Astaxanthin Induces Mitochondria-Mediated Apoptosis in Rat Hepatocellular Carcinoma CBRH-7919 Cells

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We designed to study the role of mitochondria in astaxanthin-induced apoptosis in hepatocellular carcinoma cells. Effect of astaxanthin on cell proliferation was studied by using methyl thiazolyl tetrazolium (MTT) in three tumor cell lines (CBRH-7919, SHZ-88 and Lewis) and normal human hepatocyte HL-7702 cell. Cell apoptosis rate, changes of mitochondrial morphology, mitochondrial transmembrane potential and electron transport chain were evaluated respectively. Expressions of B cell lymphoma/leukemia-2 (Bcl-2) and Bcl-2 associated X protein (Bax) were detected by Western blot. Results as following, astaxanthin had little effect on HL-7702 cell, however its inhibition was most pronounced in CBRH-7919 cell line with an IC_{50} of 39 μM. This dose of astaxanthin and CBRH-7919 cell line were chosen for further studies. Astaxanthin could induce cell apoptosis and mitochondrial membrane damage. The mitochondrial transmembrane potential and function of electron transport chain were decreased. The expression of Bcl-2 protein was down-regulated but that of Bax protein was up-regulated. In conclusion, astaxanthin showed anticancer effect by inducing cell apoptosis through the regulation of mitochondrial-dependent manner.

Key words astaxanthin; hepatocellular carcinoma; cell apoptosis; mitochondria

The marine environment is usually characterized by high-salt, high pressure, low temperature, less or no illumination and poor nutrition. The marine organisms living under these conditions are well adapted to such adverse conditions, regardless of the size, shape, presence or absence of shells, and running fast or slow. Marine organisms have their own metabolism and defense systems which are different from terrestrial organisms in many ways. They produce a large number of metabolites with novel structures and potent activities.[1—3] Since the 1960s, marine natural products have been considered as an attractive source for the discovery of new drugs to prevent and treat many human diseases including malignant tumors. Since the land resources are shrinking over the years at an alarming rate, scientists have predicted that the most promising anticancer drugs might be discovered from the marine biota.[4,5] Therefore, isolation of marine natural products and study of their pharmacological activities have attracted great interest.

Astaxanthin was first isolated from lobsters in 1938, and since then it has been found in a diverse array of organisms such as shrimp, sea bream, crab, salmon, algae and other marine organisms. It is a member of the carotenoid family and the only known keto-carotenoid which can be transported into the brain by transcytosis through the hematoencephalic barrier.[6,7] Its bioavailability can be enhanced in the presence of fat: the elimination half-life was 15.9 ± 5.3 h ($n=32$).[8] Recent studies[9—11] have shown that astaxanthin has anticancer activity, which is attributed to its ability to trigger specific cellular immune response and its antioxidant activity. Jyonouchi *et al.*[12] investigated the pharmacological activity of astaxanthin by feeding astaxanthin containing diet to the mice with methylcholanthrene-induced fibrosarcoma and found that astaxanthin could significantly inhibit proliferation of tumor cells by stimulating the immune response against tumor antigens. Ishikawa *et al.*[10] reported inhibition of adult leukemia T-cell proliferation by feeding diets containing astaxanthin to mice harboring tumors induced by human T-cell leukemia virus type 1 (HTLV-1). They suggest that astaxanthin induced the arresting of cell cycle of infected T cells at G1 phase and apoptosis, thereby enhancing the anti-tumor immune response. In another report,[13] astaxanthin was shown to activate the pre-inflammatory gene product, nuclear factor-β, by blocking the inflammatory process. It also inhibits the generation of nitric oxide (NO) and prostaglandin E2, and promotes the effects of other factors such as pre-inflammatory cytokines, tumor necrosis factor-α and interleukin-β. However, the effect of astaxanthin on hepatoma cells and its mechanism have not yet been reported.

In the present study, we showed that astaxanthin could induce apoptosis in rat hepatocellular carcinoma CBRH-7919 cells by altering the mitochondria metabolism. Experiments were designed to reveal the relationship between astaxanthin and mitochondrial apoptosis pathway.

