

Inhibitory effect of liposomal quercetin on acute hepatitis and hepatic fibrosis induced by concanavalin A

Y. Wan, M.H. Tang, X.C. Chen, L.J. Chen, Y.Q. Wei and Y.S. Wang

State Key Laboratory of Biotherapy, West China Hospital, West China Medical School, Sichuan University, Chengdu, China

Abstract

Immune response plays an important role in the development of hepatic fibrosis. In the present study, we investigated the effects of quercetin on hepatitis and hepatic fibrosis induced by immunological mechanism. In the acute hepatitis model, quercetin (2.5 mg/kg) was injected *iv* into mice 30 min after concanavalin A (Con A) challenge. Mice were sacrificed 4 or 24 h after Con A injection, and aminotransferase tests and histopathological sections were performed. Treatment with quercetin significantly decreased the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Consistent with this observation, treatment with quercetin markedly attenuated the pathologic changes in the liver. A hepatic fibrosis model was also generated in mice by Con A challenge once a week for 6 consecutive weeks. Mice in the experimental group were treated with daily *iv* injections of quercetin (0.5 mg/kg). Histopathological analyses revealed that treatment with quercetin markedly decreased collagen deposition, pseudolobuli development, and hepatic stellate cells activation. We also examined the effects of quercetin on the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and transforming growth factor beta (TGF- β) pathways by immunohistochemistry and real-time reverse transcriptase-polymerase chain reaction (RT-PCR). NF- κ B and TGF- β production was decreased after treatment with quercetin, indicating that the antifibrotic effect of quercetin is associated with its ability to modulate NF- κ B and TGF- β production. These results suggest that quercetin may be an effective therapeutic strategy in the treatment of patients with liver damage and fibrosis.

Key words: Quercetin; Concanavalin A; Acute hepatitis; Hepatic fibrosis; Liposome

Introduction

Hepatic fibrosis is a common response to liver injury caused by various etiologies (viral, toxic, metabolic, and autoimmune) that ultimately lead to cirrhosis and related complications (such as portal hypertension, liver failure, and hepatocellular carcinoma) characterized by increased deposition of extracellular matrix (ECM) (1). Liver fibrosis is a major health problem worldwide. It is estimated that up to 1% of the populations might have histological cirrhosis (2). To date, there are however few therapeutic strategies for the treatment of patients with liver fibrosis and liver cirrhosis (3).

It has been shown that the immune response plays an important role in the development of hepatic fibrosis (1,3). Interaction among hepatic stellate cells (HSCs), macrophages, and CD4 T cells (Th2) occurs within a micro-environment of growth factors and cytokines, which activate HSCs, possibly leading to the production of ECM and subsequently to liver fibrosis (4). Moreover, it has been reported that repeated flares or continuous

recruitment of inflammatory cells to the liver ultimately results in fibrosis, cirrhosis, and possible hepatocellular carcinoma (5). Hepatic fibrosis induced by administration of concanavalin A (Con A) was reported to be caused by continuous cellular injury after immune response without direct hepatotoxic activity, resembling chronic hepatitis in humans (6).

The flavonoid quercetin (3,3,4,5,7-pentahydroxy flavone) has shown anti-inflammatory and anti-fibrotic effects such as experimental allergic encephalomyelitis, airways allergic inflammatory model, and ultraviolet (UV)-irradiated primary human keratinocytes (7-9) *in vivo* and *in vitro*. Moreover, our previous study reported that quercetin could inhibit pulmonary fibrosis (10). The effects of quercetin on liver damage induced by carbon tetrachloride, hepatotoxins, or bile duct obstruction have been previously studied (11-14). However, the effect of quercetin on liver damage based on immunological mechanisms is not yet known. In the present study, we

Correspondence: Y.S. Wang, State Key Laboratory of Biotherapy, West China Hospital, West China Medical School, Sichuan University, Gaopeng Street, Keyuan Road 4, Chengdu, 610041, China. Fax: +86-28-8516-4060. E-mail: wangys75@gmail.com

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Table 1. Primers and TaqMan probe sequences.

