Original Article

Effects of Shenqi Neijin powder on activation and apoptosis of hepatic stellate cells in rats with hepatic fibrosis

Hongdong Xie, Wei Hou, Yide Yang, Ying Yu, Fengling Wang, Juanjuan Mao

Department of Infectious Disease, Taizhou Municipal Hospital, Taizhou 318000, China

Received November 19, 2014; Accepted January 9, 2015; Epub February 15, 2015; Published February 28, 2015

Abstract: Traditional herbal medicine is usually administrated according to experiences and practices. We aimed to investigate the anti-fibrotic effects of Shenqi Neijin powder (SQNJP) in hepatic fibrosis rats induced by carbon tetrachloride (CCl4). A total of 32 rats were divided into control group, model group, and SQNJP group. The hydroxyproline content was assayed. Histological features of liver tissues were determined with different staining methods. Western blotting analysis and immuno-fluorescence staining were performed to determine the expression of alpha-smooth muscle actin (α-SMA) and the activation of hepatic stellate cells (HSCs). Serial sections were stained with α-SMA immuno-fluorescence staining and the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling method (TUNEL) in turn to detect the apoptosis of HSCs. Fatty degeneration, deposition of collagen, and interval of fibers were noticed in rats induced by CCl4. After administration of SQNJP, remarkable decrease of fatty degeneration, deposition of collagen, and hydroxyproline content were noticed. Compared with the model group, significant decrease of α-SMA protein was noticed after administration of SQNJP, and remarkable apoptosis of HSCs was noticed after treating with SQNJP. SQNJP showed anti-fibrotic effects through inhibiting HSCs activation and inducing apoptosis of HSCs.

Keywords: Apoptosis, hepatic fibrosis, hepatic stellate cells, SQNJP

Introduction

Hepatic fibrosis refers to the accumulation of extracellular matrix (ECM) or scar in response to acute or chronic liver injuries [1]. Hepatic stellate cells (HSCs) have been considered as the main source of mesenchymal cells involved in hepatic fibrosis. To our knowledge, liver fibrosis is closely related to activation of HSCs, a process characterized by increased cellular proliferation, transformation of fibroblasts into myofibroblasts, increased expression of alpha-smooth muscle actin (α-SMA) and excessive accumulation of ECM [2, 3]. Currently, termination of the proliferation of activated HSCs by cell apoptosis has been considered as a promising strategy for hepatic fibrosis treatment. For example, Wang et al reported that ursolic acid could ameliorate the hepatic fibrosis in rat model through specific induction of HSCs apoptosis [4]. Meanwhile, apoptosis of hepatic stellate cells played an important role in the spontaneous recovery of biliary fibrosis in rats subjected to bile duct ligation [5]. Unfortunately, no effective and targeted anti-fibrotic drugs have been approved by FDA, demonstrating an extreme need for the investigation of hepatic fibrosis treatment.

Traditional herbal medicine with a history of more than thousands of years is based on experiences and practices. To date, several herbal medicines have been used to treat liver fibrosis, and have been approved to be efficacious in clinical practices [6, 7]. Shenqi Neijin powder (SQNJP), a traditional Chinese medicine formula consisted of Panax quinquefolius, Radix notoginseng, and endothelium corneum gigeriae galli, has been widely used for the treatment of various liver conditions in China mainland [8]. Despite its popularity for the treatment of hepatic diseases, data related to its quality standardization or the mechanism are still lacking. In our previous study, SQNJP plus lamivu-
Shenqi Neijin powder and hepatic fibrosis
dine has been used for the hepatic cirrhosis of
d the results were satisfactory [9]. On this basis, we
strongly believe that SQNJP may play an impor-
tant role in the control of hepatic fibrosis. Thus,
we investigate the potential efficacy of SQNJP
on the cellular activation and/or apoptosis in
experimental hepatic fibrosis rats in this study.

Materials and methods

Animals

Male Sprague Dawley rats weighing 160±10 g
were purchased from Shanghai Laboratory
Animal Center. The animals were housed under
controlled temperatures and a 12 h/12 h light/
dark cycle with food and water. All the experi-
ments were performed adhered to the Prin-
ciples of Laboratory Animal Care (NIH Publi-
cation No. 86-23, revised 1985) and the regu-
lation of the Committee on the Use and Care of
Animals of Fudan University (Shanghai, China).
This study was approved by the Ethics Com-
mittee of the Taizhou Municipal Hospital
(Taizhou, China).

