Original Research

Hepatoprotective effect of genistein against dimethylnitrosamine-induced liver fibrosis in rats by regulating macrophage functional properties and inhibiting the JAK2/STAT3/SOCS3 signaling pathway

Ying Xu\textsuperscript{1,2,†}, Dingqi Zhang\textsuperscript{2,†}, Hailin Yang\textsuperscript{2}, Yu Liu\textsuperscript{2}, Linzhang Zhang\textsuperscript{1,2}, Congcong Zhang\textsuperscript{1,2}, Gaofeng Chen\textsuperscript{2}, Yonghong Hu\textsuperscript{2}, Jiamei Chen\textsuperscript{2}, Hua Zhang\textsuperscript{2}, Yongping Mu\textsuperscript{2}, Ping Liu\textsuperscript{1,2,*}, Wei Liu\textsuperscript{1,2,*}

\textsuperscript{1}Experiment Center of Teaching & Learning, Institute of Chinese Materia Medica, Institute of Interdisciplinary Integrative Medicine Research, Shanghai University of Traditional Chinese Medicine, 201203 Shanghai, China, \textsuperscript{2}Institute of Liver Diseases, Key Laboratory of Liver and Kidney Diseases (Ministry of Education), Shanghai Key Laboratory of Traditional Chinese Clinical Medicine, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, 201203 Shanghai, China

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
   3.1 Main materials
   3.2 Animals and experiment design
   3.3 Cell culture and treatment
   3.4 CCK-8 cell viability assay
   3.5 EdU cell proliferation assay
   3.6 Cell cycle analysis
   3.7 Serological index and hepatic hydroxyproline (Hyp) content assays
   3.8 Histopathological assay
   3.9 Immunohistochemistry assay
   3.10 Immunofluorescent assay
   3.11 Western blot analysis
   3.12 Quantitative real-time PCR (qRT-PCR) analysis
   3.13 Statistical analysis
4. Results
   4.1 Genistein regulates the cell viability, proliferation, and cell-cycle arrest in LX2 cells
   4.2 Genistein inhibits the expressions of α-SMA and Col1A1 in LX2 cells
   4.3 Genistein ameliorates liver injury and collagen deposition in DMN-induced hepatic fibrosis rats
   4.4 Genistein regulates the expressions of α-SMA, Col1A1, MMP2/9, and TIMP1 in DMN-induced rats
   4.5 Genistein regulates inflammatory infiltration and macrophage functional properties in rats induced by DMN
   4.6 Genistein inhibits the JAK2/STAT3/SOCS3 signaling pathway
5. Discussion
6. Conclusions
7. Author contributions
8. Ethics approval and consent to participate
9. Acknowledgment
10. Funding
11. Conflict of interest
12. References
1. Abstract

**Background:** Liver fibrosis is a dysregulated wound-healing process in response to diverse liver injuries, and an effective drug therapy is not yet available. Genistein, which is one of the most active natural flavonoids mainly derived from soybean products (e.g., *Cordyceps sinensis* mycelium), exhibits various biological effects, including hepatoprotective and anti-inflammatory properties. However, the anti-hepatic fibrosis mechanisms of genistein are poorly understood. The aim of our research is to explore the effect and the possible mechanism of genistein against liver fibrosis. **Materials and methods:** Cell counting kit-8, EdU, and flow cytometry assays were applied to evaluate the effects of genistein on cell viability, proliferation, and cell cycle arrest in human hepatic stellate cell (HSC) line LX2 cells. HSC activation was induced by transforming growth factor-β1 in LX2 cells and liver fibrosis model was established by the intraperitoneal injection of dimethylnitosamine (DMN) in rats to assess the anti-fibrosis effects of genistein in vivo and in vitro models. HSC activation was assessed by qRT-PCR, Western blot, immunohistochemistry, and immunofluorescent assay. Liver injury and collagen deposition were evaluated by histopathological assay, serum biochemistry, and hepatic hydroxyproline content assays. The mRNA expressions of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), and inflammation related-factors were assessed by qRT-PCR assay. Furthermore, the functional properties of macrophage in the liver were assessed by immunohistochemistry assay. The expression levels of the JAK2/STAT3/SOCS3 signaling pathway related-protein were assessed by Western blot analysis. **Results:** Genistein significantly inhibited cell viability and proliferation and induced cell cycle arrest at G0/G1 phase in LX2 cells, respectively. Furthermore, oral administration of genistein significantly ameliorated liver injury and the collagen deposition in rats with DMN-induced fibrosis model. Genistein suppressed the expression levels of HSC activation marker α-smooth muscle actin and collagen type I alpha 1 in vivo and in vitro. Genistein significantly decreased the mRNA expression levels of extracellular matrix degradation genes MMP2/9 and TIMP1 in rats. Genistein alleviated the mRNA expression levels of IL-1β, IL-6, TNF-α, and MCP-1 and regulated the protein expressions of CD68, CD163, and CD206 in the liver. Moreover, genistein attenuated the expressions of p-JAK2/JAK2, p-STAT3/STAT3, and SOCS3 protein both in vivo and in vitro. **Conclusion:** Taken together, our results showed that genistein could be improved liver fibrosis both in vivo and in vitro, probably through regulating the functional properties of macrophage and inhibiting the JAK2/STAT3/SOCS3 signaling pathway.

