Abstract. Astragaloside IV (AS-IV) in improving liver cirrhosis injury in rats and its effect on the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mechanistic target of rapamycin (mTOR) signaling pathway were observed. Rat model of liver cirrhosis was induced by injection of carbon tetrachloride (CCL4). A total of 36 Sprague-Dawley (SD) rats were randomly divided into three groups: the normal control group (n=10), the model control group (n=13), and the AS-IV group (n=13). The normal control group was injected with olive oil and given carboxymethyl cellulose (CMC)-Na (10 ml/kg/day), the model control group was given CMC-Na (10 ml/kg/day), and the AS-IV group underwent intragastric administration of AS-IV (20 ml/kg/day). The content of alanine transaminase (ALT) and aspartate transaminase (AST) of rats was detected via enzyme-linked immunosorbent assay (ELISA). Hematoxylin and eosin (H&E) staining was applied to observe morphological changes in liver tissues. The expression of collagens in liver tissues was detected via Masson's trichrome staining. Additionally, the expression of proteins in liver tissues was detected via western blotting. Compared with those in the blank group, the levels of AST, ALT, TNF-α, IL-6, IL-1β were higher, the expression level of collagen in liver tissues was increased, and the expression ratios of phosphorylated (p)-PI3K/PI3K, p-Akt/Akt and p-mTOR/mTOR proteins were increased in the model group. Compared with the model group, AS-IV could significantly decrease the content of AST, ALT, TNF-α, IL-6 and IL-1β in serum of rats, obviously inhibit the expression of collagens in liver tissues and decrease the expression ratios of p-PI3K/PI3K, p-Akt/Akt and p-mTOR/mTOR proteins in liver tissues. AS-IV can inhibit the inflammatory response so as to reduce the expression of collagens, and its mechanism may play a key role by inhibiting the PI3K/Akt/mTOR signaling pathway.

Introduction

Liver cirrhosis is a common chronic liver disease in China, which is characterized by diffuse fibrosis as well as pseudo-lobular and nodular formation. According to statistics, patients with liver cirrhosis account for ~10% of the total number of patients in China (1). A study has revealed that the pathogenic factors of liver cirrhosis include hepatitis viruses, drugs and poisons, cholestasis, metabolism and inheritance, schistosomiasis and alcohol. These pathogenic factors stimulate liver cells for a long time, induce the activation of hepatic stellate cells, and make the generated extracellular matrices more than degraded ones, thus resulting in the extracellular matrix deposition and liver tissue remodeling (2).

The phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mechanistic target of rapamycin (mTOR) signal transduction pathway can promote cell growth, proliferation, differentiation and invasion, and it also plays key roles in anti-apoptosis, immune regulation and pro-angiogenesis. In addition, a study has evidenced that this pathway exerts crucial effects on the occurrence and development of liver fibrosis (3). Inflammation is a key factor triggering liver fibrosis and stimulating the progression of liver cirrhosis (4). PI3K and mTOR can upregulate anti-inflammatory cytokines, inhibit the expression of pro-inflammatory cytokines, the inflammatory response of liver cells, and the proliferation and invasion of hepatic stellate cells, and promote the degradation of the liver extracellular matrix (5).

A study has demonstrated that astragaloside IV (AS-IV), one of the active components of Astragalus saponins in Astragalus membranaceus, has various pharmacological effects such as antioxidation, anti-inflammation and anti-infection (6). AS-IV can alleviate the inflammatory damage induced by orthotopic liver transplantation in rats through inhibiting the transcriptional activity of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) (7). According to another study,
AS-IV pretreatment can reduce cerebral ischemia-reperfusion injury in rats, which is mainly due to its ability to inhibit neutrophil adhesion-associated molecules, thereby inhibiting inflammation (8). Recent studies have manifested that AS-IV pretreatment can protect kidney injury, and its mechanisms include reducing inflammation and inhibiting apoptosis (9). In the present study, a rat model of liver cirrhosis was established to investigate whether AS-IV pretreatment can protect rats from liver cirrhosis and the effect of AS-IV on the PI3K/Akt/mTOR signaling pathway, providing basic research on the prevention and treatment of liver cirrhosis using AS-IV.