Preparation of SQNJP

SQNJP was made from 30 g Panax quinquefo-
lus, 30 g Radix notoginseng, and 60 g endo-
thelium corneum gigeriae galli. The mixture was
ground into powder, and was kept at room tem-
perature until usage.

Induction of hepatic fibrosis in rats

The induction of rats with hepatic fibrosis was
carried out as previously described [10]. In
brief, single administration of carbon tetrachlo-
rade (CCl4, Sinopharm Chemical Reagent Co.,
Ltd. Shanghai, China) was given via subcutane-
ous injection at a dose of 5 ml/kg body weight
for the first time. For the following induction,
40% CCl4 in olive oil was administrated via sub-
cutaneous injection at a dose of 3 ml/kg body
weight. The administration was performed twi-
ce a week for 6 weeks.

Experimental design

The animals were divided into: a) control group
(n=8), which were subjected to intragastric
administration of physiological saline daily for
two weeks; b) model group (n=12), which were
subjected to intragastric administration of
physiological saline daily after the induction of
hepatic fibrosis using CCl4 for two weeks; and
c) SQNJP group (n=12), which were subjected to
intragastric administration of SQNJP powder at
a dose of 0.8 g/kg body weight daily after the
induction of hepatic fibrosis for two weeks.

Histological determination

Neutral formalin-fixed liver tissues were embed-
ded using paraffin. These samples were then
cut into a 5 μm in depth. Subsequently, the
samples were stained using hematoxylin and
eosin and sirius red, respectively.

Measurement of hepatic hydroxyproline con-
ten

The level of hydroxyproline was detected using
Jamall’s method [11]. Briefly, 100 mg liver tis-
sues were homogenized at 4°C. After the affili-
ation of 12 mol/L HCl, the mixture was incubat-
ed at 105°C for 18 hours. After hydrolysis,
samples were neutralized with 10 mol/L NaOH,
oxidized with chloramine-T, and incubated in
Ehrlich’s perchloric acid solution at 50°C for 90
minutes. A wavelength of 558 nm was chosen
for the absorbance. Hepatic collagen content
was analyzed by Sirius red staining of paraffin-
embedded sections.

Western blotting analysis of α-SMA protein

Western blotting analysis was performed as
routinely described [12]. In brief, the tissues
sections were homogenized in RIPA lysis buffer
containing protease and phosphatase inhibi-
tors. Proteins were separated by electrophore-
sis on a 10% SDS-PAGE gel and transferred to a
Hybond-P PVDF membrane. Subsequently, the
membrane was blocked in 5% nonfat milk and
incubated with an α-SMA primary antibody
(1:1000 dilution, Sigma-Aldrich Inco., St. Louis,
MO, USA) overnight at 4°C, followed by incuba-
tion with the peroxidase-conjugated secondary
antibody (1:1000 dilution, Sigma-Aldrich Inco.,
St. Louis, MO, USA) for 1 h at room tempera-
ture. After washing with PBS, the bound prima-
ry antibody was visualized with the ECL western
blotting substrate kit. Then the images were
captured using a Bio-Rad Imager system, and
were analyzed with Quantity One software (Bio-
Rad Inco., Hercules, CA, USA). The same mem-
brane was probed for GAPDH for loading con-
trol. The relative density of α-SMA to GAPDH
represented the expression of α-SMA. All the
experiments were carried out in triplicates.
Shenqi Neijin powder and hepatic fibrosis

Immuno-fluorescence staining of α-SMA

Tissue sections were incubated with a monoclonal goat anti-mice α-SMA primary antibody (1:1000 dilution, Sigma-Aldrich Inco., St. Louis, MO, USA) overnight at 4°C, followed by incubation with the rose Bengal-conjugated goat anti-mice IgG (1:1000 dilution, Sigma-Aldrich Inco., St. Louis, MO, USA) for 2 h at room temperature. Then the sections were observed using a TCS-SP2 confocal laser microscopy (Heidelberg, Germany).