MATERIALS AND METHODS

Cell Culture CBRH-7919 cell line, rat breast cancer SHZ-88 cell line, mouse Lewis lung cancer cell line and normal human hepatocyte HL-7702 cell line were purchased from Cell Bank of Chinese Academy of Sciences. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C under a humidified atmosphere of 5% CO_2 and 95% air and subcultured when cell density reached to 90% every 2—3 d.

Preparation of the Astaxanthin Stock Solution Ten millimolars astaxanthin solution was prepared by dissolving 53.08 mg astaxanthin (Santa Cruz Biotechnology Inc., U.S.A.) using 9 ml DMEM and 1 ml dimethyl sulfoxide (DMSO). The solution was filtered through 0.22 μm membrane and stored in 4 °C for future use.

Effect of Astaxanthin on Cell Proliferation The effect of astaxanthin on cell proliferation was determined by methyl

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thiazolyl tetrazolium (MTT) assay. The cell concentration of the logarithmic growth phase was adjusted to 1×10^5 cells/ml and 100 μl aliquots were transferred to 96-well plates. 0, 10, 20, 40, 60, 80 and 100 μM astaxanthin were added to each well after 24 h. Ten replicates were done for each concentration. After 24 h of incubation, 15 μl MTT solution was added to each well and incubated for additional 4 h. The reaction was stopped by the addition of 100 μl of isopropanol to each well and the plate was put at room temperature for 30 min. Absorbance was measured with an ELISA Reader (Tosoh, Japan) at 570 nm reference wavelength. Inhibition rate= [1−(optical density (OD) of astaxanthin group/OD of control group)]×100%.

Flow Cytometry Measurement of Apoptosis 1×10^6 cells/ml were seeded in 6-well plates and exposed to 39 μM astaxanthin for 0, 12, 24 and 48 h. Suspended and adherent cells were collected and washed with cold phosphate-buffered solution (PBS). Cells were then fixed with chilled 70% alcohol overnight. The fixation fluid was washed with PBS before the test. Twenty microliters RNase A was added and incubated for 30 min at 37 °C and 0.5% propidium iodide staining solution was added for overnight staining at 4 °C away from light. Apoptosis rate was measured by flow cytometry (Beckman, U.S.A.).

Observation of Mitochondrial Morphology 1×10^7 cells/ml were cultured in 182 cm^2 culture flask overnight and then treated with 39 μM of astaxanthin for 0 and 24 h, fixed in 3% glutaraldehyde for 5 h, washed with PBS buffer, and then post-fixed with 1% osmium tetroxide for 1.5 h. The fixed samples were dehydrated in a gradient alcohol-acetone dehydration solution, embedded and polymerized with Epon812. Around 70 nm ultra-thin sections were cut using the ultracut E ultramicrotome. The sections were then stained with both uranyl acetate and lead citrate prior to observation under JEM-1400 transmission electron microscope (TEM) (JEOL Ltd., Japan).

Mitochondrial Transmembrane Potential Assay 1×10^6 cells/ml were plated in 6-well plates overnight and were exposed to 39 μM astaxanthin for 0, 6, 12 and 24 h. A standard protocol was used in accordance with the instruction of mitochondrial membrane potential detection kit (Beyotime, China). Each sample was treated with 1 ml of 5 μg/ml JC-1 and placed in a 37 °C incubator for 45 min. The samples were then washed twice with PBS, and then 1 ml of 70% ethanol (−20 °C) was added before the flow cytometric analysis.

Detection of Intracellular Ca^{2+} Concentration 1×10^6 cells/ml were plated in 6-well plates overnight and were exposed to 39 μM astaxanthin for 0, 6, 12 and 24 h. Cells were collected and 50 μl of 10 μmol/l Fura-2 AM was added and incubated at 37 °C under dark condition for 40—50 min. The tubes were shaken slightly to get Fura-2 AM into cells and covered with aluminum foil during all the experimental procedure. Samples were then centrifuged at 1000 rpm for 5 min to terminate the incubation. The supernatant was discarded, while the pellet was washed with 0.1% PBS buffer. The pellet was washed twice times to remove the remnants of extracellular Fura-2 AM. Then observed and photographed by fluorescence microscope. The average optical density of the positive cells was analyzed using the optical density analysis software.

