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Astaxanthin Inhibits Proliferation and Induces Apoptosis and Cell Cycle Arrest of Mice H22 Hepatoma Cells

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Background: It is widely recognized that astaxanthin (ASX), a member of the carotenoid family, has strong biological activities including antioxidant, anti-inflammation, and immune-modulation activities. Previous studies have confirmed that ASX can effectively inhibit hepatoma cells *in vitro*.

Material/Methods: MTT was used to assay proliferation of mice H22 cells, and flow cytometry was used to determine apoptosis and cell cycle arrest of H22 cells *in vitro* and *in vivo*. Moreover, anti-tumor activity of ASX was observed in mice.

Results: ASX inhibited the proliferation of H22 cells, promoted cell necrosis, and induced cell cycle arrest in G2 phase *in vitro* and *in vivo*.

Conclusions: This study indicated that ASX can inhibit proliferation and induce apoptosis and cell cycle arrest in mice H22 hepatoma cells *in vitro* and *in vivo*.

MeSH Keywords: Apoptosis • Cell Cycle Checkpoints • Cell Proliferation

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Background

The incidence of malignant tumors has shown an increasing trend due to the deterioration of the environment and increasing life pressures. Liver cancer is one of the most common malignant tumors accounting for 600,000 cases each year worldwide [1]. Although a number of new anti-tumor drugs have been developed, the lack of tissue-specificity and drug side effects remain a major problem. Therefore, finding a low toxicity, natural anti-tumor drug would help to partially solve this problem.

Astaxanthin (3, 3'-dihydroxy-β, β'-carotene-4, 4'-dione), (ASX), is a red carotenoid extracted from phaffia yeast and haematococcus algae. ASX is the product of the highest level of carotenoid biosynthesis. It has been called a super antioxidant, as the antioxidant activity of ASX far exceeds existing antioxidants. Its reactive oxygen species (ROS) scavenging is 800 times that of coenzyme Q10, 550 times that of Vitamin E, and 75 times that of α-lipoic acid [2]. Most important, no apparent side effects have been reported for ASX [3,4]. Compared with other carotenoids, ASX contains two keto groups on each ring structure, which results in enhanced antioxidant properties. As a dietary carotenoid, ASX is extracted from algae, shrimp, salmon, lobster, and some other organisms [5,6]. A large number of studies have shown ASX has strong biological activities including anti-lipid peroxidation, anti-inflammation, and immune-modulation, as well as anti-oxidation [7–9]. Therefore, ASX has been considerable a potential treatment for various diseases such as inflammatory diseases, metabolic diseases, and neurodegenerative diseases [10–14]. Recently, the anti-tumor role of ASX has been attracting attention. Emerging data indicate that ASX may play a role in therapy for cancers such as breast cancer, prostate cancer, colon cancer, and neuroblastoma [15–18]. A large number of studies have confirmed that ASX can effectively inhibit hepatoma cells *in vitro*. In 2010, researchers explored the effects of ASX on early hepatocarcinogenesis in rats [19] ASX has been found to induce apoptosis of hepatoma cells through NF-κB p65 and Wnt/β-catenin signaling pathways [20]. In addition, an experiment using rat hepatoma cells, found ASX to inhibit invasion of mesothelial cells derived from co-cultured rat mesenteric and liver cancer cells [9]. However, few studies have been done on the effects of ASX in hepatoma cells *in vivo*. In order to confirm the anti-tumor effect of ASX in liver cancer, we observed the effects of ASX on the proliferation, apoptosis, and cell cycle arrest of mice H22 hepatoma cells *in vitro* and *in vivo* using MTT flow cytometry.

Material and Methods

Reagents

ASX and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Acridine orange staining kit, Annexin V-PI apoptosis detection kit, and cell cycle detection kit were purchased from Kaiji Biological Co., Ltd., Nanjing, China. The mouse H22 cell line was purchased from Shanghai Cell Bank, Shanghai, China.

Animals and grouping

Male Kunming (KM) mice (weighting 18–22 g) were obtained from the Experimental Animal Center of Binzhou Medical College. All experimental procedures were conducted in accordance with the Guidelines for Animal Experiments of the Chinese Academy of Medical Sciences and with approval from the Ethics Committee for Animal Care at Binzhou Medical College. All animals were housed in a standard animal-grade room with three animals in each cage. The temperature was maintained at 23±2°C, the relative humidity at 55±5% and the light cycle at 12 hours/day.

Animals were randomly divided into three groups (all n=10): control, low-dose ASX, and high-dose ASX. In the ASX groups, mice were treated with either 2 μg/kg body weight or 4 μg/kg body weight through intragastric administration every day. ASX was administered for 7 days. The control group was administered with equivalent saline. In the *in vitro* experiment, there were four groups: control (0.1% DMSO), cisplatin (40 μM), low-dose ASX (20 μM), and high-dose ASX (40 μM). ASX was dissolved in DMSO. Cisplatin was administered as the positive control.

