

Research Article

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Dynamic Changes in MMP1 and TIMP1 in the Antifibrotic Process of Dahuang Zhechong Pill in Rats with Liver Fibrosis

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Abstract: On the basis of carbon tetrachloride (CCl₄)-induced liver fibrosis in rats, this study aims to investigate the dynamic changes in matrix metalloproteinase 1 (MMP1) and the tissue inhibitor of metalloproteinase 1 (TIMP1) in the antifibrotic process of Dahuang Zhechong Pill (DHZCP). A total of 50 male Sprague Dawley rats, aged 8 weeks, were randomly divided into 3 groups: the control group, the model group (the group treated with CCl₄), and the treatment group (the group treated with CCl₄ and DHZCP). Rats were sacrificed at Weeks 4 and 8. Liver tissues were separated for RNA sequencing and bioinformatics analysis. Real-time PCR, Western blot analysis, and histological staining were conducted to confirm the gene expression and pathological change in liver tissues. Compared with control group, rats in model group showed poor mental state and slow weight gain. The liver tissues of the rats in the model group exhibited a damaged hepatic lobule structure, fibrous connective tissue hyperplasia, and inflammatory cell infiltration among the hyperplastic tissues. DHZCP could significantly

improve the appearance of rats and alleviate CCl₄-induced fibrosis. Compared to model group, 798 differentially expressed mRNAs were found in the treatment group, of which 120 were up-regulated and 678 were down-regulated. Differentially expressed mRNAs between the CCl₄-induced group and the DHZCP-treated group were mainly focused on the following KEGG pathways: focal adhesion, phagosome, tight junction, and ECM–receptor interactions. Relative to those in the control group, MMP1 was downregulated, whereas, TIMP1 and Col1A1 were upregulated in the CCl₄-induced group at Weeks 4 and 8. DHZCP could reverse MMP1, TIMP1, and Col1A1 expression. DHZCP protects against liver injury and exerts an antifibrotic effect on liver fibrosis induced by CCl₄ in rats. Its mechanism may be related to the upregulation of MMP1, downregulation of TIMP1, and promotion of collagen degradation.

Keywords: Dahuang Zhechong Pill; liver fibrosis; MMP1; TIMP1.

Abbreviations

CCl₄: Carbon tetrachloride;
Col1A1: Collagen, type I, alpha 1
DHZCP: Dahuang Zhechong Pill;
ECM: extracellular matrix;
GO: gene ontology;
IHC: Immunohistochemical;
KEGG: Kyoto Encyclopedia of Genes and Genomes;
MMP: matrix metalloproteinase;
TIMP: the tissue inhibitor of metalloproteinase;

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1 Introduction

Hepatic fibrosis caused by chronic hepatitis virus and alcohol has been one of the most serious health problems in China [1]. Liver fibrosis, mainly shown as excessive deposition of extracellular matrix (ECM) in normal liver architecture, may ultimately progress to cirrhosis and hepatocellular carcinoma, which is closely related to high morbidity and mortality. Early-stage liver fibrosis can be reversed. ECM, the critical factor, is a macromolecular molecule synthesized and secreted by animal cells and is mainly composed of collagen, non-collagen, matrix metalloproteinase (MMP), the tissue inhibitor of metalloproteinase (TIMP), and various cytokines. The interaction between MMP1 and TIMP1, among the MMPs and TIMPs, plays a key role in maintaining the balance between ECM synthesis and degradation [2-4].

Traditional Chinese medicine has been selected as an alternative treatment for liver fibrosis because of the low efficiency and unexpected side effects of the currently used therapeutic strategy [5]. Numerous herbal medicines are used for liver fibrosis therapy in clinical practice [6-8]. The Fuzheng Huayu formula is one of the most well-studied antifibrotic agents and has a good therapeutic effect on hepatitis B-induced cirrhosis [9,10]. Dahuang Zhechong Pill (DHZCP), the widely known prescription from the book “JinKuiYaoLue” authored by Zhang Zhongjing, is recommended by “The Guideline for the Combination of Chinese and Western Medicine in the Diagnosis and Treatment of Liver Fibrosis” for the treatment of hepatic fibrosis. DHZCP contains 12 Chinese herbal medicines: Gancao, Dahuang, Tubiechong, Baishao, Dihuang, Qicao, Taoren, Huangqi, Mengchong, Shuizhi, Kuxingren, and Gouqizi. The entire prescription stimulates blood circulation and removes blood stasis and astigmatism. DHZCP has been widely used for the treatment of liver fibrosis and other diseases in clinical practice.

Wei et al., conducted a meta-analysis which included 17 trails including 1212 patients and concluded that DHZCP could reduce the serum biomarker of hepatic fibrosis in CHB patients [11]. By decreasing the secretion of TNF- α and IL-13 and regulating the phosphorylation of p38 and the ERK pathway, DHZCP can protect against liver fibrosis and reverse the expression of aspartate aminotransferase, alanine aminotransferase, hyaluronic acid, collagen, and laminin [12]. Pan et al. indicated that the drug serum containing DHZCP could markedly promote MMP1 gene expression in hepatic stellate cell [13]. DHZCP can suppress the proliferation of vascular smooth muscle cells *in vivo* or the progression of hepatic fibrosis by inhibiting the mitogen-activated protein kinase pathway. However, the

expression levels of MMP1 and TIMP1 and their role in the antifibrotic effect of DHZCP have not yet been determined *in vivo*.

Carbon tetrachloride (CCl_4) is widely used in the study of liver fibrosis, which can cause liver injury and other toxic effects. [14]. On the basis of liver fibrosis induced by CCl_4 in rats, we performed RNA sequencing and bioinformatics analysis to screen differently expressed mRNAs among groups. Real-time PCR, Western blot analysis, and histological staining were conducted to observe the expression levels of MMP1 and TIMP1 in the antifibrotic process of DHZCP and thus provide a new strategy for antifibrotic therapy.

