Riboflavin ameliorates mitochondrial dysfunction via the AMPK/PGC1α/HO-1 signaling pathway and attenuates carbon tetrachloride-induced liver fibrosis in rats

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Abstract. Hepatic fibrosis is a global health problem, with increasing evidence demonstrating that oxidative stress serves a pivotal role in fibrogenesis. Riboflavin is a vital nutrient in the human and animal diet, which enhances the activity of antioxidant enzymes and ameliorates oxidative stress. The present study evaluated the effect of riboflavin on liver fibrosis and the mechanisms underlying this process. Rats were subcutaneously injected with carbon tetrachloride (CCl_4) dissolved in sterile olive oil twice per week to induce hepatic fibrosis. The effect of riboflavin on CCl_4-induced liver fibrosis was then assessed. Blood samples and liver tissues were collected and analyzed. The liver tissue morphological changes, immunohistochemical analysis, levels of malondialdehyde (MDA) and superoxide dismutase (SOD) in the mitochondria, and the protein expression levels of α-smooth muscle actin (α-SMA), transforming growth factor-β1 (TGF-β1), extracellular signal-regulated kinase (ERK), p38, c-Jun N-terminal kinase (JNK), AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and heme oxygenase 1 (HO-1) in the liver were also analyzed. The results demonstrated that riboflavin treatment significantly decreased the levels of alanine transaminase and aspartate transaminase in the serum, increased SOD activity and modulated the MDA level in the mitochondria. Furthermore, riboflavin significantly inhibited the CCl_4-induced, upregulated protein expression levels of phosphorylated (p)-ERK, p-p38, p-JNK, TGF-β1 and α-SMA. Moreover, riboflavin significantly increased the expression of p-AMPK, PGC-1α and HO-1 in the liver tissue. These results suggested that riboflavin delays CCl_4-induced hepatic fibrosis by enhancing the mitochondrial function via the AMPK/PGC-1α/HO-1 and mitogen-activated protein kinase signaling pathways.

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Abbreviations: α-SMA, α-smooth muscle actin; ALT, alanine transaminase; AMPK, AMP-activated protein kinase; AST, aspartate aminotransferase; BCA, bicinchoninic acid; CCl_4, carbon tetrachloride; CYP2E1, cytochrome P450 2E1; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; H&E, hematoxylin and eosin; HO-1, heme oxygenase 1; HSC, hepatic stellate cell; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MDA, malondialdehyde; PBS, phosphate buffered saline; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; ROS, reactive oxygen species; SOD, superoxide dismutase; TGF-β1, transforming growth factor-β1

Key words: liver fibrosis, CCL_4, riboflavin, mitochondria, AMPK/PGC-1α/HO-1 signaling

Introduction

Long-term liver injury caused by viral, alcoholic and drug is prevalent in the world, and almost 40% of patients further develop liver fibrosis (1). The incidence of hepatic fibrosis is 4.5-9%, making hepatic fibrosis has a global health problem (2,3). Chronic liver injury caused by various factors, including viruses (such as hepatitis B and C), drugs and alcohol, increases collagen accumulation in the liver, causing an imbalance between the production and degradation of the extracellular matrix (ECM). Excessive collagen accumulation can cause hepatic fibrosis and liver cirrhosis (4,5). Hepatic fibrosis disrupts the liver functions, such as the conversion of nodules and vascular contortion, severely perturbing various physiological functions, including hepatocyte dysfunction and portal hypertension (6). Previous studies have reported that hepatic fibrosis may contribute to hepatic encephalopathy and may serve a vital role in the development and progression of hepatocellular carcinoma (7,8). The mechanisms underlying hepatic fibrosis have not yet been elucidated; however, oxidative stress is hypothesized to promote hepatic fibrosis. The role of oxidative stress in the development of several chronic

diseases, including chronic liver disease, via increased production of reactive oxygen species (ROS) that damage various organs, including the liver, has been reported (9).

The mitochondrion is a vital organelle in eukaryotic cells, supplying the energy for numerous biological functions via oxidative phosphorylation. Furthermore, mitochondria serve an essential role in the maintenance of various functions, including regulating the production of oxygen free radicals, calcium homeostasis and lipid metabolism (10). Increasing evidence demonstrates that mitochondrial dysfunction mediates certain pathological processes, including the development of neurodegenerative diseases and ischemic/reperfusion injury (11,12). Mitochondria provide the main energy supply for hepatocytes, and various acute and chronic liver pathologies, including ischemic/reperfusion and drug-induced hepatic injury, disrupt mitochondrial function (13). Mitochondrial dysfunction increases the production of mitochondrial ROS and by extension, intracellular ROS (14). Liver injury induced using carbon tetrachloride (CCl_4) is closely associated with enhanced oxidative stress (15,16). Conversion of CCl_4 into the trichloromethyl peroxyl radical triggers lipid peroxidation, which induces oxidative stress and further impairs liver function. Wound repair of chronic liver injury caused via oxidative stress stimulates excessive collagen expression, leading to liver fibrosis. However, oxidative stress serves an important role in liver fibrosis. Mitochondrial dysfunction increases the production of mitochondrial ROS and by extension, intracellular ROS, enhancing oxidative stress (14). Other studies reported that CCl_4 impairs mitochondrial function, increasing oxidative stress, accelerating liver fibrosis and impairing liver regeneration (17-19). Furthermore, it has been reported that enhanced mitochondrial function delays the progression to liver fibrosis (20,21).

Riboflavin, also called vitamin B2, is a heat-stable vitamin widely present in numerous foods, including milk, fish, dark-green leafy vegetables, fruits and rice (22,23). It has been reported that riboflavin possesses antioxidant, anti-aging, anti-inflammatory, anti-nociceptive and anticancer properties (24). Certain studies have reported that riboflavin reduces oxidative stress and oxidative DNA damage in diabetic mice (25) and relieves liposaccharide-induced shock (26). Clinical data suggest that dietary supplements with riboflavin reduce the risk of breast cancer and colorectal cancer (24), and lower the rates of microcytic anemia in men and children (27). Furthermore, riboflavin alleviates hepatocellular injury and subsequent liver ischemia/reperfusion injury via the promotion of antioxidation (28). Mouse models have demonstrated that riboflavin deficiency disrupts proper mitochondrial development and disrupts mitochondrial function in the liver, whereas riboflavin supplementation improves mitochondrial function (29). Riboflavin is a vital nutrient in the human and animal diet, and is safe even at excess levels (24). However, the effect of riboflavin on liver fibrosis is still unclear. Furthermore, the mechanism underlying the effect of riboflavin on liver fibrosis is not well understood. We hypothesized that riboflavin protects against CCl_4-induced liver injury. In the present study, an animal model of liver fibrosis was established through subcutaneous injection of CCl_4. The effects of riboflavin on liver tissue damage, including oxidative stress changes, and the mechanisms underlying this process were assessed.

