



# Astaxanthin pretreatment attenuates acetaminophen-induced liver injury in mice



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## ABSTRACT

**Background:** Acetaminophen (APAP) is a conventional drug widely used in the clinic because of its antipyretic-analgesic effects. However, accidental or intentional APAP overdoses induce liver injury and even acute liver failure (ALF). Astaxanthin (ASX) is the strongest antioxidant in nature that shows preventive and therapeutic properties, such as ocular protection, anti-tumor, anti-diabetes, anti-inflammatory, and immunomodulatory effects. The aim of present study was to determine whether ASX pretreatment provides protection against APAP-induced liver failure.

**Methods:** Male C57BL/6 mice were randomly divided into 7 groups, including control, oil, ASX (30 mg/kg or 60 mg/kg), APAP and APAP + ASX (30 mg/kg or 60 mg/kg) groups. Saline, olive oil and ASX were administered for 14 days. The APAP and APAP + ASX groups were given a peritoneal injection of 700 mg/kg or 300 mg/kg APAP to determine the 5-day survival rate and for further observation, respectively. Blood and liver samples were collected to detect alanine transaminase (ALT), aspartate transaminase (AST), inflammation, oxidative stress and antioxidant systems, and to observe histopathologic changes and key proteins in the mitogen-activated protein kinase (MAPK) family.

**Results:** ASX pretreatment before APAP increased the 5-day survival rate in a dose-dependent manner and reduced the ALT, AST, hepatic necrosis, reactive oxygen species (ROS) generation, lipid peroxidation (LPO), oxidative stress and pro-inflammatory factors. ASX protected against APAP toxicity by inhibiting the depletion of glutathione (GSH) and superoxide dismutase (SOD). Administration of ASX did not change the expression of c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and P38. However, phosphorylation of JNK, ERK and P38 was reduced, consistent with the level of tumor necrosis factor alpha (TNF- $\alpha$ ) and TNF receptor-associated factor 2 (TRAF2).

**Conclusion:** ASX provided protection for the liver against APAP hepatotoxicity by alleviating hepatocyte necrosis, blocking ROS generation, inhibiting oxidative stress, and reducing apoptosis by inhibiting the TNF- $\alpha$ -mediated JNK signaling pathway and by phosphorylation of ERK and P38, which made sense in preventing and treating liver damage.

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## 1. Introduction

Drug-induced liver injury (DILI) is one of the most familiar and critical adverse drug reactions (ADR) resulting in ALF. DILI has become the leading cause of ALF in the United States and United Kingdom, as well as in some other countries and regions [1]. Based on statistics from the World Health Organization (WHO), DILI has become the fifth fatal cause of death in the worldwide.

Acetaminophen (APAP) is a conventional drug widely used in the clinic as antipyretic analgesic. However, accidental or intentional APAP overdoses contribute to liver injury. Recent data suggest that APAP-induced ALF leads to approximately 30,000 cases of hospitalization and

500 deaths each year in America [2]. The mechanism of this hepatotoxicity has not been entirely clarified. The ultimate hepatotoxic metabolite, *N*-acetyl-*p*-benzoquinoneimine (NAPQI), is the initial step and is detoxified by glutathione (GSH). NAPQI combines with cellular target proteins during APAP overdoses [3]. Evidence suggests that exhaustion of GSH and binding between NAPQI and proteins contribute to cell injury [4,5].

Mitochondrial dysfunction triggered by APAP overdose results in oxidative stress [6,7]. In addition to ROS, increased lipid peroxidation also indicates oxidative stress in APAP-induced liver injury [8]. Recently, it was shown that inflammation, apoptosis and autophagy were also associated with APAP-induced hepatotoxicity [9]. Oxidative stress leads to activation of c-Jun N-terminal kinase (JNK) [10]. Moreover, various studies suggest that pro-inflammatory cytokines, such as TNF- $\alpha$  and interleukin-6 (IL-6), activate JNK and P38, which are related to ERK [11]. Activation of JNK plays an important role in APAP-induced hepatotoxicity [10]. The JNK signaling pathway up-regulates apoptosis and autophagy by activating TNF receptor-associated factor 2 (TRAF2) [12].

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As a type of nature liposoluble carotenoid pigment, astaxanthin is present in aquatic animals, microalgae, flamingoes, and *Pfaffia* (yeast), and possesses potent antioxidant ability [13]. Additionally, ASX also has many pharmacological properties, such as ocular protection, anti-tumor, anti-diabetes, anti-inflammatory, and immunomodulatory effects [14]. Previous studies showed that ASX effectively inhibits peroxyl radical-dependent lipid peroxidation, scavenges singlet oxygen, and blocks ROS generation [15]. The antioxidative capacity of ASX makes it a potential pharmacologic resource. ASX pretreatment attenuates hepatic ischemia reperfusion-induced apoptosis and autophagy via the ROS/MAPK pathway in mice [16]. ASX inhibits neural progenitor cellular apoptosis modulated by the P38 and MEK signaling pathways [17]. ASX was found to protect the liver against ConA-induced autoimmune hepatitis by reducing the phosphorylation of Bcl-2 modulated by the JNK signal pathway [18]. According to data, the antioxidant capacity of ASX is 100–500 times greater than vitamin E, and 10 times greater than beta-carotene [19]. Several studies showed that ASX has better capacity in relevant diseases. In *N*-methyl-*N*-nitrosourea (MNU)-induced mammary cancer, high concentrations of ASX showed an anti-cancer effect, whereas canthaxanthin did not suppress carcinoma [20]. In ischemia reperfusion (I/R) injury after intestinal transplantation, ASX was effective compared to other antioxidants, such as 4,5-dihydroxy-1,3-benzene-disulfonic acid (Tiron) and epigallocatechin gallate (EGCG) [21]. In diet-induced insulin resistance and nonalcoholic steatohepatitis (NASH), ASX played a more effective role in NASH than vitamin E [22]. Moreover, ASX showed no apparent inhibition of seven major human hepatic UGTs so that it is safe for further clinical use [23].