2 Materials and methods

2.1 Dahuang Zhechong Pill

DHZCP was manufactured by Xi'an C. P. Pharmaceutical Co., Ltd. (Xi'an, China, Lot No. 61021089). It contains 12 Chinese herbal components, as listed in Table 1. All herbs were dried and ground into powder. The powder was mixed with honey to form the pills, which were then dissolved in saline to suspend before use.

2.2 Establishment of hepatic fibrosis rat models

Fifty 8-week-old Sprague Dawley rats with 200–240 g were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). Rats were raised in common feeding conditions at 22°C, relative humidity of 55%, and a 12 h light/dark cycle for 7 d prior to drug administration.

By using the table of random numbers, 50 rats were randomly divided into three groups: the control group (16 rats), the model group (17 rats induced with CCl_4), and the treatment group (17 rats treated with CCl_4 +DHZCP). The model rats were injected intraperitoneally with 0.6 mL/kg CCl_4 (Fuyu Fine Chemical Reagents Co., Ltd., Tianjin, China) and a corn oil mixture (Equivalent volume mixing of CCl_4 and corn oil) twice a week. Rats in control group were only administrated by the same amount of corn oil. The treatment group received 3.0 g/kg DHZCP dissolved in normal saline by intragastric administration daily. Finally, the rats were sacrificed at Weeks 4 and 8, with their liver tissues separated for subsequent experiments.

Liver tissue sections of rats were preserved in 4% paraformaldehyde for histological staining. Fresh liver

Table 1: The ingredients of Dahuang Zhechong pill.

Chinese name	Latin name	Dosage (g)
Da Huang	<i>Rheum officinale</i> Baill, Polygonaceae	300
Di Huang	<i>Rehmannia glutinosa</i> Libosch, Scrophulariaceae	300
Tu Bie Chong	<i>Eupolyphaga sinensis</i> Walker, Polyphagidae	30
Meng Chong	<i>Tabanus bivittatus</i> Mats., Tabanidae	45
Ku Xing Ren	<i>Prunus armeniaca</i> Lam., Rosaceae	120
Huang Qi	<i>Scutellaria baicalensis</i> Georgi, Labiatae	60
Qi Cao	<i>Holotrichia diomphalia</i> Bates, Scarabaeidae	45
Gan Cao	<i>Glycyrrhiza uralensis</i> Fisch., Leguminosae	90
Bai Shao	<i>Paeonia lactiflora</i> Pall., Ranunculaceae	120
Tao Ren	<i>Prunus persica</i> Batsch, Rosaceae	120
Shui Zhi	<i>Hirudo nipponica</i> Whitman, Hirudinidae	60
Gan Qi	<i>Toxicodendron vernicifluum</i> F.A. Barkl., Anacardiaceae	30

tissues were preserved in TRIzol Reagent (Invitrogen, MA, USA) for RNA detection. Animal experiments in this study strictly follow the Guidelines for Animal Nursing and Use. All animal experiments were authorized by the Animal Ethics Commission of Peking University Shenzhen Hospital.

2.3 Histological staining

After being fixed in 4% paraformaldehyde at room temperature for 48 h, the liver tissue samples from the rats were wrapped in paraffin and cut into 4 mm slices. The slices were dewaxed in xylene, then hydrated twice in 100% ethanol at room temperature for 5 minutes, and finally in 80% ethanol for 5 minutes. Finally, the samples were dyed with hematoxylin for 5 minutes and with eosin for 1 minute in accordance with the standard procedures (SBJ0447, SenBeiJia BioTech Co., Ltd., Nanjing, China). Masson staining was performed in accordance with the protocol provided by the manufacturer (SBJ0033, SenBeiJia BioTech Co., Ltd., Nanjing, China). Sections were stained as following: azure blue for 2 minutes, mayehematoxylin for 2 min, and carmine and acid fuchsin for 10 minutes.

Immunohistochemical (IHC) staining for MMP1 (SC-30069, Santa Cruz, USA), TIMP1 (SC-5538, Santa Cruz, USA), and Col1A1 (SC-25974, Santa Cruz, USA) was conducted using the common IHC streptavidin–peroxidase method with an antibody (1:100). After staining, five random fields from each section were taken under 100× or 200× magnification with an optical microscope.

2.4 RNA extraction and real-time PCR

The experimental procedure was similar to our previous study [15]. We extracted total RNAs from liver tissues by TRIzol Reagent, and detected the purity and concentration by NanoDrop 2000 (Thermo Fisher Scientific, Inc.), and defined a effective value by an optical density value $260/280 > 1.90$. Total RNA was then reverse-transcribed by commercial kit with gDNA Eraser (Cat. no. RR047A, Takara Bio, Inc., Shiga, Japan). We performed real-time PCR to detect gene expression by using ABI PRISM 7500 system (Thermo Fisher Scientific, Inc., USA) with SYBR kit containing Tli RNaseH (Cat. no. RR820A, Takara Bio, Inc., Otsu, Japan). Thermocycling conditions were as follows: 95°C for 5 minutes, 40 cycles at 95°C for 15 seconds, 56°C for 30 seconds, and 72°C for 15 seconds, final extension at 72°C for 7 minutes. The GAPDH was used to normalize. All experiments were repeated three times. The $2^{-\Delta\Delta C_q}$ method was used to determined the fold changes of mRNAs [16]. The primers used were the following: MMP1 forward primer 5'- CCACTAACATTCGAAAGGGTTT -3', reverse primer 5'- GGTCCATCAAATGGGTTATTG -3'; TIMP1 forward primer 5'- CAGCAAAGGCCTTCGTAAA -3', reverse primer 5'- TGGCTGAACAGGGAACACT -3'; GAPDH forward primer 5'- CACGGCAAGT TCAACGGCACAGT-3', and reverse primer 5'-AGCGGAAGGGGCGGAGATGAT-3'.

