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Basic Study

Yinhuang granule alleviates carbon tetrachloride-induced liver fibrosis in mice and its mechanism

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Abstract

BACKGROUND

Liver fibrosis is a formidable global medical challenge, with no effective clinical treatment currently available. Yinhuang granule (YHG) is a proprietary Chinese medicine comprising *Scutellariae* Radix and *Lonicerae Japonicae* Flos. It is frequently used for upper respiratory tract infections, pharyngitis, as well as acute and chronic tonsillitis.

AIM

To investigate the potential of YHG in alleviating carbon tetrachloride (CCl_4)-induced liver fibrosis in mice.

METHODS

To induce a hepatic fibrosis model in mice, this study involved intraperitoneal injections of 2 mL/kg of CCl_4 twice a week for 4 wk. Meanwhile, liver fibrosis mice in the low dose of YHG (0.4 g/kg) and high dose of YHG (0.8 g/kg) groups were orally administered YHG once a day for 4 wk. Serum alanine/aspartate aminotransferase (ALT/AST) activity and liver hydroxyproline content were detected. Sirius red and

Masson's trichrome staining assay were conducted. Real-time PCR, western-blot and enzyme-linked immunosorbent assay (ELISA) were conducted. Liver glutathione (GSH) content, superoxide dismutase (SOD) activity level, reactive oxygen species (ROS) and protein carbonylation amount were detected.

RESULTS

The administration of YHG ameliorated hepatocellular injury in CCl_4-treated mice, as reflected by decreased serum ALT/AST activity and improved liver histological evaluation. YHG also attenuated liver fibrosis, evident through reduced liver hydroxyproline content, improvements in Sirius red and Masson's trichrome staining, and lowered serum hyaluronic acid levels. Furthermore, YHG hindered the activation of hepatic stellate cells (HSCs) and ameliorated oxidative stress injury and inflammation in liver from CCl_4-treated mice. YHG prompted the nuclear accumulation of nuclear factor erythroid 2-related factor 2 (Nrf2) and upregulated the expression of Nrf2-dependent downstream antioxidant genes. In addition, YHG promoted mitochondrial biogenesis in liver from CCl_4-treated mice, as demonstrated by increased liver adenosine triphosphate (ATP) content, mitochondrial DNA (mtDNA) levels, and the expression of peroxisome proliferator-activated receptor gamma coactivator 1alpha (PGC1α) and nuclear respiratory factor 1 (NRF1).

CONCLUSION

YHG effectively attenuates CCl_4-induced liver fibrosis in mice by inhibiting the activation of HSCs, reducing inflammation, alleviating liver oxidative stress damage through Nrf2 activation, and promoting liver mitochondrial biogenesis.

INTRODUCTION

Liver fibrosis is a complex process of continuous hepatic injury and subsequent tissue repair in response to various types of chronic liver insults, resulting in the pathological accumulation of extracellular matrix (ECM) components within the hepatic

microenvironment [1,2]. In the absence of timely intervention, the relentless cycle of liver injury and futile regeneration persists, ultimately leading to the gradual progression of liver fibrosis into advanced cirrhosis and the potential development of hepatocellular carcinoma [1,2]. Notably, liver fibrosis can arise from diverse etiologies, encompassing viral hepatitis, alcoholic liver disease, non-alcoholic steatohepatitis, cholestasis, autoimmune hepatitis, *etc.* [3,4]. Epidemiological data suggest that liver fibrosis affects approximately 18.0%-27.0% of individuals afflicted by various chronic liver diseases [5]. Indeed, liver fibrosis represents a significant global health concern, underscored by the current absence of an efficacious pharmaceutical intervention in clinical practice.

A plethora of studies have underscored the pivotal role of hepatic stellate cells (HSCs) activation in the progression of liver fibrosis [1,6,7]. Activated HSCs manifest an exuberant production of diverse ECMs including fibronectin, proteoglycan, collagen I, and laminin, culminating in the formation of scar in liver tissue [1,7]. Furthermore, activated HSCs secrete pro-inflammatory cytokines and chemokines, thereby recruiting immune cells from the periphery into the liver, thus exacerbating hepatic inflammatory injury [8,9]. Aside from inflammation, the significance of oxidative stress-induced liver injury in the relentless progression of liver fibrosis has been underscored for decades, fostering the notion that enhancing cellular antioxidant capacity may present a promising therapeutic avenue for liver fibrosis management [10,11].

