Basic Study

Morin enhances hepatic Nrf2 expression in a liver fibrosis rat model

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AIM
To investigate whether morin can reduce hepatic fibrosis by activating the NF-E2-related factor 2 (Nrf2) signaling pathway.

METHODS
Twenty male Sprague-Dawley rats were randomly divided into four groups: control group, morin group, carbon tetrachloride (CCl₄) group, and morin + CCl₄ group. Rats in both the CCl₄ and morin + CCl₄ groups were injected intraperitoneally with CCl₄ at a dose of 2 mL/kg twice a week. Rats in both the morin and morin + CCl₄ groups were treated orally with morin at a dose of 50 mg/kg twice a week. Control rats were treated with vehicle only twice a week. At the end-point of the 8 wk of the experimental period, serum AST, ALT, and ALP were measured, and the liver specimens...
We constructed a liver fibrosis rat model of liver fibrosis are significantly related to oxidative stress in which a large number of free radicals lead to cell metabolic disorders and subsequent destruction of normal liver cells. Although there is currently no effective therapy for curing liver fibrosis, previous studies showed that the pathological changes in liver fibrosis could be reversed.

Oxidative stress is closely related to the occurrence of liver disease. A large number of studies have shown that oxidative stress may promote the activation of hepatic satellate cells (HSCs) and increase collagen production. In the past decade, numerous studies proved that NF-E2-related factor 2 (Nrf2) plays a role as an important transcription factor in normal liver cells, and its activation could increase the expression of the downstream specific genes, such as the quinone oxidoreductase 1 (NQO1), heme oxygenase (HO-1), and glutathione, which play a role against oxidative stress. Studies have shown that Nrf2 activation could resist oxidative stress caused by hepatic ischemia and injury, liver fibrosis, and drug-induced liver damage.

Flavonoids are rich in a variety of fruits, vegetables, and components of herbal-containing dietary agents and play an important role in preventing many kinds of diseases. Morin (3, 5, 7, 2′, 4′-pentahydroxyflavone) is a kind of flavonoid that consists of a yellowish pigment found in onion and apple (Figure 1). It has been shown that morin possesses biological properties, including antioxidant,[22–24] anti-inflammatory,[22] anti-apoptosis,[23,24] and anticancer[19] activities. Morin also protects various human cells, such as myoblasts,[25] hepatocytes,[26] and erythrocytes, against oxidative damages.[27]

Carbon tetrachloride (CCl₄) intraperitoneal injection is a classical method for establishing an animal model of hepatic fibrosis, and the toxicity of CCl₄ leads to liver cell necrosis and mitochondrial damage along with aggravating oxidative stress. In addition, the abundant release of inflammatory and fibrogenic cytokines induced by CCl₄ could further augment the degree of hepatic fibrosis. A previous study demonstrated that morin protected against acute liver damage[29] and ameliorated liver fibrosis[30] induced by CCl₄, where morin inhibited proliferation and induced apoptosis of activated HSCs by suppressing the Wnt/β-catenin and NF-κB signaling pathways. However, there is no molecular evidence of the effects of morin on the Nrf2 signaling pathway. To our knowledge, in vivo investigation of the effect of morin on the Nrf2 signaling pathway and Nrf2 expression in the CCl₄-induced liver fibrosis model has not been reported. The purpose of this study was to investigate whether morin could reduce hepatic fibrosis by inducing the expression of Nrf2 and its downstream antioxidant enzymes using pathology as a gold standard in a rat...
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Figure 1 Chemical structure of morin. (https://pubchem.ncbi.nlm.nih.gov/compound/morin).

model of CCl₄-induced hepatic fibrosis.

MATERIALS AND METHODS

Chemicals and reagents
The chemical agents used in this study included CCl₄ and olive oil (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) as well as morin (Sigma Chemical Co., St Louis, MO, United States). Serum aspartate transaminase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The antibodies against Nrf-2, HO-1, NQO1, collagen I, collagen III, and α-SMA were obtained from Proteintech Group Inc. (Chicago, IL, United States). All other reagents used were in the purest form available commercially.

