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Lycium barbarum polysaccharides improve CCl_4-induced liver fibrosis, inflammatory response and TLRs/NF-kB signaling pathway expression in wistar rats

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ABSTRACT

Lycium barbarum polysaccharides (LBPs) have multiple biological and pharmacological functions, including antioxidant, anti-inflammatory and anticancer activities. This research was conducted to evaluate whether LBPs could alleviate carbon tetrachloride (CCl_4)-induced liver fibrosis and the underlying signaling pathway mechanism. Fifty male wistar rats were randomly allocated to five groups (n = 10): control, CCl_4 and CCl_4 with 400, 800 or 1600 mg/kg LBPs, respectively. Each wistar rat from each group was used for blood and tissue collections at the end of experiment. The results showed that CCl_4 induced liver fibrosis as demonstrated by increasing histopathological damage, α-smooth muscle actin expression, aspartate transaminase activities, alkaline phosphatase activities and alanine aminotransferase activities. LBPs supplementation alleviated CCl_4-induced liver fibrosis as demonstrated by reversing the above parameters. In addition, CCl_4 treatment induced the oxidative injury, increased the mRNA levels of tumor necrosis factor-α, monocyte chemoattractant protein-1 and interleukin-1β, and up-regulated the protein expressions of toll-like receptor 4 (TLR4), TLR2, myeloid differentiation factor 88, nuclear factor-kappa B (NF-kB) and p-p65. LBPs supplementation alleviated CCl_4-induced oxidative injury, inflammatory response and TLRs/NF-kB signaling pathway expression by reversing the above some parameters. These results suggest that the alleviating effects of LBPs on CCl_4-induced liver fibrosis in wistar rats may be through inhibiting the TLRs/NF-kB signaling pathway expression.

1. Introduction

Hepatic fibrosis has been recognized as a reversible wound-healing response to a variety of chronic stimuli [32]. The etiologies of hepatic fibrosis include alcohol abuse, viral infection, persistent exposure to chemicals and drugs and so on [33]. Hepatic fibrosis is characterized by the excessive depositions of extracellular matrix (ECM) whose major sources are activated hepatic stellate cells (HSCs) [26,38]. HSCs participate in the collagen synthesis [34] and activate into α-smooth muscle actin (α-SMA)-expressing contractile myofibroblasts during liver fibrosis [24]. In addition, persistent inflammatory responses promote the activation of HSCs and eventually aggravate hepatic fibrosis. Toll-like receptors (TLRs) are a class of transmembrane receptors and ubiquitously expressed on HSCs, and play an important role in mediating inflammatory responses. The mechanism underlying TLRs effects is via the downstream adaptor myeloid differentiation primary response 88 (MyD88), which result in inflammatory cytokines and chemokines production such as tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β) and monocyte chemoattractant protein-1 (MCP-1) [14,23]. Previous studies showed that hepatic fibrosis could induce TLRs/NF-kB signaling pathway expression [9,28]. In addition, liver fibrosis and inflammation were significantly alleviated in TLR2-konckout and TLR4-mutated mice [15,30].

The activated hepatic stellate cells (HSCs) are major targets for antifibrotic therapy. So, inhibiting the activation of HSCs to alleviateα-smooth muscle actin (α-SMA) levels and TLRs/NF-kB signaling pathways might contribute to the treatment of hepatic fibrosis [20]. Liu et al. indicated that selenium-enriched probiotics alleviated CCl_4-induced liver fibrosis by preventing HSCs activation in rats [17]. Zhao et al. indicated that polysaccharide of *Larimichthys crocea* swim bladder protected CCl4-induced hepatic damage [38]. Arauz et al. found that nicotinic acid prevented experimental liver fibrosis by blocking the expression of α-SMA [3]. Peng et al. reported that Cordyceps polysaccharide significantly inhibited DMN-induced liver fibrosis in rats

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[21]. He et al. reported that docosahexaenoic acid attenuated CCl_4-induced hepatic fibrosis in rats [12]. Although many methods on antifibrotic are reported, there is few research about the antifibrotic of polysaccharides which have less side-effect. Previous study indicated that *Lycium barbarum* L. polysaccharides (LBPs) possessed antifibrotic activity but mechanistic studies needed to be performed [36].

LBPs, a traditional chinese medicine, are the major bioactive components of the *Lycium barbarum* (*L. barbarum*, Goji). LBPs have multiple biological and pharmacological functions, including antioxidant, anti-inflammatory, anticancer and anti-radiation activities [2,6,16]. It has been reported that LBPs could effectively alleviate non-alcoholic steatohepatitis-injury of liver in mice [36]. In addition, LBPs also have been reported to protect mice liver from carbon tetrachloride (CCl_4)-induced oxidative stress and necroinflammation [1,37]. However, the effects of LBPs on CCl_4-induced TLRs/NF-kB signaling pathway in wistar rats have not been reported until now.

Thus, the objectives of the present study are to evaluate the alleviating effects of LBPs on CCl_4-induced liver fibrosis and to explore the underlying TLRs/NF-kB signaling pathway mechanism.

2. Materials and methods

2.1. Materials

CCl_4 (analytical grade, CAS 56-23-5) was purchased from Jiangsu Qiangsheng Chemical Co. (Shanghai, China). Commercial kits for AST, ALT, ALP, SOD, GSH-Px, GSH and MDA were provided by the Institute of Jiancheng Biotechnology (Nanjing, China). Monoclonal antibody of α-SMA was purchased from Abcam (Cambridge, U.K.). Other reagents and chemicals were commercially available and of high quality.