With the continuous deepening of research, there is increasing evidence that numerous traditional Chinese patent medicines, natural products and ingredients have demonstrated efficacy in effectively ameliorating liver injury and treating liver dieaseas [12-19]. Yinhuang granule (YHG) is a Chinese patent medicine comprising *Scutellariae* Radix and *Lonicerae Japonicae* Flos. Previous study has demonstrated the potential hepatoprotective effects of the individual components of YHG, with the water extract of *Lonicerae Japonicae* Flos ameliorating liver fibrosis in CCl_4-treated mice, and the methanol extract of *Scutellariae* Radix inhibiting liver fibrosis induced by bile duct ligation or CCl_4 in rats [17,18]. Additionally, baicalin and chlorogenic acid, the primary bioactive compounds within YHG, have also exhibited promising hepatoprotective

effects against liver fibrosis [19-23]. Although YHG is traditionally employed for the management of chronic and acute tonsillitis or pharyngitis, as well as upper respiratory tract infections in clinical practice in China, its potential application for the therapy of liver fibrosis remains unexplored. The study aims to investigate the hepatoprotective effects of YHG against liver fibrosis induced by CCl_4 in mice and to uncover the underlying mechanisms through which YHG exerts its protective actions.

MATERIALS AND METHODS

Reagents

YHG was provided by Prof. Lili Ji, Institute of Chinese Medicine, Shanghai University of Traditional Chinese Medicine. The reagents used in this study are listed in Table 1.

Experimental animals

SPF male C57BL/6 mice (20±2 g), obtained from the Shanghai Experimental Animal Center of Chinese Academy of Sciences, were kept at a controlled environment, and received humane care following the institutional animal care guidelines approved by the Experimental Animal Ethical Committee of Shanghai University of Traditional Chinese Medicine (Approval No. PZSHUTCM190912010).

Mice were divided into 5 groups (n = 6 per group) including control group, CCl_4 model group, CCl_4+YHG (0.4 g/kg) group, CCl_4+YHG (0.8 g/kg) group, YHG (0.8 g/kg) group. CGA (dissolved in 0.5% CMC-Na solution) was orally administered to mice every day, and CCl_4 (mixed 1:3 in olive oil, 2 mL/kg) was i.p. injected into mice twice a week for a total of 4 wk. The selection of the CCl_4 dose followed a previous study [24]. Following the treatment period, the mice were euthanized, and samples were collected for subsequent analysis.

Liver histological observation

Liver samples were sectioned and stained with H&E, Sirius red and Masson's trichrome for histological evaluation of liver injury and hepatic collagen deposition.

Measurement of Serum alanine/aspartate aminotransferase (ALT/AST) activity, liver hydroxyproline content, glutathione (GSH), adenosine triphosphate (ATP), superoxide dismutase (SOD), activity protein carbonylation amounts and Enzyme-linked immunosorbent assay (ELISA)

We performed these experiments following the manufacturer's instructions.

Hepatic reactive oxygen species (ROS) amount analysis

Hepatic ROS level was measured previously described [25].

Mitochondrial DNA extraction

Mitochondrial DNA was extracted following the manufacturer's instruction.

Real-time PCR analysis

Real-time PCR was performed as previously described [25]. The primer sequences are shown in Table 2.

Western-blot analysis

Western-blot was detected as previously described [25]. The quantification of protein bands was standardized by calculating the average ratio of integrated optical density. Internal controls such as β-Actin or Lamin B1 expression were used for normalization, and further standardized to the control group.

Statistical analysis

The data is presented as the Mean ± SEM. Group differences were assessed using non-parametric one-way ANOVA, followed by the least significant difference post hoc test when ANOVA indicated a significant F-value and homogeneity of variance. In cases where homogeneity of variance was not met, the Mann-Whitney U non-parametric ANOVA was employed. Statistical significance was set at $P < 0.05$.

RESULTS

YHG reduced liver injury induced by CCl_4 in mice.

As depicted in Figure 1A, YHG (0.4, 0.8 g/kg) effectively decreased the elevated serum ALT activity in CCl_4-treated mice. Furthermore, YHG at a dosage of 0.8 g/kg also effectively decreased the elevated serum AST activity in CCl_4-treated mice (Figure 1B). Notably, YHG (0.8 g/kg) did not exert any impact on ALT or AST activity alone (Figure 1A,B). Evaluation of liver histology unveiled that CCl_4 administration induced obvious liver injury in mice, which was characterized by immune cell infiltration, as well as hepatocyte swelling and necrosis (Figure 1C). However, YHG (0.4, 0.8 g/kg) effectively alleviated these pathological changes.