Materials and methods

Materials and reagents. AS-IV (Aladdin Reagent Co., Ltd., Shanghai, China), alanine transaminase (ALT) and aspartate transaminase (AST) detection kits, as well as interleukin (IL)-6, IL-1β and tumor necrosis factor-α (TNF-α) (cat. nos. H052, H007, H002; all from Nanjing Jincheng Bioengineering Institute, Nanjing, China), rabbit anti-rat phosphorylated (p)-PI3K, PI3K, p-Akt, Akt, p-mTOR, mTOR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) polyclonal antibodies, secondary goat anti-rabbit polyclonal antibody (cat. nos. bs-6417R, bs-10657R, bs-3043R, bs-0115R, bs-5329R, bs-1992R, bs-0755R, bs-0295G; all from Bioss, Beijing, China) and Masson's staining solution (Wuhan Sanying Biotechnology, Wuhan, China), and hematoxylin and eosin (H&E) staining kit, bicinchoninic acid (BCA) protein quantitation kit and tissue lysate (all from Beyotime Institute of Biotechnology, Nantong, China).

Experimental animal grouping and treatment. A Sprague-Dawley (SD) male rat model (4 months of age, 210-240 g; Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) of liver cirrhosis was induced by the intra-peritoneal injection of 50% carbon tetrachloride (CCl₄) (the volume ratio of CCl₄ to olive oil was 1:1) twice a week, and given intragastric administration of 0.5% carboxymethyl cellulose (CMC)-Na (10 ml/kg/day); and the model control group was intraperitoneally injected with olive oil (210-240 g; Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) and Masson's staining solution (Wuhan Sanying Biotechnology, Wuhan, China), and hematoxylin and eosin staining (H&E) staining kit, bicinchoninic acid (BCA) protein quantitation kit and tissue lysate (all from Beyotime Institute of Biotechnology, Nantong, China).

Detection of the liver histopathology of rats. The fixed liver tissues in each group were selected. These tissues underwent dehydration and transparency at first, immersed in wax and embedded into wax blocks next, and cut into blank slices finally. After that, H&E staining and Masson’s trichrome staining were carried out based on the histopathological conventional methods. After the stained slices were dehydrated with gradient alcohol and made transparent via xylene, they were sealed with gum. Then the histopathological changes in the liver of rats in each group were observed using a microscope (Olympus Corp., Tokyo, Japan).

Statistical analysis. Statistical Product and Service Solutions (SPSS) 17.0 software (IBM Corp., Armonk, NY, USA) was applied for data processing, and the data were expressed as mean ± standard deviation. The t-test was implemented for the intergroup comparison. ANOVA was used for comparison between multiple groups and LSD test was the post hoc test. P<0.05 was considered to indicate a statistically significant difference.
Results

Effects of AS-Ⅳ on AST and ALT in serum of rats. Compared with those in the normal control group, the levels of AST and ALT in serum of rats in the model group were significantly increased (p<0.01). Compared with those in the model group, the levels of AST and ALT in serum of rats in the AS-Ⅳ administration group were remarkably decreased (p<0.01), indicating that the degree of rat liver injury in the AS-Ⅳ administration group was lowered than that in the model group (Fig. 1).

Effects of AS-Ⅳ on inflammatory cytokines in serum of rats. Compared with those in the normal control group, the content of TNF-α, IL-6 and IL-1β in serum of rats in the model group was obviously increased (p<0.01). Compared with those in the model group, the expression levels of TNF-α, IL-6 and IL-1β significantly declined in serum of rats in the AS-Ⅳ group (p<0.01), indicating that the inflammatory response of rats in the AS-Ⅳ group was reduced compared with that of rats in the model group (Fig. 2).

Effect of AS-Ⅳ on the liver histopathology of rats. There were intact liver lobule structures and central veins in the liver of rats in the normal control group. Destructed liver structures and inflammatory cell infiltration appeared in the liver of rats in the model group, while the above symptoms of rat liver tissues in the AS-Ⅳ treatment group were remarkably improved (Fig. 3).

Effect of AS-Ⅳ on the expression of rat collagens. Compared with the blank control group, obvious collagen hyperplasia was observed in liver tissues of the model group, and the fiber rope formed was relatively thick; whereas collagen deposition in liver tissues in the AS-Ⅳ treatment group was significantly reduced compared with that of the model group, and the fiber rope formed was relatively thin (Fig. 4).

Effect of AS-Ⅳ on the expression of proteins relevant to the PI3K/Akt/mTOR signaling pathway in liver tissues of rats. Compared with those in the control group, the expression levels of p-PI3K/PI3K, p-Akt/Akt, and p-mTOR/mTOR proteins...
in liver tissues of the model group significantly increased (p<0.01). Compared with the model group, AS-Ⅳ obviously reduced the expression of p-PI3K/PI3K, p-Akt/Akt, and p-mTOR/mTOR proteins in rat liver tissues (p<0.01) (Fig. 5).

