# Original Article

# Astaxanthin Induces Apoptosis in Human Osteosarcoma MG-63 Cells

(astaxanthin / MG-63 cells / apoptosis / cytochrome *c* / mitochondria)

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**Abstract. We explored the mechanism of human osteosarcoma MG-63 cell apoptosis induced by astaxanthin. The MTT assay was used to detect the effect of astaxanthin on cell viability. Morphological changes associated with apoptosis were observed after DAPI staining. Early and late stages of apoptosis were detected by flow cytometry with annexin V-FITC/PI staining. Activation of caspases-8, -9 and -3 was detected by enzyme activity *in vitro*. Changes in the mitochondrial membrane potential were detected by MitoCapture staining. Western blot was used to detect the cleavage of PARP, which is a caspase-3 substrate, the release of cytochrome *c* and Smac into the cytosol, the translocation of pro-apoptotic proteins Bax and Bak, and the expression of mitochondrial pathway-related proteins. The translocation of Bax was also detected by immunofluorescence assay. Astaxanthin significantly inhibited the viability of human osteosarcoma MG-63 cells with an $IC_{50}$ value of 12.36 μg/ml. The DAPI-stained cells showed characteristic apoptotic morphological changes – cell shrinkage, cell membrane blebbing, nuclear condensation, and apoptotic body formation. Cytochrome *c* and Smac were released from mitochondria to the cytosol. Pro-apoptotic proteins Bax and Bak were rapidly translocated to mitochondria after six hours of astaxanthin action. Caspases-9 and -3 were activated and PARP was cleaved. The expression of anti-apoptotic proteins Bcl-2, Bcl-xL and XIAP was significantly decreased. Astaxanthin induced human osteosarcoma MG-63 cell apoptosis through the mitochondria-mediated endogenous apoptosis pathway.**

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Abbreviations: DAPI – 4',6-diamidinyl-2-phenylindole, FITC – fluorescein isothiocyanate, MTT – 3-(4,5-dimethyl thiazole-2)-2,5-diphenyl tetrazolium bromide, PARP – poly(ADP-ribose) polymerase, PI – propidium iodide, Smac – second mitochondria-derived activator of caspases.

## Introduction

Apoptosis plays an important role in body development, homeostasis maintenance and clearance of damaged cells (Brill et al., 1999; Johnstone et al., 2002; Sharma et al., 2014). Studies have shown that many anticancer drugs can kill tumour cells by inducing apoptosis (Ding et al., 2014; Fu et al., 2019; Liu et al., 2019; Fang et al., 2020). Two main molecular mechanisms, mitochondrial pathway and death receptor pathway, are closely related to drug-induced apoptosis (Ouyang et al., 2012; Aziz et al., 2018; Jo et al., 2019). In the intracellular pathway mediated by mitochondria, different apoptosis-stimulating signals cause changes in mitochondrial membrane permeability, including release of cytochrome *c*, formation of Apaf-1 heptameric apoptosome complex, and activation of caspases (Wuerstle et al., 2012; Elena-Real et al., 2018). Bcl-2 family proteins play a role in regulating mitochondrial permeabilization, and damage to the mitochondrial outer membrane releases cytochrome *c* and DIABLO (Dewson and Kluck, 2009).

Astaxanthin, a carotenoid, is the most antioxidant natural product found in nature known so far (Guerin et al., 2003). Astaxanthin is produced by microorganisms such as algae and bacteria, through which it is ingested and accumulates in fish, shrimp, shellfish and birds that feed on aquatic life. Astaxanthin is used in the prevention of aging, cancer and cardiovascular diseases because of its wide sources and strong antioxidant capacity (Visioli and Artaria, 2017; Davinelli et al., 2018; Faraone et al., 2020). Compared with the extensive studies on astaxanthin in the health care, there are relatively few studies on its anti-tumour activity. In this study, human osteosarcoma MG-63 cells were used to investigate the molecular mechanism of astaxanthin-induced apoptosis, providing a theoretical basis for development of new anticancer drugs.