Western Blot Analysis Cells were collected after treatment with 39 μM of astaxanthin for 0, 6, 12 and 24 h and lysed by cell lysis buffer. Protein extracts (40 μg) were separated on 12% polyacrylamide gel and then transferred electrophoretically onto polyvinylidene difluoride membranes. After blocking for 1 h with blocking buffer, the membranes were incubated overnight at 4 °C, with antibodies against β-actin (goat anti-mouse actin monoclonal antibody, 1 : 500, Santa Cruz Biotechnology Inc., U.S.A.) or B cell lymphoma/leukemia-2 (Bcl-2) (rabbit anti-mouse Bcl-2 antibody, 1 : 100, Santa Cruz Biotechnology Inc., U.S.A.) or Bcl-2 associated X protein (Bax) (Bax polyclonal antibody, 1 : 50, Santa Cruz Biotechnology Inc., U.S.A.). The primary antibodies were detected by using of HRP labeled goat anti-rabbit immunoglobulin G (IgG) secondary antibodies (1 : 1000, Boster, China). Visualization of the immunoreactive proteins was accomplished by using of enhanced chemiluminescence (ECL) plus reagents (Amersham Bioscience) and exposed to X-ray film. β-Actin was used as a loading control.

Detection of ATP Content 1×10^5 cells/ml were plated in 96-well plates overnight and treated with 39 μM astaxanthin for 0, 2, 4, 8, 16 and 24 h. ATP content was measured by microplate reader according to the standard protocol of ATP detection kits (Beyotime, China).

Detection of Reactive Oxygen Species (ROS) 1×10^6 cells/ml were plated in 6-well plates overnight and were exposed to 39 μM astaxanthin for 0, 2, 4, 8, 16 and 24 h. Then cells treated were co-activated with 5 μM 2′-7′-dichlorofluorescein-diacetate for 30 min. Colorimetrical intensity was determined with fluorescence spectrophotometer (excitation at 484 nm, emission at 531 nm).

Cytochrome *c* Oxidase (COX) Activity Assay 1×10^6 cells/ml were plated in 75 cm^2 cell culture flasks overnight, and treated with 39 μM astaxanthin for 0, 2, 4, 8, 16 and 24 h. Cells were collected after treatment. Mitochondria were isolated as the standard protocol of the mitochondria extraction kits (Beyotime, China). The isolated mitochondria were lysed by mitochondrial lysis buffer containing protease inhibitors. Protein content was measured with bicinchoninic acid (BCA) assay. Mitochondrial protein was calculated according to the standard curve, which was produced under the same analytical conditions. COX activity was measured based on the principle that COX can oxidize reduced cytochrome *c* (Cyt C) (highest absorption peak at 550 nm) to oxidized Cyt C (lowest absorption peak at 550 nm). In the latter assay mitochondria sample isolated above was added to the reduced Cyt C. The OD value was detected at the beginning (0 s) and 60 s-reaction (60 s), respectively, by using colorimetric spectrophotometer. COX activity was shown as the OD value difference between 0 s and 60 s.

Statistical Analyses Results are presented relative to untreated controls. Values represent mean±standard deviation of three or more replicate tests. Data were analyzed by Duncan test following the ANOVA-procedure when multiple comparisons were made. $p<0.05$ was considered statistically significant.

RESULTS

Effect of Astaxanthin on Cell Proliferation Results from inhibition of cell proliferation by MTT assay showed