2.5 RNA sequencing

The protocol provided by the manufacturer for RNA sequencing in rats was introduced in our previous study [15]. Nine rats at Week 8 from the control group, model group, and treatment group were selected to isolate total RNA from the liver tissues. We then depleted ribosomal RNA from total RNA, generated cDNA libraries, and performed sequencing on the Illumina HiSeq 3000 at RiboBio Co., Ltd. (Guangzhou, China). We filtered raw reads to clear low-quality sequences, then used TopHat v2.0.13 to compare the clean reads with the genome Rnor_6.0 (asia.ensembl.org/Rattus_norvegicus/Info/Index). Gfold v1.1.2 was applied to determine the number of reads of

each gene. The Cufflinks with fragment/kb transcription were used to evaluate the differential expression of each million fragments. The reading count was used as input. Differentially expressed mRNAs (DEMs) were defined as following criteria: fold change >2 and adjusted P value <0.001.

2.6 Bioinformatics analysis

In a previous study [15], DEMs in fibrotic liver tissues were analyzed via gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). GO (<http://geneontology.org/>) was used to perform enrichment analysis on gene sets. DEMs were classified by enrichment analysis into three items, namely, molecular function, biological process, and cellular component. KEGG pathway analysis (www.genome.jp/kegg/) was conducted to research the potential pathways of DEMs enrichment.

2.7 Western blot analysis

Liver tissues were lysed in RIPA with proteinase inhibitors (Roche, Penzberg, Germany). A BCA protein detection kit was used to quantify the total protein (Boster, Wuhan, China). The equivalent amount of protein was separated by SDS-PAGE electrophoresis and transferred to PVDF membrane (Millipore, Billerica, MA, USA). After incubating with defatted milk for 1 hour, the membrane was probed with primary antibodies (diluted with defatted milk 1:1000) against rat MMP1 (SC-30069, Santa Cruz, USA), TIMP1 (SC-5538, Santa Cruz, USA), Collagen, type I, alpha 1 chain (Col1A1) (BS60771, Bioworld Technology Inc., China) and α -tubulin (BS1699, Bioworld Technology Inc., China) for 1 h. Secondary antibodies (Beyotime Biotechnology, Shanghai, China) labeled with horseradish peroxidase (diluted with defatted milk 1:1000) were used to conjugate with a primary antibody. After washing, the protein was incubated with chemiluminescence detection kit (Thermo Scientific, Waltham, MA, USA). The immunoreactive signals of the protein was detected using an automatic chemiluminescence imaging system (Tanon 5200, Shanghai, China).

2.8 Statistical analysis

Data were represented by mean \pm standard deviation. One-way ANOVA was used to compare differences between groups. Student's t -test was used to analyze the data

obtained by real-time PCR and Western blot analysis. $P < 0.05$ was considered statistically significant. All statistical data were analyzed using SPSS 19.0 (version 19.0. Armonk, NY, USA).

3 Results

3.1 Role of anti-hepatic fibrosis by Dahuang Zhechong Pill

Two rats in the model group and one rat in the treatment group were dead throughout the experimental period, whereas the rats in the control group were normal. The rats in the model group showed lethargy, fatigue, loss of appetite, slow movement, yellow and dirty fur, and slightly increased weight. The rats in the treatment group exhibited a better appearance than those in the model group.

To assess the pathological status of the liver tissue between groups, HE staining was conducted. The results are shown in Figure 1. The structures of the control rats at Weeks 4 and 8 were normal (Figures 1A, 1D). At Week 4, significant hepatic steatosis, including bubble-like liquid drops in the cytoplasm and cell swelling, was observed in the model rats (Figure 1B). At Week 8, hepatocytes with dissolved putrescence and severe hepatic steatosis were observed in model rats. Inflammatory cell infiltration and pseudolobules were also found between liver tissues (Figure 1E). Compared with the rats in the model group exhibiting pathological features, the rats in the treatment group alleviated liver fatty denaturation, inflammatory cell infiltration, and cell death (Figures 1C, 1F).

Hepatic fibrosis in rats at Weeks 4 and 8 was assessed by Masson staining. No apparent ECM deposition was found in the liver tissues of the control rats (Figures 1G, 1J). Hepatic fibrosis appeared in the portal tract of the model rats at Week 4 (Figure 1H). An increased hepatic fibrosis area and a destructed liver structure were observed at Week 8 (Figure 1K). DHZCP could significantly alleviate hepatic fibrosis at Week 4s and 8 (Figures 1I, 1L).

3.2 Bioinformatics analysis of differentially expressed mRNA

We have identified 35,362 mRNAs by RNA sequencing. We then performed GO and KEGG analysis on the DEMs among the three groups. Our previous study suggested that the DEMs between the control and model groups were