## Material and Methods

### *Main reagents*

Astaxanthin and 3-(4,5-dimethyl thiazole-2)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from

Sigma (Burlington, MA); Roswell Park Memorial Institute (RPMI)-1640 medium and foetal bovine serum were purchased from GIBCO (Carlsbad, CA). The BCA Protein Assay Reagent Kit, Mitochondria Isolation Kit and horseradish peroxidase-labelled secondary antibody were all purchased from Thermo Fisher Scientific (Waltham, MA). The annexin V-fluorescein isothiocyanate (FITC) apoptosis assay kit, FITC- and Cy3-labelled fluorescent secondary antibodies were purchased from Beyotime (Haimen, China). Rabbit anti-poly(ADP-ribose) polymerase (PARP), second mitochondria-derived activator of caspases (Smac) and Bak antibodies, mouse anti-cytochrome *c*, β-actin, α-tubulin and Bax antibodies were all purchased from Santa Cruz (Dallas, TX). Rabbit anti-COX IV antibody was from Cell Signaling (Danvers, MA). Enhanced chemiluminescence (ECL) reagents were from Tanon (Shanghai, China). The Caspase Assay Reagent and MitoCapture Apoptosis Detection Kit were obtained from Merk (Darmstadt, Germany). Astaxanthin was dissolved in DMSO solution to 50 mg/ml.

### *Cell line and cell culture*

Human osteosarcoma MG-63 cells were purchased from KeyGEN BioTECH (Nanjing, China). RPMI-1640 medium containing 10 % (by volume) heat-inactivated foetal bovine serum and 1 % antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) was used to culture human osteosarcoma MG-63 cells in a moist incubator containing 5 % $CO_2$ at 37 °C.

### *MTT assay was used to detect cell viability*

MG-63 cells in logarithmic growth phase were inoculated into 96-well plates in the amount of $1 \times 10^4$ cells/well, three replicates per group. After 24 h of culture, astaxanthin in different concentrations (0, 1.25, 2.5, 5, 10, 20, 40, 80 μg/ml diluted in RPMI-1640 medium) was added and culturing continued for 48 h. Twenty μl MTT (5 mg/ml) was added 4 hours in advance and incubated. The resulting purple crystals were dissolved in 200 μl DMSO, and the absorption value was measured at the wavelength of 565 nm.

### *DAPI staining was used to observe cell morphology*

MG-63 cells in logarithmic growth phase were inoculated into a 24-well plate at $5 \times 10^4$ cells/well. After 24 h of culture, 15 μg/ml astaxanthin was added, with different effect times (0, 6, 12, 24 h). The medium was removed and 1 ml precooled 95 % ethanol was added to each well to fix the cells for 10 minutes. The fixing solution was removed and 500 ml of 4',6-diamidinyl-2-phenylindole (DAPI) (0.1 mg/ml) solution was added into each well. Fluorescence microscopy was used to observe and take fluorescence images under UV excitation light.

### *Flow cytometry analysis*

MG-63 cells in logarithmic growth phase were inoculated into a culture dish in the amount of $1.5 \times 10^6$ cells. After 24 hours of culture, 15 μg/ml astaxanthin was added and left to act for different times (0, 6, 12 h). The cells (including supernatant of floating cells) were digested by trypsin and collected, then centrifuged for 5 min at 4 °C, 845 × *g*. Precooled PBS was used to wash once, and the precipitate was resuspended in 500 μl binding buffer. Five μl annexin V-FITC and 5 μl propidium iodide (PI) were added and mixed, avoiding light for 15 min at room temperature, for flow cytometry detection.

### *Determination of in vitro caspase activity*

After protein concentration was determined with the BCA Protein Assay Reagent Kit, 50 μg of whole-cell lysate protein was added to a 96-well plate, and then 200 ml of reaction buffer containing 25 mM caspase-3 substrate (Ac-DEVD-AFC), caspase-8 substrate (Ac-IETD-AFC), and caspase-9 substrate (Ac-LEHD-AFC) was added and cultured at 37 °C for 1 h. The fluorescence level generated by the substrate fraction was measured at the excitation wavelength of 405 nm and the emission wavelength of 505 nm with a microplate reader.

### *Measurement of mitochondrial membrane potential*

MG-63 cells in logarithmic growth phase were inoculated into a 24-well culture plate at $5 \times 10^4$ cells/well. After 24 h of culture, 15 μg/ml astaxanthin was added, with different effect times (0, 6, 12, 24 h). According to the specification of MitoCapture Apoptosis Detection Kit, the MitoLight working solutions were prepared. The culture medium in the 24-well plate was discarded and the cells were washed once by PBS. Three hundred μl of working solutions were added along the well wall and the cells were incubated at 37 °C for 30 min. Fluorescence microscopy (Olympus, Tokyo, Japan) was used to observe and photograph the excitation light at wavelengths of 500 nm and 570 nm.

