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PATHOLOGY

Mechanism of astaxanthin relieving lipopolysaccharide (LPS)-induced acute liver injury in mice

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ABSTRACT: Acute liver injury (ALI) is an important medical problem that requires effective therapy. Astaxanthin (AST) is a carotenoid, and the beneficial effects of astaxanthin, including anti-oxidative, anti-inflammatory and anti-tumour activities, have been identified. The present study was designed to elucidate the protective effects of astaxanthin against ALI and their underlying mechanisms. RAW264.7 macrophages were treated with dimethyl sulfoxide combined with different doses of astaxanthin for 12 h. Mice were fed with or without astaxanthin for up to 7 days. LPS was administered to induce inflammation. We assessed histopathology, oxidative stress, inflammation and apoptosis .The results indicated that astaxanthin attenuated LPS-induced oxidative stress, inflammation and cell apoptosis both *in vivo* and *in vitro*. *In vivo* and *in vitro* experiments showed that astaxanthin down regulated the nuclear factor-kappa beta (NF-κB), nuclear factor erythroid 2-related factor 2 (Nrf2) and NLR family pyrin domain containing 3 (NLRP3) signalling pathways, inhibiting the LPS-induced inflammatory response, oxidative stress and cell apoptosis, and alleviating LPS-induced ALI in mice.
Key words: Astaxanthin, Acute liver injury, LPS, NF-κB, Nrf2.

Mecanismo de alívio da astaxantina lipopolisacarídea (LPS) induzido lesão hepática aguda em ratos

RESUMO: A lesão hepática aguda (ALI) é um problema médico importante que requer terapia eficaz. A astaxantina (AST) é um carotenóide, e os efeitos benéficos da astaxantina, incluindo atividades antioxidantes, anti-inflamatórias e antitumorais, foram identificados. O presente estudo foi desenhado para elucidar os efeitos protetores da astaxantina contra ALI e seus mecanismos subjacentes. Macrófagos RAW264.7 foram tratados com dimetil sulfóxido combinado com diferentes doses de astaxantina por 12 h. Os camundongos foram alimentados com ou sem astaxantina por até sete dias. O LPS foi administrado para induzir a inflamação. Histopatologia, estresse oxidativo, inflamação e apoptose foram avaliados. Os resultados indicaram que a astaxantina atenuou o estresse oxidativo induzido por LPS, inflamação e apoptose celular *in vivo* e *in vitro*. Experimentos *in vivo* e *in vitro* mostraram que a astaxantina regulou negativamente as vias de sinalização do fator nuclear-kappa beta (NF-κB), fator nuclear 2 relacionado ao eritróide 2 (Nrf2) e domínio de pirina da família NLR contendo 3 (NLRP3), inibindo o LPS-resposta inflamatória induzida, estresse oxidativo e apoptose celular e alívio da ALI induzida por LPS em camundongos.
Palavras-chave: Astaxantina, lesão hepática aguda, LPS, NF-κB, Nrf2.

INTRODUCTION

Liver diseases and injuries are important medical problems worldwide. In particular, acute liver injury (ALI), is associated with high mortality rates (DAI et al., 2018). ALI is a terrible affliction caused by many factors, such as large-scale necrosis or apoptosis of hepatocytes, steatosis of hepatocytes, inflammation, oxidative stress and liver function damage (FYFE et al., 2018). The liver, which mainly controls the metabolism of substances, is the most vulnerable to attack by toxic substances. Intake of drugs is the first way to attack the liver and easily damage liver cells (KUNA et al., 2018). ALI resulting in the development of liver disease that progresses to terminal liver failure is the initiating factor and a common pathway to numerous liver diseases. The resulting long-standing hepatic injury often leads to liver fibrosis, cirrhosis and even liver cancer, and these diseases have a profound impact on the body (LIU et al., 2014). Liver transplantation is currently the most efficient therapy for liver failure and end-stage liver disease. However, it is constrained by the scarcity of donors, expensive medical costs, surgical risk and requiring life-long immunosuppressant agents (CHAN et al., 2012).

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Excessive or persistent inflammation characterizes a range of liver diseases (ROBINSON et al., 2016). Excessive formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) can overload antioxidant defence mechanisms, which can be translated into oxidative stress (FEITOSA et al., 2018). The liver is a major organ attacked by ROS, and various liver diseases are thought to be caused by excessive oxidative stress leading to their initiation and development (URSO & CLARKSON, 2003). The liver disease progresses due to apoptosis which is a prominent pathologic feature of most liver injuries (WANG, 2015).

Marine *Rhodotorula* are unicellular eukaryotic microorganisms widely existing in numerous environments in nature. They belong to the fungi of hemiacetal, spores, cryptococcosis and *Rhodotorula* and are the dominant flora in marine yeast (JIANNAN et al., 2015). Marine *Rhodotorula* are rich in astaxanthin. Astaxanthin has strong antioxidant activity and cannot be underestimated in various aspects, such as its anti-inflammatory effects, anti-tumour activities, improving the body's immunity and scavenging of free radicals in the body. Experiments *in vivo* and *in vitro* have proven that astaxanthin can inhibit biomarkers related to oxidative stress and inflammation (BROWN et al., 2017).

In this study, astaxanthin was extracted from *Rhodotorula mucilaginosa* ZTHY2 by the acid-heating dimethyl sulfoxide (DMSO) method, and then we evaluated the effects of astaxanthin on intraperitoneal injections of lipopolysaccharide (LPS) in C57BL/6 mice, a commonly used animal model, and explored the underlying mechanisms.

