

Quercetin prevents alcohol-induced liver injury through targeting of PI3K/Akt/nuclear factor- κ B and STAT3 signaling pathway

MINGLIN ZHU, XUEFENG ZHOU and JINPING ZHAO

Department of Thoracic and Cardiovascular Surgery, Zhongnan Hospital of Wuhan University,
Wuhan, Hubei 430071, P.R. China

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Abstract. Quercetin is a type of flavonoid compound, which has potent antioxidant and anti-inflammatory activities, capable of treating a variety of diseases including neurodegenerative diseases, tumors, diabetes and obesity. The present study selected alcohol-induced liver injury model mice and aimed at studying the protective role of quercetin in preventing alcohol-induced liver injury. In alcohol-induced liver injury mice treated with quercetin, it was demonstrated that levels of aspartate transaminase, alanine transaminase, total bilirubin and triglyceride were reduced. In addition to this, the activities of the antioxidant enzymes superoxide dismutase and glutathione peroxidase were increased, malondialdehyde was inhibited, and interleukin (IL)-1 β , IL-6, IL-10 and inducible nitric oxide synthase were suppressed. Quercetin additionally suppressed the protein expression levels of B-cell lymphoma (Bcl)-2, Bcl-2 associated X apoptosis regulator, Caspase-3, poly ADP-ribose polymerase, and signal transducer and activator of transcription (STAT) 3 phosphorylation, nuclear factor (NF)- κ B and protein kinase B (Akt) phosphorylation levels in alcohol-induced liver injured mice. These results suggested that the protective role of quercetin prevents alcohol-induced liver injury through the phosphoinositide 3-kinase/Akt/NF- κ B and STAT3 pathway.

Introduction

Alcohol abuse is a worldwide problem, and about 3.2% of deaths each year in the world are associated with alcohol, causing great medical burdens. Alcoholic liver disease (ALD) is the most important clinical manifestation of alcohol abuse, of which the spectrum includes alcoholic fatty liver, alcoholic hepatitis and alcoholic cirrhosis (1). Alcoholic fatty

liver is present in almost all the heavy drinkers, with the pathological changes reversible, and the patients can recover after the alcohol temperance of a few weeks (2). 15-20% of patients with alcoholic fatty liver disease will develop into alcoholic hepatitis, and the course is usually progressive; even if complete temperance is adopted, only 27% patients show pathological self-healing, and about 20-50% alcoholic hepatitis patients will be further developed into cirrhosis (3).

After the activated STAT enters into the nucleus, it activates the expressions of various genes including the abnormal genes, which can effectively reduce liver inflammation and cell damage in alcohol liver (4). While after the knockout of STAT3 gene, liver regeneration capacity is weakened, and insulin resistance is increased (5). Interestingly, the selective knockdown of STAT3 in murine hepatocyte may aggravate the degree of ALD, whereas the selective knockdown of STAT3 from endothelial cells in mice can significantly reduce endothelial and hepatic damage (6). The studies in the effects of STAT3 on different cells show that STAT3 plays a role of proinflammatory cytokine in hepatocytes, which may be related to the release of various cytokines and regulatory proteins in stimulated hepatocytes; STAT3 in macrophages and neutrophils from bone marrow has the anti-inflammatory effect, and plays a leading role in ALD (5,7).

Akt gene is in the core of PI3K/Akt signaling pathway, and Akt is involved in cell growth and proliferation. It is both a signal of cell survival and an essential factor for cell survival. The continuous existence of Akt can avoid cell damage (8). Akt signal is activated under the stimulation of hypoxia or cytokine (9). The main targets of Akt signal are the apoptotic family members, Bax/Bcl-2 and caspases. In addition, Akt can also regulate intracellular glucose metabolism, in order to make the cells adapted to environmental changes, and enhance cell viability (9).