### *Isolation and preparation of mitochondrial and cytoplasmic proteins*

MG-63 cells in logarithmic growth phase were inoculated into a culture dish at $1.5 \times 10^6$ cells/well. After 24 h of culture, 15 μg/ml astaxanthin was added, with different effect times (0, 6, 12, 24 h). The cells and culture medium were placed in a centrifuge tube and centrifuged for 5 min at 4 °C, 845 × *g*. The supernatant was discarded and the cells were washed once with precooled PBS. According to the kit instructions, mitochondria were extracted and the cytoplasmic proteins were separated. The cell lysates were added to the mitochondrial precipitate, placed on ice for 1 h, and centrifuged for 15 min at 4 °C, 13,523 × *g*. The supernatant represented the extracted mitochondrial protein.

### *Western blot*

Polyacrylamide gel electrophoresis was performed with the same amount of samples to be tested, and the

protein was transferred to PVDF membrane. The membranes were blocked with 5 % skin milk powder for 1 h at room temperature. Rabbit anti-PARP, Smac, Bak and COX IV antibodies, mouse anti-cytochrome *c*, β-actin, α-tubulin and Bax antibodies were added, respectively (all diluted at 1 : 1000). Incubation was done for 3 hours at room temperature or 4 °C overnight. PBST (containing 0.1 % twain) was used to wash the membranes three times, each time for 5 min. Horseradish peroxidase-labelled antibody (diluted at 1 : 5000) was added, followed by incubation for 1 h at room temperature. Membranes were washed with PBST three times, each time for 5 min. The enhanced chemiluminescence (ECL) mixture was prepared in a ratio of 1 : 1 and then added to the membranes. After 1 min reaction, the membranes were exposed.

### *Immunofluorescence*

MG-63 cells in logarithmic growth phase were inoculated in an amount of $5 \times 10^4$ cells/well onto aseptic glass slides of a 24-well culture plate and cultured overnight. Fifteen μg/ml astaxanthin was added to treat the cells for 6 h. Medium was discarded, cells were fixed with 4 % paraformaldehyde for 20 min and washed three times with PBST. Cells were permeabilized with 0.1 % Triton X-100 for 15 minutes and washed with PBST three times, and then blocked with PBST solution containing 5 % BSA at room temperature for 1 h. Proportionally diluted primary antibody was added, incubated for 3 h at room temperature, and washed three times with PBST. Proportionally diluted secondary antibodies (with corresponding fluorescent dye markers, such as FITC and rhodamine) were added, incubated at room temperature for 1 h, and washed with PBST three times. One drop of Antifade Mounting Medium was added to the slide and the coverslip containing cells was carefully covered, stored at 4 °C and protected against light. Images were taken with a fluorescence confocal microscope (Olympus, Tokyo, Japan).

### *Statistical analysis*

SPSS 19.0 was used for statistical analysis. The test results were expressed as mean ± standard deviation (x ± s), and the independent sample *t*-test was used. $P < 0.05$ was considered statistically significant.

## Results

### *Effect of astaxanthin on growth inhibition of human osteosarcoma MG-63 cells*

In order to detect the growth inhibition effect of astaxanthin on human osteosarcoma MG-63 cells, we used the MTT assay to detect the lethality of the cells after astaxanthin treatment. After 48 h of cell culture, we detected the changes in cell survival rate. By detecting the absorbance (OD value), we calculated the 50 % inhibitory concentration ($IC_{50}$ value) for the cells.

The MTT results showed that with an increasing astaxanthin dose, the survival rate of MG-63 cells decreased gradually. Astaxanthin could inhibit cell growth in a dose-dependent relationship, and its lethal concentration $IC_{50}$ was 12.36 μg/ml (Fig. 1B).

### *Astaxanthin induces apoptosis of human osteosarcoma MG-63 cells*

We observed apoptosis morphologically, detecting apoptosis of annexin V-FITC/(propidium iodide) PI double-stained cells, and we examined the cleavage of PARP to detect whether the killing effect of astaxanthin on human osteosarcoma MG-63 cells was achieved by inducing apoptosis.