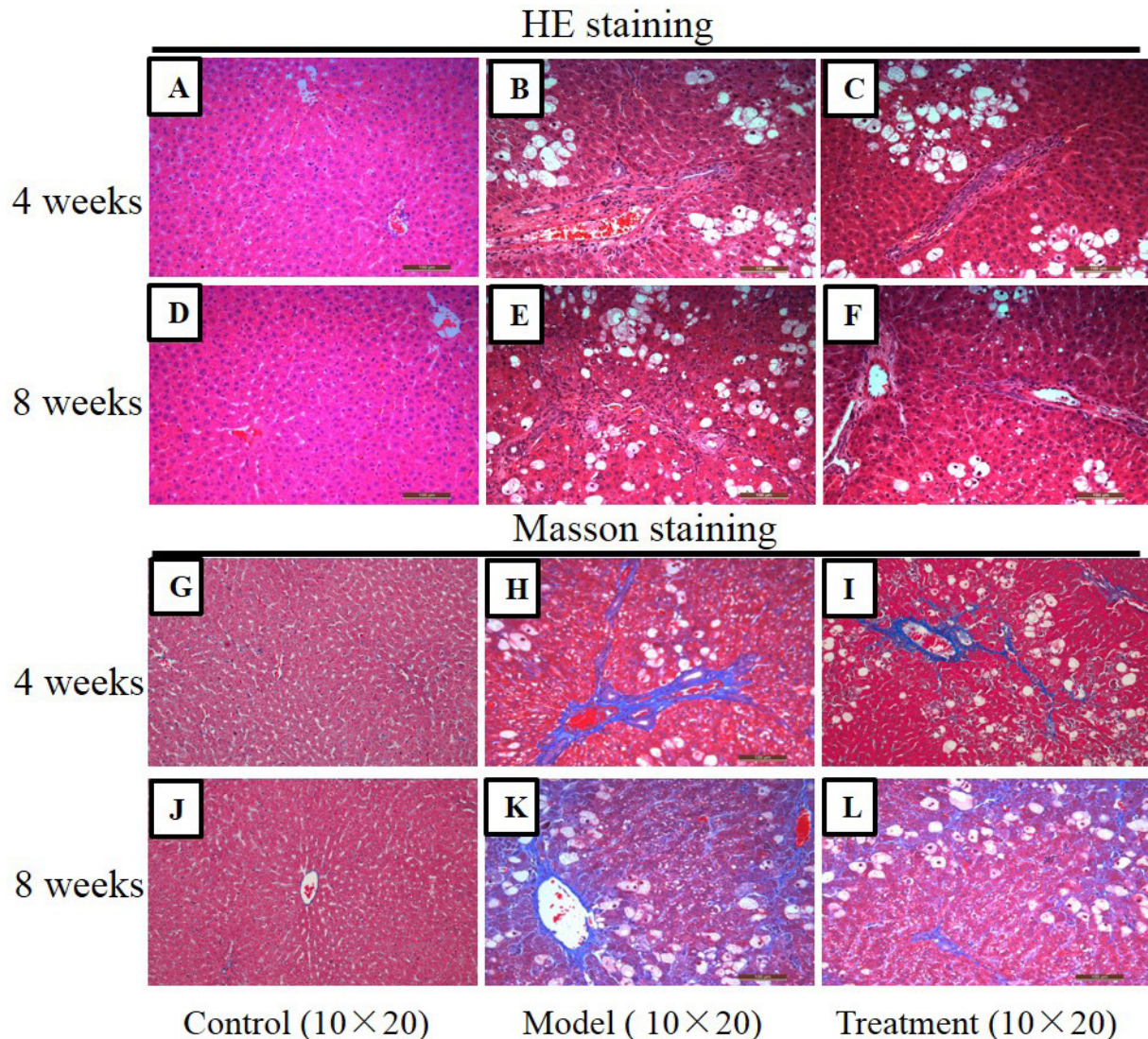


Figure 1: Hematoxylin–eosin staining and Masson staining of rat liver tissues at Weeks 4 and 8. A, D, G, J represent the normal liver tissues; B, E, H, K represent the CCl_4 -induced fibrotic liver tissue; C, F, I, L: DHZCP-treated group. (magnification, $\times 200$).

mainly enriched in ECM–receptor interaction, PI3K–Akt, and focal adhesion pathway, which were essential for liver fibrosis [15].

Compared to model group, a total of 798 DEMs were found in the treatment group of which 120 mRNAs were up-regulated and 678 mRNAs were down-regulated. DEMs were mainly focused on the following GO items: “cytoskeletal protein binding,” “muscle structure contraction,” and “contractile fiber and myofibril,” which might be related to cell migration (Figure 2).

The KEGG database was used to analyze the pathways. Twenty five pathways with significant differences in gene expression were found in the liver tissues of rats in CCL_4 treatment group and treatment group ($p < 0.05$). As shown in Figure 3, DEMs were mainly abundant in

pathways including “local adhesion”, “Phagosome” and “Tight junction.” In addition, differences were observed between “ECM–receptor interactions,” “cell cycle,” and “p53 signaling pathway,” which were strongly related to fibrosis (Figure 4).

3.3 mRNA and protein expression of MMP1 and TIMP1 in liver tissues

We performed real-time PCR to evaluate the MMP1 and TIMP1 mRNAs in different rats (Figures 4A, 4B). Relative to those of the control group, the MMP1 expression decreased significantly, whereas TIMP1 expression increased at Weeks 4 and 8. DHZCP could upregulate MMP1 expression