The pathogenic factor of ALD is long-term large-quantity alcohol intake. But its pathogenesis is not entirely clear (10). It has been confirmed that long-term alcohol intake can activate NF- κ B in liver tissue, and the activated NF- κ B enters into the nucleus, bond to the κ B sequence in promoter regions of various inflammatory factors, to promote the transcriptions of inflammatory cytokines (TNF-1, IL-2, IL-6, etc.), adhesion molecules and NO synthase, leading to further inflammation, necrosis, apoptosis and fibrosis of hepatocytes (11). In addition, the metabolism of alcohol in the liver

Correspondence to: Dr Jinping Zhao, Department of Thoracic and Cardiovascular Surgery, Zhongnan Hospital of Wuhan University, 169 Donghu Road, Wuhan, Hubei 430071, P.R. China
E-mail: whuwill@gmail.com

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results in a large number of free radicals, which inhibits fatty acid oxidation, accumulates intrahepatic fat when beyond the body's clearing ability, and also leads to lipid peroxidation of liver cell membrane, resulting in liver cell damage (12). At the same time, these reactive oxygen species will increase the level of endotoxin, and lead to the release of inflammatory mediators, which can also make NF- κ B activated, thus generating a large number of inflammatory mediators, to have an inflammatory cascade amplification effect, aggravating liver damage (7).

Quercetin (Fig. 1) is a kind of flavonoid antioxidant substance, present in a variety of plants such as apples, green tea, onions, grape skins and so on. The red wine also contains a lot, and it is not easily soluble in water (13). It is regarded to have protective effect on cardiovascular and respiratory systems. Moreover, it has been shown that quercetin can reduce carcinogens and inhibit the growth of cancer cells (14). It is found that the quercetin flavors extracted from French red wine can effectively inhibit the proliferation of prostate cancer cells and only a small dose is enough, which is equivalent to the amount contained in two cups of red wine every day (15). Quercetin has significant antioxidant and anti-inflammatory activities, so as to be used to treat a variety of diseases (16). Therefore, these fruits were selected to evaluate the protective role of quercetin prevents alcohol-induced liver injury.

Materials and methods

Animal and grouping. Eight-week-old male C57 mice (n=30) were obtained from Anhui Medical University. The animal protocol was approved by the Institutional Animal Care and Use Committee of Anhui Medical University. Mice were maintained in a room with a controlled temperature of $22\pm 0.5^{\circ}\text{C}$, 50-60% relative humidity and were allowed free access to a basal pellet diet and tap water. 30 mice were randomly divided into three groups (very group=10): Control group, model group and quercetin group. Mice of model group and quercetin group were irrigated with 35% v/v ethanol (3 g/kg) for 1 week, 40% v/v ethanol (4 g/kg) for 1 week, and 52% v/v ethanol (5 g/kg) for 1 week. Meanwhile, we used staining for alcohol-induced liver injury model. Meanwhile, mice of quercetin group were irrigated with 60 mg/kg of quercetin for 3 weeks. Then, the animals were anesthetized, and liver tissues and blood samples were collected.

Liver oil red O staining. After anesthesia, liver tissue was acquired, washed with PBS and fixed in 10% formalin for 24 h. Then, tissue was embedded in paraffin, cut into $0.4\ \mu\text{M}$ and tained with Oil Red O (17).

Measurement of hepatic injury in the serum. The blood samples were centrifuged at $2,000 \times g$ for 10 min to separate the serums. ELISA assays to determine AST, ALT, TBIL, TG, SOD, GSH-Px, MDA, IL- 1β , IL-6, IL-10, iNOS and caspase-3 contents.

Western blot analysis. Liver tissues samples were collected and homogenized and treated with RIPA lysis buffer (Dingguo, China) and protease inhibitor (1:100, Dingguo, China).

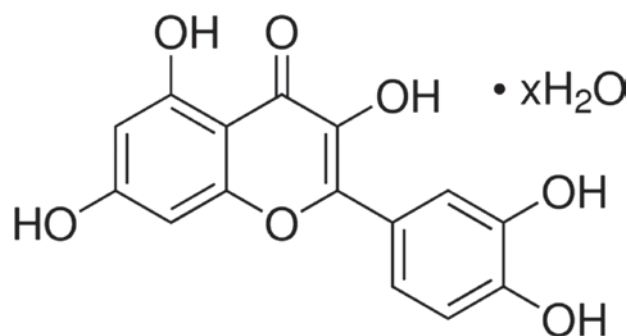


Figure 1. The chemical structure of berberine hydrochloride.

