Effect of Rougan Huaqian granules combined with human mesenchymal stem cell transplantation on liver fibrosis in cirrhosis rats

Zhen-Chang Wang¹, Shan Yang², Jing-Jing Huang¹, Song-Lin Chen¹, Quan-Qiang Li², Yuan Li²*

¹Liver Disease Center, The First Affiliated Hospital of Guangxi University of Traditional Chinese Medicine, Nanning 530023, China
²Guangxi University of Traditional Chinese Medicine, Nanning 530007, China

ARTICLE INFO

Objective: To observe the effect of Rougan Huaqian granules combined with human mesenchymal stem cell (hMSC) transplantation on the liver fibrosis in carbon tetrachloride-induced cirrhosis rats. Methods: Sixty SD rats were randomly divided into five groups. The rats in control group received intraperitoneal injection of saline, while those in model control group, treatment group A, group B and group C received intraperitoneal injection of carbon tetrachloride oily solution to induce liver cirrhosis within 8 weeks. Then, the rats in the model control group, treatment group A, treatment group B, treatment group C received vein tail injection of saline, Rougan Huaqian granules, hMSC suspension and Rougan Huaqian granules combined with hMSC suspension. Results: The treatment groups had significantly different liver function (AST levels), liver fibrosis index (laminin and HA), hepatic sinusoidal walls (α-smooth muscle actin, IV collagen, and laminin protein expression and I, III collagen) from the model group (P<0.05). The transplanted cells showed human hepatocyte-like cells differentiation trend in the liver. Conclusions: The Rougan Huaqian granules combined with hMSC transplantation can alleviate liver fibrosis in cirrhosis rats.

1. Introduction

Cirrhosis is a complex pathological process. In clinic, the only effective treatment method is liver transplantation, but restricted by the donor, surgery costs, limited cure rate and other factors. Recent studies suggest that bone marrow-derived mesenchymal stem cells (MSCs) as a member of adult stem cells may regulate the liver function of the liver failure animal. MSCs have lots of advantages such as easy obtainment, strong copy and proliferative capacity, and mature culture in vitro technology[1]. Rougan Huaqian granules are a new prescription which is a combination of the treatment of prominent TCM doctors on hepatitis cirrhosis. It has a good liver protection and anti-hepatic fibrosis effects[2]. However, its mechanism is still not clear. In this study, we transplanted the Rougan Huaqian granules combined with human MSCs (hMSCs) into the carbon tetrachloride-induced liver cirrhosis rats and observed the survival rate, liver function, hepatic stellate cells (HSC) activation and pathological changes after this treatment.

2. Materials and methods

2.1. Rats

Sixty healthy SPF grade SD rats (male or female) weighing 160–200 g were selected. All animals were provided by the Experimental Animal Center of Guangxi Province and housed in the SPF animal room (n=5) with autoclaved water for free drink, a constant temperature of 25 °C, and constant humidity of 40%–50%.
2.2. Reagents and instruments

An incubator was purchased from Eppendorf company, Germany. An inverted microscope was purchased from Nikon company, Japan. FACS was purchased from BD Biosciences. Rabbit anti-mouse α-smooth muscle actin (α-SMA) monoclonal antibody, IV collagen (Col IV) monoclonal antibody, rabbit anti-mouse laminin (LN) monoclonal antibody, and anti-mouse CD29, CD34, CD44, CD45, CD105, CD133, CD147 monoclonal antibody were purchased from American BioLegend company. RT-PCR kits were purchased from Bioaiss Biological Technology Co., Ltd. Collagen type I (Col I), collagen type III (Col III), MMP-13 and β-actin antibodies were purchased from Wuhan Boster biotechnology company. CCl4 was purchased from Jiangsu Hengrui Corporation, ALT, α(1,3) glucan, collagen type II (Col II) and MMP-13 antibodies (concentration of 5 g/kg) and received from Bioasis Biological Technology Co., Ltd. Collagen type I (concentration of 5 g/kg) and received vein tail injection of 1×10^6 hMSCs once a day. The rats in the treatment group B were filled Rougan Huaqian granules into the stomach (concentration of 5 g/kg) and received vein tail injection of 1×10^6 hMSCs once a day. Finally, all rats were sacrificed.