Materials and methods

Antibodies, reagents and materials. Riboflavin was purchased from Nanjing Chemical Reagent Co., Ltd. The bicinchoninic acid (BCA) kit was purchased from Beyotime Institute of Biotechnology. Alanine transaminase (ALT) microplate assay kit (cat. no. C009-2), aspartate transaminase (AST) microplate assay kit (cat. no. C010-2), glucose assay kit (cat. no. F006-1-1), malondialdehyde (MDA) assay kit (TBA method; cat. no. A003-1) and superoxide dismutase (SOD) assay kit (Hydroxylamine method; cat. no. A001-1) were purchased from Nanjing Jiancheng Bioengineering Institute. Standard laboratory rodent food was purchased from Nanjing Qinglongshan Experimental Animal Feed Technology Co. Carbon tetrachloride (CCl_4) was purchased from Sinopharm Chemical Reagent Co., Ltd. Primary polyclonal antibodies, including AMP-activated protein kinase (AMPK; cat. no. ab32047), phosphorylated (p)-AMPK (cat. no. ab133448) and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α; cat. no. ab106814) were purchased from Abcam, heme oxygenase (HO)-1 (cat. no. 82206), c-Jun N-terminal kinase (JNK; cat. no. 9252S), p-JNK (cat. no. 4668P), extracellular signal-regulated kinase (ERK)1/2 (cat. no. 4695S), p-ERK1/2 (cat. no. 4370P), p38 (cat. no. 8690S) and p-p38 (cat. no. 9211S) were purchased from Cell Signaling Technology Inc., and β-actin (cat. no. bs-0061R), α-smooth muscle actin (α-SMA; cat. no. bs70000) and transforming growth factor β1 (TGF-β1; cat. no. bs1361) were purchased from Bioworld. HRP-conjugated goat anti-rabbit IgG secondary antibodies (cat. no. BST11112B54) and rabbit anti-goat IgG secondary antibodies (cat. no. BA1060) were purchased from Wuhan Boster Biological Technology, Ltd.

Animals and experimental protocol. All animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals (30) and the experimental protocol was approved by the Animal Experimental Ethics Committee of Wannan Medical College (Wuhu, China; approval no. LISC-2018-001). The rats received humane care and all efforts were made to alleviate suffering.

A total of 30 male Sprague-Dawley rats (weighing 240-260 g; 8-10 weeks) were purchased from the Changsha Tianqin Biotechnology Co., Ltd.. The rats were housed in a standard facility at 22±2˚C with 50±5% humidity and a 12/12 h light/dark cycle. The animals had access to water and food *ad libitum*. After a week of acclimation, rats were randomly divided into groups (n=10) as follows: i) Control (CON) group; ii) model (MOD) group; and iii) riboflavin (RIB) group.

CCl_4 is catabolized into the trichloromethyl radical by cytochrome P450 2E1 (CYP2E1) in the liver. Trichloromethyl radicals and oxygen are further converted into the trichloromethyl peroxyl radical. These radicals cause liver injury, and chronic liver injury further progresses into hepatic fibrosis and cirrhosis of the liver (31). In addition to its anesthetic effect, phenobarbital enhances the activity of CYP2E1, promoting conversion of CCl_4 into the trichloromethyl radical and the subsequent development of hepatic fibrosis (32). Phenobarbital has been reported to upregulate CYP2E1 when administered via drinking water (33,34).

In the present study, liver fibrosis was induced according to the aforementioned methods with minor revisions. Briefly,

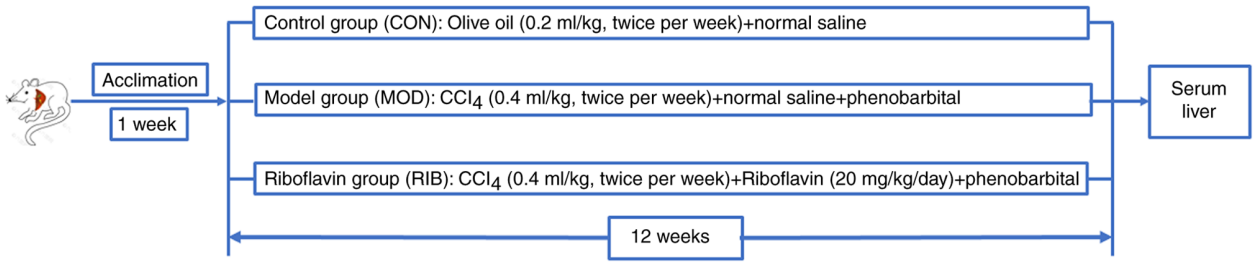


Figure 1. Experimental design. Weight-matched rats were randomly allocated to three groups (n=10) and treated as indicated. CON, control; MOD, model; RIB, riboflavin; CCl_4, carbon tetrachloride.

the rats in the RIB and MOD groups received a subcutaneous injection of CCl_4 dissolved in sterile olive oil (0.4 ml/kg; v/v, 1/1) twice per week and phenobarbital (0.35 g/l) dissolved in drinking water for 12 weeks (Fig. 1). Oxidative stress was induced using riboflavin as previously described (24). Briefly, the RIB group received an oral riboflavin dose (20 mg/kg/day). The animals in the CON group received a subcutaneous injection of olive oil (0.2 ml) twice per week (Fig. 1). To observe effect of riboflavin on the state of rats treated with CCl4, general characteristics of the rats, including mental state, activity, and eating and drinking status, were observed every day.

Collection of samples. The amount of food consumed every day was recorded. The rats were weighed once every 2 weeks over the experimental period. The rats were anesthetized after 12 weeks using an intraperitoneal injection of sodium pentobarbital (40 mg/kg). Blood (3 ml) was then collected from the carotid artery. To minimize pain, the rats were sacrificed via intraperitoneal injection with sodium pentobarbital (100 mg/kg) and bleeding. Rats were confirmed dead when there was no autonomous respiration and no reflex activity, and no heart activity. The liver, spleen and pancreas were immediately removed and weighed. A portion of the left lobe of the liver was removed and fixed in 10% neutral formalin for 2 days at room temperature. The remaining portion of the liver was stored at -80˚C until use. The epididymal adipose tissue was separated and weighed to assess the amount of visceral fat.