Measurement of cell proliferation using the MTT assay

Cell proliferation was determined using MTT assay. The cell concentration was adjusted to 1×10^5 cells/mL and 100 μL aliquots were transferred to 96-well plates. ASX (20 μM and 40 μM), DMSO (0.1%) and cisplatin (40 μM) were added to each well as appropriate and then washed twice with sterile saline at 6 hours, 12 hours, and 24 hours. According to the manufacturer's instructions, absorbance was measured with an ELISA Reader (Tosoh, Japan) using 560 nm reference wave length.

Measurement of cell apoptosis by acridine orange staining

Visualization of cell apoptosis was performed using acridine orange staining as previously described [21]. According to the manufacturer's instructions, cells were seeded in 96-well plates with 10,000 cells/well. After 24 hours of incubation, cells were stained with acridine orange solution and incubated. The confocal microscope (Leica, Germany) was used to observe the presence of apoptosis.

Measurement of cell apoptosis and cell cycle by flow cytometry analysis

Cell apoptosis and cell cycle were determined using flow cytometry as previously described [9]. H22 cells were incubated

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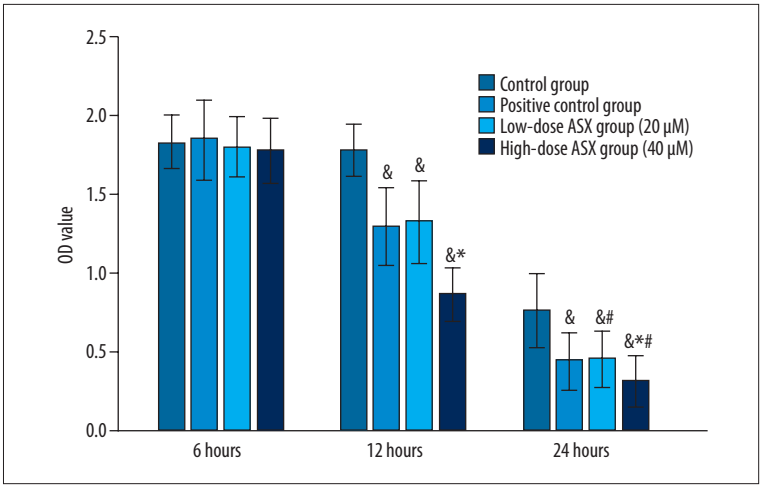

Figure 1. Effect of astaxanthin (ASX) on H22 cells proliferation by MTT assay. H22 cells were treated with 0.1% DMSO (the control group), 40 μM cisplatin (positive control group), 20 and 40 μM ASX for 6 hours, 12 hours, and 24 hours. The low-dose and high-dose ASX groups could obviously inhibit H22 cells proliferation compared to the control group at 12 hours and 24 hours. Low-dose ASX inhibited cells proliferation as effectively as the classical chemotherapy drug cisplatin. In addition, the inhibitory effect of ASX was dose-dependent, and the high-dose ASX group had a higher inhibitory effect than the low-dose group at 12 hours and 24 hours ($p<0.05$). With time increasing, the inhibitory effect was gradually improved. At 24 hours, the low-dose ASX group and high-dose ASX group had higher inhibitory effect than those at 12 hours ($p<0.05$). $^{\&}$ $p<0.01$ versus the control group; * $p<0.05$ versus the low-dose ASX group; $^{\#}$ $p<0.05$ versus the low-dose ASX and high-dose ASX groups at 12 hours.

with various concentrations of ASX (20 μM and 40 μM) for 6 hours, 12 hours, and 24 hours. According to the manufacturer's instructions, cell cycle distribution and apoptotic cells were measured by flow cytometry (Beckman, USA.). *In vivo* mice were treated with ASX (2 and 4 μg/kg body weight) through intragastric administration every day for 7 days, and the control group was administered with equivalent saline. After H22 cells were administered by intraperitoneal injection, H22 cells in the ascites were measured.

Measurement of amount of ascites and cell numbers

After ASX was administered for 7 days, each mouse was administered H22 cells (0.5 mL, 1×10^6 cells/mL) by intraperitoneal injection. After an additional 7 days, the ascites were extracted and recorded. Then the ascites were centrifuged, flushed, and diluted with sterile saline, and the H22 cell numbers were detected using flow cytometry.

Tumor-bearing test

After ASX was administered for 7 days, each mouse was injected with 0.1 mL of H22 cells (concentration of 5×10^6 cells/mL) into their back subcutaneously. After an additional 28 days, the mice were sacrificed and the transplanted tumors were resected and weighed.

Statistical analysis

All data were expressed as mean ±SD. Statistical analysis was performed with SPSS 16.0. Comparisons between groups were performed by one-way ANOVA with a p value < 0.05 being considered significant.