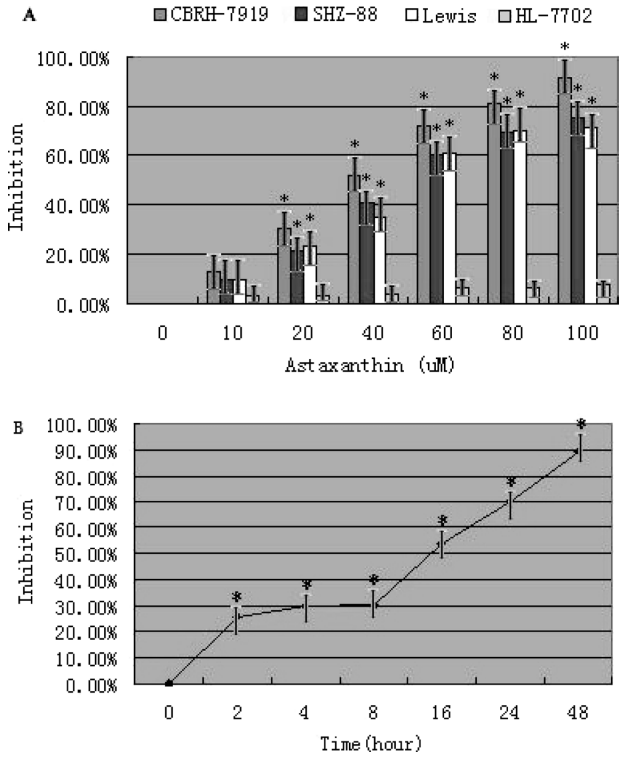


Fig. 1. Effects of Astaxanthin on Cell Proliferation

(A) MTT assay of effect of astaxanthin on cell proliferation of the three tumor cell lines (CBRH-7919, SHZ-88, Lewis) and the normal human hepatocyte HL-7702 cell line with a series of concentration (0—100 μM) for 24 h. Astaxanthin had little effect on HL-7702 cell line, but its inhibition was most pronounced in CBRH-7919 cell line with an IC_{50} value of 39 μM. This dose of astaxanthin and CBRH-7919 cell line were chosen for further studies. (B) The effect of 39 μM astaxanthin on CBRH-7919 cell proliferation at a series of co-culture time intervals (0—48 h). * Represents statistically significant at $p<0.05$.

that astaxanthin had little effect on HL-7702, however it could significantly inhibit the proliferation of three tumor cell lines: CBRH-7919, SHZ-88, and Lewis, after 24 h of treatment. In all three cases, the inhibitory effect showed a dose-dependent effect (Fig. 1A). The difference was statistically significant at $p<0.05$ compared to the control (0 μM of astaxanthin). The most sensitive cell line, CBRH-7919, with an IC_{50} value of 39 μM astaxanthin was selected for further study. Results indicated that the inhibitory effect of astaxanthin on CBRH-7919 cells increased with an increase in treatment time. When the cells were treated with 39 μM astaxanthin for series of co-culture time 0, 2, 4, 8, 16, 24 and 48 h (Fig. 1B), the inhibitory effect also increased, suggesting that the inhibitory effect of astaxanthin is time-dependent. The difference was statistically significant at $p<0.05$.

Effects of Astaxanthin on Cell Apoptosis A significant peak of hypodiploid indicative of apoptosis was detected by flow cytometry when the cells were treated with 39 μM astaxanthin for 12 or 24 h. The apoptotic rate of CBRH-7919 cells treated with 39 μM astaxanthin for 12 h was 15.16%, and it increased to the maximum of 74.87% after 24 h of treatment. However, many of the CBRH-7919 cells showed necrosis with necrosis rate of 59.40%, while the apoptotic rate was 12.50% when cells treated with 39 μM for 48 h (Fig. 2).

Changes in Morphology of Mitochondria TEM showed that a relatively lesser number of mitochondria were observed in cells treated with astaxanthin (Fig. 3C) compared to control cells (Fig. 3A). The ultrastructures of the control cells showed that structural integrity of mitochondrial membrane (inner and outer membranes), and the mitochondria cristae were not affected (Fig. 3B), while the ultrastructures of the astaxanthin treated cells showed altered membrane structural integrity. Some of the mitochondria were swollen with vacuole-like structure, some showed less number of cristae or deformed cristae, and few mitochondria had

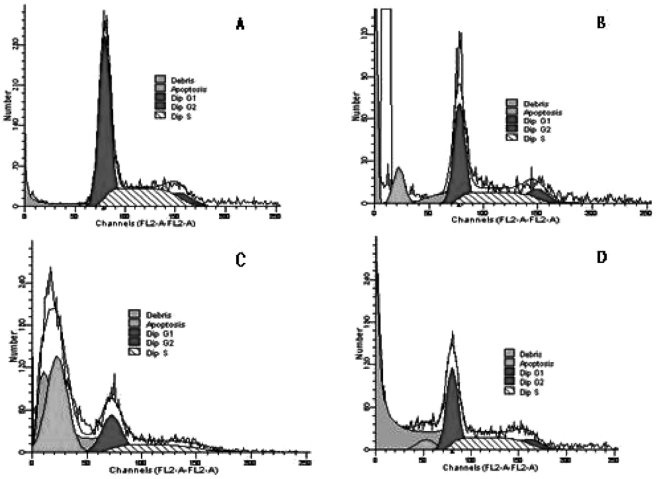


Fig. 2. Apoptosis Rate Assay

(A) Control cell, apoptosis rate 0.02%. (B) Cells treated for 12 h, apoptosis rate 15.16%. (C) Cells treated for 24 h, apoptosis rate 74.87%. (D) Cells treated for 48 h, apoptosis rate 12.50%.