**Discussion**

Liver cirrhosis results from many chronic liver diseases developing to the late stage. The long-term stimulation of damage factors leads to diffuse necrosis of liver cells, and promotes the proliferation of fibrous tissues and the regeneration of nodular hepatocytes, thus ultimately contributing to the remodeling of liver lobule structures and blood vessels (10). A large number of basic and clinical studies have manifested that treatments can reverse the progression of liver fibrosis (11,12). A great controversy currently exists in the treatment of liver cirrhosis, and some studies have revealed that early treatment can reverse liver cirrhosis (13-16).

PI3K phosphorylates phosphoinositides via the phosphorylation of serine 308 and threonine 473 by phosphoinositide-dependent kinase-1 (17). PI3 on the inositol ring generates phosphatidylinositol 4,5-bisphosphate (PIP$_2$) and phosphatidylinositol (3,4,5)-trisphosphate (PIP$_3$), and then acts as the second messenger in cells. Akt acts as a serine/threonine protein kinase, and can bind to PIP$_2$ and PIP$_3$ under the action of the phosphatidylinositol-dependent protein kinase, which promotes the transfer of Akt from the cytoplasm to the cell membrane (18). According to a study, platelet-derived growth factors can induce the phosphorylation of PI3K and Akt. Activated Akt can promote the phosphorylation of downstream substrates such as mTOR, Bad and the caspase family, having a variety of biological effects (19). Inhibiting the PI3K/Akt signaling pathway can obviously reduce the proliferation of hepatic stellate cells and the expression levels of type I collagen messenger ribonucleic acid (mRNA) and protein (20).

mTOR/p70S6K protein plays a crucial role in the activation process of hepatic stellate cells (21). Inhibiting the activation of mTOR/p70S6K protein, a downstream target of the PI3K/Akt signaling pathway, can inhibit the proliferation and activation of hepatic stellate cells as well as the synthesis and secretion of collagens (22). This study showed that low-dose rapamycin can suppress the progression of liver fibrosis, whose mechanism is to inhibit the activation of mTOR/p70S6K so as to suppress the activation of hepatic fibroblasts (17). Another study has revealed that the mTOR inhibitors, sirolimus and everolimus, can significantly slow the development of liver fibrosis, and inhibit the migration of hepatic stellate cells and the production of collagens (23).

In the present study, the rat model of liver cirrhosis was established through the intraperitoneal injection of CCl$_4$, and then the protective role of AS-IV intervention in the liver of rats and its effect on the PI3K/Akt/mTOR signaling pathway were investigated. Compared with those in the normal control group, the levels of AST, ALT, TNF-α, IL-6 and IL-1β in serum of rats in the model group were obviously increased. Compared with those in the model group, the levels of TNF-α, IL-6 and IL-1β significantly declined in serum of rats in the AS-IV group, indicating that the liver injury degree and inflammatory response of rats in the AS-IV group were lower than those in the model group. The histopathological detection revealed that there were intact liver lobule structures and central veins in the liver of rats in the normal control group. Destructed liver...
structures, inflammatory cell infiltration, and the significant increase in collagen expression appeared in the liver of rats in the model group, while the above symptoms of rat liver tissues in the AS-IV treatment group were remarkably improved, and collagen deposition in liver tissues in the AS-IV treatment group was significantly reduced compared with that in the model group. Based on western blotting results, compared with those in the control group, the expression levels of p-PI3K/PI3K, p-AKT/Akt, and p-mTOR/mTOR proteins in rat liver tissues in the model group increased significantly. AS-IV obviously reduced the expression ratios of p-PI3K/PI3K, p-AKT/Akt, and p-mTOR/mTOR proteins in rat liver tissues, thus inhibiting the PI3K/Akt/mTOR signaling pathway.

In summary, AS-IV protects CCL2-induced liver cirrhosis, and its mechanism may play a role in inhibiting the PI3K/Akt/mTOR signaling pathway. This study provides a clear research basis for the treatment of liver cirrhosis with AS-IV in clinic.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RW and HL wrote the manuscript and conducted the experimental animal grouping and treatment. RC analyzed the biochemical indexes. YS and TL contributed to western blotting. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Sixth People's Hospital of Qingdao (Qingdao, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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