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# Autophagy Inhibition Promotes Quercetin Induced Apoptosis in MG-63 Human Osteosarcoma cells

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**Quercetin is a natural flavonoid phytochemical that is extracted from various plants. Having an advantages due to its varied biological properties, such as anti-inflammatory, anti-viral, anti-oxidant, and anti-cancer effects, quercetin is used to treat many diseases. Recently, it has been reported that autophagy inhibition may play a key role in anti-cancer therapy. Therefore, in this study, we investigated the molecular mechanisms and anti-cancer effects of quercetin in human osteosarcoma cells via autophagy inhibition. We ascertained that quercetin inhibited cell proliferation and induced cell death, these process is demonstrated that apoptosis via the mitochondrial pathway and the caspase cascade. Quercetin also induced autophagy which was inhibited by 3-MA, autophagy inhibitor and the blockade of autophagy promoted the quercetin-induced apoptosis, confirming that autophagy is a pro-survival process. Thus, these findings demonstrate that quercetin is an effective anti-cancer agent, and the combination of quercetin and an autophagy inhibitor should enhance the effect of anti-cancer therapy.**

**Key words: quercetin, apoptosis, autophagy, osteosarcoma, 3-MA**

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## Introduction

Osteosarcoma is the most common bone tumor and is highly malignant. While the incidence of osteosarcoma is low, it predominately occurs in children and young adults [1,2]. The tumor most commonly arises in the metaphysis of long bones (thigh bones and shin bones) around the knee or the shoulder. The five-year survival rate for osteosarcoma patients with localized tumors is 60%–70%. In cases where tumors metastasize to the lungs, the survival rate decreases sharply to 20%–30% [3,4]. Typical treatments for cancer are resection of the tumor and chemotherapy. Despite the number of chemotherapeutic agents available, there is still a need to develop novel biological agents for osteosarcoma treatment [5,6].

Quercetin is a flavonoid phytochemical found in many kinds of fruits and vegetables, such as berries, apples, brassica vegetables, and onions [7,8]. Recently, quercetin has received much attention because of its various biological advantages, such as its anti-bacterial, anti-inflammatory, anti-oxidant, and anti-cancer properties [9-11]. A previous study reported that quercetin treatment results in cell death in human hepatoma HepG2 cells [12], and a number of studies have indicated that quercetin is an effective anti-cancer agent in various types of

cancer [13-15]. However, few studies have discussed the effect of quercetin on osteosarcoma cells.

Autophagy, or type II programmed cell death, is an evolutional process related to the degradation and recycling of cytoplasmic components by the formation of autophagosomes in response to stress or nutritional deficiencies [14,16]. Lately, this process has emerged as an increasingly important concept in cancer therapy. Dysregulated autophagy may lead to many diseases, including tumorigenesis. It is uncertain to what extent this mechanism participates in tumor growth, and thus this needs to be researched further [17-19].

In this study, the aim is to determine the underlying mechanisms and the effect of quercetin on MG-63 cells. Here, we provide some evidence that quercetin executes its anti-tumor effects in osteosarcoma by regulating apoptosis and autophagy.

## Materials and Methods

### Cell culture

The human osteosarcoma cell line MG-63 was purchased from ATCC (Rockville, MD, USA). Cells were maintained at 37°C in a humidified atmosphere containing with 5% $CO_2$ in DMEM (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin-steptomycin (WELGENE, Daegu, Korea).

### Treatment of Quercetin

Quercetin (Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO; Duchefa, haarlem, NED) at a stock solution of 100 mM and was kept frozen at -20°C until use. The stock was diluted to their concentration with DMEM when needed. Prior to quercetin treatment, cells were grown to about 70%–80% confluence and then exposed to quercetin at different concentrations (0–1000 μM) for 24 h–72 h. Cells grown in medium containing an equivalent amount of DMSO without quercetin served as controls. For autophagy control, cells were grown in Earle's Balanced Salt Solution (EBSS; Sigma).

### Hoechst staining

After quercetin treatment, cells were harvested and cytocentrifuged onto a clean, fat-free glass slide with a cytocentrifuge. Cells were stained in 1 μg/ml Hoechst 33342 (Sigma) for 10 min at 37°C in the dark and washed twice in phosphate buffered saline (PBS; Sigma). The slides were mounted with glycerol. The samples were observed and photographed under an epifluorescence microscope (Axioskop, Carl Zeiss, Göettingen, Germany). The number of cells that showed condensed or fragmented nuclei was determined by a blinded observer from a random sampling of $3\times10^2$ cells per experiment. Three independent experiments were conducted.