2. Introduction

Liver fibrosis, the excessive accumulation of extracellular matrix (ECM) proteins, is a pivotal structural basis of the pathogenesis in many chronic liver diseases [1, 2]. If left untreated, progressive fibrosis eventually develops into cirrhosis, liver failure, and hepatocellular carcinoma [1, 3]. In recent years, although great progress has been made in understanding the pathogenesis, effective anti-liver fibrosis drugs have not been developed [4]. Hepatic stellate cells (HSC), the main cell population of liver-synthesizing ECM proteins, not only secrete some ECM components including proteoglycan and glycoprotein, but also synthesize several collagenases to maintain normal basement membrane structure, which plays an important role during the development of hepatic fibrosis [4, 5]. In addition to ECM synthesis, the pathological process of hepatic fibrosis also involves ECM degradation, which is synergistically regulated by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) [6]. Studies have shown that activated HSC can increase the synthesis and decrease degradation of ECM, scar tissue from excessive deposition of ECM gradually distorts the normal parenchymal structure and impairs its function in this process, and finally leads to the development of liver fibrosis [7]. Furthermore, inflammation is a hallmark of liver fibrosis. Many studies have demonstrated that chronic liver inflammation drives hepatic fibrosis by accelerating liver macrophage infiltration through the secretion of cytokines and chemokines, such as interleukin-1β (IL-1β) and monocyte chemotactic protein-1 (MCP-1/CCL2). Furthermore, liver inflammation is regulated by different phenotypes of macrophages, including pro-inflammatory M1 and anti-inflammatory M2 macrophages [8–11]. Thus, the regulation of HSC activation and inflammatory infiltration are promising therapeutic strategies for the treatment of liver fibrosis.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway specifically mediates the signal transduction of many cytokines and growth factors from the cell membrane to the nucleus [12]. The activation of JAK2/STAT3 signaling is among the distinctive hallmarks of HSC activation, and it is involved in the development of liver fibrosis [13, 14]. Studies in HSCs shown that the inhibition of JAK2/STAT3 pathway prevents HSC morphological trans-differentiation and downregulates the expression levels of pro-fibrotic genes [15]. The suppressor of cytokine signaling 3 (SOCS3), an inhibitor protein of STAT3 signaling, can inhibit JAK/STAT pathways and thereby exert important actions in liver fibrosis [16, 17]. The expression of SOCS3 gradually increases with the aggravation of liver fibrosis and decreases during the reversal process [16].

Dimethylnitosamine (DMN), a potent hepatotoxin, can induce liver fibrosis and cirrhosis. In rats, DMN administration can lead to severe necrosis and ECM pro-
tein deposition in the liver [18, 19]. Thus, in investigating human liver fibrosis and cirrhosis, the DMN induced liver injury rat model is reproducible and potentially valuable animal model. Genistein (4′,5,7-trihydroxyisoflavone, Fig. 1), a biologically active isoflavone that is mainly extracted from soybean products, including Cordyceps sinensis mycelium, exhibits multiple biological effects, such as anti-inflammatory, hepatoprotective, and anti-cancer properties, which has aroused great interest in the field of pharmacy [20]. Genistein ameliorates many chronic liver diseases, such as hepatocellular carcinoma [21] and nonalcoholic fatty liver disease [22]. In the present study, we aimed to evaluate the anti-hepatic fibrosis effects of genistein and its potential mechanism both in vitro and in vivo. First, the effects of genistein on cell viability, proliferation, cell cycle and activation were investigated in human immortalized HSC LX2 cells. Furthermore, liver fibrosis model was established by DMN in rats. Subsequently, liver injury, liver fibrosis, HSC activation, the expressions of MMPs and TIMPs, and the functional properties of macrophage, as well as the JAK2/STAT3/SOCS3 signaling pathway were evaluated.

Fig. 1. Chemical structure of genistein.

3. Materials and methods

3.1 Main materials

Genistein was obtained from Dalian Meilun Biotechnology Technology Co., Ltd. (Dalian, China). Dimethyl-nitrosamine was obtained from TCI (Shanghai) Chemical Industry Development Co., Ltd. Dulbecco’s modified Eagle’s medium (DMEM, high glucose) and fetal bovine serum (FBS) were obtained from Gibco Life Technology (Gibco Invitrogen Corporation, Barcelona, Spain). Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total bile acid (TBA) test kits, hematoxylin and Eosin (H&E) staining kit, and hydroxyproline (Hyp) assay kit were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Reverse transcriptase assay kit was purchased from Applied Biological Materials Inc. (abm, Vancouver, Canada). TRIzol reagent and all primer were obtained from Shanghai Sangon Biological Engineering Co. Ltd. (Shanghai, China). SYBR Green Realtime PCR master mix was obtained from Toyobo Co., Ltd. (Osaka, Japan) Cell counting kit-8 (CCK-8) was obtained from Dojindo Laboratories Co., Ltd. (Kumamoto, Japan). BeyoClick™ EdU cell proliferation kit with Alexa Fluor 594 and cell cycle and apoptosis analysis kit were obtained from Beyotimebiotechnology Co., Ltd. (Shanghai, China). RIPA lysis buffer, proteinase and phosphatase inhibitor, BCA protein concentration assay kit and QuickBlock™ blocking buffer for western blot were obtained from Beyotimebiotechnology Co., Ltd. (Shanghai, China). Antibodies JAK2 (#3230), p-JAK2 (#3771), STAT3 (#9139) and p-STAT3 (#9145) were obtained from Cell Signaling Technology (Danvers, MA, United States); α-smooth muscle actin (α-SMA, #ab7817, for Western blot; #ab124964, for immunofluorescence), collagen type I alpha 1 (Col1A1, #ab34710), SOCS3, and CD68 (#ab125212), as well as Goat Anti-Rabbit IgG H&L (Cy3 ®) preadsorbed (#ab6939) were obtained from Abcam, Inc. (Cambridge, UK). CD163 (#16646-1-AP), CD206 (#60143-1-Ig) and GAPDH (#60004-1-Ig) were purchased from Proteintech (Wuhan, China). HSC line LX2 cells was provided by Professor Lieming Xu.