Primer	Sequence
NF- κ B p50	
Forward	5'-CCTGCTTCTGGAGGGTGATG-3'
Reverse	5'-TCCGGCCGCTATATGCA-3'
Probe	5'-FAM-TGGACAGTACCACCTATGATGGGACTACACCT-TAMRA-3'
TGF- β	
Forward	5'-AAACGGAAGCGCATCGAA-3'
Reverse	5'-GGGACTGGCGAGCCTTAGTT-3'
Probe	5'-FAM-CCATCCGTGGCCAGATCCTGTCC-TAMRA-3'
GAPDH	
Forward	5'-CACCCCATTTGATGTTAGTG-3'
Reverse	5'-CCATTTGCAAGTGGCAAAG-3'
Probe	5'-FAM-CAAGGCCGAGAATGGGAAGCTTGTC-TAMRA-3'

investigated the effects of liposomal quercetin on hepatitis and hepatic fibrosis induced by Con A.

Material and Methods

Experimental animals

Female BALB/c mice (6-8 weeks; 18-20 g) were obtained from the Sichuan University Laboratory Animal Center. The experimental procedures were approved by the Ethics Committees of State Key Laboratory of Biotherapy, West China Hospital, West China Medical School, Sichuan University.

Preparation of liposomal quercetin

Liposomal quercetin was prepared as previously described (10,15). Briefly, lecithin, cholesterol, polyethylene glycol (PEG) 4000 and quercetin (weight ratios: 13:4:1:6) were dissolved in chloroform/methanol (15 mL, 3:1, v/v) in a round-bottomed flask and dried in a rotary evaporator (Rotavapor R-200, BUCHI Labortechnik AG, Switzerland) to form a thin lipid film. The final products were concentrated, lyophilized under vacuum (5 h) and stored (-20°C). The free liposomes were prepared the same way as liposomal quercetin was, except quercetin was not added. The diameter of liposomal quercetin was typically in the range 170 ± 20 nm. In the following experiments, the listed dose of liposomal quercetin is based on the content of quercetin.

Effect of liposomal quercetin on acute hepatitis

Acute hepatitis was induced by injecting Con A (20 mg/kg; Sigma-Aldrich, USA) *iv* through the tail vein of female BALB/c mice. To investigate the role of quercetin in Con A induced-hepatitis, liposomal quercetin (2.5 mg/kg), free liposomes (3.75 mg/kg), or the same volume of saline were administered 30 min after Con A administration. Mice were sacrificed 4 or 24 h after Con A injection and blood samples and liver tissues were

obtained. The levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured (Cobas Integra 400, F. Hoffman-La Roche Ltd., Switzerland).

Effects of liposomal quercetin on hepatic fibrosis

The hepatic fibrosis mouse model was induced as previously described (6,16). Briefly, Con A (12.5 mg/kg) was injected *iv* into the tail vein of mice once a week for 6 consecutive weeks. Mice were treated with daily *ip* injections of liposomal quercetin (0.5 mg/kg), free liposomes (0.75 mg/kg), or the same volume of saline before being sacrificed 1 week after the final Con A injection.

Histopathology and immunohistochemistry

Liver tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin. Three-micrometer thick sections were stained with either hematoxylin and eosin or Sirius red for light microscopic evaluation. In sections that stained for collagen (Sirius red positive; magnification $100\times$), the areas were quantified by image analysis using the software Image-pro plus 5.0 (Media Cybernetic, USA). In the immunohistochemistry assay, an anti-nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) p65 monoclonal antibody (Santa Cruz Biotechnology, USA) was used as the primary antibody to detect the expression and distribution of NF- κ B in liver tissue. An anti- α -SMA monoclonal antibody (Santa Cruz Biotechnology) was used as the primary antibody to analyze the activity of hepatic stellate cells.

RNA extraction and real-time RT-PCR

Frozen liver tissues were mechanically pulverized and total RNA was isolated with the Trizol reagent (Life Technologies, USA) to analyze NF- κ B and transforming growth factor beta (TGF- β) mRNA expression. Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) of TGF- β and NF- κ B was