The objectives of the present study were to determine whether ASX provides preventive and therapeutic effects during APAP-induced liver injury. We also assessed the potential mechanism of action.

## 2. Materials and methods

### 2.1. Preparation of animals and reagents

Male C57BL/6 mice were purchased from the Animal Feeding Center of Xi'an Jiaotong University Health Science Center. Experimental animals included in this study were 4–5 weeks old and weighed  $23.0 \pm 1.4$  g. Mice were housed in an air-conditioned room under a 12-h light/dark cycle (lights on 6:00 AM to 6:00 PM) and allowed food and water ad libitum. All experimental protocols followed the criteria of the Ethics Committee of Xi'an Jiaotong University Health Science Center. Mice were randomly allocated into the following groups: control (saline intraperitoneal (i.p.) injection); oil (olive oil by gavage); ASX (30 mg/kg/d or 60 mg/kg/d by gavage, dissolved in olive oil) [16]; APAP (700 mg/kg or 300 mg/kg i.p. injection), and ASX administered after APAP (APAP + ASX; doses same as described above). Saline,

olive oil and ASX were administered for 14 days. APAP and ASX were obtained from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. Experimental protocols

Blood samples were collected at selected time points (8 h and 24 h) after APAP administration and centrifuged at  $3000\times$  for 15 min to obtain supernatants. Serum samples were stored at  $-80^\circ\text{C}$  for further biochemical detection. Sections of liver tissues were fixed in 10% formaldehyde for histology. Blocks of liver tissues were placed in 2.5% glutaraldehyde for scanning electron microscopy (SEM). Additionally, a portion of the remaining liver was immediately homogenized for later analysis.

### 2.3. Survival

The survival study was conducted using the following three groups: control, APAP, and APAP + ASX groups. Mice received an i.p. injection of 700 mg/kg APAP. To observe the impacts of ASX on APAP-induced liver injury, the 5-day survival rate was determined.

### 2.4. Serum biochemistry

Serum ALT and AST were selected as sensitive indicators for hepatotoxicity, and ALT and AST levels were measured with an automated biochemical analyzer in the Department of Inspection, the First Affiliated Hospital of Xi'an Jiaotong University.

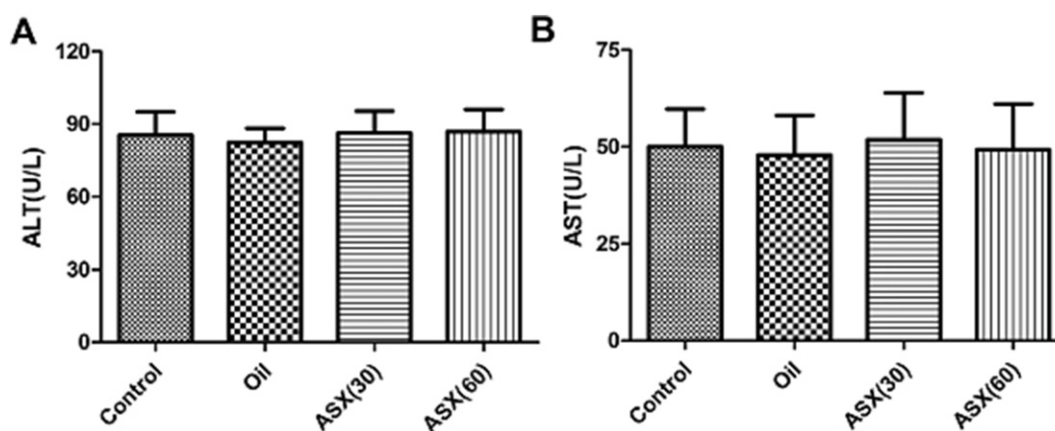
### 2.5. Determination of cytokines

Levels of TNF- $\alpha$  and IL-6 were measured using commercial ELISA kits according to the manufacturer's instructions (Dakewe, Shenzhen, China).

### 2.6. Histology

Liver samples were fixed in 10% formaldehyde and prepare to be embedded in paraffin. Serial liver sections (5  $\mu\text{m}$  thick) were stained with H&E or terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (Roche Molecular Biochemicals, Indianapolis, IN) kits and were observed under a microscope (Olympus Optical Co., Tokyo, Japan).

Portions of liver tissues were prefixed in 2.5% glutaraldehyde and washed with 0.1 M phosphate buffer. The samples were post-fixed with 1% OsO<sub>4</sub> and again washed with 0.1 M phosphate solution. The fixed tissues were dehydrated with graded ethanol and dried using the critical point drying method. After conductive treatment, ultrathin



**Fig. 1.** Olive oil and ASX cause no damage to normal liver tissues. (A) Serum ALT levels were measured in four groups of male C57BL/6 mice respectively treated with 0.9% saline, olive oil, ASX (30 mg/kg/d), and ASX (60 mg/kg/d) for 14 days; (B) Serum AST levels were detected as in (A). Data are presented as the mean  $\pm$  SD.

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