Immuno-fluorescence staining and TUNEL staining of α-SMA and apoptosis cells

Three rats were sacrificed at 72 h after the last intragastric administration in each group. The optimal cutting temperature (OCT) compound embedded liver tissues were fixed using frozen acetone. Subsequently, the sections were incubated with 5% goat serum to reduce nonspecific background staining, followed by α-SMA primary antibody (1:1000 dilution, Sigma-Aldrich Inco., St. Louis, MO, USA) for overnight incubation at 4°C. After that, the sections were incubated with rose Bengal linked goat anti-mice IgG (1:1000 dilution, Sigma-Aldrich Inco., St. Louis, MO, USA) for 2 h at room temperature. TUNEL staining was performed using a commercial kit (Dead End® Fluorometric TUNEL system,) purchased from KeyGen Biotech Co., Ltd. Finally, specimen were observed and photographed under a FV1000 confocal laser microscopy (Olympus Inco., Tokyo, Japan).

Statistical analysis

All the data were presented as mean ± standard deviation. SPSS 12.0 software was used for the data analysis. ANOVA post hoc analysis (q test) was carried out for the inter-group comparison. \( P < 0.05 \) demonstrated statistical difference.

Results

Histological measurements

The anatomical structure of hepatic lobule and hepatocytes was normal and slight collagen

Table 1. Effects of SQNJP on Hydroxyproline content

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Concentration (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>8</td>
<td>203.39±30.05</td>
</tr>
<tr>
<td>Model group</td>
<td>10</td>
<td>610.64±68.40**</td>
</tr>
<tr>
<td>SQNJP group</td>
<td>11</td>
<td>314.85±52.07##</td>
</tr>
</tbody>
</table>

**, P < 0.01, compared with control group; ##, P < 0.01, compared with model group.
deposition was identified in the peripheral blood vessels of portal area in control group. Swelling of hepatocytes was noticed in model group. Meanwhile, significant vacuolar degeneration was observed in hepatic fatty tissues. Ballooning degeneration and dispersed necrosis were noticed in majority of hepatocytes. Inflammatory cell infiltration was noticed in the portal area and interval of fibers. Significant fibroplasia and deposition were observed in the collagen fibers located in the portal area and the hepatocytes with severe fatty degeneration, partial of which transformed to interval of fibers to separate the hepatic lobule. For the SQNJP group, significant amelioration was noticed in the fatty degeneration, inflammation, necrosis, and infiltration of inflammatory cells. Meanwhile, deposition of collagen fiber showed remarkable decrease (Figure 1).

Expression of α-SMA protein attenuated after administration of SQNJP

As indicated in Table 1, significant increase was noted in the concentration of hydroxyproline in hepatic tissues after hepatic fibrosis compared with normal group (P < 0.01, Table 1). However, the level of hydroxyproline showed remarkable decrease in SQNJP group compared with the model group (P < 0.01). No statistical difference was noted in the concentration of hydroxyproline in hepatic tissues in the SQNJP group compared with the control group (P > 0.05).

Hydroxyproline content decreased after administration of SQNJP

Results of immunohistochemical staining showed slight expression of α-SMA protein in the vessel walls in the animals of control group (Figure 2). Nevertheless, enhanced expression of α-SMA protein was noticed in model group. The expression was mainly distributed in the
interval of liver fibers, especially the hepatic tissues with fatty degeneration. Compared with the model group, significant decrease was noticed in the staining intensity of α-SMA protein in the SQNJP group, and at the same time, the expression of α-SMA protein in the SQNJP group showed a strip-like pattern (Figures 2 and 3).

Apoptosis of HSCs after administration of SQNJP powder

To investigate whether SQNJP induces apoptosis in hepatic fibrosis rats, TUNEL assay was performed. Few α-SMA positive staining cells were noticed in the control group. No positive staining cell of TUNEL was noticed in control group. For the model group, few TUNEL-positive staining cells were identified. Nevertheless, at 72 h after administration of SQNJP, significant increase of TUNEL-positive staining cells was noted compared with those of the model group and control group (Figure 4).