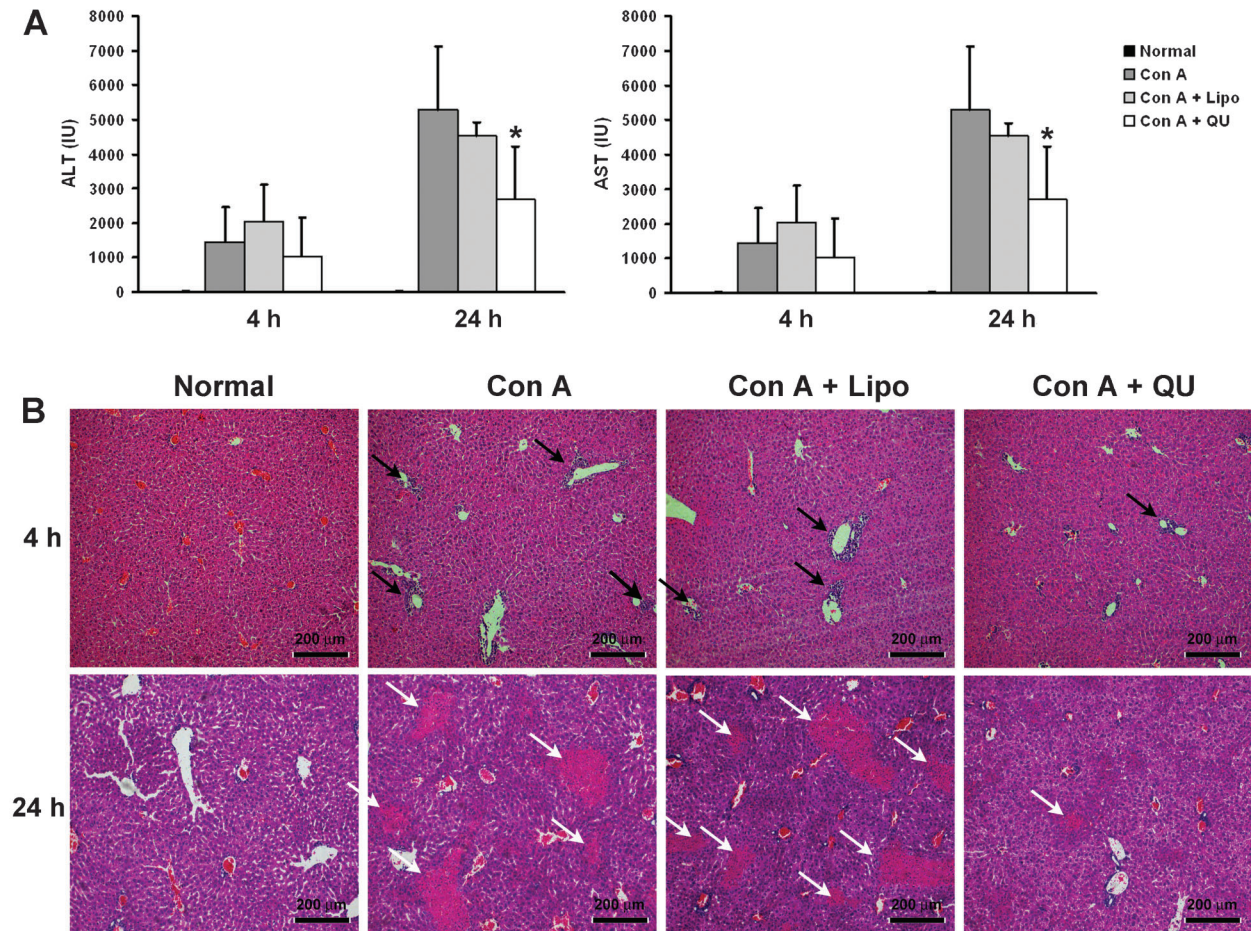


Figure 1. Liposomal quercetin (QU) inhibited acute hepatitis induced by Con A. Murine hepatitis was induced by *iv* injection of Con A. Liposomal QU, free liposomes (lipo), or saline were administered *iv* 30 min after Con A administration. Mice were sacrificed 4 or 24 h after Con A injection, and blood and liver tissue samples were collected. **A**, Levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) 4 and 24 h after Con A injection. Data are reported as means \pm SD for 6 mice per group. * $P < 0.01$, compared to the Con A control (one-way ANOVA followed by the LSD test). **B**, Photomicrographs of representative H&E-stained liver sections. Black arrows indicate infiltration of inflammatory cells, and white arrows indicate necrotic areas.

performed using a one-step kit (TaKaRa Biotechnology [Dalian] Co., Ltd., China). Samples were run in triplicate and quantitative analysis was performed according to the kit protocol. The sequences of the primers and TaqMan probes for mouse GAPDH, NF- κ B p50 subunit, and TGF- β employed in this study are shown in Table 1. The reactions were performed at 42°C (15 min), 95°C (2 min), followed by 45 cycles at 95°C (30 s) and 50°C (31 s). Values were normalized to those of mouse GAPDH expression. Relative expression levels were analyzed using the comparative Ct method according to the TaqMan manual.