YHG reduced hepatic collagen deposition and the increased serum hyaluronic acid content in CCl_4-treated mice.

As shown in Figure 2A, YHG (0.8 g/kg) decreased the increased hydroxyproline content in liver of CCl_4-treated mice. Additionally, YHG (0.4, 0.8 g/kg) significantly reduced the increased serum hyaluronic acid levels induced by CCl_4 (Figure 2B). YHG (0.8 g/kg) alone did not affect liver hydroxyproline content or serum hyaluronic acid levels (Figure 2A-B). Furthermore, as depicted in Figs.2C-D, the treatment with YHG (0.4, 0.8 g/kg) effectively decreased hepatic collagen deposition in CCl_4-treated mice. It's worth noting that YHG (0.8 g/kg) alone did not induce any significant changes in the staining patterns, as demonstrated by Masson's trichrome staining and Sirius red staining.

YHG reduced HSCs activation in CCl_4-treated mice.

Fig.3A illustrated that YHG (0.4, 0.8 g/kg) significantly reduced the enhanced hepatic of Col1a1, Col3a1, and fibronectin (Fn1) mRNA expression in CCl_4-treated mice. Additionally, YHG (0.4, 0.8 g/kg) significantly attenuated the increased hepatic mRNA expression of transforming growth factor (TGF)-β in CCl_4-induced mice (Figure 3B). The typical biomarker for HSCs activation, a-smooth muscle actin (a-SMA), showed

reduced hepatic mRNA and protein expression upon treatment with YHG (0.4, 0.8 g/kg) in CCl_4-treated mice (Figure 3B, Figs.3C-D).

YHG ameliorated hepatic oxidative stress damage and inflammation induced by CCl_4 in mice.

As demonstrated in Figure 4A, CCl_4 caused a decline in hepatic GSH content in mice, which was reversed by YHG (0.4, 0.8 g/kg). Furthermore, as depicted in Figs.4B-C, YHG (0.4, 0.8 g/kg) effectively reduced the increased levels of hepatic ROS and liver protein carbonylation in CCl_4-induced mice. Moreover, CCl_4 decreased hepatic SOD activity in mice, which was restored by YHG (0.8 g/kg) (Figure 4D). Additionally, Figure 4E shows that YHG (0.4, 0.8 g/kg) suppressed the hepatic mRNA expression of TNFα, IL-1β, IL-6, and inducible nitric oxide synthase (iNOS) in mice treated with CCl_4.

YHG induced the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) antioxidant signaling pathway in CCl_4-treated mice.

As demonstrated in Figs.5A-B, YHG (0.8 g/kg) promoted the nuclear accumulation of Nrf2 in livers from mice exposed to CCl_4. Additionally, YHG (0.8 g/kg) increased hepatic mRNA expression of glutamate-cysteine ligase (GCLC), modifier subunit of glutamate-cysteine ligase (GCLM) and NAD(P)H:quinone oxidoreductase-1 (NQO1). Furthermore, YHG (0.4 g/kg) also elevated mRNA expression of GCLM in livers of mice exposed to CCl_4 (Figure 5C). Notably, YHG (0.8 g/kg) increased the hepatic protein expression of GCLC, GCLM, and NQO1 in livers of mice exposed to CCl_4 (Figs.5D-E).

YHG induced mitochondrial biogenesis in livers from CCl_4-treated mice.

As depicted in Figure 6A, YHG (0.4, 0.8 g/kg) obviously elevated the decreased expression of hepatic mtDNA copy in liver from mice exposed to CCl_4. Additionally, YHG (0.4, 0.8 g/kg) significantly increased ATP content in liver from CCl_4-treated mice (Figure 6B). Furthermore, YHG (0.4, 0.8 g/kg) elevated the reduced hepatic expression

of peroxisome proliferator-activated receptor gamma, coactivator 1alpha (PGC1a) protein, while YHG (0.8 g/kg) enhanced the decreased expression of nuclear respiratory factor1 (NRF1) protein in livers from CCl_4-induced mice (Figs.6C-D).