Serum biochemical analysis. Blood samples were centrifuged at 10,000 x g at 4˚C for 10 min to extract the serum. The levels of ALT, AST and glucose levels in the serum were quantified using a Hitachi 7600-120 automated biochemical analyzer (Hitachi High-Technologies Corporation). Serum was mixed with 2,4-dinitrophenylhydrazine and incubated for 30 min at 37˚C, then 0.4 mol/l sodium hydroxide was added into mixture. Absorbance at 505 nm was used to measure AST and at 510 nm for ALT.

Assessment of oxidative stress in the mitochondria. MDA (a product of lipid peroxidation) and SOD (an antioxidant enzyme) levels in the mitochondria were used to assess changes in the level of oxidative stress.

Mitochondria in the liver were separated as previously described (35). Briefly, liver tissues were immersed in a precooled buffer (10 mM Tris, 210 mM mannitol, 70 mM sucrose, 1 mM EDTA and 0.5 mM EGTA; pH 7.4) and immediately cut into 1-mm portions. Portions were homogenized and centrifuged at 1,000 x g at 4˚C for 10 min. The supernatant was collected and centrifuged at 10,000 x g for 15 min at 4˚C. The precipitate was resuspended in the buffer and the precipitate suspension was centrifuged at 10,000 x g and 4˚C for 15 min.

The precipitate was resuspended in normal saline to determine the levels of SOD and MDA in liver mitochondria using commercially available kits according to the manufacturer's protocol.

Morphological examination. The liver was fixed in 4% neutral paraformaldehyde for 2 days at room temperature, embedded in paraffin after dehydration in an ascending ethanol series in turn (from 75 to 100% ethanol) and serially cut into 5-μm thick sections to observe morphological features and fibrosis. For histological examination, the sections were stained with hematoxylin (15 min) and eosin (3 min) (H&E) at 25˚C. For assessment of the presence of collagen in the livers, the sections were stained with Masson's Trichrome. Staining in Weigert hematoxylin for 8 min, ponceau for 10 min and aniline blue for 2 min at 25˚C.

Immunohistochemical analysis. Immunohistochemical staining of TGF-β1 and α-SMA was performed to examine the activated hepatic stellate cells (HSCs). The liver was embedded in paraffin and cut into 5-μm thick sections. After deparaffinization with xylene for 10 min and hydration in a descending alcohol series at room temperature, the sections were incubated with boiled 10 mM sodium citrate buffer for 5 min for epitope retrieval. The sections were treated with 3% hydrogen peroxide to inhibit endogenous peroxidase activity for 15 min at room temperature. The sections were washed with phosphate buffered saline (PBS) for 2 min three times at room temperature and the sections were treated with PBS containing 2% bovine serum albumin (BOMEI Biotechnology CO., LTD. Hefei, China) to block non-specific sites for 1 h at 37˚C. Sections were incubated with anti-α-SMA (1:100) and anti-TGF-β1 antibodies (1:100) overnight at 4˚C. The sections were then washed with PBS and incubated with HRP-conjugated goat anti-rabbit IgG secondary antibodies (1:100) for 1 h at 25˚C. Antigen staining was visualized using the Enhanced HRP-DAB Chromogenic kit (BaSo Biotechnology Co., Ltd.) and observed under a Moticam S6 microscope (Motic China Group Co., Ltd.).

Western blotting. Western blotting was performed as described previously (36). Briefly, liver tissues were lysed in ice-cold lysis

Table I. Effects of riboflavin on food consumption, blood glucose and epididymal adipose.

Parameter	CON	MOD	RIB
Food consumption, g/day	23.64±1.85	18.69±1.49[a]	19.94±1.72[b]
Blood glucose, mM	4.11±0.65	4.53±0.41	4.33±0.51
Epididymal adipose, g	3.08±0.50	2.17±0.39[a]	2.21±0.44
Epididymal adipose to BW, %	0.69±0.08	0.60±0.09[a]	0.59±0.10

Values are presented as mean ± SD. [a]P<0.01 vs. CON; [b]P<0.01 vs. MOD (n=8). CON, control; MOD, model; RIB, riboflavin; BW, body weight.

buffer (10 mM HEPES, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 20 mM sodium fluoride, 10 mM EDTA, 2 mM PMSF, 1% Triton X-100) and centrifuged at 13,000 x g at 4˚C for 15 min. After quantification using the BCA method, the proteins (30 μg) in lysates were separated using a 10% SDS-PAGE gel and transferred onto nitrocellulose membranes. The membranes were blocked in 2% bovine serum albumin (dissolved in PBS; Hefei Bomei Biotechnology Co., Ltd.) for 1h at room temperature. Then, the membranes were incubated with the following specific antibodies: β-actin, HO-1, AMPK, p-AMPK, PGC-1α, ERK, p-ERK, JNK, p-JNK, p38, p-p38, TGF-β1 (all 1:1,000) and α-SMA (1:2,000) overnight at 4˚C. The membranes were washed three times with PBS and incubated with HRP-conjugated goat anti-rabbit IgG secondary antibodies (1:10,000) for 90 min at room temperature, and the membrane for PGC-1α, with rabbit anti-goat IgG secondary antibodies. Protein bands were visualized using the ECL chemiluminescence substrate kit (Beijing labgic Biotechnology CO., LTD. Beijing, China) according to the manufacturer's protocol and analyzed using Image J software (version 1.8; National Institutes of Health). β-actin was used as the loading control.

Statistical analysis. Data were analyzed using SPSS version 20.0 (IBM Corp.). Results are presented as the mean ± standard deviation. Differences between groups were analyzed using one-way ANOVA and Dunnett's post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

General characteristics of the rats. After 4 weeks of treatment with CCl_4, the rats in the MOD group were depressed and dull. Administration of riboflavin markedly improved these characteristics. The rats in the MOD group were also less active and had a poor appetite. Food consumption was significantly higher for rats in the CON group than in the MOD group (P<0.01; Table I, the food consumption in the MOD group was significantly decreased compared with the RIB group (P<0.01). Granular protrusions were observed on the liver surface of rats in the MOD group (Fig. 2A) and fewer granular protrusions in the RIB group. Riboflavin supplementation improved the appetite of rats in the RIB group.

Effects of riboflavin on body weight and liver index. At the beginning of the experiment, there was no statistical difference in rat weight when comparing among the three groups (P>0.05; Fig. 3A). However, after 2 weeks of CCl_4 treatment, bodyweight in the CON group was significantly higher compared with that of rats in the MOD group (P<0.05). However, there was no significant difference in the body weight of rats in the MOD group compared with that in the RIB group (P>0.05). Further analysis demonstrated that there was no significant difference with regard to liver weight for rats in the three groups (P>0.05; Fig. 3B). However, the liver weight/body weight (LW/BW) ratio was significantly higher for rats in the MOD group compared with that in the CON group (P<0.05; Fig. 3C). Furthermore, riboflavin treatment significantly decreased the LW/BW ratio of the RIB group when compared with the MOD group (P<0.05). The results suggested that riboflavin attenuated liver fibrosis induced by CCl_4.

