4 h incubation. After that, culture media were discarded and 150 µl of dimethyl sulfoxide (DMSO) was added followed by 15 min shaking. The optical density (OD) value was measured at 490 nm in a microplate-reader. The whole process was repeated for three times.

4.4. Flow cytometric analysis of cell cycle

Cells of each group were harvested by centrifugation with the supernatant fluid being removed. The cells were washed with ice cold PBS twice and added with 70% cold ethanol for fixation at 4 °C overnight. The cells were resuspended by centrifugation and washed once with 1 ml PBS. Cell suspension (100 µl) at 1×10^6 cells/ml were collected and treated with 50 µg/ml propidium iodide (PI), 100 µg/ml RNase A and 0.2% Triton X-100 PBS. After 30 min of incubation at 4 °C protected from light, the cells were analyzed on the flow cytometer (Epics-XL II, Beckman Coulter, Inc. USA). The experiment was repeated in triplicate.

4.5. Flow cytometric analysis of cell apoptosis

Apoptotic rates of cells were carried out by using Annexin V-FITC/PI detection kit (Jiamei, Beijing, China), according to the instruction. The cells were resuspended and adjusted to a concentration of 10^6 cells/mL. A working solution consisting of 5 µl of Annexin V-FITC and 10 µl of PI was added to the cells. After 15 min incubation at room temperature protected from light, the cells were detected for early and late apoptotic rates on the flow cytometer (Epics-XL, Beckman Coulter, Inc. USA). The assay was conducted for three times.

4.6. Wound assay

The cells were seeded at 5×10^4 cells/mL in 24-well plates and cultured until confluent. After another 48 h incubation, a mark of scratch was created with a 200 µl pipette tip following the aspiration of media. The cell culture was resuspended in serum-free media for another 24 h. After that, cells were rinsed with PBS three times for removal of cell debris. The cells were observed under microscope for measuring the distance of migration. The procedure was repeated in triplicate.

4.7. Transwell chamber assay

The cell invasion assay was conducted using 24-well plate, transwell chamber inserted with a pore size of 8 µm on the PC membrane. The top of the membrane was coated by 100 µl of Matrigel and solidified under UV radiation for 2 h. MG63 and U2OS cells suspension was prepared when they were growing in the exponential phase. The cells were seeded at 1×10^6 cells/ml in 6-well plates. They were added with biochanin A or DMSO after the yield reached 60-70%. After a 24 h culture, 200 µl of cell solution from each group were aspirated and plated in the upper chamber in 24-well plates. In the lower chamber, EMDM medium containing 10% fetal bovine serum was added. Following 24 h culture, the Matrigel and the remaining cell on the top of upper chamber were removed by cotton-tipped applicators. The transwell insert then placed into ethanol for 10 min allowing cell fixation. After that, crystal violet staining was performed for visualization. The transwell membrane was viewed under microscope and counted for the number of cells in five random fields of view. The assay was conducted three times.

4.8 Quantitative fluorescence RT-PCR

Total RNA was extracted using the Trizol single-step method and reverse transcribed with 2 µg into cDNA template. Two µl of products of reverse transcription was used to examine the expression levels in target molecules. GAPDH was used as internal reference gene. The PCR system in total 20 µl was established according to the manufacturer's protocol, consisting of 2 µl of reverse transcription product, 10 µl of SYBR Green Mix (Applied Biosystems, Foster City, CA), 0.5 µl of forward and reverse primers (10 µmol/l) respectively. PCR was run for 45 cycles under such cycling conditions as 95 °C for 5min followed by denaturation at 94 °C for 30 s and annealing at 60 °C for 30 s. The primers were designed by using Primer 5.0 and then BLASTed to test the specificity. The sequences of primers are listed in the Table. The result of RT-PCR was analysed using 1.5% agarose gel electrophoresis. The result of fluorescence quantitative PCR was quantified by using $2^{-\Delta\Delta Ct}$ method with β-actin as the endogenous reference gene.

4.9. Western-blot assay

Total protein from each group was extracted and the concentration was determined using concentration Bradford reagent. Protein samples (100 µl) from each group was obtained and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on to polyvinylidene fluoride membrane (PVDF). The membrane was then blocked in 5% skim milk solution containing TBST for 1 h at room temperature. Each group of membrane was subsequently treated with dilutions of primary antibody specific for molecules of interest or of endogenous reference β-actin and incubated at 4 °C overnight. After that, it was washed for 3 times with TBST and treated with horseradish peroxidase conjugated secondary antibodies for 1-hour incubation at room temperature. The blots were then developed using chemiluminescence for colorimetric detection. The band was scanned for absorbance.

4.10. Caspase-3 activity assay

The caspase-3 activity in MG63 and U2OS cells was detected using N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (ac-DEVD-AFC) (Calbiochem, USA) method. A 15µl of protein extraction was added with caspase-3 assay buffer (50 mM of HEPES, pH 7.4, 100 mM NaCl, 1 mM EDTA, and 10 mM DTT) and incubated at 37°C for 2 h. Following that, the buffer was added with 15 µl of ac-DEVD-AFC (2 mM). The AFC liberated from the Ac-DEVD-AFC was then measured using a spectrofluorometer (400 nm excitation wavelength and 505 nm emission wavelength). The experiment was conducted three times.

4.11. Statistical analysis

Data were expressed as ±standard error of mean. Analysis of variance (ANOVA) and Dunnet *t* test were carried out using SPSS 19.0 to cross analyze the effect and the molecular mechanism of biochanin A on MG63 and U2OS cell. $P < .05$ was considered as statistically significant.

Conflicts of interest: All authors have no relevant financial interests to disclose in this paper.

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