Proteins contents were determined using BCA kit (Dingguo, China). 25-50 μg protein were electrophoresed on 6-10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred onto PVDF membranes ($0.45\ \mu\text{m}$, Millipore, BioRad). Membranes were blocked with 5% BSA in TBST for 1 h at 37°C and incubated with iNOS, Bax/Bcl-2, PARP, p-STAT3, NF- κ B, p-Akt and GAPDH overnight at 4°C . Membranes washed with TBST for 3 times and ncubated for 2 h at room temperature with secondary HRP-conjugated sheep anti-rabbit antibody. Immunoreactivity was detected by enhanced chemiluminescence (ECL kit, Millipore, USA) and measured using the Bio-Rad Laboratories Quantity One software.

Statistical analysis. The data were presented as mean \pm standard deviation. Differences among multiple groups were analyzed by D'Agostino's K-squared test followed by Tukey's test. $P < 0.05$ was considered to be statistically significant.

Results

The effect of quercetin on liver injure. As showed in Fig. 2, AST, ALT, TBIL and TG content of ALD model group were higher than those of control group. Quercetin significantly reduced AST, ALT, TBIL and TG content in ALD mice, compared with ALD model group (Fig. 2). Meanwhile, we used red oil assay to stained liver tissue and found that liver injure were significantly increased in ALD model group, compared with control group (Fig. 3). However, treatment with quercetin significantly inhibited liver injure in ALD mice, compared with ALD model group (Fig. 3).

The effect of quercetin on SOD, GSH-Px and MDA contents. To investigate the antioxidant effects of quercetin in alcohol-induced liver injury mice, SOD, GSH-Px and MDA contents were measured using ELISA Kits. The SOD and GSH-Px contents were reduced and MDA content was increased in ALD model group, compared with control group (Fig. 4). Quercetin significantly increased SOD and GSH-Px contents and inhibited MDA content in ALD mice, compared with ALD model group (Fig. 4). These data indicate that quercetin might inhibit oxidative stress of ALD mice.

The effect of quercetin on IL- 1β , IL-6, IL-10 contents. To investigate the anti-inflammation effects of quercetin in

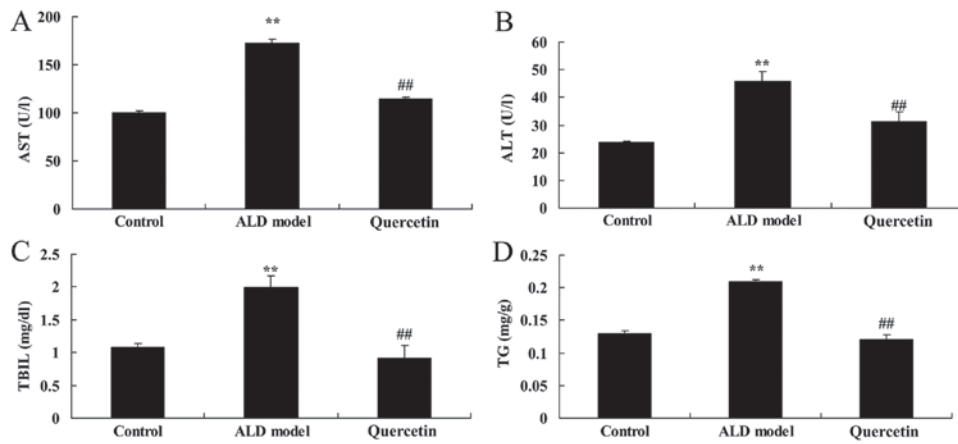


Figure 2. The effect of quercetin on AST, ALT, TBIL and TG levels. The effect of quercetin on AST (A), ALT (B), TBIL (C) and TG (D) levels. Control, control group; ALD, ALD model group; Quercetin, quercetin group. **P<0.01 compared with control group; ##P<0.01 compared with ALD model group.

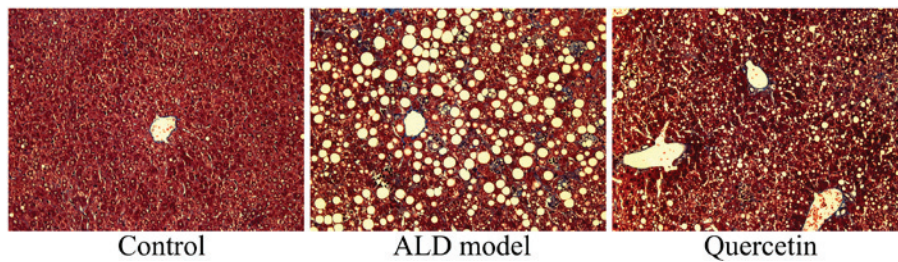


Figure 3. The effect of quercetin on liver injure stained with red oil assay. Control, control group; ALD, ALD model group; Quercetin, quercetin group.