Effects of riboflavin on serum parameters. The liver function markers ALT and AST in the serum were quantified to assess the effects of riboflavin on liver function. The results demonstrated that the activities of ALT and AST were significantly higher in the MOD group compared with that in the CON group (P<0.05; Fig. 4) and that ALT and AST activities were significantly lower in the RIB group compared with the MOD group (P<0.05). These results demonstrated that oral administration of riboflavin significantly improved function impaired by CCl_4.

Changes in blood glucose and visceral fat. The level of blood glucose was not significantly different when compared among the groups (P>0.05; Table I). The level of epididymal fat, a marker of the visceral fat, was significantly higher in the CON group compared with that in the MOD group (P<0.05). Furthermore, the epididymal fat to BW ratio was significantly higher in the CON group compared with that in the MOD group (P<0.05); however, the ratio was not significantly different when compared between the MOD and RIB groups (P>0.05).

Effects of riboflavin on mitochondrial function. SOD exerts its antioxidant activity by scavenging superoxide. MDA, a lipid peroxidation product, reflects the oxidant-induced lipid peroxidation level. Therefore, SOD and MDA levels were quantified to evaluate oxidative stress. SOD activity in the liver was significantly lower in the MOD group compared with that in the CON group (P<0.01; Fig. 5A). Furthermore, the MDA level was significantly higher in the MOD group compared with that in the CON group (P<0.05; Fig. 5B). Treatment with

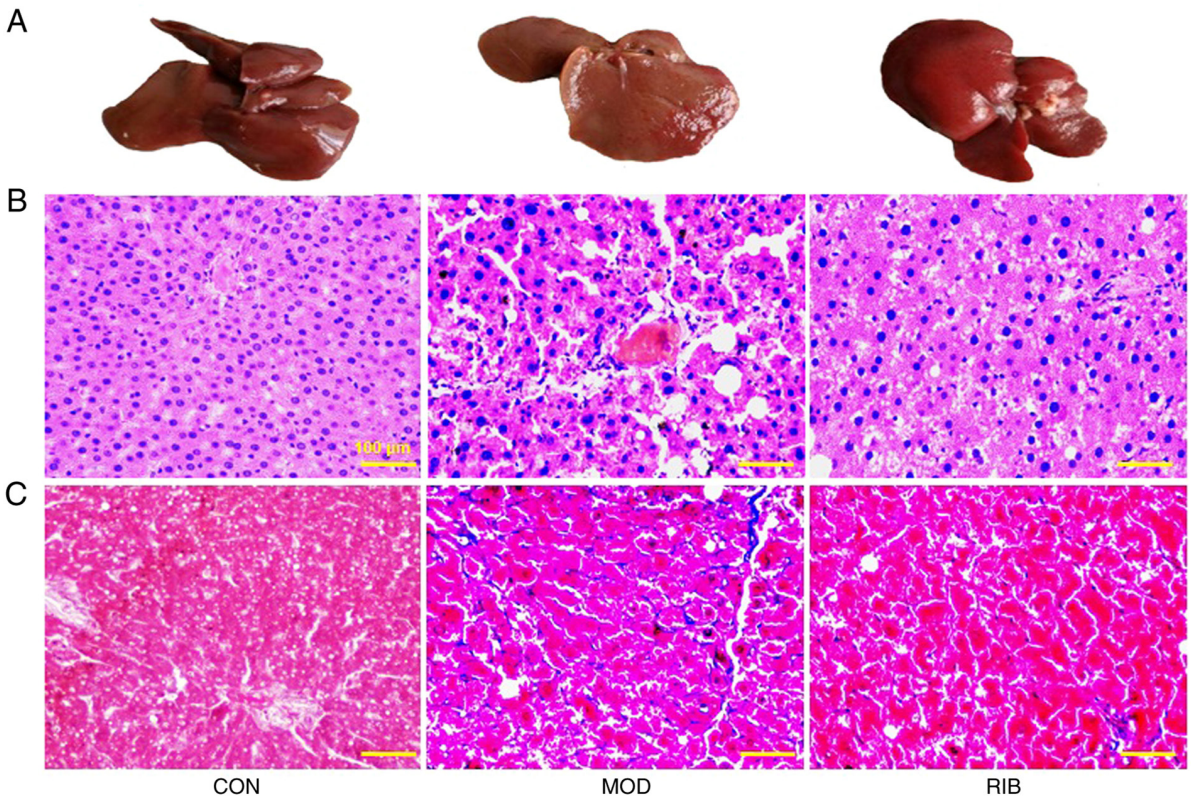


Figure 2. Histopathological changes in the liver of rats. Liver tissues were collected at the end of the experiment. (A) Changes in the liver tissues were assessed. Tissues were cut into thin sections and stained with (B) hematoxylin and eosin, and (C) Masson's Trichrome. Scale bar, 100 μm. CON, control; MOD, model; RIB, riboflavin.

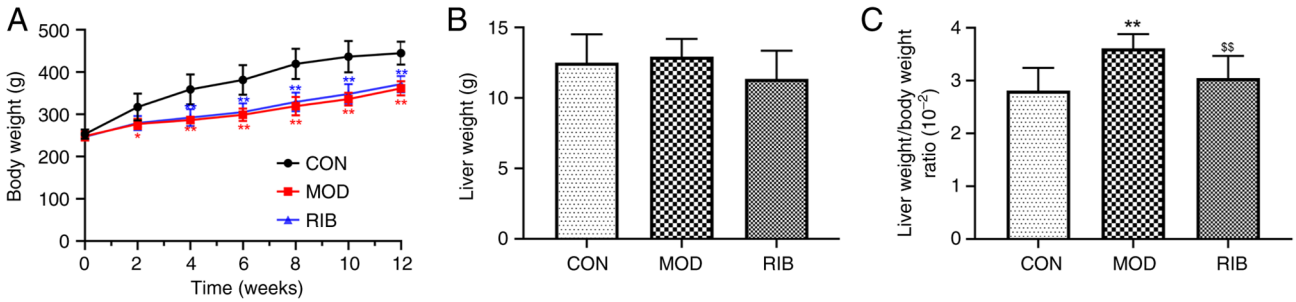


Figure 3. Changes in body and liver weight and liver weight/body weight ratio. (A) Body weight and (B) liver weight of the rats. (C) Liver weight/body weight ratio. Values are presented as mean ± SD. *P<0.05, **P<0.01 vs. CON; $^{\$\$}$P<0.01 vs. MOD (n=8). CON, control; MOD, model; RIB, riboflavin.