MATERIALS AND METHODS

Extraction method of astaxanthin

Our research team isolated and identified 71 strains of marine *Rhodotorula* from marine organisms, seawater, sea mud and other samples collected from the coastal waters of Leizhou Peninsula; the representative 36 strains of *Rhodotorula* spp. belong to five species and two suspected new species. The acid heat, dimethyl sulfoxide (DMSO), lactic acid and grinding freezing-thawing methods were compared and analysed. It was found that the acid heat method had the highest total carotenoid yield. The steps of the acid heat method were as follows. We weighed 0.1 g of the dry cells into a centrifuge tube, added 5 mL of 3 mol/L HCL, stirred evenly, and allowed the mixture to stand still for 40 min. We treated the mixture with a boiling water bath for 3 min, cooled it rapidly, centrifuged it at 3000 r/min for 10 min and discarded the supernatant. Then, we washed the pellet with distilled water 3 times, added 5 mL of acetone solution to extract for 30 min, centrifuged at 4000 r/min for 15 min and repeatedly extracted until the cells were colourless. We then put the extract into a 20 mL graduated test tube, diluted the acetone to 15 mL and detected the astaxanthin content at 480 nm.

The extracted astaxanthin was identified by HPLC. Mobile phase A was methanol/ tert-butyl methyl ether/1% phosphoric acid at a ratio of 81:15:4. Mobile phase B was methanol/tert-butyl methyl ether/1% phosphoric acid at a ratio of 16:80:4. The detection wavelength was 474 nm, the temperature was 35 °C, and the sample loading was 20 μL.

Cell culture and treatment

The RAW264.7 monocyte/macrophage murine cells were purchased from ATCC Cell Bank (USA). The cells were cultured in a high-sugar DMEM (GIBCO) medium containing 10% FBS (Clark), 1% penicillin and streptomycin at 37 °C in a 5% CO_2 cell culture incubator. Astaxanthin (AST) was dissolved in DMSO. Cultured cells were treated with 10, 20 and 40 μM astaxanthin for 12 h, with APCC as a positive control, negative control without any treatment. Treated cells were exposed to 40 μg/mL LPS (Sigma) for 12 h to induce inflammation. Exposed cells were divided into six groups: (1) the negative control, (2) the LPS group, (3) the 10 μM astaxanthin (AST) group, (4) the 20 μM AST group, (5) the 40 μM AST group and the (6) 300 μM APCC group.

CCK8 assay

RAW264.7 cells were plated at a density of 1×10^5 cells·mL^{-1} into 96-well plates (100 μL medium per well) with five replicates. Plated cells were then treated with different concentrations of astaxanthin and 40 μg/mL LPS. After incubation for 24 h, cell viability at 450 nm was measured using the CCK8 assay (AbMole) according to the manufacturer's instructions.

Animal experiments and design

All animal care and experimental protocols complied with the guidelines of the China National Institutes of Health and were approved by the Animal Care and Use Committee of Guangdong Ocean University, China (Animal Experiment Ethics Committee of Guangdong Ocean University. Approval No.: GDOU-LAE-2022-014. Date:2021/03/08). All efforts were made to minimize the suffering of experimental mice in this research.

Male C57BL/6 mice weighing between 20 g ± 2 g (6–8 weeks old) were bought from Beijing

Huafukang Biotechnology Co. (Beijing, China) and were housed under controlled temperature, humidity and lighting with ad libitum food and water. Acclimatization of mice was carried out for one week before experimentation. The concentrations of astaxanthin, aspirin and LPS were screened in the pre-experiment. The mice were randomly distributed into the following six groups.

Negative control group (n = 5): mice were fed the standard chow diet. The LPS group (n = 5) mice were gavaged with 0.2 mL of olive oil daily and injected intraperitoneally with 10 mg/kg of LPS solution after 7 days. The 25 mg/kg AST +LPS group (n = 5) mice were gavaged with 25 mg/kg AST daily, and 10 mg/kg of LPS solution was injected intraperitoneally after 7 days. The 50 mg/kg AST + LPS group (n = 5) mice were gavaged with 50 mg/kg AST daily, and 10 mg/kg of LPS solution was injected intraperitoneally after 7 days. The 100 mg/kg AST+LPS group (n = 5) mice were gavaged with 100 mg/kg AST daily, and 10 mg/kg of LPS solution was injected intraperitoneally after 7 days. The positive control group (n = 5) mice were gavaged with 25 mg/kg APCC daily, and 10 mg/kg of LPS solution was injected intraperitoneally after 7 days. Each group of mice was placed in a cage. AST was dissolved in olive oil, APCC was dissolved in distilled water, and LPS was dissolved in saline.

After 12 hours of LPS treatment, cervical dislocation execution of mice, collected serum and liver tissue. One part of the liver was fixed in 4% paraformaldehyde, and the other part was immediately put into a -80 °C.

Histological observation of liver pathology in mice

Liver tissue samples, which were fixed in 4% paraformaldehyde, were embedded within paraffin. Serial sections were sliced at 5 μm and stained with hematoxylin-eosin (H&E).

Biochemical analysis

Cell culture supernatant was obtained, and the levels of TNF-α, IL-6, IL-18 and IL-1β (Qisong Biology) were determined at 450 nm. Mouse blood was collected and serum was separated by centrifugation at 3000 rpm for 10 min. The total bilirubin (TBIL) was measured at 450 nm, alkaline phosphatase (ALP) was measured at 405 nm, aspartate aminotransferase (AST) was measured at 510 nm, and alanine aminotransferase (ALT) levels were measured at 510 nm using commercially available kits (Nanjing Jiancheng Bioengineering Institute). The assays were carried out according to the manufacturer's protocols.