### Flow cytometry analysis and mitochondrial membrane potential($\Delta\Psi m$)

Cells were seeded in a 6-well plate, incubated for 24 h, and treated with various dosages of quercetin (0–1000 μM) for 24 h–72 h. For the quantification of DNA hypoploidy, cells were harvested by trypsinization, and ice cold 95% ethanol with 0.5% Tween 20 was added to the cell suspensions to a final concentration of 70% ethanol. After the cells were fixed overnight, they were washed in 1% bovine serum albumin (BSA; Sigma)-PBS solution, resuspended in PBS containing 40 mg/mL RNase A, and incubated at 4°C for 30 min. The cells were washed with BSA-PBS and resuspended in propidium iodide (PI; Sigma) solution (10 μg/ml). After the cells were incubated at 4°C for 5 min in the dark, the DNA content was measured using a Cytomics FC500 flow cytometry system (Beckman Coulter, CA, USA) and data was analyzed using MultiCycle software that allowed a simultaneous estimation of cell-cycle parameters and apoptosis. The mitochondrial membrane potential (MMP) was measured using $DiOC_6$ dye. The cells were loaded with a final concentration of 1 μM $DiOC_6$ solution at 37°C for 30 min and then analyzed using flow cytometry. Three independent experiments were conducted.

### Immunofluorescent staining

The cells were plated on coverslips and treated with quercetin. After 48 h, the cells were stained with 50 nM MitoTracker Red at 37°C for 30 min. After washing two times with PBS, the cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min and washed three times with PBS. After permeabilization with Triton X-100 and blocking with 10% goat serum in PBS, the cells were incubated with primary antibodies in 1% BSA overnight at 4°C. After washing with PBS, the cells were

incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibodies in 1% BSA-PBS for 60 min and rinsed in PBS. Fluorescent images were observed and analyzed using a Zeiss LSM 750 laser-scanning confocal microscope (Carl Zeiss, Göettingen, Germany).

### Fluorescence microscopy

The cells were grown on coverslips and treated with quercetin. After 48 h, they were stained with 1.25 μM monodansylcadaverine (MDC; Sigma), a selective fluorescent marker for autophagic vacuoles, at 37°C for 30 min. The cellular fluorescence changes were observed using a fluorescence microscope (Axioskop, Carl Zeiss). As an autophagy control, cells were starved using EBSS. For further detection of the acidic cellular compartment, we used acridine orange (AO; Sigma), which emits bright red fluorescence in acidic vesicles but fluoresces green in the cytoplasm and nucleus. The cells were stained with 1 μg/mL AO for 5 min and washed with PBS. The formation of acidic vesicular organelles (AVOs) was observed using an LSM 700 confocal microscope (Carl Zeiss).

### Western blot analysis

The cells ($2\times10^6$) were washed twice in ice-cold PBS, resuspended in 200 μl ice-cold lysis buffer (300 mM NaCl, 50 mM Tris-Cl [pH 7.6], 0.5% Triton X-100, 2 mM PMSF, 2 μl/ml aprotinin and 2 μl/ml leupeptin), and incubated at 4°C for 30 min. The lysates were centrifuged at 13,200 rpm for 30 min at 4°C. Protein concentrations of cell lysates were determined using a Bradford protein assay (Bio-Rad, Richmond, CA, USA), and 25 μg of proteins were resolved in 10% SDS/PAGE gel. The gel was transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and reacted with appropriate primary antibodies. Immunostaining with secondary antibodies was detected using SuperSignal West Femto enhanced chemiluminescence substrate (Pierce, Rockford, IL, USA) and detected with Alpha Imager HP (Alpha Innotech, Santa Clara, CA, USA). Antibodies against caspase-3, PARP, Beclin-1, Atg5, and LC3 were purchased from Cell Signaling Technology (Beverly, MA, USA). The p62/SQSTM1, Bcl-2, Bak, caspase-9, caspase-7, GAPDH, mouse anti-actin antibody, mouse anti-rabbit IgG antibody, and rabbit anti-mouse IgG antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA).

### Statistic analysis

The data are expressed as mean ± SE. Statistical significance between groups was analyzed using one-way analysis of variance (ANOVA) and a Dunnett's comparison. Probability (p) values less than 0.05 were considered statistically significant.