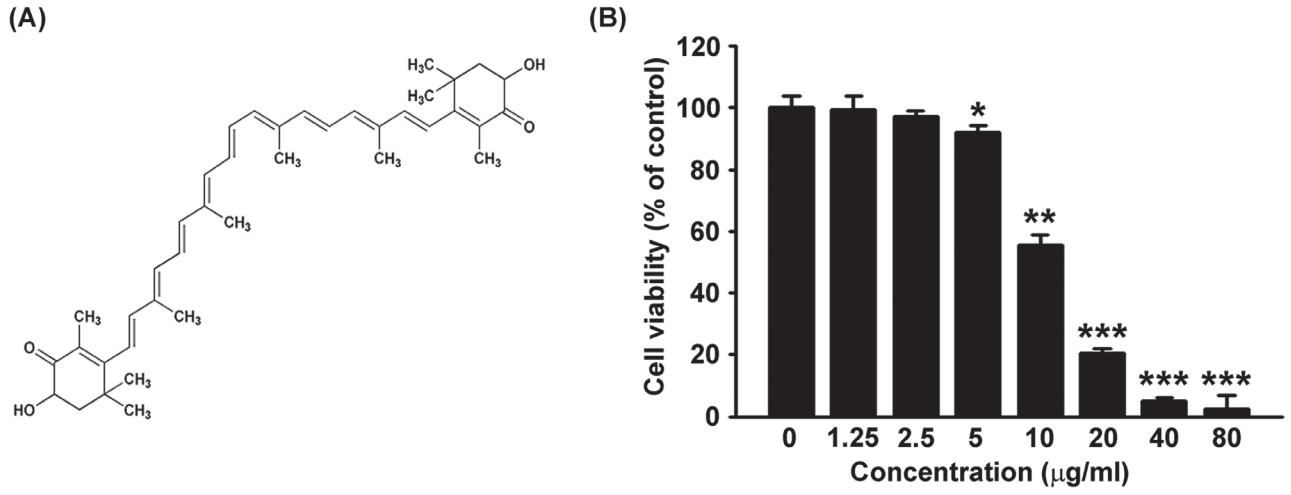


*Fig. 1.* Dose-dependent effects of astaxanthin on cell viability of MG-63 cells. (**A**) Chemical structure of astaxanthin. (**B**) MG-63 cells were treated with indicated concentrations of astaxanthin for 48 h. Cell viability was detected by the MTT assay. All experiments were performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

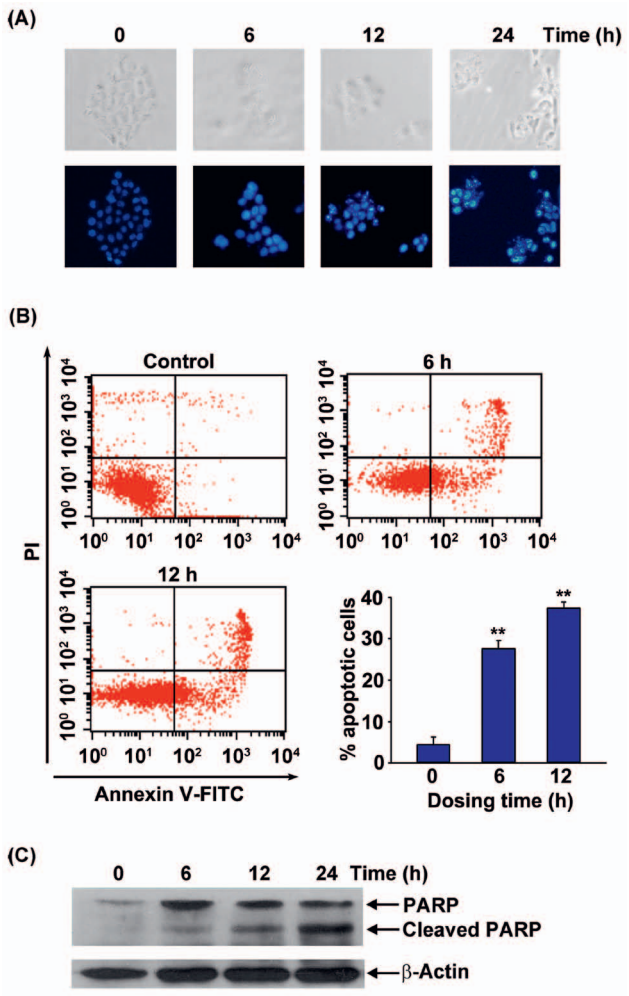


*Fig. 2.* Astaxanthin induced apoptosis in MG-63 cells. MG-63 cells were treated with 12.36 mg/ml astaxanthin for the indicated time periods. (**A**) The cells were stained with DAPI and imaged by a fluorescence microscope (100×). The light photos (upper panel) and the fluorescent images (lower panel) indicate apoptotic bodies and chromatin condensation, respectively. (**B**) The cells were analysed by flow cytometry with annexin V-FITC/PI staining. The percentage of apoptotic cells was represented by a total number of annexin V-FITC/PI-positive cells. (**C**) Whole-cell lysates were resolved by SDS-PAGE and analysed by Western blotting using a specific antibody against PARP. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

MG-63 cells were treated with 15 μg/ml astaxanthin. After 6 h, DAPI-stained nuclei with ruptured fragments and pyknosis of chromatin were observed (Fig. 2A, upper panel). The morphological characteristics of apoptosis were observed under a light microscope – cell shrinkage, cell membrane blebbing, and apoptotic body formation (Fig. 2A, lower panel).