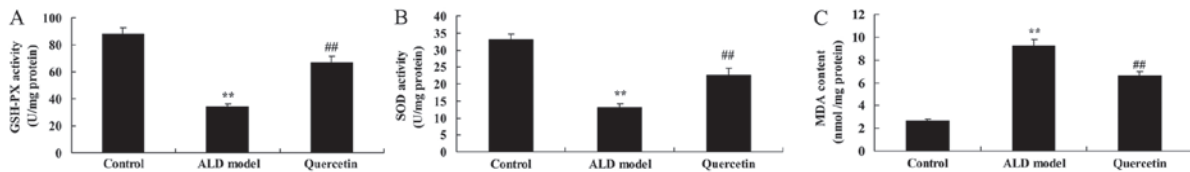


Figure 4. The effect of quercetin on SOD, GSH-Px and MDA levels. The effect of quercetin on GSH-Px (A), SOD (B), and MDA (C) levels. Control, control group; ALD, ALD model group; Quercetin, quercetin group. **P<0.01 compared with control group; ##P<0.01 compared with ALD model group.

alcohol-induced liver injury mice, IL-1 β , IL-6, IL-10 contents were measured using ELISA Kits. The levels of IL-1 β and IL-6 were promoted and IL-10 level was decreased in ALD model group, compared with control group (Fig. 5). Quercetin significantly also reduced IL-1 β and IL-6 levels and increased IL-10 level in ALD mice, compared with ALD model group (Fig. 5). Our study showed that quercetin reduced inflammation of ALD, and it mechanism need to measure.

The effect of quercetin on iNOS contents and protein expression. Nevertheless, we also found that iNOS contents and protein expression of ALD model group were higher than that of control group (Fig. 6). Treatment with quercetin significantly increased iNOS contents and protein expression in ALD mice, compared with ALD model group (Fig. 6), suggesting that quercetin inhibited iNOS contents and protein expression to prevent ALD.

The effect of quercetin on caspase-3 contents and Bax/Bcl-2 protein expression. In order to explore the anti-apoptosis of

quercetin in alcohol-induced liver injury mice, caspase-3 contents and Bax/Bcl-2 protein expression were measured in ALD mice. As showed in Fig. 7, caspase-3 contents and Bax/Bcl-2 protein expression were promoted in ALD model group, compared with control group. Treatment with quercetin significantly suppressed caspase-3 contents and Bax/Bcl-2 protein expression in ALD mice, compared with ALD model group (Fig. 7). The results indicate that quercetin possess anti-apoptosis effects in treatment of ALD.

The effect of quercetin on p-STAT3, NF- κ B, PARP and p-Akt protein expression. In order to determine the potential mechanism of magnesium isoglycyrrhizinat in alcohol-induced liver injury mice, p-STAT3, NF- κ B, PARP and p-Akt protein expression was measured using Western blot analysis. As showed in Fig. 8, p-STAT3, NF- κ B, PARP and p-Akt protein expression was induced in ALD model group, compared with control group. p-STAT3, NF- κ B, PARP and p-Akt protein expression was significantly suppressed by magnesium isoglycyrrhizinat in ALD mice, compared with

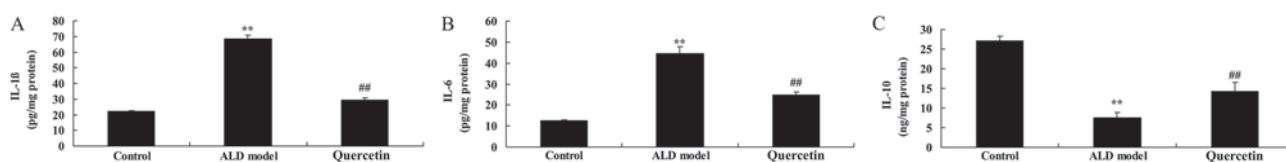


Figure 5. The effect of quercetin on IL-1 β , IL-6, IL-10 levels. The effect of quercetin on IL-1 β (A), IL-6 (B) and IL-10 (C) levels. Control, control group; ALD, ALD model group; Quercetin, quercetin group. ** $P < 0.01$ compared with control group; ## $P < 0.01$ compared with ALD model group.