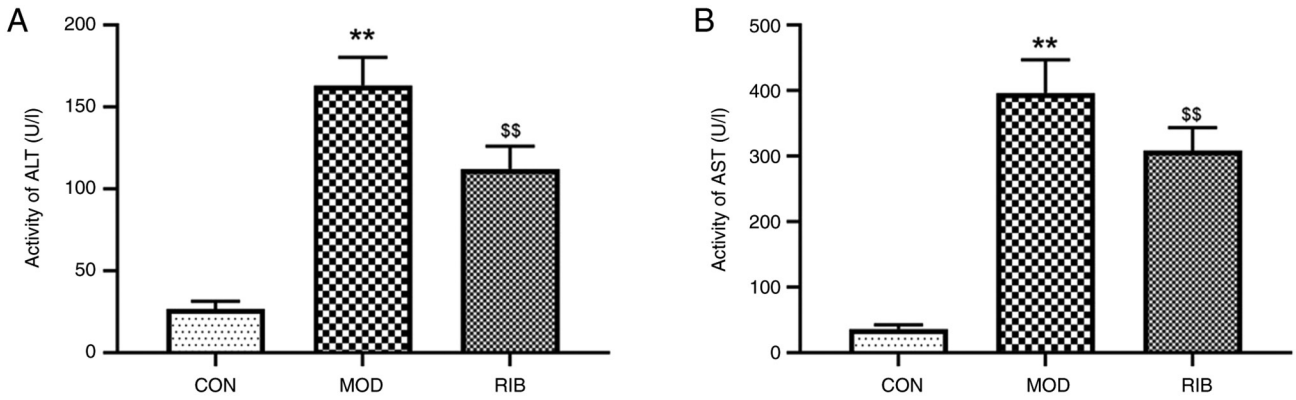


Figure 4. Parameters of liver function in serum. The serum (A) ALT and (B) AST levels were determined using an automated biochemical analyzer. Values are presented as mean ± SD. **P<0.01 vs. CON; $^{\$\$}$P<0.01 vs. MOD (n=8). ALT, alanine transaminase; AST, aspartate aminotransferase; CON, control; MOD, model; RIB, riboflavin.

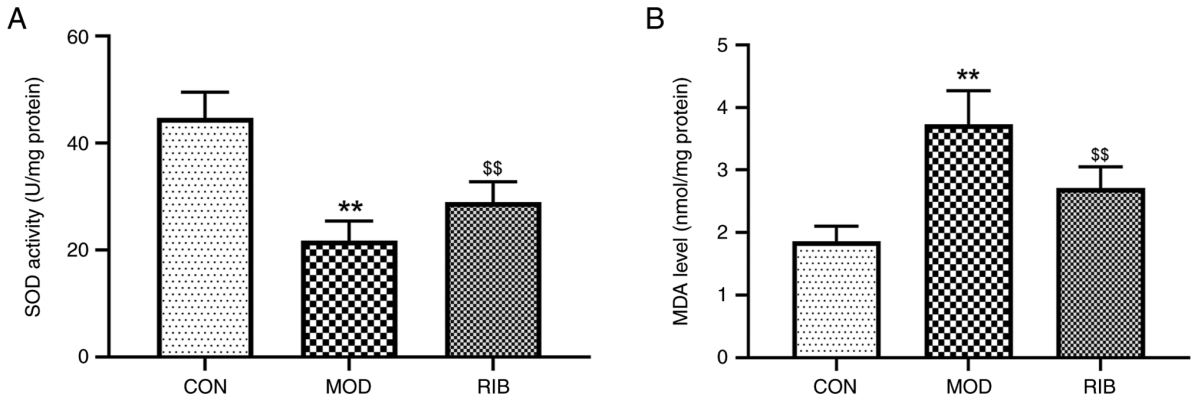


Figure 5. Changes of oxidation in the liver mitochondria. The (A) SOD activity and (B) MDA level in the liver mitochondria. Values are presented as mean ± SD. **P<0.01 vs. CON; $^{\$\$}$P<0.01 vs. MOD (n=8). SOD, superoxide dismutase; MDA, malondialdehyde; CON, control; MOD, model; RIB, riboflavin.

riboflavin significantly decreased the MDA level (P<0.01) and significantly increased SOD activity in the liver (P<0.01) in the RIB group when compared with the MOD group. The results indicated that riboflavin ameliorated CCl_4-induced mitochondrial dysfunction in rats.

Histological analysis. Histological examination is one of the best methods for evaluating the severity of hepatic fibrosis (37). For rats in the MOD group, the liver adhered to the surrounding tissues, and there is no obvious adhesion between the liver and surrounding tissues in the CON and RIB groups. The liver surface was significantly rougher and more nodular compared with the CON group. However, riboflavin treatment markedly reduced the number of diffuse nodules on the liver surface. H&E staining demonstrated a lobular architecture, clear sinusoids and hepatocytes radiating around the central vein for rats in the CON group (Fig. 2B). However, rats in the MOD group displayed a poor liver structure, infiltration of inflammatory cells, and swollen and deformed hepatocytes. However, riboflavin treatment reduced the infiltration of inflammatory cells and necrosis in the liver for rats in the RIB group compared with the MOD group. Furthermore, Masson's Trichrome staining demonstrated that the deposition of collagen fibers was higher in the MOD group compared with that in the CON group. However, riboflavin treatment markedly decreased the deposition of collagen fibers in the liver of rats in the RIB group compared with those in the MOD group (Fig. 2C). Therefore, riboflavin treatment attenuated CCl_4-induced liver injury in rats.

α-SMA and TGF-β1 levels in the liver. TGF-β1 is a key profibrogenic cytokine in hepatic fibrosis. HSCs activated via TGF-β1 serve a vital role in liver fibrogenesis by promotion of the production of α-SMA. Therefore, α-SMA is a marker of activated HSCs. Immunohistochemical staining demonstrated that CCl_4 treatment markedly increased the expression of TGF-β1 and α-SMA in the MOD group compared with that in the CON group (Fig. 6A and B). The administration of riboflavin markedly decreased the expression of TGF-β1 and α-SMA in the RIB group compared with that in the MOD group. The protein expression levels of TGF-β1 and α-SMA in the liver were assessed using western blotting (Fig. 6C). The results demonstrated that compared with the CON group, CCl_4 treatment significantly increased the protein expression levels of TGF-β1 and α-SMA in the MOD group (P<0.01; Fig. 6D and E). However, riboflavin treatment significantly decreased the protein expression levels of TGF-β1 and α-SMA compared with the MOD group (P<0.01). The results showed that riboflavin treatment attenuated CCl_4-induced liver fibrosis via decreasing the expression of fibrogenic cytokines.