Apoptosis was determined by flow cytometry with annexin V-FITC/PI double staining. Compared with the control group, the proportion of cells undergoing early/late apoptosis was 1.01 % and 3.45 %, and in the experimental group it increased to 17.25 % and 12.05 %, respectively, after 6 h of 15 μg/ml astaxanthin application to MG-63 cells (Fig. 2B). The results showed that the cell numbers with early apoptosis and late apoptosis increased with prolongation of the treatment time.

Western blot results showed that after 15 μg/ml astaxanthin treatment of MG-63 cells for 6 h, the PARP substrate of caspase-3 was cleaved, and with extending treatment time, the cleavage band became more visible (Fig. 2C).

## *Astaxanthin induces activation of caspases-9 and -3 during MG-63 cell apoptosis*

The activation of the initial caspase is an important basis to determine whether the mitochondrial apoptosis pathway or the membrane death receptor apoptosis pathway is initiated (Shi, 2002). In the next experiment, we examined the activation of caspase-8 and caspase-9 and the downstream effect caspases-3/-7. The results showed that the activity of caspase-9 and caspase-3 began to increase after 6 h treatment of MG-63 cells with 15 μg/ml astaxanthin, and both reached their peak at 24 h (Fig. 3B–C). However, the caspase-8 activity did not change (Fig. 3A). The results showed that astaxanthin-induced MG-63 cell apoptosis represented caspase-9-mediated mitochondrial apoptosis.

## *Astaxanthin induces changes of mitochondrial membrane potential and release of cytochrome c and Smac during MG-63 cell apoptosis*

The decrease of mitochondrial membrane potential is an early event of apoptosis. When the cell is in the normal state, the mitochondrial membrane potential voltage is higher, forming polymers and emitting red spot fluorescence, while when the mitochondrial membrane potential is reduced, the membrane depolarizes, forming monomers and presenting green fluorescence (Heiskanen et al., 1999). As shown in Fig. 4A, after treatment of MG-63 cells with 15 μg/ml astaxanthin for 6 h, the red spots gradually weakened with the extension of treatment time, while on the contrary, the intensity of green fluorescence gradually increased. The results showed that the mitochondrial membrane potential decreased significantly during MG-63 cell apoptosis induced by astaxanthin.

To elucidate the specific molecular mechanism of astaxanthin-induced MG-63 cell apoptosis, Western blot was used to detect cytochrome *c* and Smac proteins in whole-cell lysates, mitochondria and cytoplasm. As shown in Fig. 4B, cytochrome *c* and Smac were released from mitochondria to the cytoplasm after astaxanthin treatment of MG-63 cells for 6 h, and the content of these two proteins in the cytoplasm increased significantly with the prolonging drug action time. However, there was no significant change in the contents of these two proteins in whole-cell lysates (Fig. 4D).

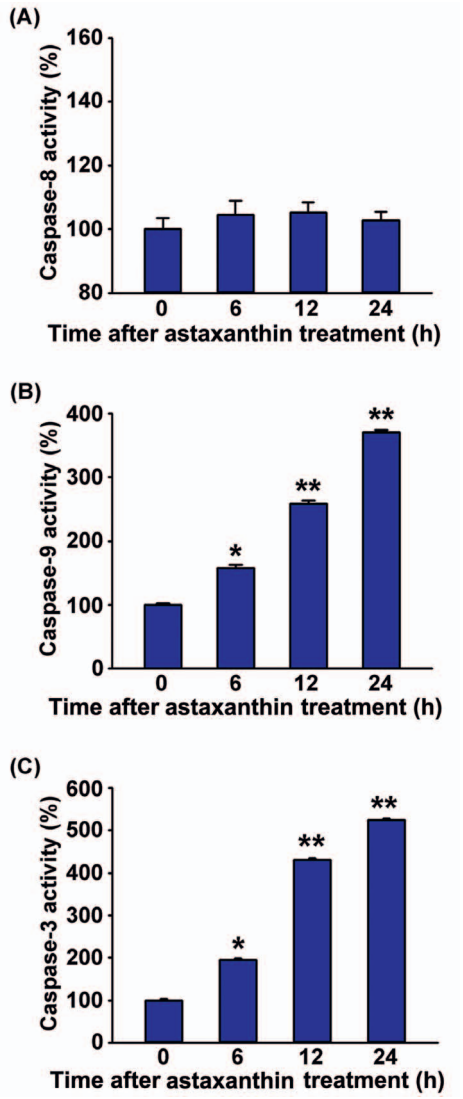


*Fig. 3.* Activation of caspases-8, -9, and -3 in astaxanthin-induced apoptosis. MG-63 cells were treated with 12.36 mg/ml astaxanthin for different times. The cell-free caspase-8 (**A**), -9 (**B**), and -3 (**C**) activities were analysed using specific substrates. The values are expressed as the mean ± SD of triplicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