3.2 Animals and experiment design

Forty male Wistar rats (SPF grade, weight of 140 ± 20 g) were provided by Shanghai Xipuer-Bikai Experimental Animal Co., Ltd. Animal experiment was carried out in Experimental Animal Center of Shanghai University of Traditional Chinese Medicine. The animal study was reviewed and approved by the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine (PZSHUTCM190322007; Approval date: July 27, 2018). All rats (weighing 160–180 g) were randomly divided into the normal control (n = 8) and liver fibrosis model group (n = 32). An experimental model of liver fibrosis was established by intraperitoneal injection of 0.5% DMN saline (2 mL/kg body weight, 3 consecutive days per week) for 4 weeks according to the previous study [23]. After the second week, 32 rats with liver fibrosis were further divided into four groups with eight rats in each group as follows: DMN model, genistein-5 mg/kg, genistein-20 mg/kg, and sorafenib (5 mg/kg, as a positive control drug) group. From weeks 3 to 4, rats received treatment with 0.4% CMC-Na or correspondence drugs by gavage per day. After the fourth week, all rats were anesthetized by 3% pentobarbital sodium through intraperitoneal injection, and the samples were collected for further studies.

3.3 Cell culture and treatment

LX2 cells were seeded in 12-well plates with 5 × 10^4 cells and 30 mm petri dishes with 2 × 10^6 cells and were maintained in 10% FBS-containing DMEM medium. The cells were incubated with genistein at concentrations of 20, 40, and 80 µM. The divided experimental groups were as
follows: DMSO control group, TGF-β1 (5 ng/mL) group, TGF-β1 + SB431542 (10 µM, an TGF-β1 inhibitor) group, TGF-β1 + 20 µM genistein group, TGF-β1 + 40 µM genistein group, TGF-β1 + 80 µM genistein group. The mRNA and protein expressions were evaluated by qRT-PCR, Western blot and immunofluorescent assay.

3.4 CCK-8 cell viability assay

LX2 cells were cultured at 5 × 10^3 cells/well into 96-well plate and incubated in 10% containing DMEM medium at appropriate times. Subsequently, cells were incubated with genistein at concentration of 1.56, 3.13, 6.25, 12.5, 25, 50, 100 and 200 µM. And then, 10 µL CCK-8 solution was quickly added to each well at two hours before the scheduled times (24 h). Ultimately, the absorbance of 96-well plates was detected at 450 nm by microplate reader (BioTek, Hercules, CA, USA) at the scheduled times.

3.5 EdU cell proliferation assay

LX2 cells were grown in 12-well plates and were treated with genistein (20, 40 and 80 µM) for 24 h. Then, the cells were incubated with EdU labeling medium with a final concentration of 10 µM for additional 2 h at 37 °C. Two hours later, the cells were fixed with 4% formaldehyde for 10 min, were permeabilized with 0.3% Triton X-100 for 15 min, and were stained with click reaction solution for 30 min, respectively. The cell nuclei were stained using hoechst for 10 min. Finally, the cells were observed using an inverted fluorescence microscope DP80 (Olympus, Tokyo, Japan).

3.6 Cell cycle analysis

Briefly, LX2 cells, seeded into the 6-well plate at 1 × 10^5 cells per well, were cultured with genistein (20, 40 and 80 µM) for 24 h. The cells were then washed using ice-cold PBS, harvested and fixed in EP tube with cold 70% ethanol for 24 h, and followed by staining with PI for 30 min 37 °C, respectively. Added 500 µL propidium iodide staining solution and incubated for 30 min at 37 °C in the dark. Finally, the samples were analyzed by flow cytometry (FACS AriaIII, BD, USA). The percentage of cells in different phases of the cell cycle was analyzed by ModFit LT 5.0 software (Verity Software House, Inc., Topsham, ME, USA).

3.7 Serological index and hepatic hydroxyproline (Hyp) content assays

Serum AST, ALT, ALP and TBA levels, and the content of hepatic Hyp were measured using a matched test kit. In short, the serum samples were thawed at room temperature, and then placed in an automatic analyzer according to the labeling sequence. Subsequently, the corresponding detection reagents were added into the analyzer detection reagent bottle to detect serum AST, ALT, ALP, and TBA levels. Furthermore, hepatic Hyp content was assessed by alkaline hydrolysis method. Approximately 50 mg of liver tissues were weighed and hydrolyzed, and pH was adjusted to 6.0–6.8. The detection steps were performed according to the manufacturer’s instructions.