2.2. Preparation of L. barbarum polysaccharides

L. barbarum fruits, validated by pharmacologists according to the Pharmacopoeia of the People's Republic of China, were purchased from Nanjing Zelang medical Technology Co. Ltd. (Nanjing, China). The voucher specimen was deposited at the Nanjing Agriculture University (Nanjing, China). LBPs were extracted and purified as previously described [19]. Briefly, *L. barbarum* fruits were dried at 60 °C. The dried fruits were ground to a fine powder and refluxed two times to remove lipids with chloroform: methanol solvent (2:1) and then refluxed with 80% ethanol solvent at 80 °C to remove oligosaccharides. After filtering, the residues were extracted four times in a 4-fold volume of hot water and then filtered. The combined filtrates were concentrated by a rotary evaporator at 60 °C, and then precipitated with 95% ethanol, 100% ethanol and acetone. The precipitates were collected and vacuum-dried to obtain LBPs extraction. LBPs extraction was mainly composed of glucose, arabinose, mannose, rhamnose, galactose and xylose and the purity of LBPs was > 90%.

2.3. Animals and feeding experiment

Fifty healthy male wistar rats, weighing 200 ± 20 g, were purchased from Experimental Animal Center of Yangzhou University. All animals were kept under similar conditions of climate, ventilation, temperature, humidity and 12 h light-dark cycle in rooms. All animals had free access to tap water. All the basal diets which were purchased from Qinglongshan animal breeding grounds (Nanjing, China) for the wistar rats were replenished daily and fresh water was accessible at all the time throughout the trial.

After a week acclimatization, rats were randomly segregated into five groups (n = 10): group a served as a control group and rats in group a received olive oil (2 mL/kg body weight) through gavage; rats in group b, c, d, e received olive oil with 40 v/v% CCl_4 at a dose of 2 mL/kg twice a week for 8 weeks through gavage; rats in group c, d, e at the same time received LBPs at three different doses of 400 mg/kg, 800 mg/kg and 1600 mg/kg, respectively. LBPs were administrated via gavage everyday since the first day of the experiment and should be given no < 4 h after giving CCl_4. Each rat was weighed at the beginning and the end of the experiment.

This study met the standards of the European Guidelines for Animal Welfare and the study protocol was approved by the Committee for the Care and Use of Experimental Animals at Agriculture University of Nanjing.

2.4. Sample collection and preparation

At the end of the experiment, blood samples of wistar rats in five different groups were collected for separating serum. Blood was collected in a syringe without EDTA and gently ejected into 2 mL Eppendorf tubes. Then, 2 mL of whole blood without EDTA was kept in a slanting position at 37 °C for 2 h and then at 4 °C for overnight, followed by centrifugation at 700*g* for 15 min. The resulting supernatant was the serum sample required, and stored at − 20 °C until analysis of AST, ALP and ALT levels.

Livers from all animals were collected, weighted and the liver index was calculated and expressed as liver weight/final body weight. Then, one-quarter of livers were immediately collected and preserved in 10% neutral formaldehyde buffer for histological examination and immunohistochemistry. Other liver tissues were rapidly excised and rinsed with ice-cold isotonic saline, then snap-frozen in liquid nitrogen and stored at − 70 °C until analysis of GSH-Px and SOD acitivities, GSH and MDA levels, andα-SMA, Col-I, TGF-β1, TNF-α, IL-1β, MCP-1, TLR2, TLR4, p65, and p-p65 expressions.

2.5. Determination of serum AST, ALT and ALP levels

The levels of serum AST, ALT and ALP were determined by automatic biochemical analyzer (Abaxis, Vetscan, USA) and expressed as U/L.

2.6. Histological examination

Liver samples were collected and fixed in 10% neutral buffered formalin for histopathological analysis. The samples were embedded in paraffin wax, and the blocks were sectioned at 5 μm. Hematoxylin-eosin (H&E, Jiancheng Biotechnology, China) and Sirius red staining (Sigma-Aldrich) were used for highlighting the liver damage and collagens depositions, respectively. Extents of fibrosis were analyzed by an image-analysis system with Image-Pro Plus version 6.0 (Media Cybernetics, MD, USA).

2.7. Immunohistochemistry

Liver tissues were fixed with 10% neutral buffered formalin and then embedded, cut into serial sections (5 μm), dehydrated, and incubated with 1% hydrogen peroxide. After rinsing in phosphate-buffered saline, sections were blocked with 5% BSA for 30 min at room temperature. Next, liver tissues were incubated at 4 °C for overnight with anti-α-SMA antibody. Slides were washed with PBS (pH = 7.4) for three times and then incubated with HRP-labeled secondary antibody at room temperature for 1 h. Slides were washed with PBS (pH = 7.4) for three times, and incubated with diaminobenzidine solution and then counterstained with hematoxylin. Stained slides were visualized by microscope camera and determined by using Image-Pro Plus image analysis software.

2.8. Determination of SOD activities, GSH-Px activities, GSH levels and MDA levels

The activities of SOD and GSH-Px and levels of GSH and MDA in liver were determined using the commercial kits in accordance with the