Statistical analyses

All data are reported as means \pm SD. Data were analyzed by one-way analysis of variance (ANOVA) and the least significant difference (LSD) test. Differences

between means were considered significant at $P < 0.05$. The experiments were performed at least in duplicate.

Results

Liposomal quercetin inhibited Con A-induced acute hepatitis

To evaluate acute hepatic injury after Con-A challenge, serum ALT and AST levels were measured. As shown in Figure 1A, *iv* administration of Con A resulted in a significant increase in serum ALT and AST 4 and 24 h after Con A administration. Liposomal quercetin treatment significantly decreased ALT and AST concentrations. Consistent with this observation, histological examination of liver sections showed that Con A challenge resulted in massive infiltration of inflammatory cells (4 h) and widespread areas of necrosis (24 h) in liver after Con A

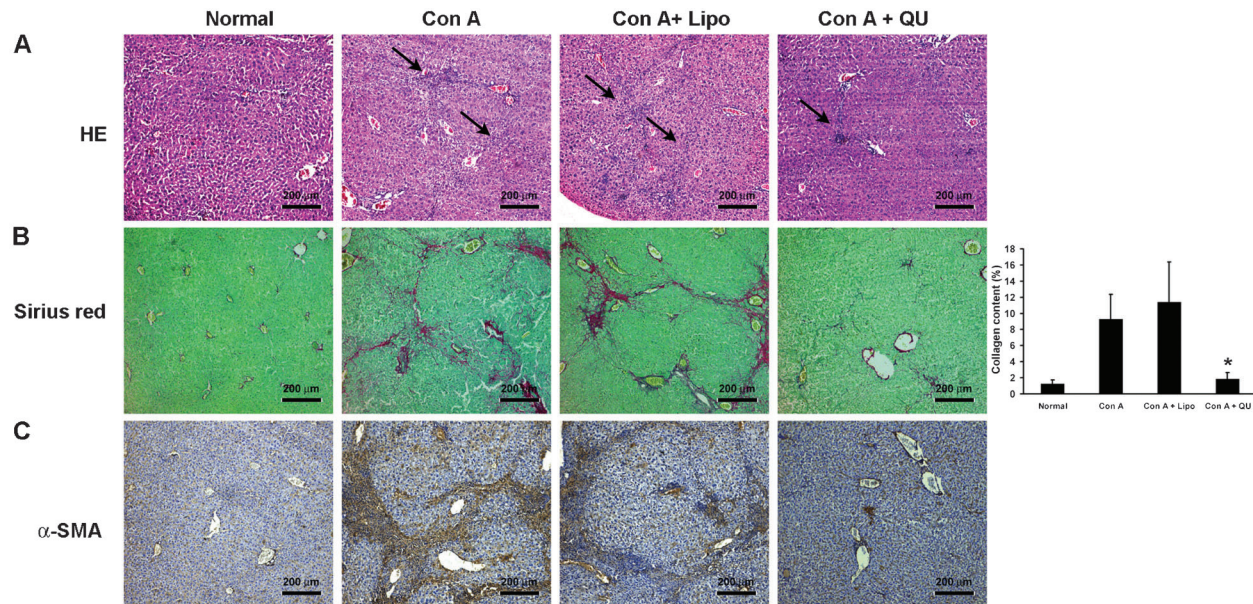


Figure 2. Liposomal quercetin (QU) inhibited Con A-induced hepatic fibrosis. Fibrosis was induced by *iv* injection of Con A once a week for 6 consecutive weeks in the tail vein of mice, which were treated by daily *ip* injections of liposomal QU, free liposomes (lipo), or saline. Mice were sacrificed 1 week after the last injection of Con A. **A**, Photomicrographs of representative H&E-stained liver sections. Black arrows indicate collagen deposition and development of early-stage pseudolobuli. **B**, Liver sections were stained with Sirius red to assess collagen deposition. To further quantitatively analyze collagen deposition, Sirius red-stained areas were analyzed using the Image-pro plus 5.0 software. Data are reported as means of the percentage of collagen positive areas and standard deviation. * $P < 0.01$, compared to Con A control (one-way ANOVA followed by the LSD test). **C**, Liver sections were immunostained for α -SMA to assess HSCs activation.

administration. Treatment with liposomal quercetin markedly attenuated these pathologic changes (Figure 1B). Moreover, free liposomes showed no effect on transaminase levels or pathologic changes. Our data indicate that liposomal quercetin reduced hepatocellular injury induced by Con A injection.