Detection of oxidative stress

The cells (1×10^5) were treated with AST and LPS for 24 h. Mouse hepatocyte suspensions were prepared. The cells were collected, the absorbance was measured at 532 nm according to the manufacturer's plan (Beyotime), and the MDA content was calculated according to the standard curve. Briefly, cells were incubated with 1 mL of the diluted probe for 20 min at 37 °C. Cells were washed and 200 μL PBS was used to resuspend the cells, then the absorbance at the excitation wavelength of 488 nm and emission wavelength of 525 nm was measured immediately through the fluorescent enzyme marker (BioTek), and the ROS ratio was calculated (Beyotime). The collected mouse serum is used to measure the absorbance at 450 nm with a commercially available kit, and the SOD level was calculated according to the instruction manual. Liver tissue homogenate was prepared to measure the level of GSH at 412 nm (Solarbio).

Western blotting

Protein was extracted from cultured cells or frozen liver samples using standard techniques. A total of 20 μg protein was loaded onto SDS-polyacrylamide gels, and the separated proteins were transferred to PVDF membranes. The membranes were incubated overnight at 4 °C with primary antibodies, followed by incubation with a secondary antibody (1:5,000). Finally, the blots were scanned using a Gel Imaging System (Tanon Shanghai Corporation, China).

Apoptosis analysis

RAW264.7 cells (1×10^5) were plated into 6-well plates and treated with AST and LPS for 24 h. The cells were then washed and trypsinised. Mouse hepatocyte suspensions were prepared. Cells were resuspended with 100 μL of binding buffer containing Annexin V (5 μL) and PI (5 μL). The cells were further incubated at room temperature for 15 min in the dark. The samples were analysed by flow cytometry (Beckman Coulter).

Data and statistical analysis

The experimental data were evaluated by calculating the mean ± SD. Student's t-test, and one-way analysis of variance (ANOVA), followed by the Tukey test when F was significant, were performed to compare the differences between the experimental groups according to their characteristics. Statistical significance was assumed at $P < 0.05$. All statistical analyses were calculated using GraphPad Prism Software version 6.0 for Windows (GraphPad, San Diego, USA).

RESULTS

Identification results of astaxanthin

Among the types collected, *Rhodotorula mucilaginosa* ZTHY2 had the highest astaxanthin content, 11.641 μg/mL, and the purity was 96.8%. Therefore, astaxanthin extracted from *Rhodotorula mucilaginosa* ZTHY2 was used in this study. The identification results of astaxanthin are shown in figure 1. The retention time of the astaxanthin standard was 5.080 min, and the retention time of astaxanthin extracted from *Rhodotorula mucilaginosa* ZTHY2 was 5.081 min, which is similar to the retention time of the astaxanthin standard, so it was determined as astaxanthin.

Astaxanthin suppression of LPS-induced injury in RAW264.7 cells

RAW264.7 cells, a macrophage-like cell line, are considered a suitable model for screening and evaluating candidate drugs that possess anti-inflammatory potential (YANG et al., 2016). We first utilized CCK8 to screen for safe concentrations of AST and APCC and to determine the modelling concentration of LPS (Figure 2a). The results showed that the safe concentration of AST was 80 μM and the safe concentration of APCC was 1200 μM; therefore, cell models were established by incubating LPS at a concentration of 40 μg/mL for 12 h. We pre-treated the cells with different doses of AST and APCC for 12 h, exposed the dosed cells to LPS for 12 h, and then measured the effects using a CCK8 assay (Figure 2b). From these results, we found the beneficial effects of AST were concentration-dependent over the range of 10–40 μM, with the best effect at 40 μM; meanwhile, APCC was most effective at 300 μM. Thus, in subsequent work, we used AST at 10, 20 and 40 μM and APCC at 300 μM for 12 h. In comparison with the negative control, the cell culture supernatant TNF-α, IL-6, IL-18 and IL-1β levels were elevated from the LPS group ($P < 0.01$). The AST pre-treatment markedly decreased all LPS-induced TNF-α, IL-6, IL-18 and IL-1β in the cell supernatant (Figure 2c) ($P < 0.05$). We also measured the levels of LDH, MDA and ROS. As shown in figure 2d, LDH, MDA and ROS were up-regulated by exposure to LPS, and this effect was inhibited by AST. The proportion of living and dead cells was determined by flow cytometry, and the results (Figure 2e) showed that incubation with LPS induced approximately 6.11% cell apoptosis, and AST reduced the cell apoptosis rate to 0.75%. These results demonstrated clearly that AST protected RAW264.7 cells against LPS-induced injury.