2.3. In vitro culture of hMSCs

Umbilical cord blood were immediately collected from newborns after delivery under sterile conditions. Cord blood monocytes were isolated with lymphocyte separating medium and inoculated on culture dish. The conditions are DMEM (Dulbecco’s Modified Eagle’s Medium) culture medium containing 10% (v/v) fetal bovine serum at 37 °C with saturation humidity in 5% (v/v) CO2. The medium was changed after 2 d. When cell confluence was 80%, the cells were sub–cultured by proportion 1:3 with the digestion of 0.25% trypsin and 0.02% EDTA.

2.4. Determination of cell phenotype

A proper amount of the 5th generation of human umbilical cord blood (hUCB) MSCs were made into single cell suspension and added the anti–human CD29, CD34, CD44, CD45, CD105, CD133, CD147 monoclonal antibody, followed by incubation at 4 °C for 30 min to determine cell phenotype by flow cytometry.

2.5. Experimental animal model and grouping method

Sixty SD rats were randomly divided into control group, model group, treatment group A, treatment group B and treatment group C (n=12). The control group received intraperitoneal injection of saline 3 mL/kg, 2 times a week for 8 weeks. Four rats were sacrificed and the blood and liver tissue were collected for liver function test and HE staining, respectively. The model control group received intraperitoneal injection 50% volume fraction of carbon tetrachloride oily solution 3 mL/kg, 2 times a week for 8 weeks. Four rats were sacrificed and the blood was obtained for liver function test. Their liver tissues were used for HE staining and immunohistochemical staining. After the injection of carbon tetrachloride olive oil for 8 weeks, the rats filled 10 mL/kg saline into the stomach also received 0.2 mL vein tail injection of saline once a day for 4 weeks. Before 8 weeks, the method of treatment group, treatment group B and treatment group C was the same as that of the model group. After 4 weeks, the rats in the treatment group A were filled Rougan Huaqian granules into the stomach (concentration of 5 g/kg) and received vein tail injection of 0.2 mL saline once a day. The rats in the treatment group B were filled 10 mL/kg saline into the stomach and received vein tail injection of 1×10^6 hMSCs once a day. The rats in the treatment group C were filled Rougan Huaqian granules into the stomach (concentration of 5 g/kg) and received vein tail injection of 1×10^6 hMSCs once a day. Finally, all rats were sacrificed.

2.6. Serological examination

Rats were anesthetized and subjected to thoracotomy. The blood from inferior vena cava was added 0.3% heparin sodium anticoagulant. Then 1 mL of blood was centrifuged and used to determine the levels of AST, ALT and hepatic fibrosis indexes.

2.7. Histological observation

Rats were anesthetized and subjected to thoracotomy. The liver tissue was collected at hepatic hila for HE staining, and the histological features were observed. Sinusoidal walls paraffin sections were dewaxed, using immunohistochemical EnVision method. Rabbit anti–rat α-SMA monoclonal antibody (working concentration 1:100), Col IV monoclonal antibodies (working concentration 1:100), LN monoclonal antibody (working concentration 1:100) and ready–to–use secondary antibody were used for staining in accordance with instructions. PBS instead of primary antibody was used as a negative control.

2.8. RT–PCR detection of Col I, Col III, and MMP–13 mRNA expression

Partial liver tissue was immediately collected after surgical resection and stored in liquid nitrogen. Total RNA was extracted using Trizol method. The primers are shown in Table 1. After agarose gel electrophoresis, the results were analyzed under DC2000 gel imaging analyzer.

The gray integral value of each stripe was automatically read and recorded by the computer. Statistical analysis was conducted based on sample integral value/internal reference ratio. Results were determined by the double–blind method,
and three slice of each group were separately judged by two experienced pathologists. Five typical slice were selected and five different visual fields were observed under ×200 magnification. Sinusoidal walls positive region of the standard measurement window area was determined.

2.9. Statistical analysis

All data are expressed as mean±SD. The data were analyzed by SPSS 13.0 statistics software. P<0.05 was considered statistically significant.

3. Results

3.1. Determination of hMSCs cell phenotype

CD29, CD34, CD44, CD133 and CD147 were positive, while CD45 and CD105 were negative.

3.2. Changes of body weight and liver weight before and after the transplantation of hMSCs

The rats in the normal control group showed normal activities. The liver was purple–red with thin sharp edges and smooth surface. The body weight and liver weight are shown in Table 1. The rats in the model control group had poor mental status, less activity and decrease of food intake and weight. Compared with the normal group, the difference was statistically significant (P<0.05). Liver volume increased significantly than the normal liver, and the surface has multiple homogeneous nodes. The rats in treatment group A had poor mental status, less activity and decrease of food intake and weight. Compared with the normal group, the difference was statistically significant (P<0.05).