Animals and experimental design
This study was performed in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institutes of Health of China (Guide for the Care and Use of Laboratory Animals, 1996) and was approved by the Animal Care and Use Committee of China Medical University. Twenty male Sprague-Dawley rats with an average body weight of 200-220 g (Changsheng Biotechnology Co., Ltd, Liaoning, China) were used in this study. All rats were fed a standard laboratory diet for a week at room temperature (20-22 °C) with a light/dark cycle of 12 h. Then, the rats were randomly divided into four groups of five rats each, i.e., control group, morin group, CCl₄ group, and morin + CCl₄ group. The control rats were treated with vehicle only (olive oil) equivalent to the treatment group. The rats in the morin group were treated with morin at a dose of 50 mg/kg (suspended in water as previously described[30]) by oral administration and 2 mL/kg of olive oil by intraperitoneal injection twice a week. The rats in the CCl₄ group were injected intraperitoneally with CCl₄ at a dose of 2 mL/kg [mixed with olive oil (40%, V/V)] twice a week. The rats in the morin + CCl₄ group were treated with the same doses of morin and CCl₄ via the same routes as the morin group and the CCl₄ group. Body weights of animals were recorded twice per week. After 8 wk of treatment, animals were kept fasting for 24 h. Under 10% chloral hydrate anesthesia, the following procedures were performed, including obtaining blood samples from the heart for biochemical tests and resecting the liver and spleen for histopathological analysis. Liver tissues were weighted and cut in 10 mm × 10 mm × 3 mm pieces. Half of the specimen was fixed in 10% formaldehyde for histopathology and the other half was immediately frozen in -80 °C for PCR and Western blot tests.

Biochemical analysis
The blood samples were centrifuged at 3000 g for 10 min at 20 °C, and the serum was collected from the supernatant. The values of AST, ALT, and ALP were measured using commercial assay kits according to the manufacturer’s protocols.

Histopathological assessment
Specimens of the liver were embedded in paraffin and cut into 5-µm-thick sections after 24 h of fixation. Then, the samples were stained with hematoxylin and eosin (HE). The degree of liver fibrosis was analyzed and determined by an experienced pathologist. The liver fibrosis was categorized into five degrees, i.e., F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis with rare septa, F3 = numerous septa without cirrhosis, and F4 = cirrhosis according to reference criteria[31].

Quantitative real-time PCR
Total cellular RNA was extracted from tissues using TRIzol (Invitrogen). Reverse transcription of 1 µg of RNA was done using RT regents (TAKARA) following the manufacturer’s instructions. Quantitative real-time PCR was done using SYBR Green PCR master mix (Applied Biosystems) in a total volume of 20 µL on the 7900HT fast Real-time PCR system (Applied Biosystems) using the following cycling parameters: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. GAPDH was used as the reference gene. The relative levels of gene expression were represented as ΔCt = CtGene - CtReference, and the fold change of gene expression was calculated by the 2-ΔΔCt method. Experiments were repeated in triplicate. The primer sequences are listed in Table 1.

Western blot analysis
Total proteins from tissues were extracted in lysis buffer (Pierce, United States) and quantified using the Bradford method. A total of 40 µg of protein were separated using 10% SDS-PAGE (80 V -120 V) and then electrophoretically transferred to a PVDF membrane.
Table 1 Primer sequences

| Name                  | Primer sequence                  |
|-----------------------|----------------------------------|
| Rat Collagen I for    | 5'-ACTGGTACATCCGCGCAAACCC-3'     |
| Rat Collagen I rev    | 5'-GGAATCCATCGTGATGCTT-3'        |
| Rat Collagen II for   | 5'-GAGCTCCTCCCATATGATGCTTGC-3'  |
| Rat Collagen II rev   | 5'-AGCAAACAGGCCAAGTCTCC-3'       |
| Rat α-SMA for         | 5'-GCTGTAACCTTGATGCTCAGG-3'      |
| Rat α-SMA rev         | 5'-CAGCCTCAGAGCTAGTCACGAA-3'     |
| Rat NQO1 for          | 5'-GCCAGAGAGCAGCATCATT-3'        |
| Rat NQO1 rev          | 5'-CCACGCAGACATGAGCCGAC-3'       |
| Rat HO-1 for          | 5'-GCTGTAACCTTGATGCTCAGG-3'      |
| Rat HO-1 rev          | 5'-CTTCCGAGGAGCATGCAAC-3'        |
| Rat Gapdh for         | 5'-GCTGTCACCTTGATGCTCAGG-3'      |
| Rat Gapdh rev         | 5'-GCCAGAGAGCAGCATCATT-3'        |

Figure 2 Changes in body weight among different groups. Body weight increased observably in the control and morin groups. The CCl group had slow weight growth, but morin treatment was associated with increased body weight.