Results

ASX inhibited H22 cells proliferation

As shown in Figure 1, the low-dose and high-dose ASX groups could obviously inhibit H22 cell proliferation compared to the control group at 12 hours and 24 hours ($p<0.01$). The low-dose ASX inhibited cell proliferation as effective as cisplatin, a classical chemotherapy drug. In addition, the inhibitory effect of ASX was dose-dependent; the high-dose ASX group had higher inhibitory effect than the low-dose ASX group at 12 hours and 24 hours ($p<0.05$). With increasing time, the inhibitory effect was gradually improved. At 24 hours, the low-dose and high-dose ASX groups had higher inhibitory effect than at 12 hours ($p<0.05$). These results suggested that the inhibitory effect of ASX was time-dependent.

ASX induced H22 cells apoptosis

Visualization of cell apoptosis was performed using acridine orange staining. As shown after ASX treatment, the H22 cells showed evidence of nuclear condensation and nuclear/cellular fragmentation, especially in the high-dose ASX group (Figure 2).

As shown in Figure 3A, the Annexin V-PI double staining flow diagram illustrated that ASX could promote apoptosis of H22 cells *in vitro*. However, the rate of cell apoptosis was not statistical difference between the low-dose ASX group, high-dose

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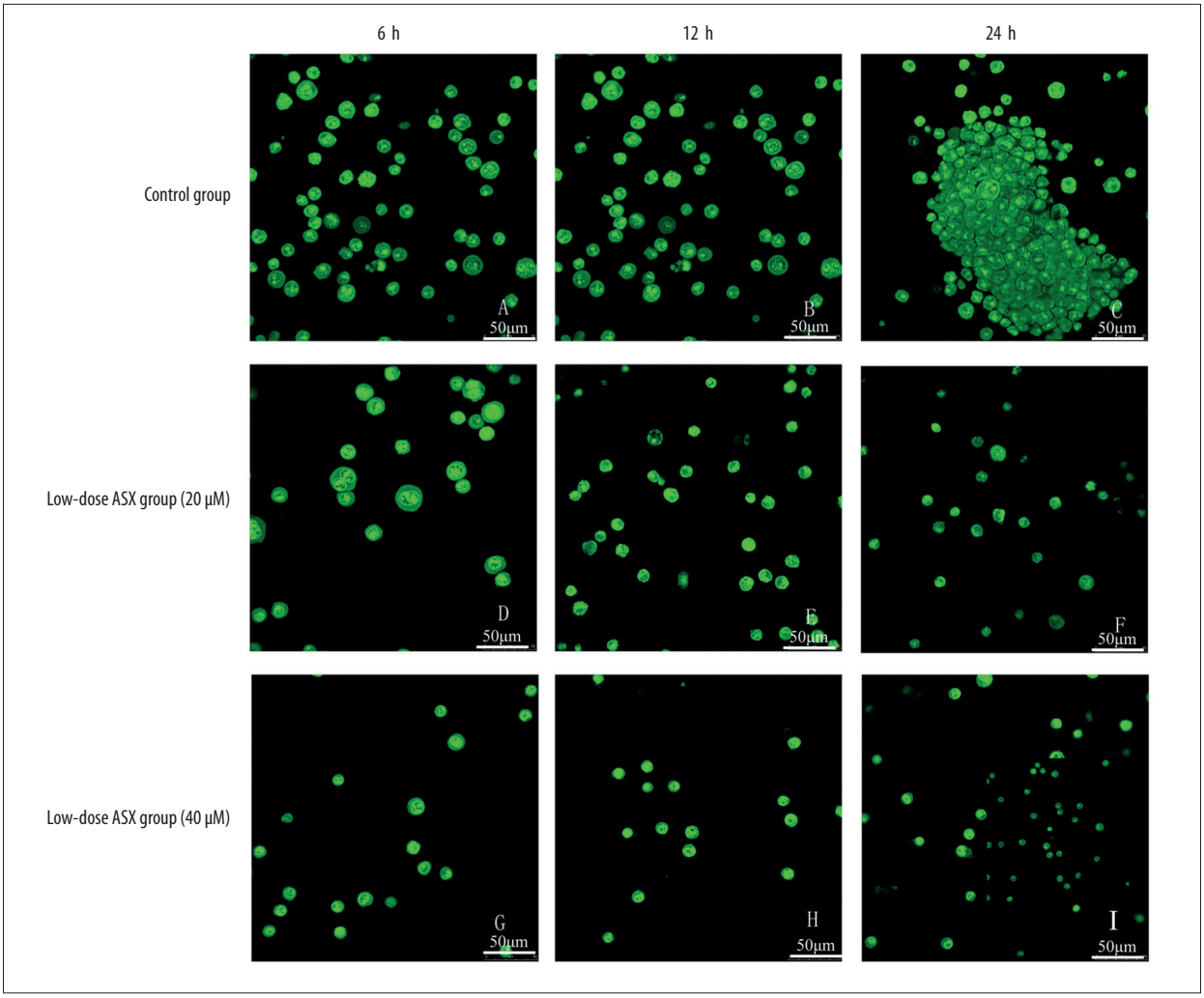