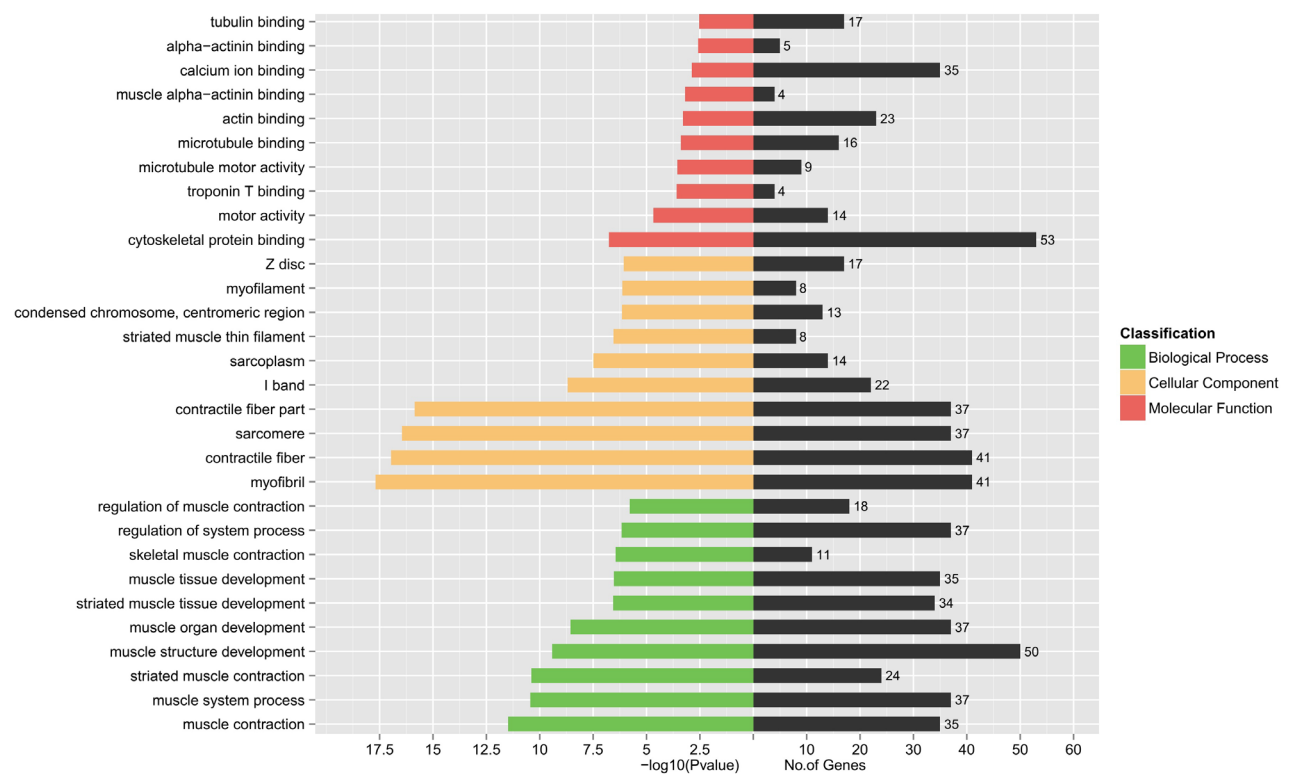


Figure 2: Differentially expressed mRNAs between the model group and the treatment group enriched in Top 10 Gene Ontology terms related to biological process, cellular component, and molecular function.

and downregulate TIMP1 expression in the hepatic liver tissues.

The protein expression levels in the liver tissues were detected by Western blot analysis. We only detected the protein expression in the liver tissues at Week 8 (Figure 3C). Similar gene expression results were determined. Relative to the expression levels in the control group, the MMP1 expression was significantly decreased, whereas, TIMP1 expression increased in the model group. TIMP1 expression was then downregulated, and MMP1 expression was upregulated with DHZCP treatment. We, also, detected the Col1A1 expression in liver tissues. On the basis of the formation of hepatic fibrosis, Col1A1 expression in liver tissues increased in the model group but decreased in the treatment group.

3.4 Expression of MMP1, TIMP1, and Col1A1 in liver tissues by IHC

To determine the expression and location of MMP1 and TIMP1 proteins in liver tissues, we performed IHC by using rats at Weeks 4 and 8. As shown in Figures 5 and 6, protein staining is found in the cytoplasm. Compared

with the control rats, the model rats showed lower MMP1 expression (Figures 5B, 5E) but higher TIMP1 expression in liver tissues (Figures 6B, 6E) at both Weeks 4 and 8; meanwhile, the rats treated with DHZCP showed higher MMP1 expression (Figures 5C, 5F) and lower TIMP1 expression (Figures 6C, 6F).

Meanwhile, we performed IHC to detect Col1A1 in the liver tissue. As shown in Figure 7, brownish-yellow granules in the cytoplasm represented positive Col1A1 expression. The liver tissues in the model group were deeper in color and exhibited more positive staining than those in the control group (Figures 7B, 7E). Staining of Col1A1 suggested a lighter color and fewer positive cells after DHZCP treatment (Figures 7C, 7F).

4 Discussion

Hepatic fibrosis is mainly manifested as deposition caused by imbalance in ECM metabolism and the reconstruction of liver tissue structure. Intervention was administered at an appropriate stage; liver fibrosis could be blocked and reversed, thus delaying its progression to cirrhosis. Numerous documents have suggested that