3.8 Histopathological assay

Formalin-fixed paraffin-embedded tissue samples were cut into 4-µm sections, and then adhered to silanized slides. The samples were then stained by H&E and Sirius red staining to evaluate histopathological features and fibrosis stage according to manufacturer’s instructions. Sirius red-positive area was further calculated by Leica LAS Image Analysis, percentage of positive staining area = positive staining area/total area × 100%.

3.9 Immunohistochemistry assay

The tissue sections were routinely de-waxed and dehydrated, and underwent antigen retrieval with mixing 4% sodium citrate buffer. Endogenous peroxidase activity and non-specific antigens were blocked with methanol containing 3% H₂O₂ for 15 min and PBS containing 10% goat serum for 30 min, respectively. Further, the sections were incubated with anti-α-SMA (1:1000), anti-Col1A1 (1:500), anti-CD68 (1:1000), anti-CD163 (1:1000), and anti-CD206 (1:1000) at 4 °C overnight. PBS was used instead of the primary antibody as the negative control. The following day the sections were incubated using secondary antibody labeled with HRP. Finally, the sections were stained by 3, 3-diaminobenzidine (DAB) and harris hematoxyl.

3.10 Immunofluorescent assay

An 8-µm-thick frozen sections of liver tissues were fixed with cold acetone for 10 min. The cells were fixed with 4% formaldehyde for 15 min and were permeabilized with 0.3% Triton X-100 for 10 min, followed by incubation with anti-α-SMA (1:1000) and anti-Col1A1 (1:500) overnight at 4 °C, respectively. Meanwhile, PBS was used instead of the primary antibody as the negative control. Subsequently, the specimens were stained using fluorescent secondary antibodies with goat anti-rabbit IgG-Cy3 (1:3000) and the nuclei were counterstained with DAPI (1:1000). Finally, these specimens were scanned under Olympus Fluoview 500 confocal microscope (Malvern, USA), and representative images were displayed.

3.11 Western blot analysis

The proteins from the liver tissues and LX2 cells were extracted using RIPA lysis buffer. Simply, LX2 cells and liver tissues were lysed with RIPA lysis buffer containing phosphatase and protease inhibitor, and lysed tissues and cells were centrifuged at 12,000 rpm for 20 min. The protein concentration was measured using the BCA protein assay kit. And then, the samples, SDS-PAGE protein loading buffer (5×) and water were mixed together to prepare loading protein samples. Subsequently, 30 µg proteins were loaded on 8% or 12% SDS-PAGE (electrophoresis...
Fig. 2. The effects of genistein on cell viability, proliferating and cell cycle. (A) CCK8 test was used to evaluate the effect of different concentrations of genistein on cell viability at 24 h in LX2 cells was detected by. (B) EdU assay was performed to detected the effect of genistein (20, 40 and 80 µM) on the proliferation at 24 h in LX2 cells, and the number of positive cells in EdU assay, Hoechst was counterstained the nuclei, representative confocal microscopy images are shown, scale bar 20 µm. (C) Flow cytometry was used to evaluate the effect of genistein (20, 40 and 80 µM) on the cell cycle at 24 h in LX2 cells. **p < 0.001, *p < 0.01, #p < 0.05 vs control group (0.1% DMSO).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-SMA</td>
<td>CTATGCCTCTGGAGCCACACACT</td>
<td>CAGATCCAGACGATGATTGCA</td>
</tr>
<tr>
<td>Col1A1</td>
<td>GACTCCCTGGAGCCTAAAGGTGC</td>
<td>AGCCCTCCATCTTTGCGACGCA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GTCTCCCTGACTTCAACACGCG</td>
<td>ACCACCCCTGTTGCTAGCCAA</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-SMA</td>
<td>AAGTATCCGATAGAAACAGAC</td>
<td>TAGATAGGCAGTTGGAAG</td>
</tr>
<tr>
<td>Col1A1</td>
<td>ACAGACCAAACACAGGAGCAAAGTCC</td>
<td>ACTTATACCCACATAGGTCTCTCAG</td>
</tr>
<tr>
<td>MMP1</td>
<td>GGAAGGTGATATTGTTGCG</td>
<td>CATATGCTCTCTTGAGAAGGCC</td>
</tr>
<tr>
<td>MMP9</td>
<td>CACCTGAACTGTGGGAAMAAGT</td>
<td>CACTTCTGTACGCTGAGA</td>
</tr>
<tr>
<td>TIMP1</td>
<td>TGGCATCCTCTCTTCTGCTATC</td>
<td>ACAGGTGGAAT CCTTTGAG</td>
</tr>
<tr>
<td>IL-6</td>
<td>AGCGAGCTGGGCTGCAAGT</td>
<td>GCAGGTGCTGGGATTTCTGAGT</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CCCAAAGGATGAAGATG</td>
<td>CACTTGAGTTTGAAGAC</td>
</tr>
<tr>
<td>IL-1β</td>
<td>GGATGAGGACATGAGCAAAC</td>
<td>AGCCCTATGGTCCACGCA</td>
</tr>
<tr>
<td>MCP-1</td>
<td>GACATCCTCAGGAGCTTTGTCTC</td>
<td>GACAGCTTCCATCTCTGAG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>CCATCAAGGACCCCTCTATT</td>
<td>GACAGCTTCCATCTCTGAG</td>
</tr>
</tbody>
</table>