Liposomal quercetin inhibited Con A-induced hepatic fibrosis

To assess the effect of liposomal quercetin on hepatic fibrosis, liver tissues were collected from mice 1 week after the sixth Con A injection and liver sections were stained with both hematoxylin and eosin and Sirius red for light microscopic analysis. Histological analysis showed collagen deposition and development of early-stage pseudolobuli after Con A challenge, whereas liposomal quercetin treatment markedly attenuated these changes (Figure 2A). To further quantitatively analyze collagen deposition, the Sirius red stained areas were analyzed using an Image-pro plus 5.0 software. Collagen deposition decreased from 7.36 to 1.75% in mice challenged with Con A and treated with liposomal quercetin ($P < 0.001$, Figure 2B).

The principal cells responsible for causing hepatic deposition of cross-linked fibrillar collagen are hepatic

myofibroblasts (HmFs), which are believed to be largely derived from HSCs (17). To further evaluate HSCs activation, immunohistochemical analysis for α -SMA was performed. As shown in Figure 2C, our results indicate that liposomal quercetin decreased the number of α -SMA positive cells, which is suggestive of inhibition of HSCs activation.

Quercetin inhibited the expression of NF- κ B in the hepatic fibrosis model

In sections immunolabeled with the NF- κ B p65 subunit, NF- κ B was barely detected in the normal control group, whereas, it was significantly increased after Con A challenge. Moreover, NF- κ B expression was observed mainly in liver HSCs/HmFs and immune cells. After mice received daily liposomal quercetin treatment, lower levels of NF- κ B expression were detected (Figure 3A). In agreement with the immunohistochemical results, real-time RT-PCR analyses showed that NF- κ B p50 subunit expression was significantly decreased in mice treated with liposomal quercetin compared with mice challenged with Con A ($P = 0.004$; Figure 3B).

Quercetin inhibited the expression of TGF- β

To estimate the relative expression of TGF- β , real-time RT-PCR was performed (Figure 4). After Con A

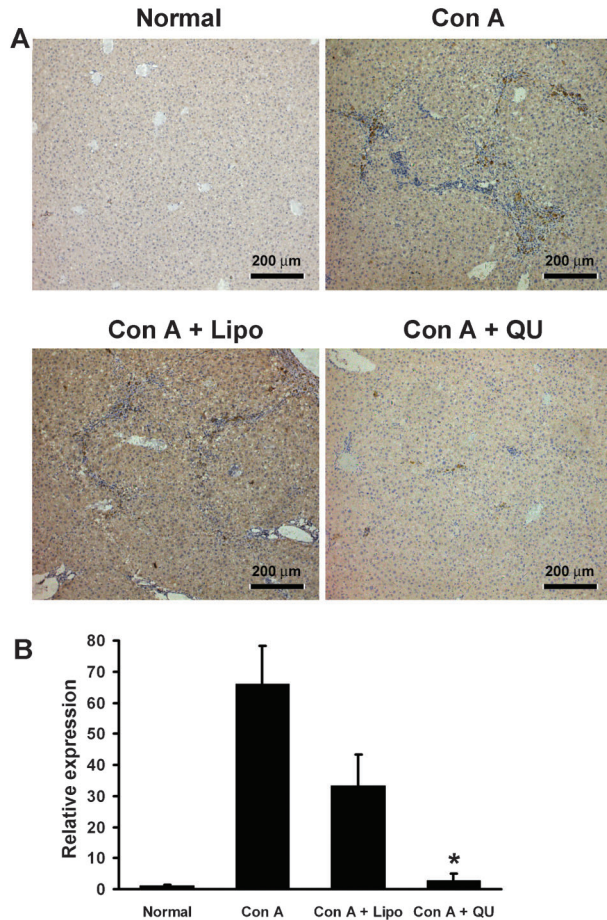


Figure 3. Quercetin inhibited the expression of NF- κ B. *A*, Sections were immunostained for the NF- κ B p65 subunit; the dark yellow or brown colors indicate NF- κ B-positive cells. *B*, Results of real-time RT-PCR for the NF- κ B p50 subunit. Data are reported as the means and standard deviation of the relative expression levels of NF- κ B. QU: liposomal quercetin; lipo: free liposomes; normal: saline. * $P < 0.01$, compared to Con A control (one-way ANOVA followed by the LSD test).