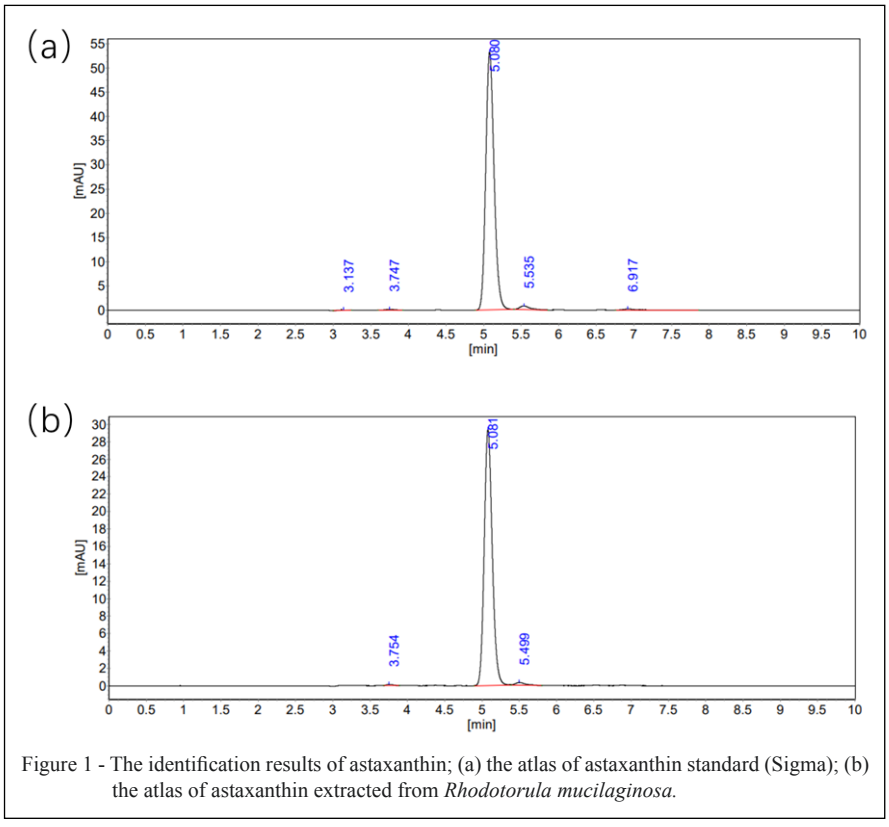

Figure 1 - The identification results of astaxanthin; (a) the atlas of astaxanthin standard (Sigma); (b) the atlas of astaxanthin extracted from *Rhodotorula mucilaginosa.*

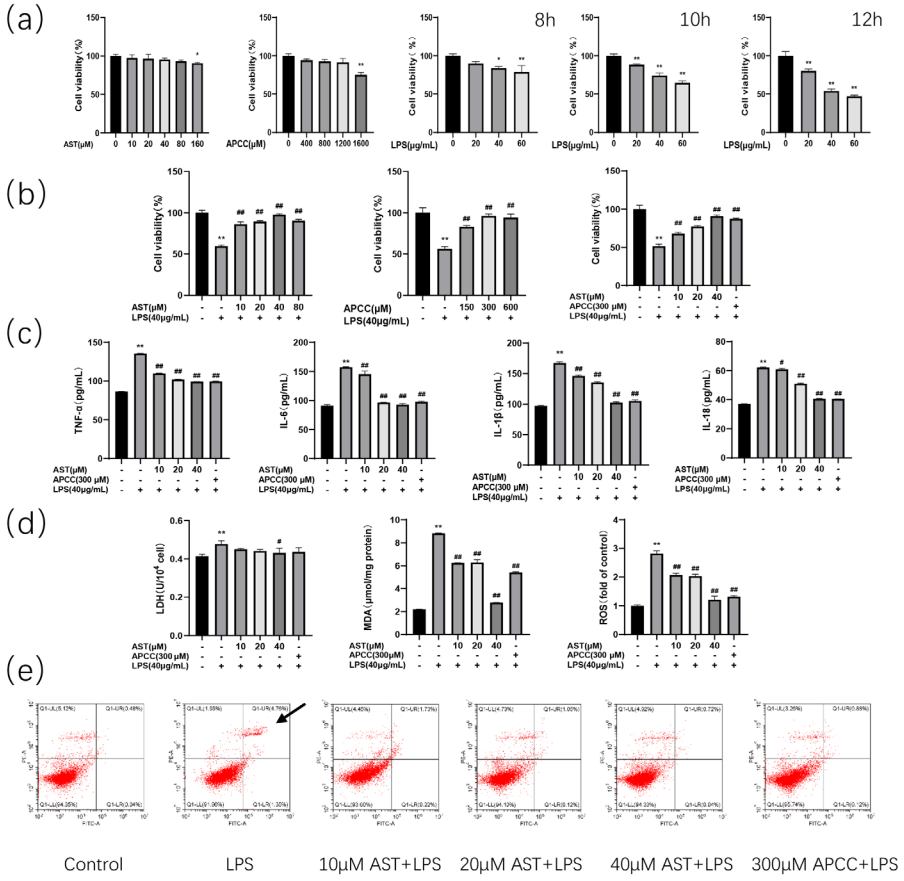

Figure 2 - Astaxanthin (AST) alleviates RAW264.7 cell damage by regulating oxidative stress and inflammation; (a) the safe concentration of drug and modelling concentration of LPS; (b) the protective effect of AST; (c) inflammatory factors; (d) LDH, MDA and ROS; (e) flow cytometry for RAW264.7 cells apoptosis; data shown are means ± SEM; n = 5 in each group; * $P < 0.05$, significantly different from control, ** $P < 0.01$, extremely significantly different from control; $^{\#}$ $P < 0.05$, significantly different from LPS, $^{\#\#}$ $P < 0.01$, extremely significantly different from LPS.

Astaxanthin attenuates LPS-induced liver injury in mice by modulating oxidative stress and inflammation

We established an LPS-induced ALI model to determine the effect of astaxanthin on ALI, and the results of the experiments are shown in figure 3. The haematology analyzer showed that astaxanthin prevented LPS-induced WBC abnormalities (Figure 3a). The levels of serum TBIL, ALP, ALT and AST are presented in figure 3b ($P < 0.05$). LPS caused an elevation of TBIL, ALP, ALT and AST levels, which could be reduced by astaxanthin in a dose-dependent manner ($P < 0.05$). Pathological changes in the liver of these mice were assessed and these results were shown in Figure 3c. The H&E-stained samples showed that the mice treated with LPS displayed the typical appearance of the infiltration of inflammatory cells. However, treatment with astaxanthin effectively alleviated these symptoms. And similar to the above, all of these changes were dose-dependent, with the group treated with 100 $mg \cdot kg^{-1}$ astaxanthin showing the greatest effects. ALI involves multiple mechanisms, including inflammation, oxidative stress and apoptosis. The levels of TNF-α, IL-6, IL-18 and IL-1β were assessed by ELISA. The expression of these pro-inflammatory cytokines was raised in the model group but decreased following astaxanthin treatment (Figure 3d) ($P < 0.01$).