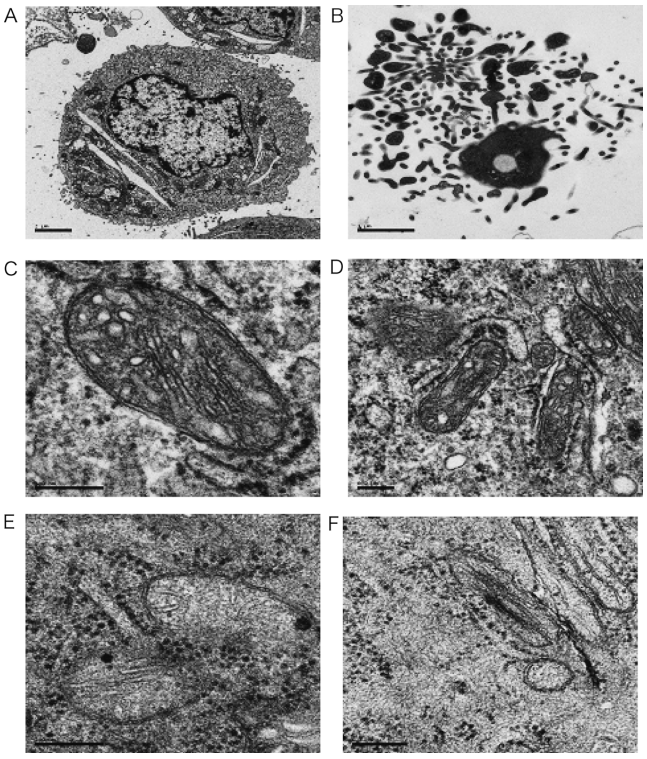


Fig. 3. Changes of Mitochondrial Morphology Observed by TEM

(A) The control normal cell. (B) The apoptotic cell treated with astaxanthin. (C) The control normal cells showed that structural integrity of inner and outer mitochondrial membranes and the mitochondria cristae were not affected. (D—F) Cells treated for 12 h, 24 h and 48 h with astaxanthin showed altered membrane structural integrity. The mitochondria were swollen with vacuole-like structure, less number of cristae or deformed cristae, and few mitochondria had cristae parallel to the long axis.