Figure 2. Effect of astaxanthin (ASX) on H22 cells apoptosis by acridine orange staining. Acridine orange staining was used to visualize apoptosis by confocal microscope *in vitro* (×400). *In vitro*, H22 cells were treated with 0.1% DMSO (the control group), 40 μM cisplatin (positive control group), 20 and 40 μM ASX for 6 hours, 12 hours and 24 hours. After ASX treatment, the H22 cells showed evidence of nuclear condensation and nuclear/cellular fragmentation, especially in the high-dose ASX group.

ASX group, and the control group at 6 hours, 12 hours, and 24 hours ($p>0.05$). We found that the rate of cell necrosis was significantly higher in low-dose and high-dose ASX groups than in the control group at 24 hours ($p<0.01$).

Compared with the control group, the low-dose and high-dose ASX groups showed no obvious difference in cell apoptosis *in vivo* ($p>0.05$), though there was a trend toward promoting apoptosis. Like the *in vitro* experiment, the rate of cell necrosis was significantly higher in the low-dose and high-dose ASX groups than in the control group ($p<0.05$) (Figure 3B).

ASX induced H22 cell cycle arrest

In vitro, DNA ploidy analysis showed the proportion of S phase was decreased, and the proportion of G2 phase was increased in low-dose and high-dose ASX groups compared to the control group (Figure 4A).

Likewise, the proportion of S phase was decreased and the G2 phase was increased in the ASX groups compared to the control group *in vivo* (Figure 4B). These results suggested that ASX could induce cell cycle arrest in G2 phase.

Effect of ASX on anti-tumor activity in mice

In order to estimate the effect of ASX on anti-tumor activity *in vivo*, the tumor-bearing test was performed. Compared with the control group, the ascites amount and the total number of H22 cells were significantly decreased in the low-dose and high-dose ASX groups ($p<0.05$ and $p<0.01$, respectively). It was obvious that anti-tumor activity in the high-dose ASX

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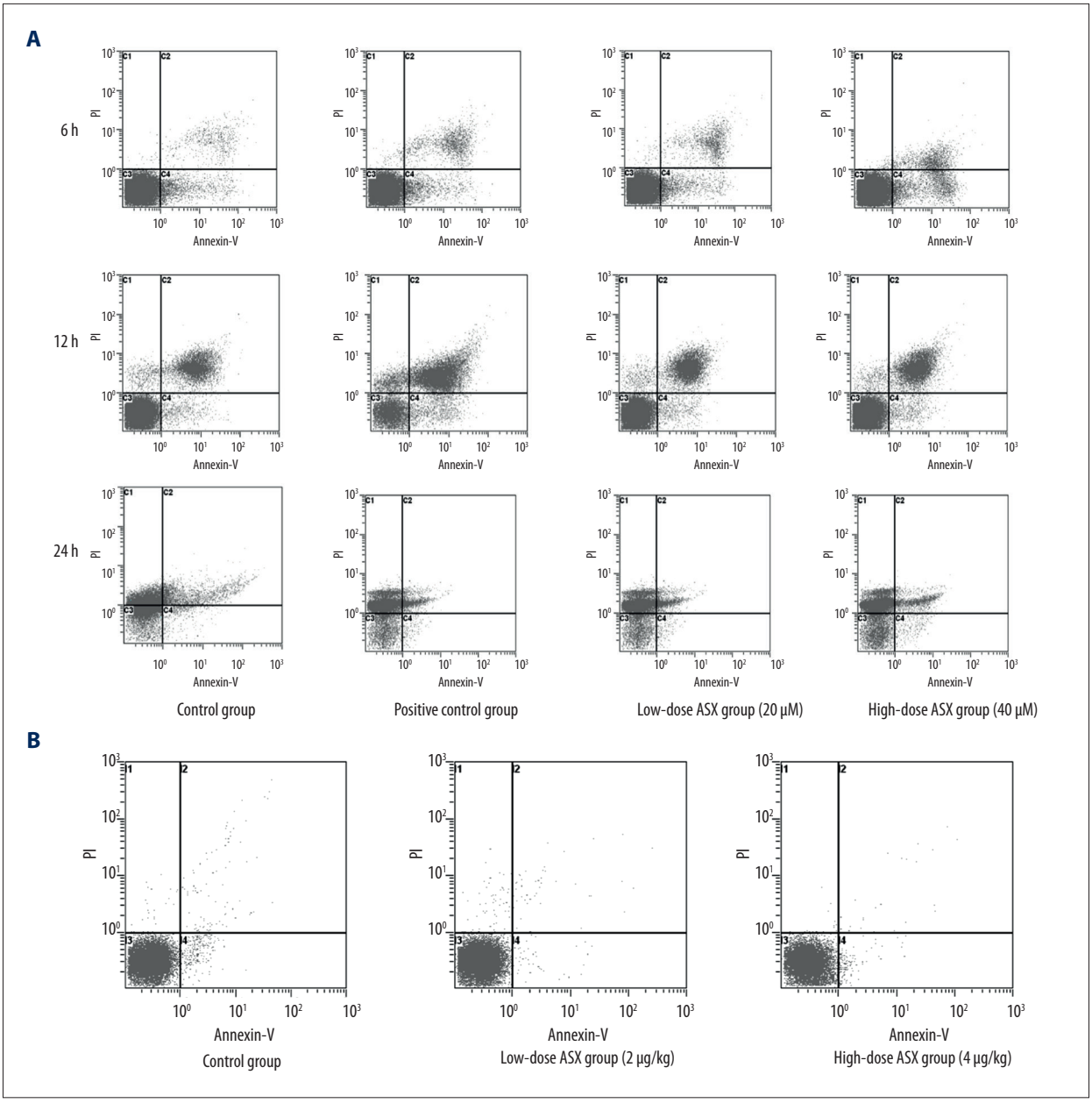