## Results

### Quercetin treatment inhibits MG−63 cell growth and induces cell death via apoptosis

To investigate the effect of quercetin on the growth of osteosarcoma cells, we used MG-63 cells treated with various doses of quercetin. The cells were incubated with different concentrations of quercetin (0−1000 μM) for 24 h −72 h. Cell morphological changes were observed using photographs, and the proliferation of MG-63 cells was inhibited at 1000 μM, 500 μM, and 250 μM after 24, 48, and 72 h, respectively (Fig. 1A).

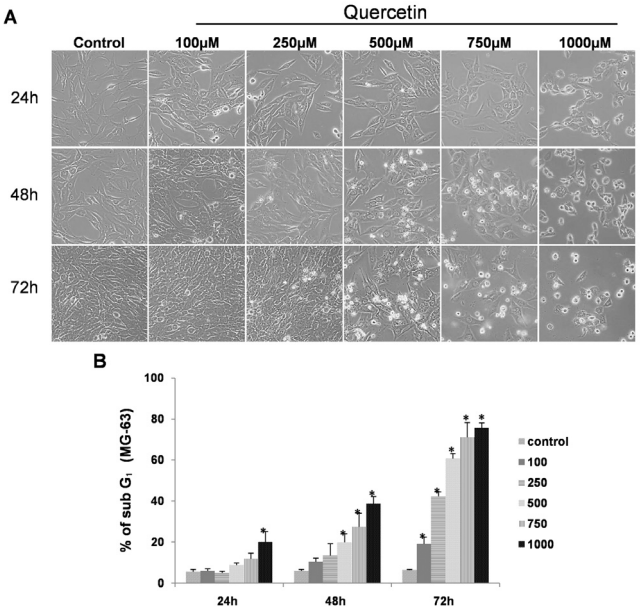


**Fig. 1.** Quercetin inhibited MG-63 cell growth via apoptotsis in dose- and time-dependent manners. (A) Representative micrographs of MG-63 cells treated with vehicle, quercetin (0−1000 μM) for 24 h-72 h. (B) The ratio of apoptotic cells was determined by flow cytometry analysis. Values are the mean ± SE from three independent experiments. * $P < 0.05$ compared to control group.

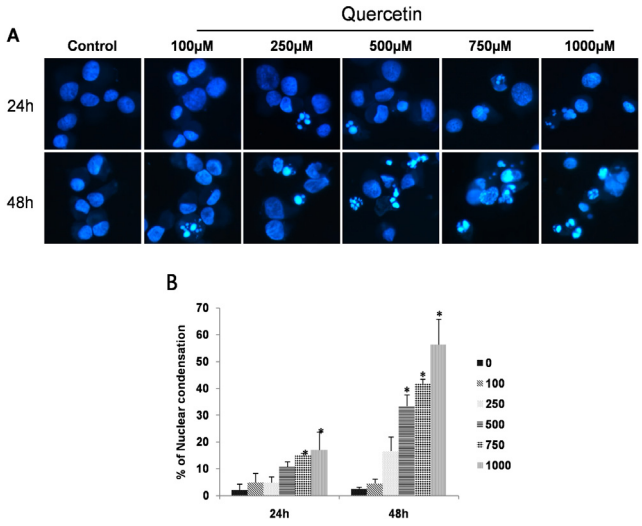


**Fig. 2.** Quercetin induced chromatin condensation and nuclear fragmentation in MG-63 cells. Cells were treated with quercetin for the range of concentrations, (A) Hoechst stained cells were observed by fluorescence microscope. (B) The values are denoted as mean ± SE of three independent experiments. * $P < 0.05$ compared to control group.

In addition, to investigate the type of cell death caused by quercetin, we performed flow cytometry analysis and Hoechst staining. As shown in Fig. 1B, flow cytometry analysis revealed that accumulations of sub-$G_1$ phase in both dose- and time-dependent manners. Quercetin-induced cell death is also associated with nuclear condensation and fragmentation, which are features of apoptosis. Quercetin-treated MG-63 cells displayed fragmented and condensed nuclei; in contrast, the control group nuclei were normal with a round shape. Significant morphological changes were detected in the 48 h group (Figs. 2A and 2B).