Discussion

Currently, HSCs-apoptosis targeting therapeutic strategies has been approved to be promising for hepatic fibrosis treatment. In this study, our results showed that a traditional Chinese medicine, SQNJP, could effectively reduce CCl4 induced hepatic fibrosis in rats. Our results indicated that hydroxyproline content and expression of α-SMA protein were significantly

![Figure 4. Apoptosis of HSCs and expression of α-SMA protein in control group (A-C), model group (D-F), and SQNJP group (G-I). Apoptosis indicated by TUNEL assay (B, E, H). Expression of α-SMA protein indicated by immuno-fluorescent assay (A, D, G). Double staining of HSCs apoptosis and expression of α-SMA protein (C, F, I).]
Shenqi Neijin powder and hepatic fibrosis

decreased after SQNJP administration. In addition, remarkable apoptosis of HSCs was noted in CCl4 induced hepatic fibrosis rats compared with the model group and control group. All these suggested that SQNJP could exert anti-fibrotic effects in hepatic fibrosis rats via inhibition of HSCs activation.

Hydroxyproline has been specifically detected in collagen, which plays a major role in hepatic fibrosis [13]. Generally, it has been considered as an effective method to evaluate the expression of collagen in vitro or in vivo [14]. To our best knowledge, elevation of liver hydroxyproline content was closely associated with hepatic fibrosis without inducing cell necrosis, and at the same time, the increase of liver collagenase activity was identified [15]. In our study, significant decrease of hydroxyproline content was noticed after administration of SQNJP compared with the model group.

To date, to remove the causative agent is still the only effective therapy to terminate or even reverse hepatic fibrosis [16, 17]. Nevertheless, the cellular and molecular mechanisms underlying hepatic fibrosis have been clearly depicted. Attempts have been carried out for the termination of the proliferation of activated HSCs through cell apoptosis for hepatic fibrosis. Although many of these approaches are experimentally effective in animal models with hepatic fibrosis, its clinical efficacy and safety in humans are still not well defined. A number of drugs are available to reduce the accumulation of scar tissue in animal models with chronic liver injury, such as rennin-angiotensin system blockers and antioxidants, however, the lack of clinical trials is still a great obstacle for their popularity in clinical practices [17].

Recently, more attention has been paid to the traditional Chinese medicine due to its characteristics with few side effects. For instance, many studies have been carried out to investigate the anti-fibrotic effects of herbal medicine. In a recent study, Parajuli et al reported PF2401-SF, a standard fraction of *Salvia miltiorrhiza*, could induce apoptosis of activated HSCs in vitro and in vivo. Additionally, a recent review summarized that traditional Chinese medicine and its effective components showed anti-fibrosis effects through inhibition of cell proliferation, regulation of cytokines and interference of signal transduction [18]. Several clinical trials have been carried out in China mainland using herbal medicine for treatment of hepatic fibrosis patients, but the results are still controversial [19]. SQNJP, an ancient traditional Chinese medicine consisted of *Panax quinquefolius*, *Radix notoginseng*, and endothelium corneum gigeriae galli, has been extensively used in clinical practices in China mainland with satisfactory clinical efficiencies [8]. Nevertheless, no study has been performed to investigate its anti-fibrotic efficiency in experimental animal models. In this study, the animals treated with SQNJP showed decreased hydroxyproline content in hepatic tissues. Meanwhile, expression of α-SMA protein decreased after treating with SQNJP compared with the model group. All these indicated that SQNJP could inhibit the activation of HSCs. Simultaneously, remarkable cell apoptosis of HSCs was noticed at 72 h after administration of SQNJP compared with the model group, which demonstrated that it may be involved in the amelioration of hepatic fibrosis through targeting the cell apoptosis of HSCs.

In conclusion, SQNJP showed satisfactory anti-fibrosis efficiency in rats with hepatic fibrosis through inhibiting cellular activation and inducing cell apoptosis of HSCs. Further randomly controlled trials are needed to investigate its clinical efficacy for treatment of hepatic fibrosis.

Acknowledgements

This study was supported by the Zhejiang TCM Scientific Research Projects (No. 2011ZA112 and No. 2012ZB173).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hongdong Xie, Department of Infectious Disease, Taizhou Municipal Hospital, 381 Zhongshandong Road, Jiaojiang District, Taizhou 318000, China. Tel: +86-576-888-58261; Fax: +86-576-88858261; E-mail: hongdong_xie@163.com

References

Shenqi Neijin powder and hepatic fibrosis