conditions: 80 V) and transferred onto 0.45 µM polyvinylidene fluoride (PVDF) membranes (transfer conditions: 2–2.5 h, 100 V). The membranes were blocked with quick sealing fluid for 30 min, and then incubated with anti-α-SMA (1:1000), anti-JAK2 (1:500), anti-p-JAK2 (1:500), anti-STAT3 (1:500), anti-p-STAT3 (1:500), anti-SOCS3 (1:500), and anti-GAPDH (1:5000) at 4 °C overnight and subsequently incubated with fluorescence-labeled secondary antibody (1:5000). The protein bands were scanned by the Odyssey infrared scanner (LI-COR Biosciences, Lincoln, NE, USA).

3.12 Quantitative real-time PCR (qRT-PCR) analysis

Total RNA from rat hepatic tissues and LX2 cells was extracted using TRIzol reagent. Briefly, liver tissues and LX2 cells were lysed using TRIzol reagents. Afterward, chloroform, isopropanol, 75% ethanol, and anhydrous ethanol were added to the solutions of homogenized tissues and lysed cells, and then centrifuged at 12,000 rpm 4 °C and finally dissolved with DEPC water. cDNA was synthesized from extracted total RNA using the reverse transcriptase PCR kit. qRT-PCR was carried out using SYBR Green PCR master mix. The relative mRNA expression levels of liver tissues and LX2 cells were measured us-
Fig. 3. The effects of genistein on the expressions of α-SMA and Col1A1 in LX2 cells. (A) qRT-PCR was used to determine the effects of genistein on the expression levels of α-SMA and Col1A1 mRNA in LX2 cells, gene expression was normalized to GAPDH mRNA. (B) Western blot was used to assess the effect of genistein on the expression level of α-SMA protein, α-SMA protein expression was normalized against GAPDH level. (C) Immunofluorescence staining of α-SMA was assessed by immunofluorescence assays, DAPI was counterstained the nuclei, representative confocal microscopy images are shown, scale bar 60 µm. #p < 0.001 vs control group (0.1% DMSO); ***p < 0.001, **p < 0.01, *p < 0.05 vs TGF-β1 group. SB43152 is an inhibitor of TGF-β1.

### 3.13 Statistical analysis

Data are expressed as mean ± standard deviation (SD). Multiple groups mean with normal distributions and equality of variance were compared by one-way ANOVA with post hoc LSD for multiple pair-wise comparisons. Non-normal distributions and unequal variance were analyzed using Kruskal-Wallis test. p < 0.05 was considered statistically significant.
4. Results

4.1 Genistein regulates the cell viability, proliferation, and cell-cycle arrest in LX2 cells

Genistein was used in HSC line LX2 cells to evaluate the effect of genistein on cell growth by CCK8 assay, EdU staining, cell cycle analysis. First, the cell viability was evaluated by CCK8 assay, compared with the control group, cell viability was not significantly inhibited within 150 μM, but was obviously inhibited by genistein treatment with 200 μM ($p < 0.05$, Fig. 2A). Further, the anti-proliferative effect of genistein was investigated in LX2 cells by EdU staining, the results showed that the proliferation of LX2 cells was significantly inhibited at 40 and 80 μM of genistein ($p < 0.05$ or $p < 0.001$, Fig. 2B). The cell cycle distribution was evaluated using flow cytometry. In comparison with the control group, the G0/G1 phase population increased, and the S phase population decreased in LX2 cells after treatment with 20, 40, and 80 μM of genistein ($p < 0.05$ or $p < 0.01$ or $p < 0.001$), suggesting that genistein induced cell cycle arrest at G0/G1 phase (Fig. 2C).