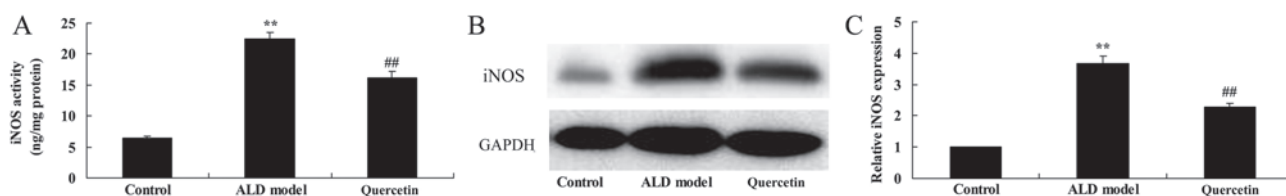


Figure 6. The effect of quercetin on iNOS content and protein expression. The effect of quercetin on iNOS content (A) and protein expression (B) by western blotting assays and statistical analysis of iNOS protein expression. (C) Control, control group; ALD, ALD model group; Quercetin, quercetin group; western blot individual samples. ** $P < 0.01$ compared with control group; ## $P < 0.01$ compared with ALD model group.

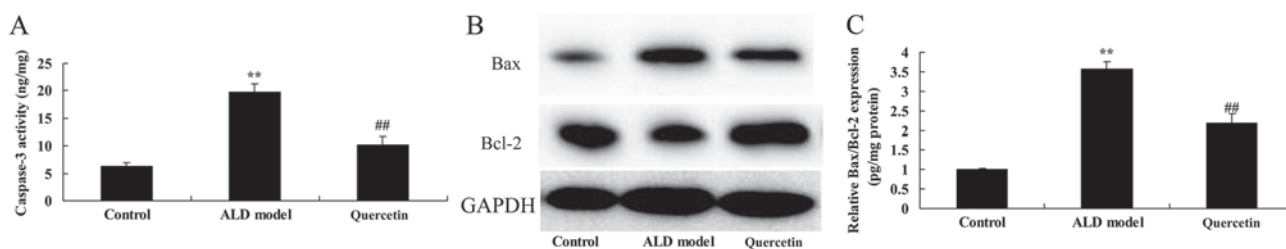


Figure 7. The effect of quercetin on caspase-3 content and Bax/Bcl-2 protein expression. The effect of quercetin on caspase-3 content (A) and Bax/Bcl-2 protein expression by western blotting assays (B) and statistical analysis of Bax/Bcl-2 protein expression. (C) Control, control group; ALD, ALD model group; Quercetin, quercetin group; western blot individual samples. ** $P < 0.01$ compared with control group; ## $P < 0.01$ compared with ALD model group.

ALD model group (Fig. 8). Ours showed that quercetin inhibited inflammation and apoptosis through STAT3/NF- κ B and PARP/Akt protein expression in treatment of ALD.

Discussion

About 90% individuals in those who have been drinking over a long period of time develop into alcoholic fatty liver disease, and only a few will develop into alcoholic hepatitis or alcoholic cirrhosis (18). The current accepted interpretation of this phenomenon is the 'second strike theory'. A large number of alcohol intake induces the formation of alcoholic fatty liver as a 'first strike', and the alcoholic liver injury requires a second strike, including the change in nutrition, the effect of drug poisons, viral infections and genetic factors (19). The result showed that Quercetin significantly reduced AST, ALT, TBIL and TG content in ALD mice.