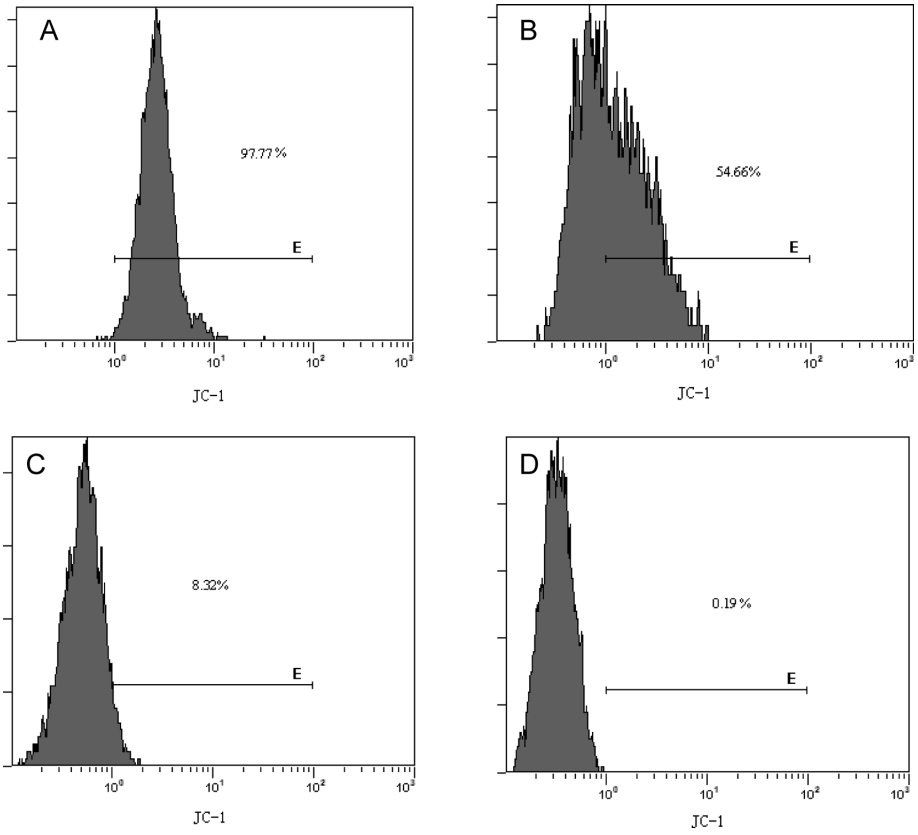


Fig. 4. Changes of Mitochondrial Transmembrane Potential

(A) Control cells. (B) Cells treated for 6 h. (C) Cells treated for12 h. (D) Cells treated for 24 h. Mitochondrial transmembrane potential was decreased with increasing time of exposure to astaxanthin.

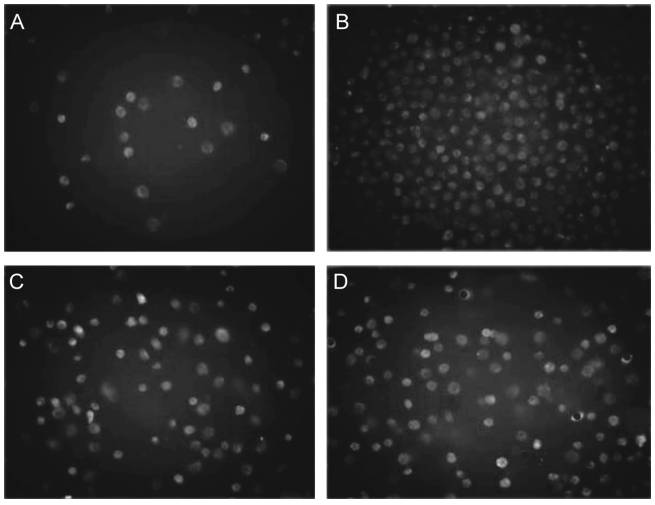


Fig. 5. Changes of Ca^{2+} Concentration

(A) Control cells. (B) Cells treated for 6 h. (C) Cells treated for 12 h. (D) Cells treated for 24 h. Ca^{2+} concentration was increased with increasing time of exposure to astaxanthin.

cristae parallel to the long axis (Fig. 3D). The vesicle-like autophagic bodies were observed which might have been formed by phagocytotic actions between mitochondria and mitochondria or endoplasmic reticulum and mitochondria (Fig. 3E). Though other organelles were degraded during the process of apoptosis, only mitochondria could be detected at this mid or late apoptotic stage, because they met the energy needs of the cell during the process of apoptosis (Fig. 3F).

Table 1. Changes in Mitochondrial Respiratory Chain

Co-cultured time with astaxanthin (h)	ATP content (nmol/mg pro.)	Complex IV activity (μmol/mg pro.)	DCF fluorescence intensity (ROS)
0	10.77±0.17	2.38±0.18	5.46±0.27
2	9.87±0.18	2.19±0.15	7.19±0.35
4	8.18±0.14	1.97±0.14*	9.69±0.38*
8	5.78±0.19*	1.57±0.23*	13.71±0.27*
16	4.66±0.12*	1.07±0.31*	17.13±0.19*
24	2.98±0.33*	0.98±0.41*	20.33±0.30*

The ATP levels showed a progressive decrease, while Cyt C and ROS levels continually increased with an increase of treatment time. * Represents statistically significant at $p<0.05$.

Change in Mitochondrial Transmembrane Potential The left shifting of image derived from flow cytometry shows reduction in mitochondrial membrane potential. Results indicated that the value of mitochondrial membrane potential in astaxanthin treated cells was lower than that of the control cells (Fig. 3A). The longer the treatment with astaxanthin was, the greater the reduction in membrane potential (Figs. 4B—D). Ca^{2+} concentration determined with Fura-2/AM fluorescence probe showed that the Ca^{2+} concentration was lower in the control cells than that of cells treated with astaxanthin (Fig. 5A), and it increased after treatment, and the increase pattern was treatment time dependent (Figs. 5B—D).