## Quercetin treatment induces disruption of the mitochondria membrane and caspase−dependent apoptosis in MG−63 cells

To address the effects of quercetin on mitochondria, we investigated various parameters associated with mitochondrial functions after quercetin treatment for 48 h. The MMP was measured using flow cytometry with $DiOC_6$ dye, and it was revealed that quercetin dose-dependently collapsed MMP in MG-63 cells (Fig. 3A). In addition, the release of cytochrome c was detected using confocal microscopy. Cytochrome c was released from mitochondria into the cytoplasm of MG-63 cells treated with 500 μM of quercetin for 48 h (Fig. 3B). We examined mitochondrial-related proteins and Bcl-2 family molecules, such as Bcl-2 and Bak, and found that the

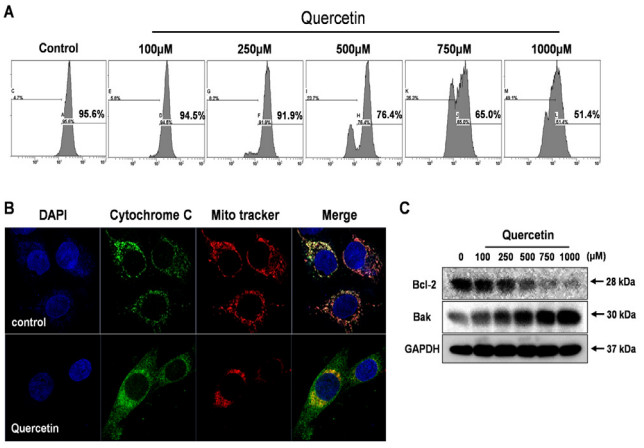


**Fig. 3.** Mitochondrial dysfunctions induced by quercetin in MG-63 cells. (A) Cells were treated with quercetin for 48 h, $DiOC_6$ stained cells were analyzed by flow cytometry. (B) Immunofluorescence of cytochrome c release after mitochondria membrane disruption by confocal microscopy. Cells were treated with 500 μM quercetin for 48 h. (C) Bcl-2 and Bak expression levels in MG-63 cells treated with quercetin was detected by western blot analysis. GAPDH was used as a loading control.

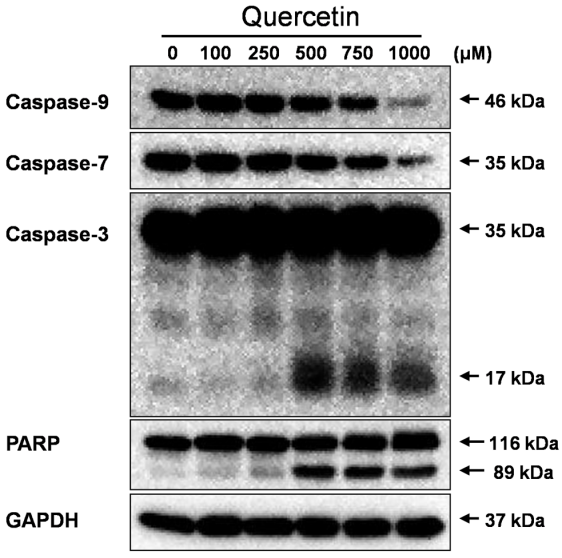


**Fig. 4.** Quercetin induced apoptosis via caspase-dependent manner in MG-63 cells. Cells were incubated with different concentrations of quercetin for 48 h. The expression levels of caspase-9, caspase-7, caspase-3 and PARP are detected by western blot analysis.

expression levels of Bcl-2 decreased and Bak increased in quercetin-treated cells in a dose-dependent manner (Fig. 3C). Caspase is known for key proteins underlying mitochondrial pathway of apoptosis. The expression levels of caspase-9, -7, -3, and PARP were measured using Western blot analysis. As shown in Fig. 4, MG-63 cells were treated with various

concentrations of quercetin for 48 h; the proform of caspase-9 and caspase-7 decreased, and cleaved caspase-3 and PARP were detected at 500–1000 μM of quercetin. These results indicate that quercetin-induced cell death is mediated by apoptosis that is regulated by the mitochondrial pathway (intrinsic pathway) and caspase cascades.

## Quercetin treatment resulted in autophagy induction in MG−63 cells

We next evaluated autophagic activity in MG-63 cells after quercetin treatment. We used AO and MDC staining as selective fluorescent markers to detect AVOs and to determine whether autophagy was triggered in quercetin-treated MG-63 cells. The cells were treated with various concentrations of quercetin for 24 h. As shown in Fig. 5A, orange-colored AVOs were observed in a concentration-dependent manner compared to the control group using fluorescence microscopy. AVOs were also detected using MDC staining with blue-colored dots, in comparison to the controls (Fig. 5B). In addition to certain quercetin-induced autophagy, a Western blot assay was used to measure the amount of various autophagy-related proteins, such as Belin-1, p62/SQSTM1, Atg5, and LC3B. All these autophagy-related proteins increased in a concentration-dependent manner, and the conversion of LC3-I protein to the LC3-II form was detected (Fig. 5C). These data suggest that quercetin-induced autophagy changed the expression levels of autophagy -associated proteins in a dose-dependent manner.