In the past, the pathogenesis of ALD has been extensively studied. The main pathogenetic causes of ALD are oxidative damage, inflammatory injury, mitochondrial damage, apoptosis damage, metabolic damage and aldehyde toxicity (20). The literature shows that the liver cells generate large amounts of reactive oxygen in the process of metabolizing alcohol through alcohol metabolism and mitochondrial pathway (21). When beyond the scope of the antioxidant defense system,

the oxidative stress occurs (21). Alcohol metabolism can also generate a large number of toxic products, acetaldehyde, and then mediate toxicity (21). Inflammatory reactions are also involved in the pathogenesis of alcoholic liver injury, and inflammatory factors can further promote oxidative stress injury (22). Excessive alcohol exposure leads to oxidative stress, acetaldehyde toxicity and inflammation, which can lead to the damage of important cellular organelle structure or function, such as endoplasmic reticulum stress and mitochondrial dysfunction, so that the liver cell function shows disorder or even induces apoptosis or necrosis of liver cell, ultimately affecting liver function and leading to ALD (20). The present study demonstrated that quercetin significantly increased SOD and GSH-Px contents and inhibited MDA content, and reduced IL-1 β and IL-6 levels and increased IL-10 level in ALD mice. Xue *et al* revealed that quercetin inhibits LPS-induced inflammation in atherosclerosis (16).

Abnormality of cytokine metabolism is another feature of ALD, especially TNF- α . Studies have shown that alcohol perfusion can cause a significant increase in serum LPS level, which is bond to the LPS CD14/toll-like receptor 4 complex on the surface of Kupffer cells to activate NF- κ B and TNF- α expression (23,24). The level of TNF- α in patients with ALD is significantly increased and the severity of ALD is positively correlated with the severity of ALD (24). Several research

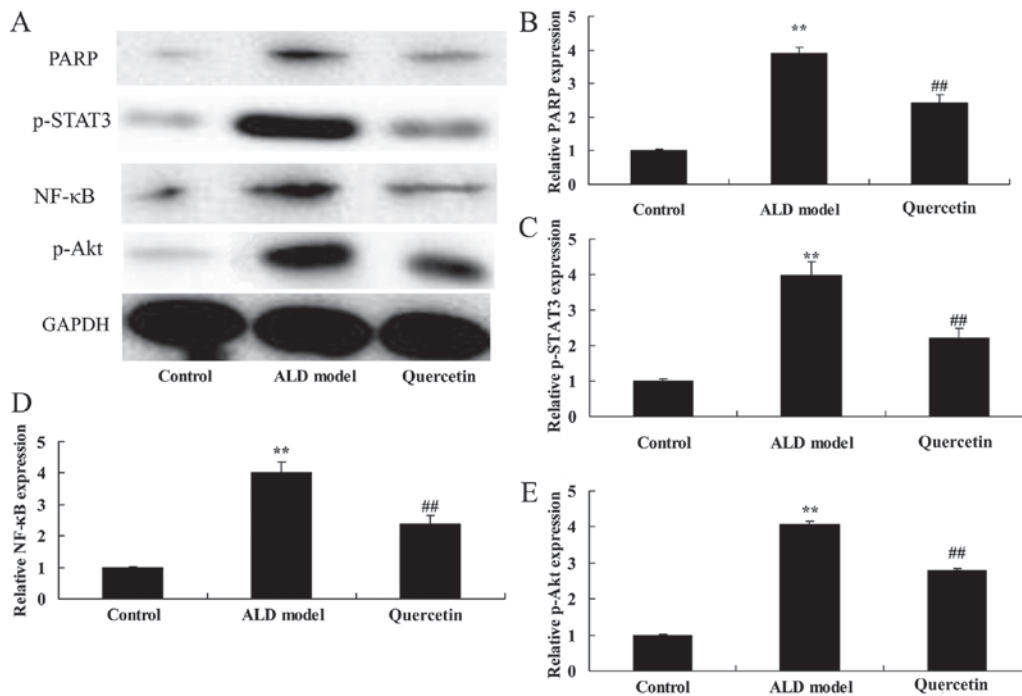


Figure 8. The effect of quercetin on p-STAT3, NF-κB, PARP and p-Akt protein expression. The effect of quercetin on p-STAT3, NF-κB, PARP and p-Akt protein expression by western blotting assays (A) and statistical analysis of p-STAT3, NF-κB, PARP and p-Akt protein expression. (B-E) Control, control group; ALD, ALD model group; Quercetin, quercetin group; western blot individual samples. **P<0.01 compared with control group; ##P<0.01 compared with ALD model group.

groups have demonstrated that TNF-α antibody therapy can prevent alcohol-induced liver injury in rats.