Astaxanthin treatment significantly relieved hepatic oxidative damage. As shown in figure 3e, in the model group, the levels of ROS,

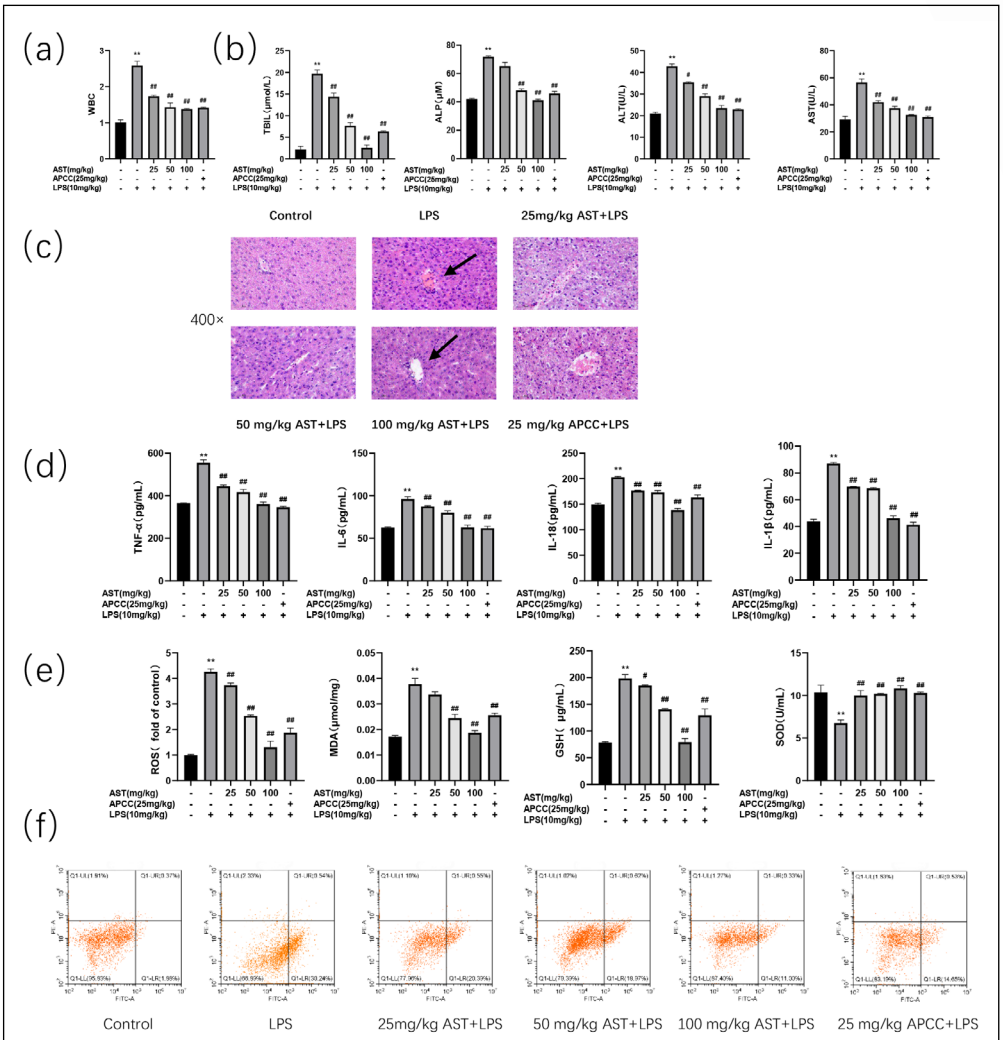

Figure 3 - Astaxanthin (AST) suppressed LPS-induced injury in hepatocytes; (a) WBC; (b) levels of serum TBIL, ALP, ALT and AST; (c) H&E staining of livers (central veins are marked by arrows); (d) serum ELISA levels of TNF-α, IL-6, IL-18 and IL-1β; (e) levels of ROS, MDA, GSH and SOD; (f) flow cytometry for liver apoptosis; mice were given different concentrations of AST (25, 50, 100 mg/kg) and 25 mg/kg APCC by gavage for 7 days, 10 mg/kg LPS solution was intraperitoneally injected and detected 12 hours later; data shown are means ± SEM; n = 5 in each group; * $P < 0.05$, significantly different from control, ** $P < 0.01$, extremely significantly different from control; $^{\#}$ $P < 0.05$, significantly different from LPS, $^{\#\#}$ $P < 0.01$, extremely significantly different from LPS.

MDA and GSH were substantially up-regulated compared with the Negative control group ($P < 0.01$), the SOD level was substantially down-regulated ($P < 0.01$), and astaxanthin reversed this phenomenon in all instances ($P < 0.01$). Hepatocyte apoptosis is one of the most prominent features of liver injury; the results (Figure 3f) showed that compared with the Negative control group, LPS significantly increased the level of apoptosis in mouse hepatocytes ($P < 0.01$). AST significantly decreased the level of apoptosis in mouse hepatocytes ($P < 0.01$); thus, we concluded that astaxanthin had protected the liver of ALI mice by regulating oxidative stress, inflammation and apoptosis.