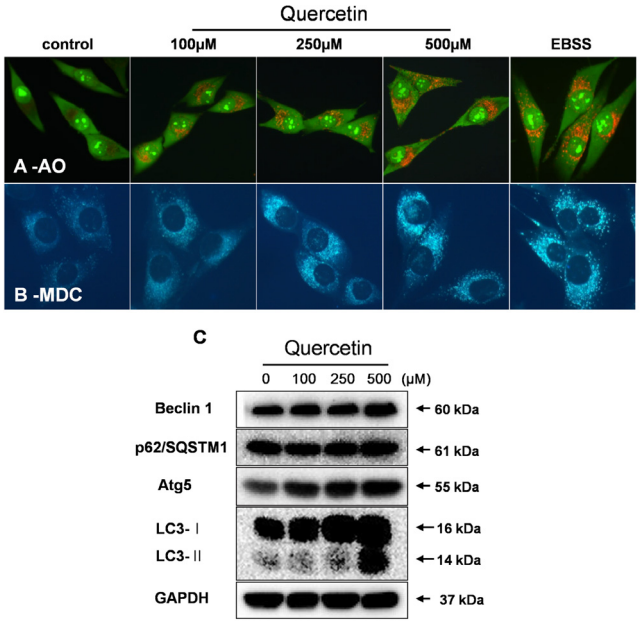


**Fig. 5.** Quercetin induced autophagy in MG-63 cells. (A) AO and (B) MDC stained cells were observed using fluorescence microscope as described in meterial and methods. Cells were cultured on coverslips and treated with quercetin (100–500 μM) for 24 h. (C) The expression levels of autophagy-related genes, Beclin-1, p62/SQSTM1, Atg5 and LC3 were analyzed by western blot.

## Inhibition of quercetin−induced autophagy augmented apoptosis in MG−63 cells

We next investigated the role of quercetin-induced autophagy in MG-63 cells using 3-MA (Sigma), a class III phosphatidylinositol 3-kinase (PI3K) specific autophagy inhibitor. The cells were pretreated with 10 mM 3-MA and then treated with 250 μM and 500 μM quercetin. Using fluorescence microscopy, we found that the 3-MA and quercetin co-treatment resulted in a decreased ratio of AO and MDC positive cells compared to the quercetin treatment alone (Figs. 6A and 6B).

To validate the correlation between quercetin-induced apoptosis and autophagy, the amount of apoptosis-related

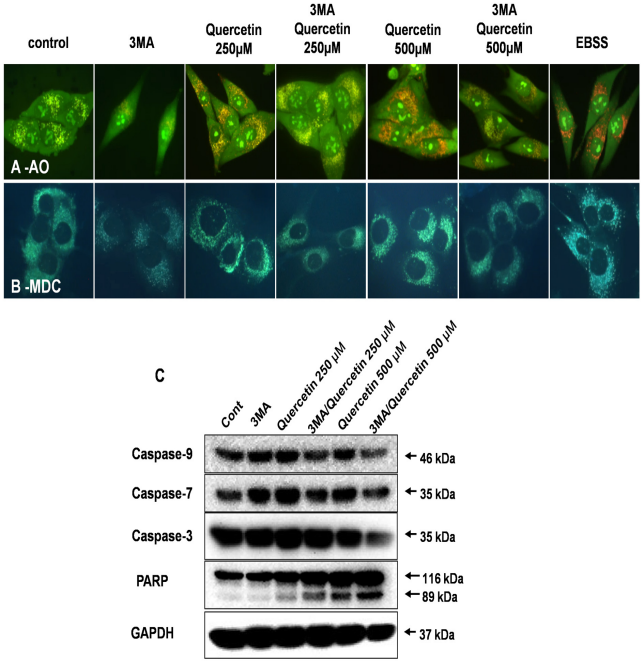


**Fig. 6.** Inhibition of quercetin-induced autophagy by 3-MA promotes the apoptosis of MG-63 cells. Representative images were from MG-63 cells that pretreated with 10 mM 3-MA for 1 h, and then quercetin for 24 h. Cells were stained with (A) AO and (B) MDC. (C) 3-MA and quercetin co-treatment group showed down-regulation of the expression levels of apoptosis-related genes, caspase-9, caspase-7, caspase-3, PARP were analyzed by western blot.