Effects of riboflavin on the protein expression levels of p-AMPK, PGC-1α and HO-1. The protein expression levels of p-AMPK, PGC-1α and HO-1 in the liver tissue were assessed (Fig. 7A) to demonstrate the antioxidant effect of riboflavin. CCl_4 treatment significantly decreased the expression of HO-1, PGC-1α and p-AMPK in the MOD group compared with that in the CON group (all P<0.01; Fig. 7B-D). However, riboflavin treatment significantly increased the protein expression levels of HO-1, PGC-1α and p-AMPK when compared with the MOD group (all P<0.01). The results indicated that riboflavin relieved liver fibrosis via the improvement of AMPK/PGC-1α/HO-1 signaling on mitochondrial function and oxidative stress.

Effects of riboflavin on the mitogen-activated protein kinase (MAPK) signaling pathway. The MAPK signaling pathway participates in the modulation of collagen expression and accelerates fibrogenesis. The protein expression levels of proteins related to the MAPK signaling pathway were assessed using western blotting to demonstrate the mechanism of riboflavin action on liver fibrosis (Fig. 8A). CCl_4 treatment of the MOD group significantly increased the protein expression levels of p-ERK, p-JNK and p-p38 compared with the CON group (all P<0.01; Fig. 8B-D). However, riboflavin treatment significantly decreased protein expression levels of p-ERK, p-JNK and p-p38 compared with the CON group (all P<0.01). The results suggested that riboflavin improved the MAPK signaling, which attenuated liver fibrosis.

Discussion

Previous research has demonstrated that CCl_4 induces acute and chronic liver injury, including hepatic fibrosis, via numerous mechanisms, and thus, is considered a hepatotoxin (38). Therefore, CCl_4 is used for inducing liver injury for related studies (39) and was used for this purpose in the present

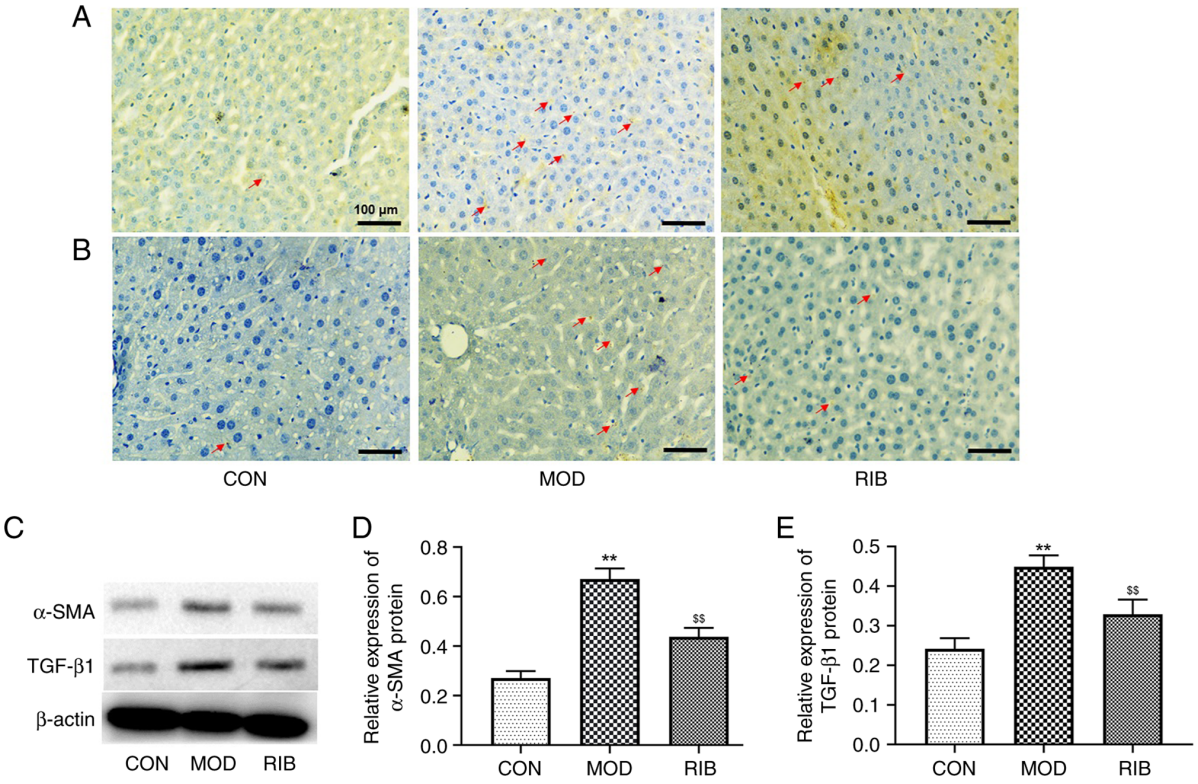


Figure 6. Immunohistochemical analysis and expression of α-SMA and TGF-β1 proteins. The staining of (A) α-SMA and (B) TGF-β1 in liver tissues. Scale bar, 100 μm. (C) Western blotting of α-SMA and TGF-β1 in liver tissues. Relative protein expression levels of (D) α-SMA and (E) TGF-β1 in liver tissues. Values are presented as mean ± SD. **P<0.01 vs. CON; $^{\$\$}$P<0.01 vs. MOD (n=6). α-SMA, α-smooth muscle actin; CON, control; MOD, model; RIB, riboflavin.