The beneficial role of astaxanthin was associated with anti-inflammatory and antioxidant activities

As inflammation and oxidative stress play an important role in the progression of ALI, we explored the protective mechanism of astaxanthin on ALI in mice in our model system. The NF-κB signalling pathway, a classical pathway of inflammation, plays

an important role in the study of the mechanisms of inflammation. NF-κB transactivation and ROS production can promote the production and activation of NLRP3 inflammatory vesicles. We measured changes in NF-κB, Nrf2 and NLRP3 signalling pathways by western blot *in vivo* (right) and *in vitro* (left), and consistent results were obtained (Figure 4). Compared with the LPS group, AST blocked p65 and IκBα phosphorylation by decreasing TLR4 expression, thereby achieving regulation of the NF-κB signalling pathway. AST attenuated the expression of NLRP3, ASC, caspase-1 and GSDMD in the NLRP3 signalling pathway. The expression of Nrf2, heme oxygenase-1 (HO-1) and NAD(P)H dehydrogenase quinone 1 (NQO1) in liver tissue increased with the increase of AST concentration. Anyway, AST inhibits NF-κB and NLRP3 signaling pathways upregulate the antioxidant pathway Nrf2 to alleviate acute liver injury in mice. The effects of astaxanthin were consistent *in vivo* (right) and *in vitro* (left) (Figure 5).

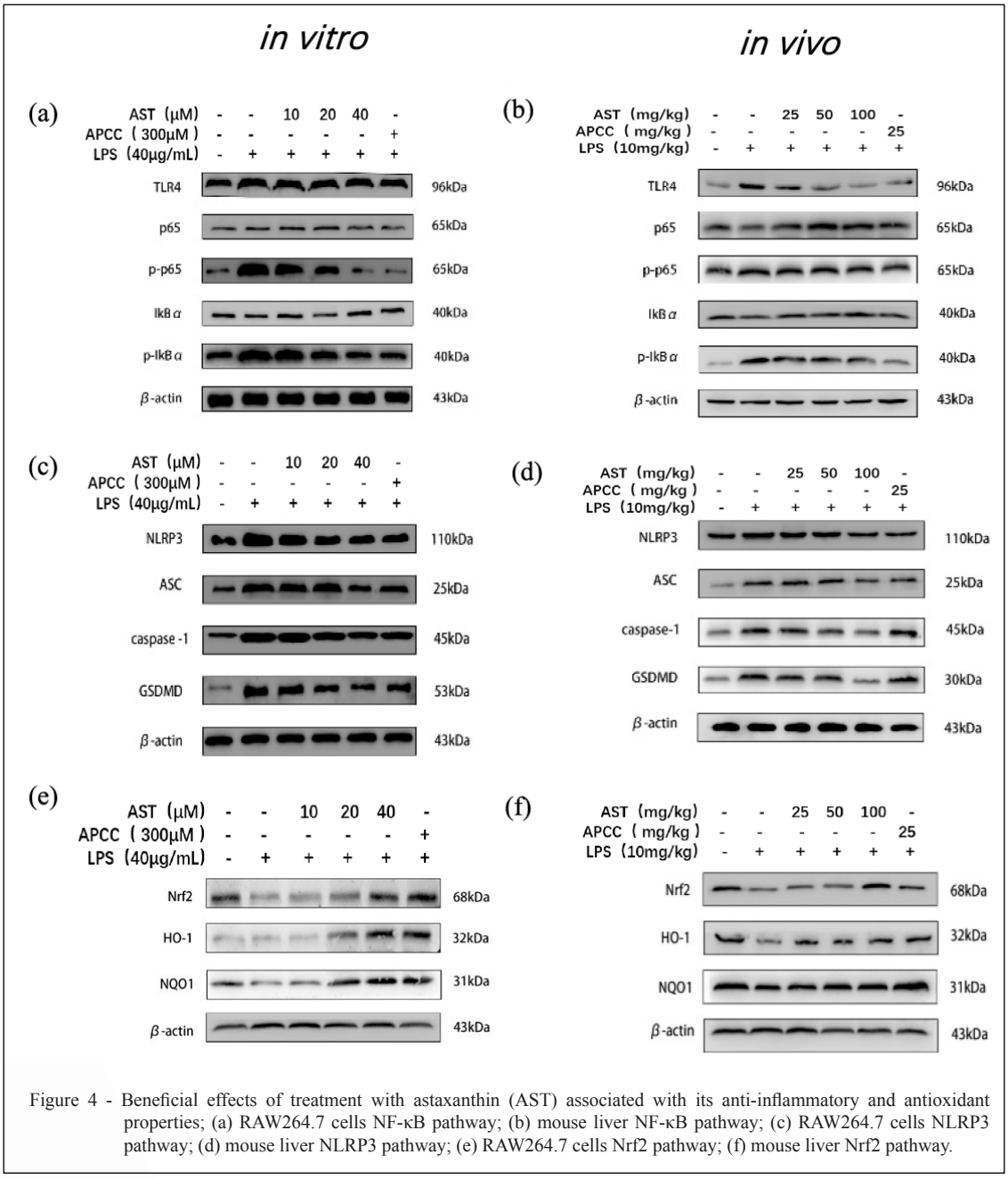

Figure 4 - Beneficial effects of treatment with astaxanthin (AST) associated with its anti-inflammatory and antioxidant properties; (a) RAW264.7 cells NF-κB pathway; (b) mouse liver NF-κB pathway; (c) RAW264.7 cells NLRP3 pathway; (d) mouse liver NLRP3 pathway; (e) RAW264.7 cells Nrf2 pathway; (f) mouse liver Nrf2 pathway.