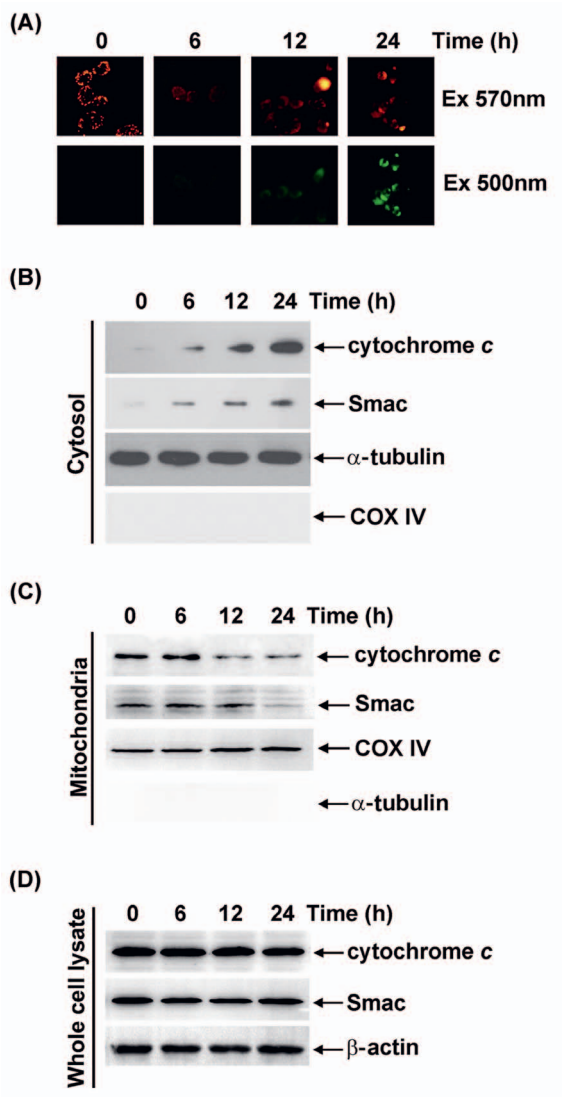


*Fig. 4.* Astaxanthin induced depolarization of mitochondrial membrane potential and release of cytochrome *c* and Smac in MG-63 cells. Cells were treated with 12.36 mg/ml astaxanthin for increasing periods. (**A**) Cells were stained with a MitoCapture specific dye and photographed under a fluorescence microscope (100×) with excitation wavelengths of 570 and 500 nm, respectively. Red fluorescence showed normal polarized mitochondria, but the diffused green fluorescence represented depolarized mitochondrial membrane potential. Cytosolic fraction (**B**), mitochondrial fraction (**C**), and whole-cell lysates (**D**) were analysed by Western blotting using specific antibodies against cytochrome *c*, Smac, α-tubulin, COX IV, and β-actin.

### *Astaxanthin induces translocation of Bax and Bak to mitochondria during MG-63 cell apoptosis*

The Bcl-2 family proteins Bax and Bak regulate mitochondrial membrane permeability and trigger the release of cytochrome *c* and Smac (Dewson and Kluck, 2009). We determined the changes of Bax and Bak in mitochondria by Western blot.

Western blot results showed that Bax and Bak aggregated on the mitochondria after 15 μg/ml astaxanthin treatment of MG-63 cells for 6 h, and the contents of Bax and Bak on the mitochondria significantly increased with the extending drug action time (Fig. 5A). Meanwhile, the contents of Bax and Bak in whole-cell lysates did not change (Fig. 5C), while the contents of Bax and Bak in the cytoplasm significantly decreased (Fig. 5B). This indicates that there was no change in the total expression of these two proteins, which was caused by the translocation of Bax and Bak from the cytoplasm to the mitochondrial membrane.