(80 V 100 min) (Millipore, Bedford, MA, United States). The membrane was blocked with 5% dry milk and incubated overnight at 4 °C with antibodies against HO-1 (1:800; Proteintech), NQO-1 (1:1000; Proteintech), Nrf2 (1:800; Proteintech), collagen I (1:800, Proteintech), collagen III (1:1000, Proteintech), α-SMA (1:1000, Proteintech), and GAPDH (1:4000, Proteintech). After washing, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) at 37 °C for 2 h. Protein bands were visualized by enhanced chemiluminescence (Pierce) and detected using BioImaging Systems (UVP, Upland, CA, United States). The relative protein levels were calculated based on GAPDH protein as a loading control. Western blot images were measured with ImageJ software, and the relative gray values of protein expression were analyzed semi-quantitatively.

Statistical analysis
The experimental data are expressed as the mean ± SD. Statistical analyses were performed using one-way analysis of variance (ANOVA) between groups, and unpaired comparisons were analyzed using the least significant difference method LSD t-test. A P-value of 0.05 or less was considered statistically significant.

RESULTS

General observation
A total of four rats died before the end-point of the study, including two in the CCl group, one in the morin + CCl group, and one in the morin group. All animals in the control group survived. Normal diet and daily activities were recorded in the control and morin groups, with body weight increasing rapidly. The CCl group presented poor feeding and daily activities with slow weight growth. The morin + CCl group presented milder symptoms compared with the CCl group, with increased body weight, which was, however, lower than that in the control and morin groups (Figure 2).

Histological changes in the liver
The results of HE staining showed that the liver cells appeared with a normal morphology and regular lobular structure in the control and morin groups. The liver tissue of CCl group rats showed inflammatory cell infiltration, with portal and central veins surrounded by fibrous tissue accompanied by fibrous septa. The lobular structure was fuzzy with clearly visible false lobules. In the morin + CCl group, the liver tissue demonstrated less hyperplasia of fiber tissue and minimal inflammatory cells compared to the CCl group (Figure 3A-D).

Liver-spleen ratio and liver weight index
Both the CCl and morin + CCl groups had increased liver-spleen ratio (LSR) and liver weight index (LWI) compared with the control and morin groups (P < 0.05). The LWI between the CCl and morin + CCl groups showed a significant difference (P < 0.05), while no statistically significant difference was found for LSR (P > 0.05) (Table 2).

Biochemical findings
The CCl and morin + CCl groups had increased ALT, AST, and ALP levels compared to the control and morin groups (P < 0.05), and CCl without morin treatment dramatically increased ALT, AST, and ALP values (Table 3).

mRNA expression of α-SMA, collagen I, collagen III, Nrf2, HO-1, and NQO1
Compared with the control and morin groups, significantly higher mRNA expression of α-SMA, collagen I, and collagen III was observed in liver tissues in the CCl and morin + CCl groups (P < 0.05). However, the mRNA expression of these molecules in the morin + CCl group was significantly less than that in the CCl group (P < 0.05) (Figure 4).

In the CCl and morin + CCl groups, mRNA expression...
values of \textit{NQO1}, \textit{HO-1}, and \textit{Nrf2} were significantly higher than those in the control and morin groups ($P < 0.05$), while these mRNA values of the morin + CCl\textsubscript{4} rats were significantly different compared to those of the CCl\textsubscript{4} group ($P < 0.05$) (Figure 5).

\textbf{Protein expression of $\alpha$-SMA, collagen I, collagen III, Nrf2, HO-1, and NQO1}

Compared with the control and morin groups, high expression of protein of $\alpha$-SMA, collagen I, and collagen III in liver tissues in the CCl\textsubscript{4} and morin +
CCl4 groups had a statistically significant difference ($P < 0.05$). However, the morin + CCl4 group had less expression of these protein factors compared to the CCl4 group ($P < 0.05$) (Figure 6).

In the CCl4 and morin + CCl4 groups, the protein expression of Nrf2, HO-1, and NQO1 was statistically higher than that in the control and morin groups ($P < 0.05$), while these protein factors of the morin + CCl4 rats had more expression compared to the CCl4 group ($P < 0.05$) (Figure 7).