DISCUSSION

YHG has excellent anti-inflammatory capacity and is generally used in clinic for clearing hotness and wind, and pharyngeal detoxification. In this study, YHG was demonstrated to alleviate hepatocellular injury, hepatic collagen deposition, and inflammation in CCl_4-treated mice. It also showed inhibitory effects on HSCs, as evidenced by the reduction in the elevated hepatic expression of α-SMA, a key indicating HSCs transdifferentiation and activation [26]. The enhanced expression of ECM components including Col1a1, Col3a1, and Fn1 in the livers of CCl_4-treated mice was decreased by YHG. Furthermore, YHG reduced the elevated expression of TGFβ, a predominant pro-fibrogenic molecule [27], in the livers of CCl_4-treated mice. These findings collectively highlight the immense potential of YHG in the clinical treatment of liver fibrosis.

Recent studies have discovered novel pathways and signals that play significant roles in regulating the activation of HSCs during the progression of liver fibrosis, including oxidative stress and inflammatory responses [28]. Oxidative stress is characterized by an imbalance between the production of ROS and the antioxidant system's ability to scavenge these harmful molecules. Free radicals generated during oxidative stress have been shown to induce the activation and proliferation of HSCs [29,30]. In this study, YHG was found to reduce the elevated hepatic levels of ROS and protein carbonylation, as well as restore the diminished hepatic GSH content and SOD activity in mice treated with CCl_4. Furthermore, YHG was found to reduce the elevated hepatic expression of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6, and iNOS. These findings collectively suggest that YHG has the ability to alleviate hepatic oxidative stress injury and inflammatory response in CCl_4-treated mice, which may contribute to its potential in alleviating CCl_4-induced liver fibrosis in mice.

Nrf2 serves as the principal transcription factor that plays a crucial role in regulating the expression of various downstream antioxidant enzymes and cytoprotective genes [31]. Numerous studies have demonstrated that enhancing Nrf2 activation to combat liver oxidative stress injury is crucial for alleviating liver fibrosis, as observed with various natural compounds such as schisandrin B, asiatic acid, Xiaochaihutang, stevia, tanshinol, and hyperoside [32-37]. In CCl_4-treated mouse livers, the nuclear accumulation of Nrf2 was decreased, but YHG was able to rescue this reduction. GCLC, GCLM, and NQO1 are known as downstream antioxidant enzymes regulated by Nrf2 [38]. The elevated hepatic expression of GCLC, GCLM, and NQO1 in CCl_4-treated mice following YHG administration indicates that YHG activates the transcription of Nrf2. The activation of Nrf2 is likely responsible for the protection against CCl_4-induced oxidative stress damage in the livers in these mice. Nrf2-regulated genes, such as those involved in the synthesis of GCLC, GCLM and NQO1, are crucial for combating oxidative stress and maintaining liver health.

Mitochondria play a core role in the production of energy and cellular metabolism, and their dysfunction can lead to a range of health issues. To maintain mitochondrial health and overall cellular function, a balance between mitochondrial turnover, fission and fusion processes, and the promotion of mitochondrial biogenesis is indeed crucial. Mitochondrial biogenesis involves the generation of the new mitochondria to replace damaged ones and maintain cellular energy production. This process helps ensure that cells have a healthy population of mitochondria and can effectively meet their energy demands [39]. Recent studies have shown that inducing mitochondrial biogenesis is beneficial in alleviating liver fibrosis in rats with secondary biliary cirrhosis or treated with carbon tetrachloride [40,41], as well as in mice with diet-induced obesity and non-alcoholic steatohepatitis [42]. Additionally, resveratrol has been reported to induce HSCs death through apoptosis, autophagy/mitophagy, and mitochondrial biogenesis [43]. The transcription of mtDNA holds a pivotal role in the process of mitochondrial biogenesis, and PGC1α and NRF1 tightly regulate this mechanism [39,44]. Furthermore, Nrf2 not only assumes a central role in protecting against oxidative stress injury but also enhances the

structural and functional integrity of mitochondria under stress conditions [45]. It has been reported that Nrf2 enhances the expression of NRF1 by binding to its promoter sites [46]. In this study, YHG was found to enhance hepatic ATP levels, increase the reduced mtDNA content, and improve the decreased expression of PGC1α and NRF1 in CCl_4-treated mice. These findings imply that YHG promotes mitochondrial biogenesis in CCl_4-induced liver fibrosis in mice, which contributes to its protective effects against liver fibrosis.

CONCLUSION

YHG effectively alleviated liver fibrosis induced by CCl_4 in mice *via* various mechanisms, including the inhibition of HSCs activation, reduction of inflammation, alleviation of liver oxidative stress damage by promoting Nrf2 activation, and promotion of liver mitochondrial biogenesis. These findings suggest that YHG has immense promise for clinical utilization in the management of liver fibrosis.

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