proteins was measured using a Western blot assay. The expression levels of caspase-9, caspse-7, and caspase-3 decreased in the 3-MA and quercetin co-treatment group. The amount of cleaved PARP increased in the co-treatment group. These results indicate that quercetin-induced autophagy has a protective role in MG-63 cells subjected to cytotoxic quercetin (Fig. 6C).

## Discussion

Quercetin is found in various fruits and vegetables, such as apples, berries, brassica vegetables, and onions. It has received significant attention due to its biological advantages, such as its anti-inflammatory, anti-oxidant, anti-viral, and anti-cancer properties. The anti-cancer properties of quercetin have been reported in many previous studies [13,15,21]. According to Cao et al, quercetin induced anti-tumor effects via the stat3 signaling pathway in melanoma cells [14]. Youn et al. reported that quercetin triggered apoptosis via the blockade of nuclear factor-kappa B signaling in lung cancer cells [15]. It has been shown that quercetin induces the degradation of oncoproteins, revealing the possibility that it may be used in targeting many types of tumors that occur due to the down-regulation of the RAS oncoprotein [20]. Thus, the aim of this research is to investigate the anti-cancer activities of quercetin and the relationship between quercetin-induced apoptosis and autophagy in human osteosarcoma MG-63 cells.

Apoptosis is described as type I programmed cell death by cell shrinkage, membrane blebbing, nuclear condensation, and DNA fragmentation [21]. In earlier research, quercetin augmented cell apoptosis due to increased ROS levels in human colon cancer cells [22]. In our study, we found that quercetin inhibited cell proliferation and induced apoptotic cell death due to the accumulation of sub-$G_1$ phase and nuclear fragmentation and condensation in both concentration- and time- dependent manners (Figs. 1 and 2).

It has been shown that quercetin-induced apoptosis is mediated by the mitochondrial pathway and that autophagy has a cytoprotective function [23]. Similarly, in this study, apoptosis induced by quercetin resulted in mitochondrial dysfunction. The MMP was dose-dependently decreased, and cytochrome c was translocated from mitochondria into cytoplasm. In addition, Bcl-2 and Bak proteins which are important mitochondria membrane regulators were down-regulated and up-regulated, respectively (Fig. 3). In order to determine the process in detail, the caspase cascade was measured using Western blot analysis. This revealed that the caspase-9 and caspase-7 expression levels significantly decreased in a concentration-dependent manner. The cleaved form of caspase-3 and PARP were detected at the 500 μM dose of quercetin (Fig. 4).

Autophagy is able to control apoptosis in various cells. The role of the autophagic process in cancer is quite controversial [19,24]. One study noted that quercetin-induced autophagy had a protective effect via AKT-mTOR signaling in gastric cancer cells [25]. Quercetin was also reported to have a protective effect against ischemia/reperfusion injury in normal renal tubular cells [9]. In this study, quercetin-induced autophagy was visualized using AO and MDC staining, and AVOs developed in a concentration-dependent manner. In addition, the expression levels of autophagy-related genes were analyzed using Western blotting. Beclin-1, p62/SQSTM1, and Atg5 expression levels increased, and LC3-I was converted to LC3-II at 500 μM of quercetin (Fig. 5).

To further clarify the role of quercetin-induced autophagy, 3-MA was used as an autophagy inhibitor. The inhibition of autophagy was detected using AO and MDC staining. The 3-MA treatment group showed fewer AVOs than the quercetin only treatment group. After the inhibition of autophagy, we investigated the amount of caspase protein using Western blotting. We found that the activation of caspase was increased by 3-MA (Fig. 6), indicating that quercetin-induced autophagy is a pro-survival process.

In conclusion, we suggest that quercetin-induced cell death via apoptosis is accompanied by mitochondrial dysfunction and a caspase cascade in human osteosarcoma MG-63 cells. Quercetin also induced autophagy, a phenomenon that is a pro-survival process in MG-63 cells. Therefore, the combination application of an autophagy inhibitor and quercetin could be a novel strategy for human osteosarcoma therapy.

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## Conflict of interest

The authors declare that they have no competing interest.

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