**DISCUSSION**

Liver fibrosis is a process of continuous damage to the liver blood vessels and hepatic cells with nodule formation, which may develop into cirrhosis and cancerous lesions. Research of fibrosis at the cellular and molecular levels suggested that the progression of liver injury was closely related to oxidative stress and lipid peroxidation$^{[32,33]}$, leading to cell destruction and inducing hepatic fibrosis. HSCs can be activated by lipid peroxides acting as products of cell damage. After HSC activation, lipid droplets and vitamin A in the cytoplasm could be reduced or exhausted with $\alpha$-SMA expression, accompanied by liver structural and functional changes resulting from redundant secretion of ECM$^{[34]}$. However, it is possible to reverse liver fibrosis and early cirrhosis with effective interventions. Previous studies have shown that antioxidants have a protective effect by inhibiting the expression of $\alpha$-SMA in HSC$^{[35]}$, thus, inhibition of oxidative stress in the liver may reduce and even reverse liver fibrosis$^{[36]}$.

Pathological features of liver fibrosis are reflected by fibrous tissue hyperplasia around the portal area and central vein and forming an interval of destruction of the lobular structure, accompanied by regenerative nodules and even early cirrhosis$^{[37]}$. The pathological findings in this study showed that liver tissue in the CCl4 group had liver cell necrosis, fibrous tissue hyperplasia, interval widening, and pseudolobuli replacing normal lobular architecture. In the morin + CCl4 group, the liver tissue showed minimal cell necrosis with less interstitial collagen fibers and lobular structure damage compared with the CCl4 group. Thus, morin could effectively protect the liver tissue by reducing inflammation and inhibiting collagen deposition and fiber hyperplasia.

There are various enzymes that take part in liver metabolism. The damaged liver cells by pathogenic factors will produce free enzymes that are released into the bloodstream$^{[20]}$. Liver function and status could be assessed by assaying the contents of serum enzymes. Aminotransferases play an important role in
hepatic metabolism. When the liver cells are damaged, the serum ALT and AST levels as well as ALP level will be increased\[38\]. In this study, in the CCl\(_4\)-induced liver fibrosis rat model, the values of serum ALT, AST, and ALP were reduced with morin administration, which implied that morin can reduce liver cell injury and thus prevent liver fibrosis. This also gives support for morin being able to condition the hepatocytes, protect against membrane frailty, and decrease the outflow of enzymes into circulation. These results are in accordance with previous studies that showed the ability of morin to inhibit hepatotoxicity\[39,40\].

The amount of collagen accounts for 5%-10% of the total protein in human liver tissue. If the liver injury leads to fibrosis, the collagen content in the liver protein will be significantly increased up to approximately 50%, becoming an important component of ECM\[41\] and ultimately leading to irreversible cirrhosis changes\[42\]. Liver fibrosis is a common histological change in liver disease, which is mainly manifested by excessive deposition of ECM, such as type I and type III collagen, and the expression of \(\alpha\)-SMA\[43\]. At present, it is believed that the ECM actively participates in the occurrence and development of fibrosis, which has a great influence on HSC activation\[44-46\]. Both in vitro and in vivo experiments found that ECM synthesis was increased when liver tissue was damaged and further caused the activation of HSCs, which was based on the secretion of type I and III collagen\[47-49\], ultimately promoting the occurrence of liver fibrosis. In our study, using both real-time PCR and Western blot methods, it was found that the control and morin groups had only minimal expression of collagen I, collagen III, and \(\alpha\)-SMA, which may represent normal physiological function of the liver, while their expression in the CCl\(_4\) group was significantly increased and had great relevance to the severity of liver fibrosis. With morin intervention reducing the expression of collagen I, collagen III, and \(\alpha\)-SMA, the degree of liver fibrosis was relieved, which was evidenced by liver histopathology and serum measurements. All these results suggested that the anti-fibrotic effect of morin may be related to the down-regulation of the expression of collagen I, collagen III, and \(\alpha\)-SMA.