The liver surface was relatively smooth with rare nodules. Compared with the normal group, the difference was not statistically significant. The rats in the treatment group B had normal spirit, activity, diet and normal weight. Liver volume was increased significantly compared with the normal liver, and the surface was relatively smooth with rare nodules. Compared with the normal group, model control group, treatment group A, the differences were statistically significant (P<0.05). The body weight and liver weight changes are shown in Table 2.

3.3. Changes of serum index before and after the transplantation of hMSCs

The ALT, AST in the model control group and treatment group showed an increasing trend (Table 3). Compared with the normal group, the difference was statistically significant (P<0.05). The AST change in the treatment group C was lower than that in the model control group, and the differences were statistically significant (P<0.05). The HA and LN in the treatment group were lower than those in the control group, and the difference was statistically significant (P<0.05). The PC I and CIV of four indicators of hepatic fibrosis did not change significantly in each group (Table 3).

3.4. Liver pathological changes

HE staining of liver tissue sections showed that normal control group rats had normal structure of hepatic lobule and no inflammatory cell infiltration, and there was only a small amount of collagen fibers around the portal area and central vein. Rat lobules in the model control group were separated by hyperplasia of liver collagen fibers and formed the hepatic pseudolobule, which was obvious around the capsule, portal area and central vein. The collagen fibers hyperplasia in the treatment group A were significantly reduced, there was still a small amount of pseudolobule, and the collagen fibers were also significantly reduced. The collagen fibers hyperplasia in the treatment group B were significantly reduced, and there was still a small amount of pseudolobule, but the collagen fibers were around the capsule, portal area and central vein. There were many morphologically normal liver cells surrounding normal

| Table 1 |
| Primer sequences for NF-κβ, MMP-2 and β-actin genes. |
| Primer name | Primer sequences | Fragment length (bp) | Annealing temperature (℃) |
| Collagen type I | 5′-cagacggaggttccttcggagct-3′ | 730 | 58 |
| Collagen type II | 5′-cagacggaggttccttcggagct-3′ | 600 | 60 |
| MMP-2 | 5′-ggctggtcagtggcttgggta-3′ | 225 | 63 |
| β-actin | 5′-ggcagagcaacgacag-3′ | 626 | 59 |
were many morphologically normal liver cells surrounding lobules. The collagen fibers hyperplasia in the treatment group C was significantly reduced, although there was still a small amount of pseudolobule. The collagen fibers around and in the lobules were significantly reduced, and there were many morphologically normal liver cells surrounding lobules.

3.5. The α-SMA, Col I and LN protein expression in sinusoidal walls

In the normal group, the rat liver α-SMA-positive staining was limited to sinusoidal walls, Col I mainly existed in the sinusoidal walls with continuous positive expression, and LN existed in sinusoidal walls with discontinuous positive expression. The α-SMA positive expression was significantly increased in the model control group and the range was significantly expanded, and Col I, LN positive expression were significant. The α-SMA, Col I and LN positive degree in each treatment group was decreased at different levels (Table 4).

3.6. Relative expression levels of Col I, Col III and MMP–2 mRNA in liver tissue

All samples were analyzed by RT–PCR, and the semi-quantitative results showed the Col I, Col III and MMP–2 expression in the model control group were significantly higher than that in the normal control group. The Col I and Col III in the treatment group A, treatment group B and treatment group C were significantly lower than those in the model control group, and the difference was statistically significant (P<0.05) (Table 5).

4. Discussion

Cirrhosis is an irreversible and incurable disease. Liver transplantation is an effective method for cirrhosis treatment, but the shortage of donor, high cost and other factors have limited the promotion of this method. In recent years, stem cell transplantation as a new treatment for cirrhosis attracts more and more attention. Rougan Huaqian granules mainly treat liver fibrosis by invigorating spleen and kidney, nourishing Yin and nourishing liver, strengthening body resistance and eliminating dampness. HMSCs can differentiate into various types of terminal potential mature cells, which have lots of advantages such as higher ability to differentiate, easy amplification in vitro and lower rejection reactions. Related studies suggest that the protein expression can still be detected after 6 weeks of liver transplantation.