Changes in Mitochondrial Respiratory Chain Results indicated that the ATP levels showed a progressive decrease,

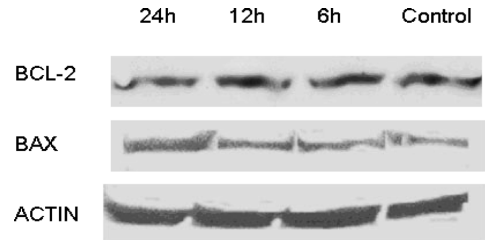


Fig. 6. Western Blot Analysis of the Apoptotic Proteins

Expression of Bcl-2 protein was down-regulated and that of Bax protein was up-regulated.

Table 2. Relative Levels of Bcl-2 and Bax to Actin in CBRH-7919 Cells

Intensity ratio	Control	6 h	12 h	24 h
BAX/ACTIN	0.05±0.02	0.08±0.01*	0.10±0.04*	0.02±0.01*
BCL-2/ACTIN	0.04±0.01	0.07±0.01*	0.09±0.03*	0.20±0.02*

The relative levels of Bcl-2 and Bax in Fig. 6 were normalized to the levels of actin using densitometric analysis. The numbers shown in Table 2 are mean number±standard deviation. * Represents statistically significant at $p<0.05$.

while Cyt C and ROS levels continually increased with an increase of treatment time (Table 1).

Effects of Astaxanthin on Apoptotic Proteins in Mitochondria The data indicated that the astaxanthin treatment did indeed affect the levels of Bcl-2 and Bax proteins by Western blot analysis (Fig. 6). Astaxanthin could activate the expression of Bax protein, and the steady increase in the expression of Bax was consistent with an extension of treatment time. Interestingly, the level of Bcl-2 displayed a different pattern under similar conditions. Bcl-2 protein expression increased significantly after 6 or 12 h of astaxanthin treatment, but decreased with a further increase in treatment time up to 24 h (Fig. 6). Results from statistical analysis using the image software indicated that the band intensity in the treated lanes displayed significant difference from that of the control lanes ($p<0.05$) (Table. 2).

DISCUSSION

Mitochondrion[14—16] is an essential eukaryotic organelle that performs many physiological and pathophysiological functions, of which ATP production is the most important one. In addition, it plays important roles in maintenance of glucose homeostasis, cell signal transduction, oxygen uptake and regulating the balance of ATP production and consumption. Its damaged function marks an irreversible stage of cell necrosis and apoptosis. In this study, the effect of astaxanthin on liver cancer cells and mitochondria were investigated by using CBRH-7919 cell. It showed that, at 39 M concentration, astaxanthin significantly inhibited CBRH-7919 cell proliferation. The apoptotic rate in CBRH-7919 cells treated with astaxanthin for 24 h was 74.87% analyzed using flow cytometry.

The cell apoptosis is initiated by signal stimulation with a series of control switches. The initiation of apoptosis can occur by multiple pathways that are caused by different intracellular signals or external factors. Most of the apoptotic signal transductions have not yet been studied in detail. However, the apoptotic signal pathway mediated by mitochondria is becoming relatively well understood recently. Mitochondria[17—19] are believed to be a core component of the cell death machinery. Mitochondria act as sensors and amplifiers in determining the execution of cell death or apoptosis through the regulation of cellular energy metabolism. In this paper, our results from transmission electron microscope indicate that astaxanthin has very destructive effect on mitochondria. The number of mitochondria was significantly reduced when the cells were treated with astaxanthin for 24 h. It also led to the alternation in mitochondrial structure such as swelling of mitochondria, disruption and collapse of mitochondrial membrane, rupture in cristae, and fewer mitochondria with cristae parallel to the mitochondria's long axis (Fig. 3). The vesicle-like autophagic bodies were also observed in the ultrastructural assay (Fig. 3). Apoptosis is a physiological process that requires energy. Therefore, although most of the organelles had completely disappeared at the mid- or late-apoptotic stage, mitochondria were still clearly discernible and active as they supplied energy to cellular processes.