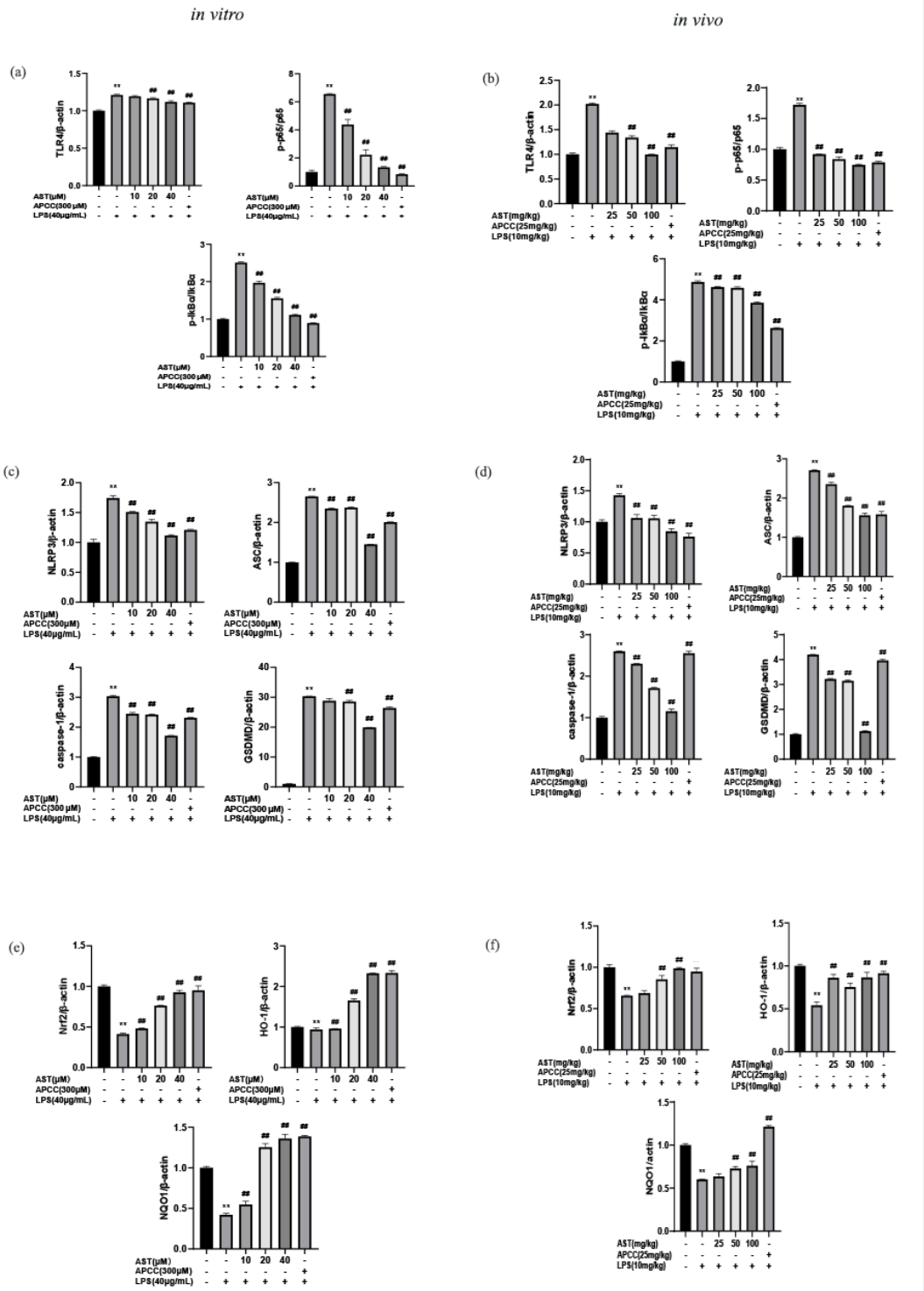

Figure 5 - Beneficial effects of treatment with astaxanthin (AST) associated with its anti-inflammatory and antioxidant properties; (a) RAW264.7 cells NF-κB pathway; (b) mouse liver NF-κB pathway; (c) RAW264.7 cells NLRP3 pathway; (d) mouse liver NLRP3 pathway; (e) RAW264.7 cells Nrf2 pathway; (f) mouse liver Nrf2 pathway; data shown are means ± SEM; n = 5 in each group; * $P < 0.05$, significantly different from control, ** $P < 0.01$, extremely significantly different from control; $^{\#}$ $P < 0.05$, significantly different from LPS, $^{\#\#}$ $P < 0.01$, extremely significantly different from LPS.

DISCUSSION

Acute liver injury is a clinical syndrome characterized by rapid loss of liver function, which may progress to life-threatening liver failure. A serious liver injury could induce excessive partial or systemic inflammation, which may culminate in liver failure and even multiple organ dysfunction. Liver transplantation is considered the only definitive treatment for liver failure, particularly, the treatment of acute liver failure (ALF), which has a high mortality. But it is limited by a shortage of graft availability, which creates the urgent need to seek complementary and promising therapies to prevent the progression of ALI (ZHOU et al., 2017; XU et al., 2021).