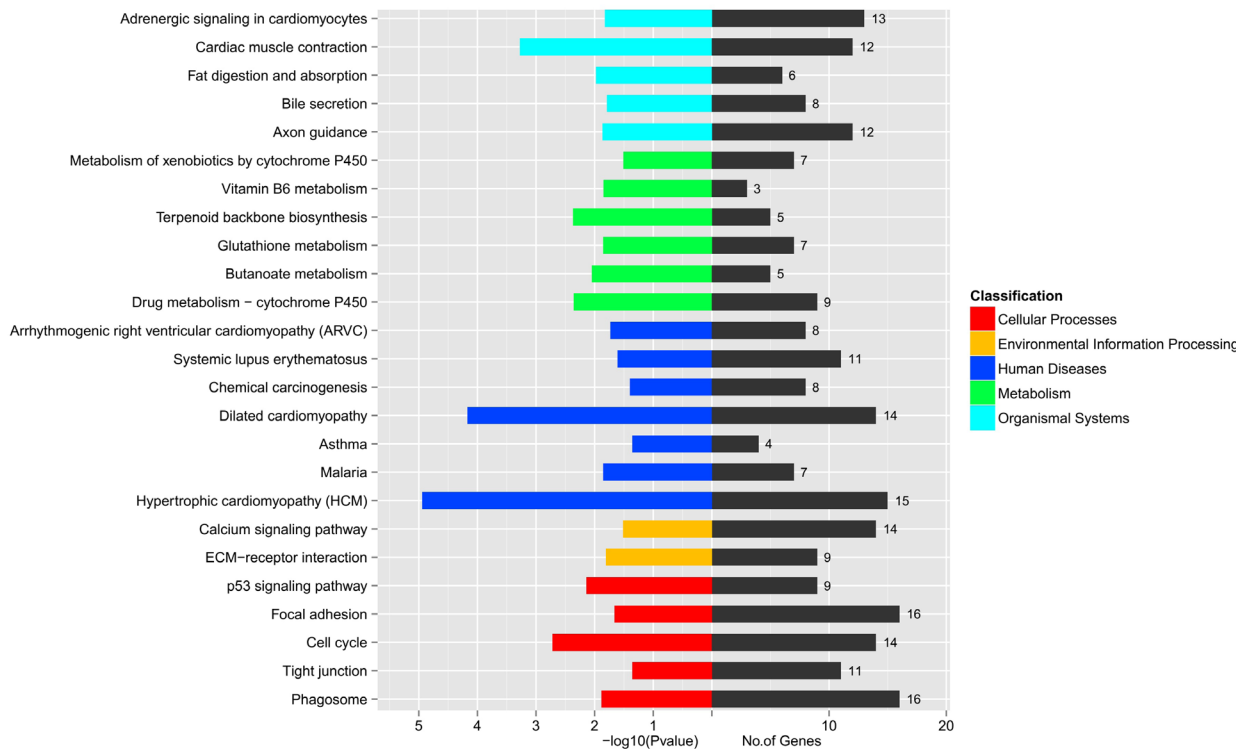


Figure 3: Kyoto Encyclopedia of Genes and Genomes pathway analysis of differentially expressed mRNAs between the model group and the treatment group

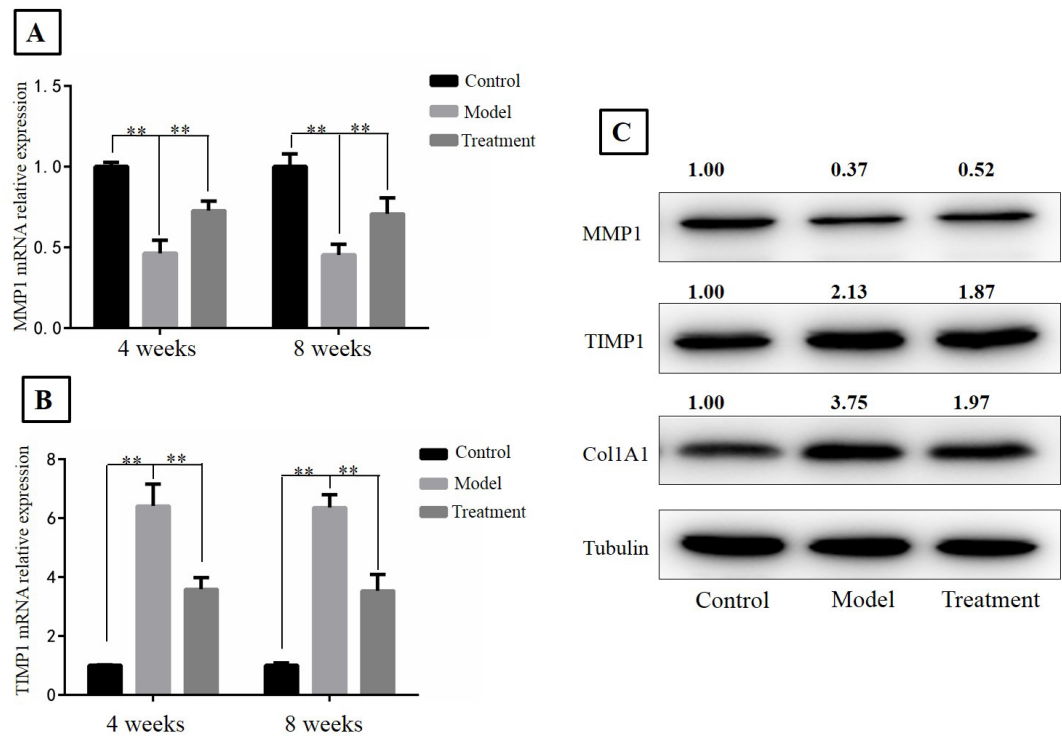


Figure 4: Relative gene and protein expression of MMP1 and TIMP1 in the liver tissues. A: gene expression of MMP1 in the liver tissues of rats at Weeks 4 and 8; B: gene expression of TIMP1 in the liver tissues of rats at Weeks 4 and 8. C: protein expression of MMP1, TIMP1, and Col1A1 in liver tissues of rats at Week 8. The relative expression levels of proteins are marked above the protein bands.