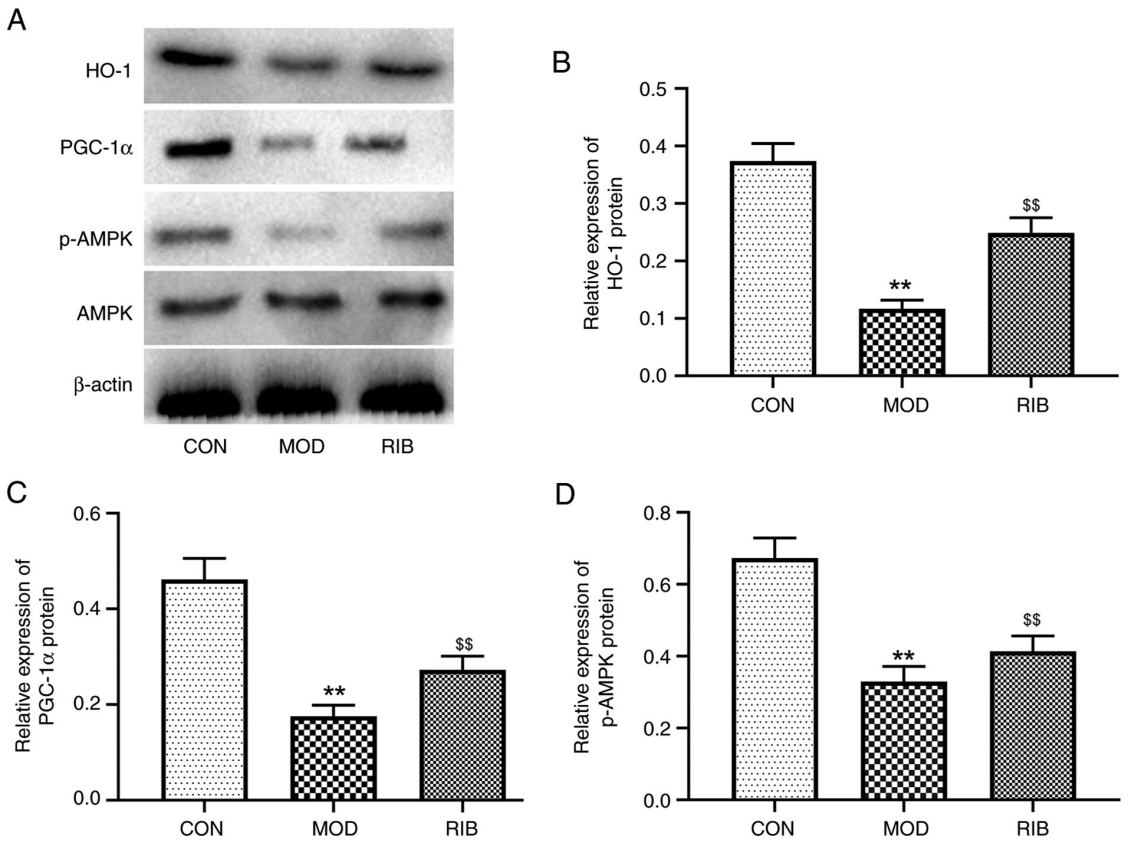


Figure 7. Effects of riboflavin on the expression of p-AMPK, PGC-1α and HO-1 proteins. (A) Western blotting of p-AMPK, PGC-1α and HO-1 protein in the liver. Relative expression of (B) HO-1, (C) PGC-1α and (D) p-AMPK in the liver. Values are presented as mean ± SD. **P<0.01 vs. CON; $^{\$\$}$P<0.01 vs. MOD (n=6). p-, phosphorylated; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1α; HO-1, heme oxygenase 1; AMPK, AMP-activated protein kinase; CON, control; MOD, model; RIB, riboflavin.

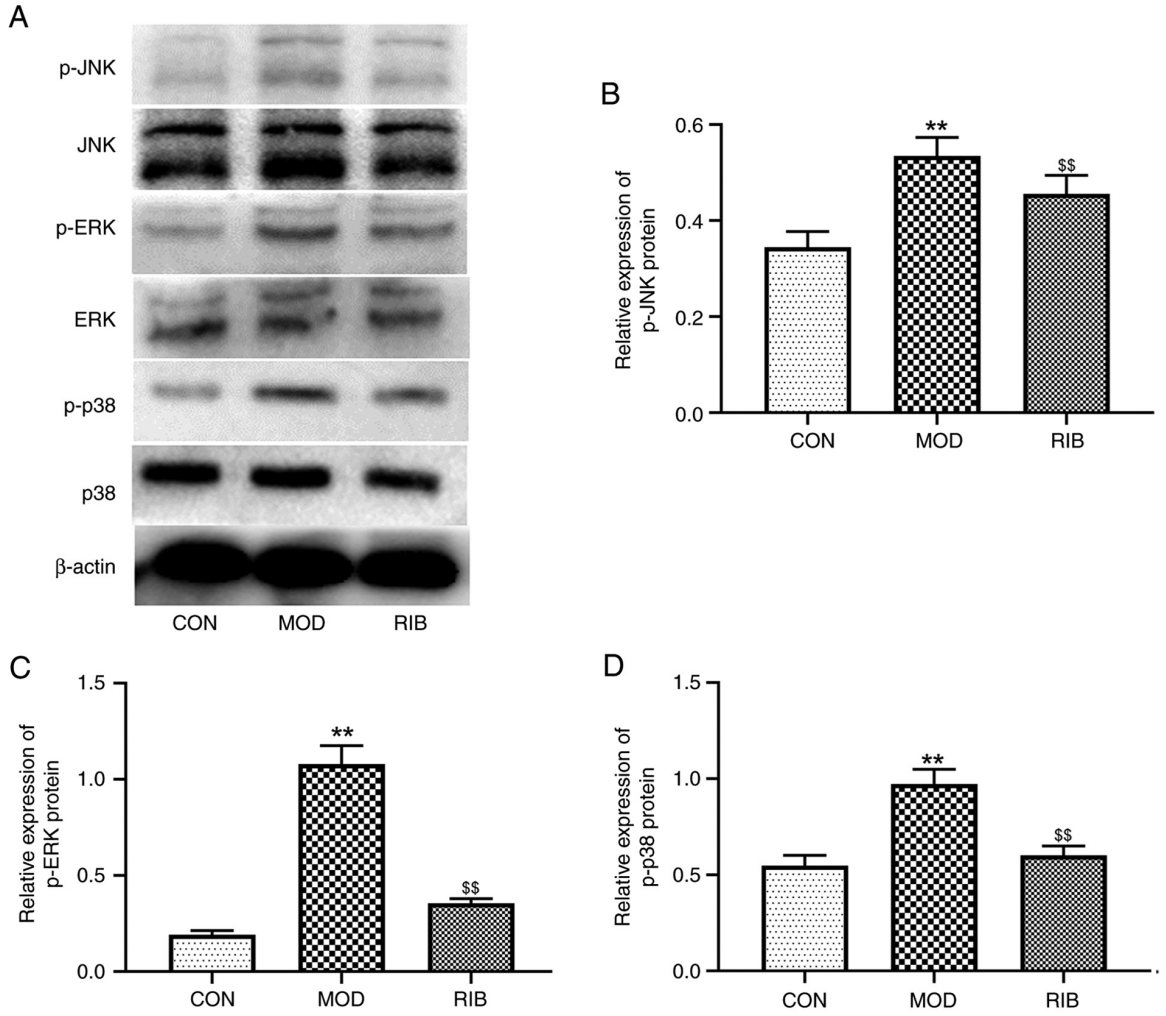


Figure 8. Effects of riboflavin on the expression of MAPK signaling pathway-related proteins. (A) Western blotting of p-JNK, p-ERK and p-p38 in liver tissues. Relative protein expression levels of (B) p-JNK, (C) p-ERK and (D) p-p38 in the liver. Values are presented as mean ± SD. **P<0.01 vs. CON; $^{\$\$}$P<0.01 vs. MOD (n=6). JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase; p-p38, phosphorylated; CON, control; MOD, model; RIB, riboflavin.

study. In the present study, the protective effects of riboflavin against chronic liver injury and the mechanisms underlying this process were evaluated. The results demonstrated that CCl_4 exposure significantly increased the activities of ALT and AST, and the LW/BW ratio. However, riboflavin treatment inhibited or reversed the CCl_4-induced damage, which suggested that riboflavin attenuated CCl_4-induced liver injury.