At the same time, ample evidence suggests the involvement of astaxanthin may be protective of liver function in liver disease. Several articles have reported the mechanisms underlying the astaxanthin treatment of ALI. It has been reported that AST protects the liver against acetaminophen (APAP) hepatotoxicity by alleviating necrosis, blocking ROS generation, and inhibiting lipid peroxidation and oxidative stress (ZHANG et al., 2017). Krill oil, which is full of astaxanthin, may counteract LPS-induced acute liver injury by inhibiting the activation of the TLR4/NF-κB/NLRP3 signalling pathway to restore antioxidant activity and the ability to attenuate inflammatory responses (DU et al., 2022). However, our knowledge of the mechanisms by which astaxanthin counters ALI is still inadequate. The increased production of reactive oxygen intermediates and lipid peroxidation, migration of activated PMNs into the liver, severe oxidative stress, and eventually extensive damage to the liver, are induced by LPS (FARZAEI et al., 2018). Our results showed that all the liver function indices exceeded the normal threshold, demonstrating that the liver injury model was established successfully. Treatments with different doses of AST significantly reduced all the serum liver function indexes (Figure 3). The data results are in agreement with those described in the literature (MA et al., 2020).

The pathogenesis of ALI is a complex multifactorial process that is inseparable from oxidative stress. In this study, MDA and ROS are boosted several times by LPS, but AST can improve this phenomenon, indicating that oxidative damage in mouse liver tissues was improved (TAKAKI et al., 2013; CHEN & KOTANI, 2016). The level of GSH in liver tissues of mice was significantly increased in the LPS group (1.98 mg/g) compared with the Negative control group and decreased in the AST group compared with the LPS group, indicating that the LPS-induced oxidative stress in the liver tissues of mice and the liver tissues secreted a large amount of GSH to resist the damage caused by oxidative stress. Astaxanthin protection restored the ability of mice to scavenge free radicals (Figures 3e). Nrf2 is a major regulator of cellular ROS and free radicals to restore redox homeostasis. Our results showed that LPS significantly decreased the expression of Nrf2, HO-1 and NQO1 and suppressed the regulation of the antioxidant pathway, while AST supplementation substantially increased their expression and regulated the protective effect of the antioxidant pathway on the liver. This was consistent with previous research results (MA et al., 2020; DONG et al., 2021; HENG et al., 2021).

ALI is an organ manifestation belonging to the systemic inflammatory response syndrome. In this study, we examined the inflammatory factors TNF-α, IL-6 and IL-1β and found that the secretion of inflammatory factors decreased after the addition of AST. Meanwhile, pathological autopsies revealed that AST could reduce inflammatory cell infiltration (Figure 3c), and the effect of the astaxanthin 100 mg/kg group was more significant. NF-κB is central to the regulation of the inflammatory response; meanwhile, Nrf2 has an important inhibitory effect on the inflammatory response by inhibiting the NF-κB pathway (HUANG et al., 2020). We also found that AST plays an important role in the NF-κB pathway and can inhibit NF-κB inflammation (Figure 4).

Pyroptosis is an important component of innate immunity, and excessive activation can also lead to excessive inflammatory reactions and disease. NLRP3 plays a major role in the classical pathway of cellular scorch death. It has been reported that AST can protect against kidney inflammation by inhibiting the activation of NLRP3 inflammasome, reducing the secretion of IL-1β and IL-18 (ZHUANG et al., 2021; SONG et al., 2022). We found that the pyroptosis pathway was activated in the liver of mice induced by LPS, as such IL-1β and IL-18 were secreted in large quantities (Figure 3d), and AST can resist this change. This is the first time investigating the NLRP3 pathway to explore the protective effect of AST on mouse liver injury has been performed. The secretion of IL-1β and IL-18 was also examined, and it was found that they were significantly reduced under the action of AST (Figure 3d). As IL-1β and IL-18 are produced as inactive precursors and require cleavage of caspase-1 to mature into bioactive cytokines, this finding further demonstrates that AST can modulate the classical pathway of pyroptosis to alleviate inflammatory responses (GREBE et al., 2018).

Apoptosis can activate numerous liver diseases (EL-HASSAN et al., 2003). High levels of ROS can promote apoptosis by altering the integrity and function of cell membranes, inducing lipid peroxidation, causing structural changes in proteins, breaking nucleic acid chains and damaging cells and organelles (DU et al., 2017). TNF-α is a multifunctional cytokine involved in apoptosis, inflammation and immunity acting via two receptors (VAN HORSSEN et al., 2006). AST can inhibit oxidative stress via the Nrf2/HO-1 pathway, thereby preventing *Cordyceps militaris* extract (CME)-induced apoptosis in cardiac myocytes (XUE et al., 2019). In this study, the apoptosis rate in the LPS group even reached 30%, but astaxanthin sharply reduced the level of hepatocyte apoptosis, and the mechanism of reducing apoptosis may be related to the increased expression of Nrf2 and the inhibition of NF-κB.

The study presents one limitation. The intracellular signalling pathways are intricate and complex, and further research is needed to explore the mechanism of AST action using protein inhibitors. Our studies confirmed that astaxanthin may become a promising drug to treat or relieve ALI. However, C57BL/6 mice are model animals, and the application of astaxanthin in the human body needs further study.

CONCLUSION

In vitro and *in vivo* experiments showed that astaxanthin alleviated LPS-induced acute liver injury in mice by modulating NF-κB, Nrf2 and NLRP3 signalling pathways to inhibit LPS-induced inflammatory responses, oxidative stress and apoptosis. 100 mg/kg astaxanthin significantly inhibited LPS-induced inflammatory factors TNF-a, IL-6, IL-18 and IL-1β in mouse serum, and reduced the secretion of AST, ALT, TBIL and ALP. It also improved the pathological damage of liver tissues and cleared the ROS and MDA produced by oxidative damage in mice. This study provides new ideas for the application of astaxanthin as a livestock feeding product in the livestock industry and for the development of new alternative antibacterial livestock feed additives.

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AVAILABILITY OF DATA AND MATERIALS

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy protection.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

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