Table 2
Body weight and liver weight changes before and after the transplantation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control group</td>
<td>388±34</td>
<td>97±11</td>
</tr>
<tr>
<td>Model control group</td>
<td>322±29</td>
<td>178±24</td>
</tr>
<tr>
<td>Treatment group A</td>
<td>322±29</td>
<td>112±15</td>
</tr>
<tr>
<td>Treatment group B</td>
<td>359±28</td>
<td>198±22</td>
</tr>
<tr>
<td>Treatment group C</td>
<td>362±30</td>
<td>216±25</td>
</tr>
</tbody>
</table>

3.5. The α-SMA, Col I and LN protein expression in sinusoidal walls

In the normal group, the rat liver α-SMA-positive staining was limited to sinusoidal walls, Col I mainly existed in the sinusoidal walls with continuous positive expression, and LN existed in sinusoidal walls with discontinuous positive expression. The α-SMA positive expression was significantly increased in the model control group and the range was significantly expanded, and Col I, LN positive expression were significant. The α-SMA, Col I and LN positive degree in each treatment group was decreased at different levels (Table 4).

Table 4
Sinusoidal walls α-SMA, Col I and LN positive area ratio.

<table>
<thead>
<tr>
<th>Group</th>
<th>α-SMA</th>
<th>Col I</th>
<th>LN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control group</td>
<td>1±0</td>
<td>6±1</td>
<td>1±0</td>
</tr>
<tr>
<td>Model control group</td>
<td>10±2</td>
<td>8±2</td>
<td>7±1</td>
</tr>
<tr>
<td>Treatment group A</td>
<td>6±2</td>
<td>8±2</td>
<td>5±1</td>
</tr>
<tr>
<td>Treatment group B</td>
<td>5±1</td>
<td>8±2</td>
<td>3±1*</td>
</tr>
<tr>
<td>Treatment group C</td>
<td>4±1</td>
<td>7±1</td>
<td>2±1*</td>
</tr>
</tbody>
</table>

3.6. Relative expression levels of Col I, Col III and MMP–2 mRNA in liver tissue

All samples were analyzed by RT–PCR, and the semi-quantitative results showed the Col I, Col III and MMP–2 expression in the model control group were significantly higher than that in the normal control group. The Col I and Col III in the treatment group A, treatment group B and treatment group C were significantly lower than those in the model control group, and the difference was statistically significant (P<0.05) (Table 5).

Table 5
Expression levels of Col I, Col III and MMP–2 mRNA.

<table>
<thead>
<tr>
<th>Group</th>
<th>Col I</th>
<th>Col III</th>
<th>MMP–2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control group</td>
<td>0.2±0.1</td>
<td>0.6±0.1</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>Model control group</td>
<td>1.6±0.8</td>
<td>1.3±0.2</td>
<td>0.6±0.3*</td>
</tr>
<tr>
<td>Treatment group A</td>
<td>1.2±0.5*</td>
<td>1.1±0.2*</td>
<td>0.6±0.2*</td>
</tr>
<tr>
<td>Treatment group B</td>
<td>1.1±0.4*</td>
<td>1.2±0.2*</td>
<td>0.5±0.2*</td>
</tr>
<tr>
<td>Treatment group C</td>
<td>0.7±0.2*</td>
<td>1.0±0.2*</td>
<td>0.5±0.1*</td>
</tr>
</tbody>
</table>

4. Discussion

Cirrhosis is an irreversible and incurable disease. Liver transplantation is an effective method for cirrhosis treatment, but the shortage of donor, high cost and other factors have limited the promotion of this method. In recent years, stem cell transplantation as a new treatment for cirrhosis attracts more and more attention. Rougan Huaqian granules mainly treat liver fibrosis by invigorating spleen and kidney, nourishing Yin and nourishing liver, strengthening body resistance and eliminating dampness. HMSCs can differentiate into various types of terminal potential mature cells, which have lots of advantages such as higher ability to differentiate, easy amplification in vitro and lower rejection reactions. Related studies suggest that the protein expression can still be detected after 6 weeks of liver transplantation.
the transplantation of the umbilical cord blood cell in vitro[9-11]. In this study, the determination of hMSCs cell phenotype showed CD29, CD34, CD44, CD133 and CD147 were positive, while CD45 and CD105 were negative, which was consistent with previous literature[12]. These results confirmed that these cells have characteristics of MSCs, which are primary stem cell. CCL4, as the main drug of cirrhosis is highly hepatotrophic, and it can cause liver cell necrosis and proliferation of collagen fibers. Studies suggest that CCL4 can lead to cirrhosis and block liver repair[13].

In this study, we injected hMSCs to cirrhotic rat model and observed the effect of MSCs on liver function. Carbon tetrachloride can cause liver cell degeneration and necrosis and promote collagen fiber proliferation.