Figure 3. Effect of astaxanthin (ASX) on H22 cells apoptosis by flow cytometry. (**A**) *In vitro*, H22 cells were treated with 0.1% DMSO (the control group), 40 μM cisplatin (positive control group), 20 and 40 μM ASX for 6 hours, 12 hours and 24 hours. The rate of cell apoptosis was not statistical difference between low-dose and high-dose ASX groups and the control group at 6 hours, 12 hours and 24 hours ($p>0.05$). The rate of cell necrosis was significantly higher in low-dose and high-dose ASX groups than in the control group at 24 hours ($p<0.01$). (**B**) *In vivo*, mice were administered ASX with 2 or 4 μg/kg body weight through intragastric administration every day for 7 days, and the control group was administered with equivalent saline. After H22 cells were administered by intraperitoneal injection, H22 cells in the ascites were measured. The rate of cell necrosis was significantly higher in the low-dose and high-dose ASX groups than in the control group ($p<0.05$).

group was higher than in the low-dose ASX group ($p<0.01$) (Figure 5A, 5B).

As shown in Figure 6B, the rate of tumor formation in the three groups was 100%, 100%, and 70%. One mouse died in the control group after H22 cells were injected. Compared with the control group, the body weight in low-dose ASX group was lighter ($p<0.05$), and tumor weight and the ratio of tumor weight to body weight was significantly decreased ($p<0.01$). Also, the body weight of mice, tumor weight, and the ratio of

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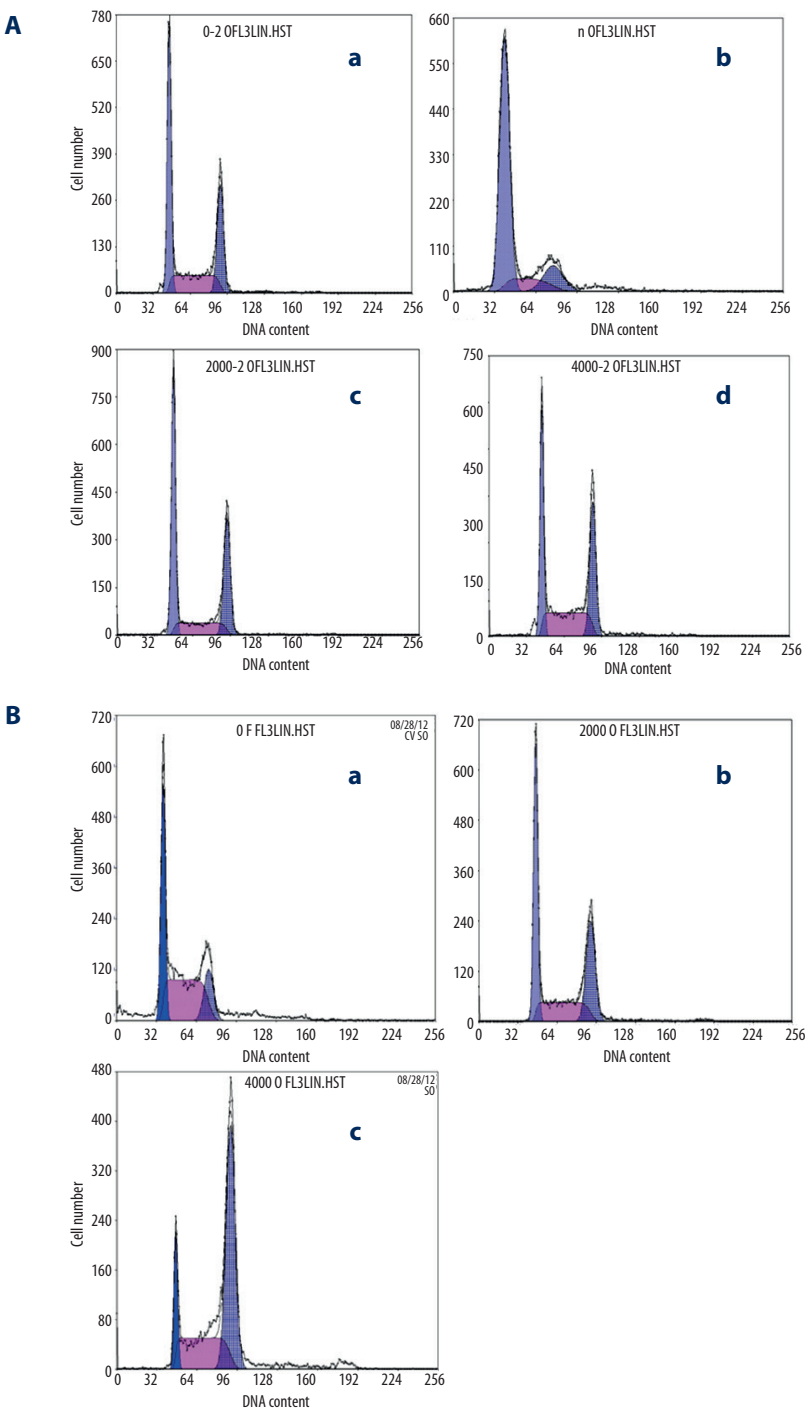