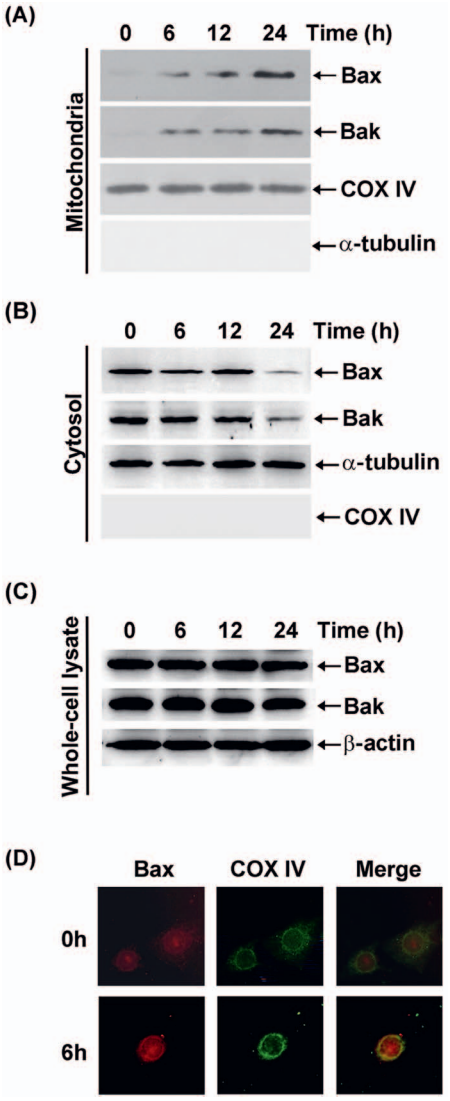


*Fig. 5.* Astaxanthin triggered both Bax and Bak translocation to mitochondria in MG-63 cells. Cells were treated with 12.36 mg/ml astaxanthin for the indicated periods. Mitochondrial fraction (**A**), cytosolic fraction (**B**), and whole-cell lysates (**C**) were analysed by immunoblotting using specific antibodies against Bax, Bak, COX IV, α-tubulin, and β-actin, respectively. (**D**) The cells were fixed and stained with anti-Bax and anti-COX IV antibodies and analysed by a laser scanning confocal microscope (LSCM) using proper filters for the visualization of green, red, or merged fluorescence resulting from the presence of FITC and Cy3 molecules.

To further demonstrate the translocation of Bax and Bak to the mitochondria during astaxanthin-induced MG-63 cell apoptosis, we performed an *in situ* immunofluorescence assay. MG-63 cells were treated with astaxanthin for 6 h. We performed *in situ* immunofluorescence staining of the cells with mouse-derived Bax antibody and rabbit-derived COX IV (COX IV is a protein located on mitochondria, which is generally used in mitochondrial localization experiments). Mouse-derived Bax antibody was fluorescently labelled with rhodamine red, and rabbit-derived COX IV was fluorescently labelled with FITC green. Immunofluorescence was detected using confocal fluorescence microscopy. The experimental results showed that, in the control group, the majority of Bax existed in the cytoplasm without translocation. In the treatment group, the results showed that Bax had transferred from the cytoplasm to mitochondria (Fig. 5D).

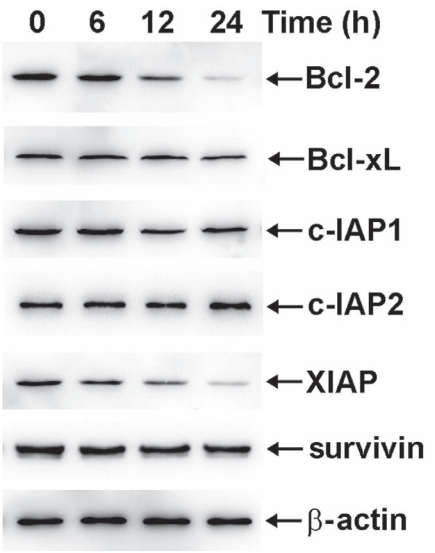


*Fig. 6.* The expression levels of mitochondrial pathway-related proteins. MG-63 cells were treated with 12.36 mg/ml astaxanthin for the designated periods. Whole-cell lysates were examined by Western blotting for Bcl-2, Bcl-xL, c-IAP1, c-IAP2, XIAP, survivin, and β-actin.

### *Study of anti-apoptotic protein family Bcl-2 and IAPs*

In order to comprehensively investigate the mechanism of astaxanthin-induced apoptosis in MG-63 cells, we examined the changes in the expression of the anti-apoptotic proteins Bcl-2 and the IAP family in astaxanthin-treated MG-63 cells for 0, 6, 12, and 24 h. As shown in Fig. 6, the expression of Bcl-2 and XIAP, Bcl-xL, cIAP-1 and survivin was decreased. However, cIAP-2, anti-apoptotic protein, showed no significant changes.

## Discussion

Extensive attention has been paid to the close relationship between apoptosis and tumours. The occurrence of many malignant tumours is often caused by the cancer cells' resistance to apoptosis or cytotoxicity of chemotherapy drugs (Panda and Biswal, 2019; Mirzayans and Murray, 2020). Astaxanthin has been reported to induce apoptosis, which is important for cancer treatment (Visioli and Artaria, 2017). In this study, the

mechanism of apoptosis induced by astaxanthin was elucidated in human osteosarcoma MG-63 cells, which provided a certain theoretical basis and data support for clinical application of astaxanthin.