The changes in mitochondrial morphology, especially the disruption in the integrity of inner and outer membrane, inevitably lead to alteration in the transmembrane potential.[20—22] Signal transduction in the cells was accompanied by changes in mitochondrial transmembrane potential. Apoptotic signal may decrease mitochondrial membrane potential by opening mitochondrial permeability transition pores (MPTP). The results from this study suggest that mitochondrial transmembrane potential of CBRH-7919 cells treated with astaxanthin was significantly lower than that of control cells (Fig. 4). Additionally, the results from the electron microscopy support the conclusion that the reduction of mitochondrial transmembrane potential is caused by the disruption in the integrity of the mitochondrial membrane after astaxanthin treatment (Fig. 3). Furthermore, this study found that Ca^{2+} concentration increased steadily with the extension of treatment time with astaxanthin, suggesting the stability of mitochondrial transmembrane potential display very important roles in maintaining the permeability of Ca^{2+}, K^+ and Na^+. This result is consistent with the previous report that any external factors or signals affecting the mitochondrial transmembrane potential might lead to abnormal changes of ion permeability. It indicated that increasing of Ca^{2+} is due to changes of mitochondrial membrane potential, which eventually leads to cell death.

The decrease in transmembrane potential is accompanied by a decline in ATP level in the cells. ATP is the final product and the cellular energy of the respiratory chain and respiratory chain is a modulator of apoptosis.[23—25] MPTP opening is a key event in the decrease of transmembrane potential and leads to Cyt C release from mitochondrial membrane into the cytoplasm, and the subsequent interruption of electron transport respiratory chain, coupling Cyt C release to the formation of free radicals. In our study, the increases in Cyt C and ROS and the decrease in the ATP levels indicated that astaxanthin might directly or indirectly lead to the interruption of electron transfer respiratory chain and subsequently the release of Cyt C from mitochondria into cytosol. Indeed, the Cyt C content in our study was increased in the cytosol when treated with astaxanthin.

The Bcl-2 family of intracellular proteins plays an important regulator role in apoptosis, and its opposing factions, *i.e.*

the anti- and pro-apoptotic members, especially the ratio of Bcl-2 to Bax, determines the life-or-death of the cells. In normal cells, anti-apoptotic member proteins are mainly located in the cell membrane system, especially mitochondrial outer membrane, while the vast majority of pro-apoptotic member proteins are mainly present in the cytoplasm. Apoptosis factors acting on the mitochondria are triggered with mitochondrial membrane damage, reduction in transmembrane potential, and deficiency of respiratory chain. The Bcl-2 family of apoptotic factors plays an important role in regulating the expression of anti-apoptotic and pro apoptotic members of the Bcl-2. The ratio of pro-apoptotic members to the Bax levels in mitochondria determines cell survival or apoptosis. After cells receive apoptosis signal, Bcl-2 family proteins change their usual localization and targeting patterns from the cytoplasm to the mitochondrial membrane, triggering mitochondrial dysfunction and release of apoptosis-inducing factors, and eventually leading to cell death.[26,27] Our Western blot analysis showed an increase in Bcl-2 protein expression within 12 h treatment of astaxanthin, and a subsequent decrease in the expression for the next 12 h of treatment, while Bax protein expression displayed a continuous increase throughout the 24 h treatment. The mechanism may be a self-defense in cells in response to the initial external signal. Furthermore, the decreased expression of Bcl-2 during the later stage (12—24 h) of astaxanthin treatment could be due to the continued signal stimulation beyond the capacity of self-defense in these cells.

Apoptotic signal is processed in an orderly fashion. Results suggest that astaxanthin induced apoptosis in CBRH-7919 cells. The apoptosis may be due to the induction of damage in mitochondrial membrane integrity, which triggered a decline in mitochondrial transmembrane potential and followed by the irreparable damage to electron transport respiratory chain and decline in cell ATP levels. The blockage in this process results in a different expression pattern of apoptotic proteins, in which the pro-apoptotic protein is induced, while the anti-apoptotic protein expression is decreased. However, apoptosis is a complex process that involves a large array of proteins. Many other apoptotic proteins may also be affected in the process of astaxanthin-induced apoptosis, which are needed to be confirmed by future studies.

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