4.2 Genistein inhibits the expressions of α-SMA and Col1A1 in LX2 cells

HSC activation is a key event in the occurrence and development of liver fibrosis. Thus, its activation is usually evaluated in the study of fibrosis. In this work, the expressions of α-SMA and Col1A1, which are typical markers of HSC activation, were induced by TGF-β1 in LX2 cells to investigate the anti-fibrosis effects of genistein in vitro. First, the expressions of α-SMA and Col1A1 mRNA were assessed by qRT-PCR analysis. As expected, the elevated mRNA expression levels of α-SMA and Col1A1 in LX2 cells induced by TGF-β1 were decreased after treatment with 20, 40, and 80 μM of genistein ($p < 0.05$ or $p < 0.01$ or $p < 0.001$; Fig. 3A). The protein expression of α-SMA was also evaluated by Western blot assay and immunofluorescent staining. Western blot assay result showed that the expression of α-SMA protein was significantly suppressed in genistein treatment group ($p < 0.05$ or $p < 0.001$; Fig. 3B). Consistent results of α-SMA protein expression were achieved by immunofluorescence staining (Fig. 3C).
Fig. 5. The effects of genistein on the expressions of HSC, MMPs and TIMPs in the DMN-induced rats. (A,B) Immunohistochemistry assay was used to determine the effects of genistein on the protein expression levels of α-SMA and Col1A1 in the liver (scale bar = 100 µm). (C) Immunohistochemistry quantification of α-SMA and Col1A1 protein with positive area. (D,E) Immunofluorescence assay was performed to determine the effects of genistein on the protein expression levels of α-SMA and Col1A1 in the liver (scale bar = 100 µm), the nuclei were counterstained with DAPI. (F) Western blot assay was applied to assess the protein expression level of α-SMA in the liver, protein expression was normalized against GAPDH level. (G) qRT-PCR was performed to evaluate the effects of genistein on the expression levels of Col1A1 and α-SMA mRNA in the liver, the expression levels of genes were normalized by GAPDH mRNA. (H) qRT-PCR was performed to assess the effects of genistein on the expression levels of MMP9, MMP2 and TIMP1 in liver, the expressions of genes were normalized by GAPDH mRNA. \( \ast \ast p < 0.001, \ast p < 0.01 \) vs control group. \( \ast p < 0.05 \) vs DMN group.

### 4.3 Genistein ameliorates liver injury and collagen deposition in DMN-induced hepatic fibrosis rats

Liver pathological histology was determined in rats by H&E staining. As shown in Fig. 4A, the structure of hepatic lobular was severely collapsed and formed more complete pseudo-lobules, an abundance of inflammatory cells infiltrated in the portal tract area, the central vein area, and the hepatic sinusoid in DMN rats compared with the control rats. However, compared with the DMN rats, these changes of liver histopathology in genistein-treated rats were significantly improved. Serum biochemistry indices: AST, ALT, ALP, and TBA levels, were also detected. As shown in Fig. 4C, the serum levels of AST, ALT, ALP, and TBA were increased significantly by administration with DMN (\( p < 0.001 \)) and were evidently reduced by genistein treatment (\( p < 0.05 \) or \( p < 0.01 \)). However, there was no significant difference in sorafenib group, compared with the DMN group.

In addition, the deposition of collagen fibers was evaluated by sirius red staining. Compared with the control rats, several collagen fibers formed fibrous septum, destroyed the structure of hepatic lobule, and formed pseudo lobule in the liver with hepatic fibrosis (Fig. 4B). After genistein treatment, several changes were improved. For instance, reduced collagen depositions and fibrous septa were observed in these rats compared with the DMN-induced rats. Further, sirius red-positive area was calculated to evaluate indirectly hepatic collagen content. The result indicated that sirius red-positive area was obviously decreased after genistein treatment, compared with the DMN model group (\( p < 0.05 \) or \( p < 0.01 \); Fig. 4D). The liver tissue Hyp content was further measured to estimate directly the collagen deposition in the liver. The results were consistent with sirius red-positive area (\( p < 0.01 \) or \( p < 0.001 \); Fig. 4E).

### 4.4 Genistein regulates the expressions of α-SMA, Col1A1, MMP2/9, and TIMP1 in DMN-induced rats

Consistent with the in vitro experiments, the expressions of α-SMA and Col1A1 were also evaluated in vivo. As shown in Fig. 5A–C, at the protein levels, the expressions of α-SMA and Col1A1 were evaluated by immunohistochemistry, immunofluorescent, and Western blot assay. As expected, immunohistochemistry and immunofluorescence results indicated that the expressions of α-SMA and Col1A1 were increased significantly in the liver with DMN-induced rats and decreased significantly in
the genistein group (p < 0.05 or p < 0.01 or p < 0.001). Similar results were obtained by Western blot assay, in which genistein could clearly reduce the expression of α-SMA (p < 0.05; Fig. 5D–F). The mRNA expressions of α-SMA and Col1A1 at transcription levels were further confirmed by qRT-PCR, showing a significant increase in the DMN model group (p < 0.001) but a remarkable reduce in the genistein group (p < 0.05 or p < 0.01 or p < 0.001; Fig. 5G).

MMPs and TIMPs are directly involved in the balance between synthesis and degradation of ECM in the liver [24]. Thus, in the present study, MMP2, MMP9, and TIMP1 at the levels of transcription were detected. The results indicated that the elevated mRNA expressions of MMP2 and MMP9 in DMN-induced rats were reversed after treatment by genistein (p < 0.05; Fig. 5H). The expression of TIMP1 mRNA was also enhanced with DMN administration (p < 0.001) but decreased in the genistein treatment group (p < 0.05; Fig. 5H).