In the MTT assay, we found that astaxanthin had a significant inhibitory effect on human osteosarcoma MG-63 cell growth in a dose-dependent relationship, and the $IC_{50}$ value was 12.36 μg/ml. After confirming the inhibitory effect of astaxanthin on MG-63 cell growth, we speculated that astaxanthin inhibited the growth of human osteosarcoma MG-63 cells by inducing apoptosis. Therefore, the cell morphology was analysed with DAPI, and compared with the control group, the treatment group's cell membrane was found to be foaming and round at 6 h, cell condensation and nuclear condensation began at 12 h, and cell fragmentation at 24 h, which were all signs of apoptosis. Secondly, the cells were examined by flow cytometry, and the percentage of apoptotic cells increased with the time of administration. Thirdly, we detected activation of the downstream effect caspase-3 of the apoptotic pathway, and the cleavage of its specific substrate PARP at 6 h after the treatment. All this indicated that astaxanthin could inhibit the growth of human osteosarcoma MG-63 cells by inducing apoptosis.

There are two main pathways involved in the process of apoptosis: caspase-8-mediated death receptor pathway and caspase-9-mediated mitochondrial pathway (Ouyang et al., 2012). Many studies have shown that mitochondria may be the target of a number of anticancer drugs (Marchetti et al., 2002; Battogtokh et al., 2018; Tian et al., 2018; Erxleben, 2019). These organelles are often closely related to cell apoptosis because the release of mitochondrial proteins, including cytochrome *c* and Smac, from mitochondria to the cytoplasm often triggers signalling of various apoptotic pathways (Hengartner, 2000; Dewson and Kluck, 2009). In order to verify whether mitochondria are involved in astaxanthin-induced cell apoptosis, we used 15 μg/ml astaxanthin to treat MG-63 cells and after 6 h, cytochrome *c* and Smac were released into the cytoplasm in a time-dependent manner. Activation of caspases-9 and -3 and cleavage of PARP were also detected along with the release of cytochrome *c* and Smac. Meanwhile, the mitochondrial membrane potential decreased and depolarization occurred. The results of MitoLight staining showed that depolarization became more apparent with prolonging the drug treatment duration. During apoptosis, Bax and Bak translocate to the mitochondrial membrane to form pores for releasing cytochrome *c* and Smac (Kim et al., 2017). The results showed that Bax and Bak were translocated to the mitochondria after treatment of MG-63 cells with 15 μg/ml astaxanthin for 6 h, suggesting that the intracellular mitochondrial pathway mediated astaxanthin-induced MG-63 cell apoptosis.

Subsequently, we detected changes in the expression levels of Bcl-2 and IAPs, family of important anti-apoptotic proteins in the mitochondrial pathway, and the expression levels of c-IAP2 and X-IAP gradually decreased with the extension of the drug treatment, indicating that the degradation of c-IAP2 and X-IAP might be accompanied by the process of apoptosis. It is speculated that Smac binds c-IAP2 and X-IAP to initiate the self-ubiquitination degradation process of these two proteins, thereby releasing the caspases-3 and -9 bound to them, which plays a role in amplifying the apoptotic signal. However, the expression levels of anti-apoptotic proteins c-IAP1, survivin and Bcl-xL did not change significantly, indicating that astaxanthin did not affect the expression of these proteins after acting on MG-63 cells, which may require further studies. There was no significant change in caspase-8 activity compared to the control group. This indicates that the caspase-8 apoptotic pathway was not involved in the MG-63 cell death.

In conclusion, astaxanthin effectively inhibits human osteosarcoma MG-63 cell growth, and this inhibition is achieved by inducing MG-63 cell apoptosis. In this process, pro-apoptotic proteins Bax and Bak translocate to the mitochondria to form pores, triggering the release of mitochondrial cytochrome *c* and Smac into the cytoplasm, and subsequently activating caspases-9 and -3. Taken together, astaxanthin has a significant inhibitory effect on the proliferation of human osteosarcoma MG-63 cells and can induce mitochondria-mediated apoptosis, providing a precursor compound and theoretical basis for the development of more efficient anti-tumour drugs.

### *Competing interests*

The authors state that there are no conflicts of interest to disclose.

### *Author contributions*

Guangyu Wang designed the study and carried it out, Xu Tian supervised the data collection, analysed the data, interpreted the data, Lintao Liu analysed part of the data and prepared figures, Jingming Dong prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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