Figure 4. Effect of astaxanthin (ASX) on H22 cells cell cycle by flow cytometry. (**A**) *In vitro*, H22 cells were treated with 0.1% DMSO (the control group), 40 μM cisplatin (positive control group), 20 and 40 μM ASX for 6 hours, 12 hours and 24 hours. (**a**) Control group; (**b**) Positive control group; (**c**) Low-dose ASX group (20 μM); (**d**) High-dose ASX group (40 μM). (**B**) *In vivo,* mice were administered ASX with 2 or 4 μg/kg body weight through intragastric administration every day for 7 days, and the control group was administered with equivalent saline. After H22 cells were administered by intraperitoneal injection, H22 cells in ascites were measured. (**a**) Control group; (**b**) Low-dose ASX group (2 μg/kg body weight); (**c**) High-dose ASX group (4 μg/kg body weight). The proportion of S phase was decreased, and the proportion of G2 phase was increased in the ow-dose ASX and high-dose ASX groups compared to the control group.

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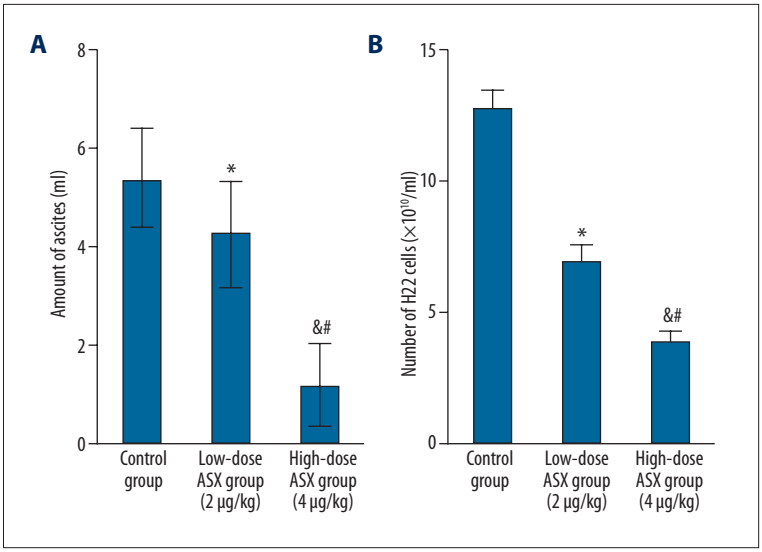

Figure 5. Effect of astaxanthin (ASX) on the amount of ascites and the number of cells in mice. (**A**) Mice were administered ASX with 2 or 4 μg/kg body weight for 7 days, the ascites amount was detected: * $p<0.05$ versus the control group; & $p<0.01$ versus the low-dose ASX group; # $p<0.01$ versus the control group. (**B**) Mice were administered ASX with 2 μg/kg or 4 μg/kg body weight for 7 days, the number of H22 cells/mL were detected: * $p<0.05$ versus the control group; & $p<0.01$ versus the low-dose ASX group; # $p<0.01$ versus the control group.