4.5 Genistein regulates inflammatory infiltration and macrophage functional properties in rats induced by DMN

Liver fibrosis is an inflammatory response. Thus, inflammation related-factors were evaluated at the transcriptional levels in this work. qRT-PCR results indicated that the increased mRNA expressions of IL-1β, IL-6, TNF-α, and MCP-1 in DMN-induced rats were decreased significantly by genistein treatment (p < 0.05 or p < 0.01; Fig. 6A). We investigated whether genistein could affect the distribution of both subtypes of macrophage in the liver. The expression levels of CD68 (M1 macrophage marker), CD163 and CD206 (M2 macrophage marker) in the liver were assessed by immunofluorescence staining. As shown in Fig. 6B–E, the expression of CD68 was evidently increased, but those of CD163 and CD206 were clearly decreased in the DMN-treated rats (p < 0.001). After genistein treatment, the expression of CD68 was markedly decreased, and those of CD163 and CD206 increased (p < 0.05 or p < 0.01 or p < 0.001).
4.6 Genistein inhibits the JAK2/STAT3/SOCS3 signaling pathway

JAK2/STAT3 and SOCS3 pathway plays an important role during liver fibrosis. In the present study, the protein expressions of JAK2, STAT3, and SOCS3 and the phosphorylation of JAK2 and STAT3 were detected. In vivo, the results indicated that the expression levels of p-JAK2/JAK2 and p-STAT3/STAT3 were clearly increased in rats with DMN-induced fibrosis ($p < 0.01$, $p < 0.001$), but genistein could depress the expressions of p-JAK2/JAK2 and p-STAT3/STAT3 ($p < 0.01$, $p < 0.001$; Fig. 7A). The expression of SOCS3 protein was also upregulated in DMN-treated rats ($p < 0.001$). After genistein treatment, the expression of SOCS3 protein was significantly inhibited ($p < 0.05$; Fig. 7A). Interestingly, consistent results were also obtained in TGF-β1-activated LX2 cells in vitro (Fig. 7B).

5. Discussion

Hepatic fibrosis is a common pathological consequence for diverse liver injuries induced by chronic viral and metabolic disorders [1]. Effective drugs are not yet available in clinic. Several studies have previously attempted to investigate the anti-fibrosis effects of genistein, and liver injury and fibrosis were significantly improved via genistein treatment in experimental models mediated by D-galactosamine [25], carbon tetrachloride [26], schistosomiasis [27], and methionine-choline-deficient diet [28]. However, these studies are focused on the current understanding of the pharmacodynamics, and research about the mechanism remains insufficient.

HSC proliferation and activation are closely related to the development of fibrosis. In the present study, we first evaluated cell viability, proliferation, and cell cycle arrest in LX2 cells. The inhibition of cell viability and proliferation, the increase of G0/G1, and the reduction of S phase population were observed in genistein-treated cells. Thus, the genistein-mediated suppression of cell prolif-
ation may be caused by cell cycle arrest, but the mechanism remains to be explored in detail in future studies. Meanwhile, HSC activation in LX2 cells was induced by TGF-β1 stimulation, the elevated expressions of α-SMA and Col1A1 mRNA and protein were suppressed substantially in genistein-treated cells, in which genistein could markedly inhibit the activation of HSCs in vitro. Further, in vivo, experimental hepatic fibrosis model in rats was established by the intraperitoneal injection of DMN to evaluate the anti-fibrosis effects of genistein. The results showed that genistein could ameliorate liver injury and collagen deposition. Meanwhile, the mRNA and protein expressions of α-SMA and Col1A1 in the liver were consistent with those in vitro. Therefore, genistein has a good therapeutic effect for the liver fibrosis in vivo and in vitro models.

To research the possible mechanism of genistein on the synthesis and degradation of ECM, we assessed the expressions of MMPs, and TIMPs in the liver. MMP2 and MMP9 are the primary MMPs that degrade normal liver matrix, while its activity is inhibited by TIMP1, a pivotal regulator in the remodeling of extracellular matrix [6]. Although MMP2 and MMP9 can accelerate degradation of ECM and its expressions are reduced in liver fibrosis, some studies also believe that the expressions of MMP2 and MMP9 substantially increased because of the body’s response to the degradation of excessive ECM during liver fibrosis [29, 30]. In the present work, at the transcriptional levels, the elevated expressions of MMP2, MMP9, and TIMP1 in the liver with DMN rats were obviously decreased by genistein treatment. Therefore, genistein contributes to degradation of ECM by regulating the expressions of MMPs and TIMPs.

Chronic liver inflammation plays a predominant role in the initiation and progression of hepatic fibrosis [31, 32]. In the present study, inflammation factors were evaluated at the transcriptional levels, the elevated expressions of IL-1β, IL-6, TNF-α, and MCP-1 in DMN-induced rats were reduced significantly by genistein treatment. In liver inflammation, intrahepatic macrophages are the most important immune cells that exhibit high dynamic plasticity, which allows them to adapt their phenotype in response to various microenvironmental signals or stimuli [8]. They are generally delineated into two major polarization states, namely, M1 and M2 macrophages. M1 macrophage display pro-inflammatory properties and release pro-inflammatory mediators that contribute to inflammation and injury in response to stimuli or polarization signals [11, 33]. By contrast, M2 macrophage show anti-inflammatory properties and release anti-inflammatory or pro-resolving mediators that accelerate wound repair and tissue remodeling [33]. To explore the effects of genistein for the functional properties of macrophage in hepatic fibrosis, we investigated the expressions of pro-inflammatory M1 macrophage marker CD68 and anti-inflammatory marker M2 macrophage CD163 and CD206 in the liver. The result showed that genistein could inhibit the expression of CD68 but upregulate the expression levels of CD163 and CD206. Therefore, genistein could regulate the functional properties of macrophage to attenuate liver inflammation.