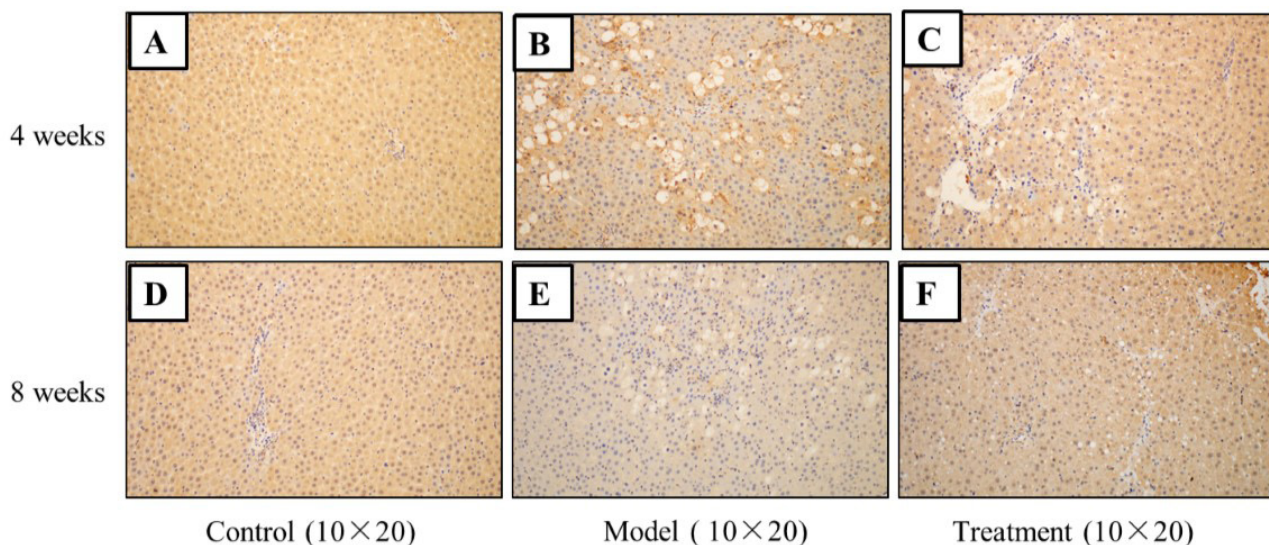


Figure 5: Immunohistochemical staining for MMP1 in liver tissues in the control group, CCl_4 -induced group, and DHZCP-treated group at Weeks 4 and 8. (magnification, $\times 200$).

compounds in Chinese Traditional medicine prescriptions play roles against hepatic fibrosis in long-term clinical practice because of their multilevel, multichannel, and multi-target characteristics [17-19]. Basic research on the intervention of active components from Chinese medicine in the molecular signaling pathway of liver fibrosis has also shown considerable progress [20,21].

A recent study demonstrated that combined with low-dose doxorubicin, DHZCP significantly increased the dose of doxorubicin in tumor tissues, thereby promoting the apoptosis of hepatoma cells and reversing the drug resistance of doxorubicin [22]. DHZCP could not only strengthen the therapeutic effects of doxorubicin but also decrease the doses of doxorubicin and the incidences of adverse reactions, providing novel strategies for the treatment of clinical liver cancer [23]. By downregulating the gene and protein expression of vascular endothelial growth factor and MMP-2, DHZCP inhibited the proliferation and migration of RF/6A cells, thus suppressing the tube formation and angiogenesis of RF/6A cells [24]. By inhibiting the syntheses of DNA and protein, DHZCP can significantly interfere with the proliferation of vascular smooth muscle cells, thus reducing the excretion of the ECM by vascular smooth muscle cells by antagonizing collagen synthesis [25].

The balance between MMPs and suppressor factors TIMPs plays a key role in hepatic fibrosis [2,26-28]. Therefore, to explore the antifibrotic mechanism and to identify new therapeutic methods, the method of regulating the balance between MMPs and TIMPs needs to be elucidated. As an important member of the MMPs

family, MMP1 can mainly hydrolyze collagen I, II, and III. MMP1 is the most active enzyme for fibrinogen degradation and is mainly regulated by TIMP1. MMP1 expression in the normal body is relatively low but can be stimulated by various cytokines, which drive liver fibrosis. In the early stages of liver fibrosis, MMP1 expression increased, and the degradation of ECM collagen was enhanced. Hepatocytes were stimulated and they proliferated. The proliferative hepatocytes released cell growth factors, which further stimulated the regeneration of hepatocytes to repair the damaged hepatocytes and maintain the function and structure of normal liver tissues. When liver injury was sustained or aggravated, TIMP1 expression increased and inhibited the function of MMP1, gradually reducing the degradation of types I, II, and III collagen in ECM, further aggravating liver fibrosis.

A large number of studies investigated the role of MMP1 and TIMP1 in Chinese herbal medicine as antifibrotic treatment. The Chinese medicine CGA formula, which consists of polysaccharide from amygdalin, gypenosides, and *Cordyceps sinensis* mycelia, alleviates dimethylnitrosamine-induced hepatic fibrosis in rats. The effect of the CGA formula on antifibrosis is likely related to the inhibition of MMP2/9 activities, reduction of TIMP1/2 protein expression, and suppression of the TGF-1/Smad signaling pathways in the liver [29]. Yiguanjian decoction inhibits CCl_4 -induced hepatic fibrosis in rats. The therapeutic mechanism may be related to the inhibition of hepatic stellate cell activation and collagen secretion by suppressing Collagen I, TIMP1/2/13/14, and MMP2/9 expression [13]. Danshao Huaxian capsules enhance

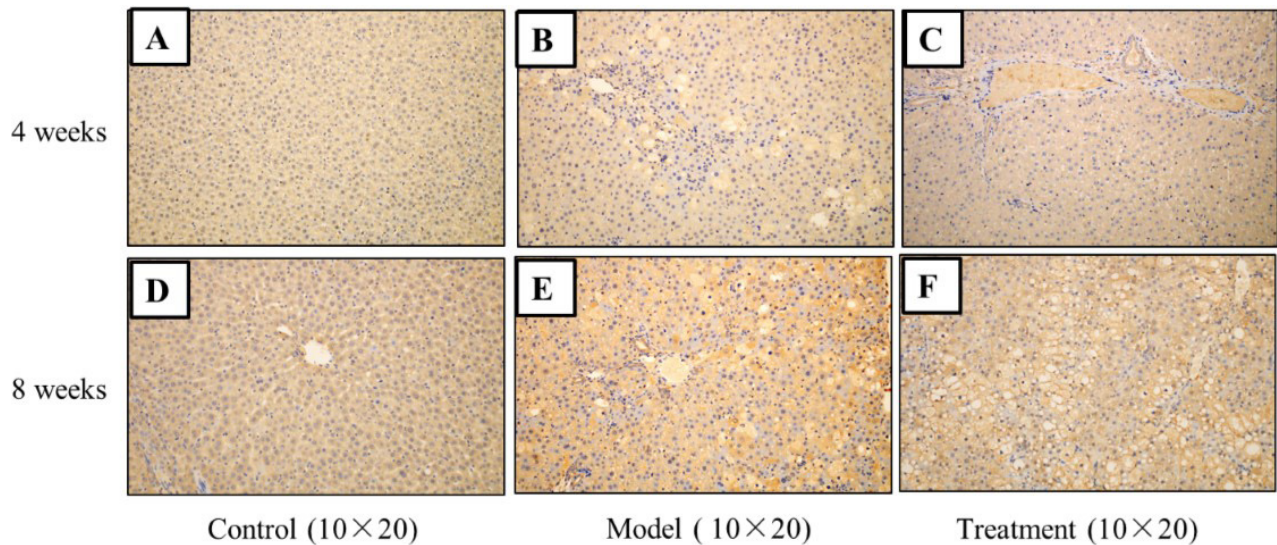


Figure 6: Immunohistochemical staining for TIMP1 in liver tissues in the control group, CCl₄-induced group, and DHZCP-treated group at Weeks 4 and 8. (magnification, x200).