Clinical experimental researches suggest that apoptosis of hepatocytes exists in ALD, and studies show the inhibition of hepatocyte apoptosis can relieve alcohol-induced liver injury in experimental animals (25). Pathologic studies show oxidative stress and hepatocyte apoptosis are involved in ALD cells apoptosis. The apoptosis of a large number of hepatocytes is a major feature of fulminant hepatitis and the inhibition of excessive hepatocyte apoptosis can be used as a means of treating fulminant hepatic failure (26). Previous studies have shown that the anti-apoptotic effect is realized by the activation of STAT3 signaling pathways to induce anti-apoptotic protein expression (26,27). In the present study, quercetin significantly suppressed p-STAT3 protein expression in ALD mice. Xue *et al* revealed that quercetin inhibits LPS-induced inflammation in atherosclerosis through suppression of STAT3 protein expression (16).

TNF-α and other inflammatory factors cause liver injury, and IL-6, IL-10 and others can protect the liver through inflammatory response (25). IL-6 up-regulates the expression of multiple anti-injury genes in liver cells by activating STAT-3 pathway; IL-10 inhibits hepatic inflammatory response by activating STAT3 in Kupffer cells (25). The imbalance and re-balance process of proinflammatory and anti-inflammatory factors is the development and regression of ALD (28).

Interestingly, although the inflammation is more obvious after the knockout of IL-10 gene from mice liver, but liver cell damage and steatosis are decreased, and the IL-6/STAT3 expression in liver is increased. This phenomenon may be related to liver cell damage and weakened steatosis (23).

Some studies have shown that IL-10 can reduce the activation of NF-κB after stimulation. IL-10 inhibits the binding of NF-κB with DNA activity from the cytoplasm to the nucleus by inhibiting the activity of nuclear factor (IKK), inhibits the NF-κB activity, and reduces the release of TNFα, thereby reducing the inflammatory response (7). Thus, the data suggest that quercetin significantly suppressed NF-κB in ALD mice.

Apoptosis refers to the cell death under a certain physiological or pathological condition, with special morphological and biochemical characteristics, which is regulated by gene (22). Physiological apoptosis is the normal life phenomenon, by which the body or cell adapts to the environment or growth and differentiation, but pathological apoptosis may cause function damage of the corresponding tissue and structure, which may lead to the failure of relevant organs (29).

Sperm exposure also leads to abnormal expression of apoptosis-related genes, such as the Bcl-2 protein family. Bax/Bcl-2 ratio can also reflect the degree of apoptosis to some extent (30). Bcl-2 protein can also promote the synthesis of GSH and inhibit the consumption of mitochondria GSH, to play an antioxidant role, in order to maintain mitochondrial redox homeostasis. It is also found that the lower expression level of Bcl-2 means the higher level of lipid peroxidation (30). Caspase-3 is an important executive molecule in the process of apoptosis (9). The activity of Caspase-3 is often indicative of the degree of apoptosis in tissues or cells. Caspase-3 activity is increased during ALD. This result suggests that quercetin significantly suppressed caspase-3 contents and Bax/Bcl-2 protein expression in ALD mice. Du *et al* indicate that quercetin attenuates neuronal apoptosis via caspase-3 and Bax levels (13).

The PI3K/Akt pathway is an important component of the insulin signaling pathway, which is also a classic cell survival signaling pathway and glucose metabolism regulation pathway that activates NF- κ B and increases the expression of anti-apoptotic proteins to improve cell survival (9). In addition, it is also reported that the activation of Akt can also inhibit the activity of Caspase-3 (9). It is also shown from literature that the activation of Akt inhibits the binding of Bcl-xL to Bcl-2 and finally inhibits apoptosis (8). In the current study, we found that quercetin significantly inhibited p-Akt protein expression in ALD mice. Lu *et al* showed that quercetin attenuates high fructose feeding-induced atherosclerosis through inhibition of PI3K/AKT signaling pathway (31). However, in this study, we not used PI3K/Akt/NF- κ B or STAT3 signaling pathway inhibitor to verify the function of PI3K/Akt/NF- κ B or STAT3 signaling pathway in effects of quercetin on alcohol-induced liver injury, which are limitations of the study and these need to execute in further study.

In summary, our study showed that quercetin prevents alcohol-induced liver injury through its antioxidant, anti-inflammation and anti-apoptosis effects by STAT3, Akt and NF- κ B pathway. These results suggest that quercetin is a promising therapeutic target for treating alcohol-induced liver injury in further clinical.

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