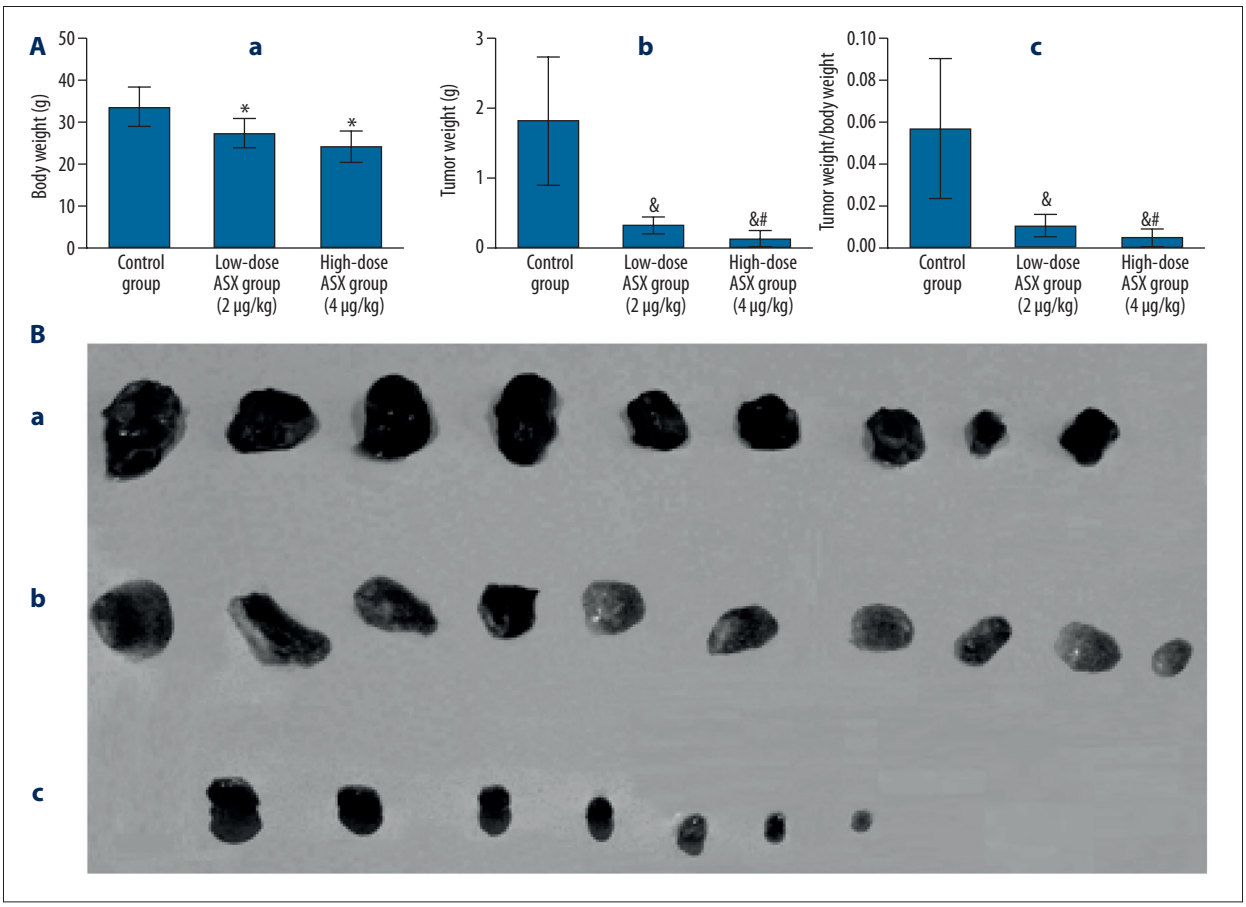

Figure 6. Effect of astaxanthin (ASX) on the tumor growth in mice. (**A**) Mice were administered ASX with 2 or 4 μg/kg body weight for 7 days. After mice were injected H22 cells into back subcutaneously, the transplanted tumors in mice were weighed. Compared with the control group, the body weight in the low-dose and high-dose ASX groups were lighter ($p<0.05$); tumor weight and the ratio of tumor weight to body weight was significantly decreased ($p<0.01$). Tumor weight and the ratio of tumor weight decreased in the high-dose ASX group compared with the low-dose ASX group ($p<0.05$): * $p<0.05$ versus the control group; & $p<0.01$ versus the control group; # $p<0.05$ versus the low-dose ASX group. (**B**) The transplanted tumors are shown in different groups. (**a**) The control group; (**b**) The low-dose (2 μg/kg) ASX group; (**c**) The high-dose (4 μg/kg) ASX group.

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tumor weight and body weight in the high-dose ASX group were decreased significantly compared to the control group ($p<0.05$, $p<0.01$, and $p<0.01$, respectively). In addition, there were statistically significant difference in tumor weight and the ratio of tumor weight to body weight between the low-dose ASX group and the high-dose ASX group ($p<0.05$) (Figure 6A).