After 8 weeks, the study showed that rats had poor mental status, less activity and decrease of food intake and weight. Anatomy showed liver weight was significantly increased. There were nodules over the liver surface, proliferation of a large number of collagen fibers and formation of pseudo-lobules. Liver biopsy microscopic observation showed liver collagen fibers and inflammatory cells increased significantly, which was obvious around the capsule, portal area and central vein. After intraperitoneal injection of MSCs, the collagen fibers were significantly reduced, and there were many morphologically normal liver cells surrounding lobules.

This study suggests that the body weight was significantly different between the model group and control group or between the model group and treatment group. The liver weight of the model control group and treatment group was significantly higher than that of the normal group, and the effect of Rougan Huaqian granules combined with hMSCs transplantation treatment was the best. The Rougan Huaqian granules combined with hMSCs transplantation treatment can resist hepatic fibrosis and promote cell regeneration. The mechanism may due to the Rougan Huaqian granules can regulate the activin A signal transduction, inhibit inflammation and necrosis, resist hepatic fibrosis, secrete the activity factor which can promote hepatocyte proliferation, and accelerate liver tissue repairment[14]. The study showed that after CCL4 intraperitoneal injection, rats produced a lot collagen fibers, formed hepatic lobe, seriously affected liver function, and then gradually formed cirrhosis resulting in the decrease of liver. This result corresponds to the related studies. Therefore, this experiment showed CCL4 is effective to promote cirrhosis, and human MSCs is feasible and effective for promoting the autogenous repairment of the liver.

Kassem et al showed that the hMSCs have a therapeutic potential for rat liver dysfunction, and its transplanted cells showed human liver–like cells differentiation trend in the tested liver[15]. hMSCs can not only improve the state of the liver function but also improve the survival rate of rats. Studies suggest that after 7 weeks, there were a large number of collagen fibers of the liver tissue of rats and pseudolobule was formed, but MSC transplantation can significantly reduce the secretion of collagen fibers in the liver tissue[16-18]. The rat model liver function index and liver fibrosis indexes were detected, and the results showed that the ALT and AST of the treatment group were lower than those of the model group. The effect of Rougan Huaqian granules combined with hMSCs transplantation treatment had the best obvious effects. The LN and HA in the treatment group were significantly lower than those in the model control group, and the difference was statistically significant. The main mechanism of its occurrence may be the hMSCs injection has the human hepatocyte–like cells differentiating tendency and also improves the blood biochemical properties and histological structure of the liver tissue in the peripheral blood. Neonatal human hepatocyte–like cells can enhance the liver function of rats and improve the spirit, activity, diet and other clinical manifestations. Some studies show that it can improve liver fibrosis, maintenance therapy and eventually reverse the trend of cirrhosis[19-23].

Therefore, hMSCs become the research direction for the improvement of liver cirrhosis. α-SMA expression is the activation marker of HSCs. Immunohistochemical staining showed α-SMA expression can significantly increased in liver fibrosis. After MSCs transplantation, the α-SMA expression can be significantly reduced, which is consistent with previous research results[24-27]. After MSCs transplantation, the α-SMA, Col I and LN protein levels were significantly reduced, and the Col I, Col III and MMP–2 mRNA levels were decreased. The Col I and Col III were decreased significantly. The Col I reduced most obviously in the Rougan Huaqian granules combined with hMSCs transplantation group. HSCs are the key link of liver fibrosis, which has a greater ability to synthesize collagen. It can synthesize a lot of collagen fiber protein and lead to excessive accumulation of ECM in liver, eventually causing liver fibrosis and cirrhosis. These results indicate one of the inhibition mechanisms of bone marrow MSCs on the HSC proliferation may be it can down-regulate Col I and Col III mRNA expression and Col I and LN protein expression, which can inhibit HSC proliferation[28,29].

In summary, the mechanism of hMSCs to promote self-repair of liver parenchymal cells is still unclear and the interaction with the surrounding cells and related factors still needs further study. These methods are still in the laboratory stage, and the specific cost and therapeutic ratio and therapeutic endpoint still need to be further confirmed.

Conflict of interest statement

We declare that we have no conflict of interest.
Acknowledgments

This work was supported by the Guangxi Scientific and Technological Project (No. 11107009–3–1) and Guangxi Natural Science Fund Projects (No. 2010 GXNSFA013211).

References


[21] Li ML, Deng MH. Progress in research of gene treatment for liver cirrhosis with bone marrow mesenchymal stem cells. Chin Arch General Surg 2011; 5: 252–255.