challenge, TGF- β mRNA increased 7.2 times relative to normal control mice. Quercetin treatment reduced the increase in TGF- β expression to 2.2-fold ($P = 0.013$, compared with mice challenged with Con A).

Discussion

Hepatic fibrosis is the result of wound-healing responses after liver injury caused by various etiologies, and immune mechanisms play an important role in this process. In the present study, we tested the effects of quercetin in hepatitis and hepatic fibrosis models induced by Con A. As shown in Figures 1 and 2, Con A challenge resulted in massive infiltration of inflammatory cells and widespread areas of necrosis. After liver injury, HSCs

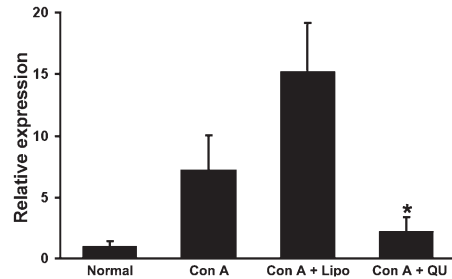


Figure 4. Quercetin inhibited expression of TGF- β . To estimate the relative expression level of TGF- β , real-time RT-PCR was performed. Data are reported as the means and standard deviation of the relative expression levels of TGF- β . * $P < 0.05$, compared to Con A control (one-way ANOVA followed by the LSD test).

undergo a response known as “activation”, during which there is a transition from quiescent cells into proliferative, fibrogenic, and contractile myofibroblasts (17). As we expected, treatment with liposomal quercetin markedly attenuated these pathologic changes.

Previous studies have suggested that NF- κ B and TGF- β signals participate in liver damage and fibrosis induced by Con A (18-20), and therefore we tested the effects of quercetin on the NF- κ B and TGF- β pathways. As shown in Figures 3 and 4, the expression of NF- κ B and TGF- β was inhibited by liposomal quercetin, suggesting that the inhibitory effect of quercetin on liver damage might be associated with its ability to modulate NF- κ B and TGF- β production.

There is growing evidence that NF- κ B acts as a key mediator of fibrosis in HSCs/HmFs, the main cells responsible for promoting hepatic deposition of cross-linked fibrillar collagen (18). HSCs/HmFs show activation of NF- κ B during culture activation (21) and in human and mouse models of liver fibrosis (22). NF- κ B has been postulated to regulate three key aspects of HSCs/HmFs biology: activation, survival, and inflammatory response (23). In the present study, a decreased NF- κ B expression was observed in liver HSCs/HmFs from mice treated with liposomal quercetin, which is in agreement with previous studies reporting the effects of quercetin on NF- κ B in other inflammatory models (9,24,25). Therefore, it is suggested that the inhibitory effect of quercetin on liver damage is associated with its ability to regulate NF- κ B.

TGF- β plays an important role in liver fibrosis. Results of studies in which TGF- β is overexpressed in liver using adenovirus or in transgenic TGF- β mice have revealed that TGF- β contributes both HSCs activation and fibrogenesis (26-28). Furthermore, blocking of TGF- β signaling protected mice and rats against liver fibrosis in several experimental models (29,30). In the present study, quercetin inhibited TGF- β expression in liver with hepatic fibrosis, which is in agreement with other results showing that quercetin treatment of fibroblasts significantly

inhibited collagen and TGF- β production (10,31,32). Accordingly, the inhibitory effects of quercetin on hepatic fibrosis may be related to its ability to decrease TGF- β generation.

In summary, our results indicate that liposomal quercetin can effectively inhibit acute hepatitis and hepatic fibrosis induced by Con A. Furthermore, the present study showed that the inhibitory effect of quercetin was associated with its ability to modulate

NF- κ B and TGF- β production. These results suggest that liposomal quercetin may be a potent strategy in the treatment of patients with liver damage and liver fibrosis.

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