Discussion

To date, several studies have confirmed that ASX can effectively inhibit the hepatoma cells *in vitro*. However, few studies have been reported on the anti-hepatoma effects of ASX *in vivo*. So, we observed the effect of ASX on anti-tumor activity *in vitro* and *in vivo*. Our results demonstrated that ASX with high doses could inhibit proliferation of hepatoma cell line H22 *in vitro* and *in vivo*. The ascites volume and total number of H22 cells in mice were significantly decreased in the low-dose ASX group and high-dose ASX group.

H22 cells are one of the portability tumor cell lines in mice. The rate of tumor formation is high, and the duration of tumor formation is short without drug intervention. Our study found that at four weeks all mice had grown tumors in the control group after H22 cells were injected, except for the one mouse that died. The rate of tumor formation was 100%. ASX could obviously inhibit the rate of tumor formation, especially in high-dose ASX group. In addition, tumor weight and the ratio of tumor weight and body weight were significantly decreased in low-dose ASX and high-dose ASX groups. Compared with the control group, body weight was low in the ASX groups, which may be due to the decrease of the ascites amount and tumor weight in a relatively short time. Our data indicated that ASX could effectively inhibit proliferation of H22 cells *in vivo*.

The flow cytometry Annexin V-PI double staining kit was chosen to detect apoptosis and necrosis, and also, if ASX could promote apoptosis and necrosis *in vitro*. However, the rate of cell apoptosis was no statistical difference between low-dose ASX and high-dose ASX groups and the control group at 6 hours, 12 hours, and 24 hours. The rate of cell necrosis was increase in low-dose ASX and high-dose ASX groups at 24 hours, and the effect of induced necrosis was as efficient as the classical chemotherapy drug cisplatin. *In vivo*, there was no statistical difference in cell apoptosis between low-dose ASX and high-dose ASX groups and the control group. Likewise, we found that the rate of cell necrosis was significantly higher in low-dose ASX and high-dose ASX groups than in the control group. Unlike other studies, our result demonstrated ASX had little impact on apoptosis *in vitro* and *in vivo*. Therefore, our data suggested that induced apoptosis is not a major role in anti-tumor effect of ASX.

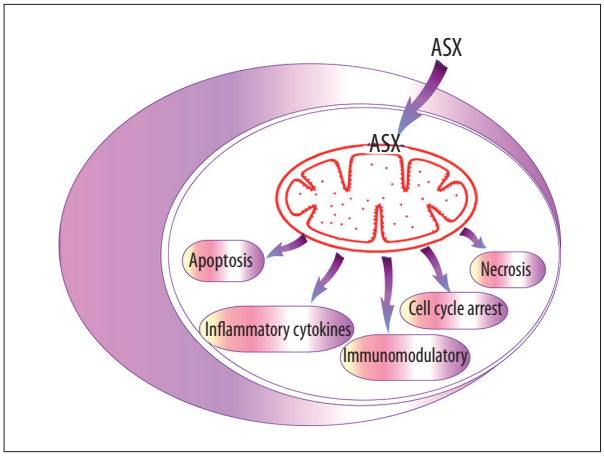

Figure 7. The mechanism of ASX in anti-tumor effect. ASX has diverse bio-activities such as anti-oxidant, anti-inflammatory and anti-apoptotic properties with minimal adverse effects.

According to the analysis of cell cycle, cultured cells *in vitro* and plated peritoneal cells showed G2 phase cells increased and G0/G1and S phase cells decreased in the ASX groups. This suggested that ASX induced cell cycle arrest in G2 phase, which might be one of the anti-tumor mechanism of ASX.

However, the anti-tumor mechanism of ASX remains unclear. It is generally accepted that multiple factors contribute to the inhibition of hepatoma cells. Previous studies found that ASX induced intrinsic apoptosis not only in breast cancer cells, but in oral cancer cells and neuroblastoma cells [16,22–24]. Other studies demonstrated that ASX played an efficient role against cancer by enhancing the immune response, and could activate T-cells and inhibit autoimmune reactions [25,26]. More specifically, ASX inhibited cyclooxygenase-2 (COX-2) enzyme activities and other cytokines [15,27]. In leukocyte cells, half of the total ASX is distributed in the mitochondria [28]. Therefore, it is a mitochondrion-permeable antioxidant. A previous study demonstrated that ASX induced mitochondria-mediated apoptosis in rat hepatoma cells via inhibition of the JAK/STAT3 signaling pathway [29] (Figure 7). Obviously, we only observed the anti-tumor effect of ASX in the present study, and further research is needed to elucidate molecular mechanisms.

Conclusions

This study indicated that ASX inhibited proliferation and induced apoptosis and cell cycle arrest of mice H22 hepatoma cells *in vitro* and *in vivo*. The present study may provide experimental evidence to enhance the anti-tumor effect of ASX. In addition, further studies will be helpful for ASX as a novel and potential drug in clinical treatment.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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