In previous studies, the mechanism of genistein against liver fibrosis was limited to TGF-β1/Smad and NF-κB pathways, so the discovery of new mechanism is very important for genistein to treat this disease. JAK/STAT pathway is key for various cytokines and growth factors in mammals [12]. During liver fibrosis, the activation of JAK2/STAT3 pathway accelerates quiescent HSC morphological trans-differentiation and the expression of profibrotic genes [13]. In the present study, JAK2/STAT3 pathway was activated by the intraperitoneal injection of DMN in rats and TGF-β1 stimulation in LX2 cells, whereas genistein could significantly inhibit the expressions of p-JAK2/JAK2, and p-STAT3/STAT3. The increase of SOCS3 expression, an inhibitor protein of STAT3 signaling that negatively regulate JAK/STAT pathways, was also reduced by genistein treatment. Therefore, genistein inhibits the progression of liver fibrosis possibly by suppressing JAK2/STAT3 pathway through regulating the expression of SOCS3, but this result still needs further study.

Furthermore, the serum and liver levels of genistein should be measured to establish concentrations that can be referred to as therapeutic. The contents of genistein in the serum and liver tissue of DMN-induced fibrosis rats were detected by LC-MS (Supplementary Fig. 1). The result is shown in Supplementary Fig. 2, in which the contents of genistein in the liver tissue of 5 mg/kg and 20 mg/kg genistein groups were successfully detected, and the contents of genistein in the serum of genistein-20 mg/kg group were detected. Notably, these samples were obtained approximately 24 h after the last oral administration. This study aimed to effectively understand the exposure of genistein in blood after oral administration genistein. The plasma concentration vs time curves of genistein in rats after oral treated 20 mg/kg genistein are shown in Supplementary Fig. 3. According of the quantitative results, the pharmacokinetic parameters were calculated and summarized in Supplementary Table 1. After oral treated 20 mg/kg genistein, genistein was detected in rat plasma at different sampling points. Genistein could be quickly absorbed into blood with $T_{max}$ of 2 h and $T_{1/2ke}$ of 2.20 ± 0.33 h. Overall, these results suggest that genistein has a good exposure in the blood or liver by oral administration.

6. Conclusions

In conclusion, the results presented here indicate that genistein efficiently attenuates hepatic fibrosis induced by DMN in rats, and this condition may be related to the regulation of the functional properties of macrophage and JAK2/STAT3/SOCS3 signaling pathway.
7. Author contributions

YX and DZ performed the experiments, analyzed the data and wrote the manuscript. HY, YL, LZ, CZ, GC and YH assisted with the experiments. JC, HZ and YM critically revised the manuscript. PL and WL designed the research, conceived the ideas, and revised the manuscript. All authors read and reviewed the manuscript.

8. Ethics approval and consent to participate

The animal study was reviewed and approved by the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine (PZSHUTCM190322007; Approval date: July 27, 2018).

9. Acknowledgment

Not applicable.

10. Funding

This work was supported by the National Natural Science Foundation of China (No. 82040162, 81703681 and 81530101), “Chen Guang” project supported by Shanghai Municipal Education Commission and Shanghai Education Development Foundation (No. 20CG50), Shanghai Sailing Program (No. 2020QNRC001), China Postdoctoral Science Foundation of China (No. 82004162, 81703681 and 81530101), “Chen Guang” project supported by Shanghai Municipal Education Commission and Shanghai Sailing Program (No. 20CG50), Shanghai Sailing Program (No. 2020QNRC001), Shanghai Sailing Program (No. 20CG50), China Postdoctoral Science Foundation (No. 2020M681367). Budget project of Shanghai University of traditional Chinese medicine (No. 2020LK029).

11. Conflict of interest

The authors declare no conflict of interest.

12. References


**Supplementary material:** Supplementary material associated with this article can be found, in the online version, at https://www.imrpress.com/journal/FBL/26/12/10.52586/5050.

**Keywords:** Genistein; Liver fibrosis; Hepatic stellate cells (HSC); Macrophage functional properties; JAK2/STAT3/SOCS3 signaling pathway

**Send correspondence to:**

Ping Liu, Experiment Center of Teaching & Learning, Institute of Chinese Materia Medica, Institute of Interdisciplinary Integrative Medicine Research, Shanghai University of Traditional Chinese Medicine, 201203 Shanghai, China, Institute of Liver Diseases, Key Laboratory of Liver and Kidney Diseases (Ministry of Education), Shanghai Key Laboratory of Traditional Chinese Clinical Medicine, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, 201203 Shanghai, China, E-mail: liuliver@vip.sina.com

Wei Liu, Experiment Center of Teaching & Learning, Institute of Chinese Materia Medica, Institute of Interdisciplinary Integrative Medicine Research, Shanghai University of Traditional Chinese Medicine, 201203 Shanghai, China, Institute of Liver Diseases, Key Laboratory of Liver and Kidney Diseases (Ministry of Education), Shanghai Key Laboratory of Traditional Chinese Clinical Medicine, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, 201203 Shanghai, China, E-mail: lwhzayl@shutcm.edu.cn

† These authors contributed equally.