Nrf2 is a key nuclear transcription factor in the oxidative stress of various cells\[50\]. Under normal circumstances, Nrf2 and Keap1 are in a binding state in the cytoplasm\[51\]; they will appear dissociated when oxidative stress is occurring\[52\] and combine with antioxidant components as dimers, which are...
involved in the synthesis of antioxidase and phase II detoxification enzymes and prevent the occurrence of liver fibrosis by improving the antioxidant capacity of the liver\(^{[53]}\). HO-1 and NQO-1 are well characterized Nrf2-dependent antioxidant defense genes. Studies have suggested that Nrf2 and its downstream antioxidant factors HO-1 and NQO1 may contribute to improvement of liver fibrosis\(^{[54]}\). It has been reported that morin could promote the nuclear translocation of Nrf2 in order to play its biological role and be used as an exogenous agonist of Nrf2\(^{[55]}\). In this study, a CCl\(_4\) induced liver fibrosis model, along with morin as an intervention, was used to observe the expression of Nrf2 and its downstream products NQO1 and HO-1 in different groups. The results showed that the expression of Nrf2, NQO1, and HO-1 was slightly increased in the CCl\(_4\) group compared with the control and morin groups (\(P < 0.05\)), and the expression levels in the morin + CCl\(_4\) group were lower than those in the CCl\(_4\) group (\(P < 0.05\)).

This study has several limitations. First, the sample size was small, which easily led to individual differences and statistical error between the groups. Second, the anti-fibrotic mechanism of morin may be related to activation of the Nrf2 antioxidant pathway and expression of its downstream antioxidases. Further experiments are needed to confirm the specific mechanism of the morin intervention.

In summary, our current study showed that morin could play a protective role by inducing the expression of Nrf2 and its downstream antioxidant factors (HO-1 and NQO1) and reducing the expression of \(\alpha\)-SMA, collagen I, and collagen III in a rat model of CCl\(_4\)-induced hepatic fibrosis. Although further studies are required, our study demonstrated that morin could effectively alleviate chronic liver damage by activation of the Nrf2 pathway.

**ARTICLE HIGHLIGHTS**

**Research background**

Previous studies have shown that the pathological changes of liver fibrosis, which refer to a series of pathogenic factors and pathological changes in the pathogenesis of a variety of liver diseases, could be reversed. In the past decade, numerous studies demonstrated that NF-E2-related factor 2 (Nrf2) as a transcription factor plays as an important role against oxidative stress in normal liver cells. Morin possesses biological properties, including antioxidant, anti-inflammatory, anti-apoptosis, and anticancer activities. To our knowledge,
Morin enhances hepatic Nrf2 expression

Figure 7  The protein expression of Nrf2, HO-1, and NQO1. (1, 2) control group, (3, 4) morin group, (5, 6) CCl4 group, (7, 8) morin + CCl4 group. *P < 0.05 vs control group; †P < 0.05 vs morin group, ‡P < 0.05 vs CCl4 group. In the CCl4 and morin + CCl4 groups, the protein expression was increased compared to the control and morin groups (P < 0.05); the morin + CCl4 group had a more significant change compared to the CCl4 group (P < 0.05).

Research motivation
Previous studies demonstrated that morin protected acute liver damage and ameliorated liver fibrosis induced by CCl4, and morin inhibited proliferation and induced apoptosis of activated hepatic satellite cells by suppressing the Wnt/β-catenin and the NF-kB signaling pathways. However, there is no molecular evidence about the effects of morin on the Nrf2 signaling pathway.

Research objectives
The purpose of this study was to investigate whether morin can reduce hepatic fibrosis by inducing the expression of Nrf2 and its downstream antioxidant factors (HO-1 and NQO1) in a rat model of CCl4-induced hepatic fibrosis.

Research methods
Twenty male Sprague-Dawley rats were randomly divided into four groups: control group, morin group, carbon tetrachloride (CCl4) group, and morin + CCl4 group. At the end-point of the experimental period, serum AST, ALT, and ALP were measured, and the liver specimens were obtained for pathological assessment.

Research results
Rats in the morin + CCl4 group had less hyperplasia of fiber tissues, minimal inflammatory cells, and less body weight loss with favorable liver enzyme measurements compared to rats treated with CCl4 only. Additionally, morin-treated rats had significantly lower mRNA and protein expression of α-SMA, collagen I, and collagen III, but significantly higher mRNA and protein expression of Nrf2, HO-1, and NQO1 compared to rats treated with CCl4 only (P < 0.05).

Research conclusions
Our study showed that morin could play a protective role by inducing the expression of Nrf2 and its downstream antioxidant factors (HO-1 and NQO1) and reducing the expression of α-SMA, collagen I, and collagen III in a rat model of CCl4-induced hepatic fibrosis.

Research perspectives
Although further studies are required, our study demonstrated that morin could effectively alleviate chronic liver damage by activation of the Nrf2 pathway.

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