Excessive production of ROS drives liver fibrosis via acceleration of the activation of HSCs (40). Mitochondria are the main sites of ROS production and the primary ROS targets. Mitochondrial ROS cause lipid peroxidation of the mitochondrial membrane, generating MDA that destroys the mitochondrial structure and disrupts mitochondrial function (41,42), MDA is used as a ROS indicator. SOD and numerous catalases exert their antioxidant functions in the mitochondria by scavenging free radicals, including superoxide anions (43). Liver fibrosis induced by CCl_4 disrupts mitochondrial function and reduces oxidative stress (44). Enhancing mitochondrial function reduces ROS production and increases energy generation (45). In the present study, chronic CCl_4 exposure increased lipid peroxidation, as demonstrated by the significant increase in MDA levels and significantly decreased SOD activity in the liver, which suggested that CCl_4 impaired mitochondrial function. A recent study reported that riboflavin promoted the degradation of superoxide to hydrogen peroxide and improved mitochondrial ROS scavenging (46). The results of the present study demonstrated that riboflavin administration significantly reduced the decrease in SOD activity induced by CCl_4 and decreased the MDA levels. The response to wound healing, such as chronic liver injury caused by ROS, triggers the release of key profibrogenic cytokines, including TGF-β1, which activate HSCs, inducing the generation of α-SMA and the excessive accumulation of ECM, which causes liver fibrosis. Therefore, TGF-β1 and α-SMA are key indicators of fibrosis. The results of the present study demonstrated that riboflavin exerted an anti-fibrotic effect via dysregulation of the expression of profibrotic factors, including TGF-β1 and α-SMA. These findings suggested that riboflavin attenuated liver fibrosis via inhibition or reversal of mitochondrial damage.

The mitochondrion serves an essential role in the regulation of cellular metabolism through oxidative phosphorylation, and riboflavin participates in mitochondrial processes such as the metabolism of amino acids and fatty acids (47,48), which

suggests that riboflavin has an important role in regulation of mitochondria. Indeed, riboflavin treatment improves mitochondrial dysfunction (49). Several studies have reported that activated AMPK stimulates mitochondrial biogenesis through the deacetylation of PGC-1α, the increase of PGC-1α expression and the destruction of the defective mitochondria (50,51). Low protein expression levels of PGC-1α in the liver reduce the expression of mitochondrial genes and impair mitochondrial function (21,52). Furthermore, PGC-1α regulates the function of ROS scavenging enzymes and improves the survival of neurons (53). AMPK-activated PGC-1α induces HO-1 expression and reduces the production of ROS (54,55). It has been reported that HO-1, an antioxidant enzyme, and its catalytic products can relieve oxidative stress and inflammatory response (56), and that the expression of HO-1 is induced by certain antioxidants as well as its own inducer (57-59). Our previous results demonstrated that riboflavin upregulates the expression of HO-1 in the heart (60). Therefore, these results demonstrated that the AMPK/PGC-1α/HO-1 signaling pathway serves a vital role in the regulation of mitochondrial biogenesis and oxidative stress. In the present study, the results demonstrated that chronic CCl_4 exposure significantly decreased the protein expression levels of p-AMPK, PGC-1α and HO-1 in the liver, and that the administration of riboflavin significantly attenuated these changes. These results suggested that riboflavin improved mitochondrial function and oxidative stress via the AMPK/PGC-1α/HO-1 signaling pathway, thus relieving liver fibrosis.

The MAPK signaling pathway regulates numerous cellular processes, including cell proliferation, differentiation and metabolism (61). Several studies reported that suppression of proteins related to the MAPK signaling pathway, including JNK, ERK and p38/MAPK, increased the expression of HO-1, improved oxidative stress and modulated inflammation (57-59). Furthermore, oxidative stress stimulated the activation of the MAPK signaling pathway, including JNKs and ERKs, which induced the overproduction of ROS, which regulated mitochondrion-mediated apoptosis (62-64). The MAPK signaling pathway also participates in the regulation of the expression of collagen and CCl_4-induced liver fibrosis (21,65). TGF-β1 activates HSCs via the p38/MAPK signaling pathway and induces hepatic fibrosis (66), whereas suppressive proteins related to the MAPK signaling pathway, including ERK and JNK, inhibit HSC proliferation and activation (67,68). The present study demonstrated that exposure to CCl_4 significantly increased the relative levels of p-JNK, p-ERK and p-p38/MAPK in the liver. However, riboflavin treatment significantly inhibited the effect of CCl_4 on the protein expression levels of p-JNK, p-ERK and p-p38/MAPK. These results demonstrated that riboflavin reduced oxidative stress and improved CCl_4-induced liver fibrosis by modulation of MAPK expression via the AMPK/PGC-1α/HO-1 signaling pathway.

However, there are several limitations that need to be further explored to clarify the mechanism underlying the effect of riboflavin on CCl_4-induced liver fibrosis. Firstly, the effect of inhibiting the AMPK/PGC-1α/HO-1 signaling pathway on liver fibrosis should be further evaluated. Secondly, the mechanisms underlying the effect of riboflavin on HSC activation and the related signaling should also be explored using an *in vitro* study.

In conclusion, the present study demonstrated that riboflavin attenuated CCl_4-induced liver fibrosis via the AMPK/PGC-1α/HO-1 signaling pathway. Furthermore, riboflavin alleviated oxidative stress and decreased the expression of TGF-β1 and α-SMA in the liver via upregulation of the expression of AMPK, PGC-1α and HO-1, and downregulation of MAPK expression via the AMPK/PGC-1α/HO-1 signaling pathway. These findings suggest that riboflavin is a potential candidate for treating chronic liver injury.

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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

NT and WL performed the laboratory experiments. FH, WH and TTY performed the tissue analyses. GGW, FH and WL collected the data. GGW, NT and WL designed the experiments. GGW, NT, FH and WL analyzed the data. NT, WL and FH supervised the project. FH, GGW, WL and NT confirm the authenticity of all the raw data. GGW, WL and FH drafted the manuscript. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

The protocols for the experiments were approved by the Animal Experimental Ethics Committee of Wannan Medical College (approval no. LISC-2018-001).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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