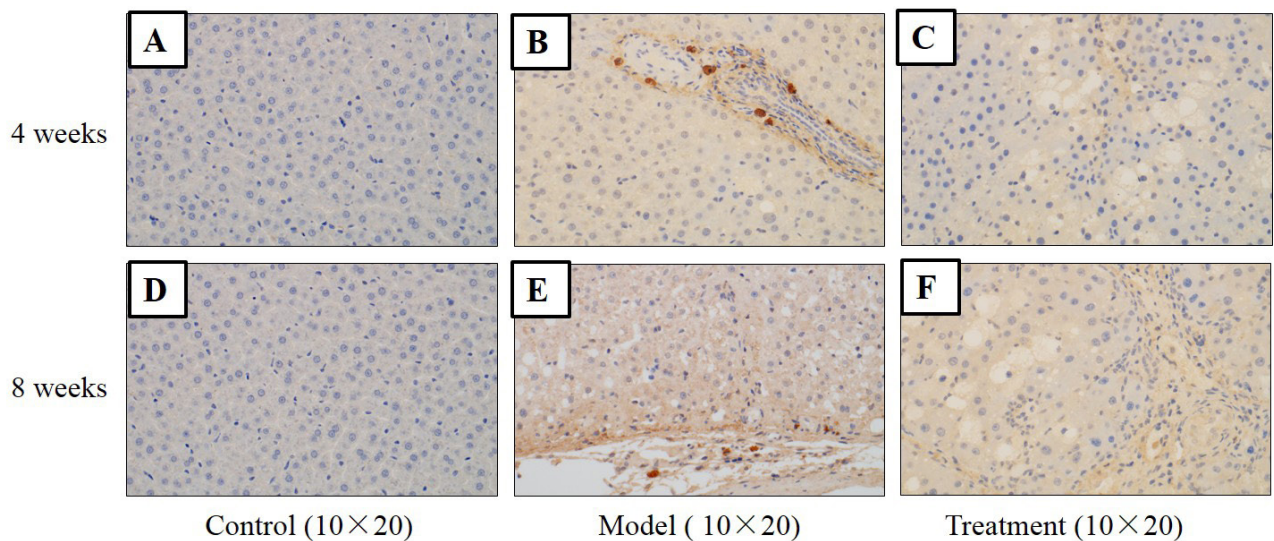


Figure 7: Immunohistochemical staining for Col1A1 in liver tissues in the control group, CCl₄-induced group, and DHZCP-treated group at Weeks 4 and 8. (magnification, x200).

MMP1 expression but decrease TIMP-1 expression in the liver tissues of CCl₄-induced hepatic fibrotic rats, which may induce its elevated activity and thereby contribute to its defense against hepatic fibrosis [30].

The CCl₄-induced liver fibrosis model has been widely used because of its advantages, such as ease of operation, high success rate, and typical lesions in the liver. In the present study, we applied the CCl₄-induced liver fibrosis model to determine whether DHZCP could significantly alleviate liver injury, fatty denaturation, and inflammatory

infiltration; decrease cell death; and prevent the formation of pseudolobules and the reconstruction of the liver tissue. These findings are in accordance with the previous study and confirm the role of DHZCP in liver protection [12,31].

In our previous study, we found that DHZCP could alleviate hepatic fibrosis by increasing the expression of long non-coding RNA growth arrest-specific 5 to suppress p-ERK and by regulating other factors to inhibit p-p38 [32]. We also conducted RNA sequencing to explore the differential genes between the normal and fibrotic liver

tissues. DEMs were mainly on ECM–receptor interaction, focal adhesion, and PI3K-Akt signal pathways [15]. The roles of MMP1 and TIMP1 in the molecular mechanism of DHZCP against liver fibrosis remain largely undetermined. Thus, we used real-time PCR, Western blot analysis, and immunostaining to analyze MMP1 and TIMP1 expression in the liver tissues of rats with CCl₄-induced fibrosis. The results indicated that CCl₄ significantly upregulated TIMP1 expression and then reduced MMP1 expression and function. Figure 6 shows that CCl₄ significantly promotes the synthesis and deposition of Col1A1 at Weeks 4 and 8. This behavior reflects the weakening function of MMP1 in degrading the ECM. Liver fibrosis then occurs. In the DHZCP-treated liver tissue, TIMP1 expression decreased while MMP1 increased, thus decreasing the synthesis and deposition of Col1A1. The fibrosis was thus significantly reduced.

In conclusion, Dahuang Zhechong Pill has the function of protecting the liver from injury and exerting an antifibrotic effect on CCl₄-induced liver fibrosis in rats. Its mechanism may be related to the upregulation of MMP1, downregulation of TIMP1, and promotion of collagen degradation. However, further research has to be conducted to elucidate the precise molecular mechanism of DHZCP.

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Author contributions: JY Lin, CW Deng, YZ Peng, and ZH Gong performed the experiments. YZ Peng and J Zheng analyzed the RNA sequencing data. ZH Gong and GX Hu wrote the manuscript. All authors have approved the final version.

Competing interests: The authors declare that there is no conflict of interest.

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