Adipose-derived mesenchymal stem cells inhibit activation of hepatic stellate cells \textit{in vitro} and ameliorate rat liver fibrosis \textit{in vivo}

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\textbf{KEYWORDS}

Adipose derived mesenchymal stem cells; fibrosis; hepatic stellate cells; proliferation

\textbf{Background/purpose:} Previous studies suggested that mesenchymal stem cells may ameliorate fibrogenesis through the inhibition of hepatic stellate cells (HSCs) activation. This study aimed to investigate whether adipose derived mesenchymal stem cells (ADSCs) could modulate the activation of HSCs and contribute to the recovery of liver fibrogenesis.

\textbf{Methods:} ADSCs and HSCs were isolated from Sprague-Dawley rats and co-cultured using a transwells insert. Cell proliferation, apoptosis and smooth muscle $\alpha$-actin ($\alpha$-SMA) expression in HSCs were examined. Rats were injected with CCl$_4$ to induce liver fibrogenesis. After injection of ADSCs through portal vein, the rats were examined for pathological changes in the liver. $\alpha$-SMA expression and hydroxyproline content in the liver and serum levels of collagen III and hyaluronic acid was detected.

\textbf{Results:} After co-culturing for 72 h, the proliferation and activation of HSCs was inhibited by ADSCs and the apoptosis of HSCs was promoted by ADSCs. Transplantation of ADSCs inhibited liver fibrogenesis in the rats.

\textbf{Conclusion:} ADSCs inhibit the proliferation and activation of HSCs \textit{in vitro} and inhibit liver fibrogenesis in rat model, suggesting the potential application of ADSCs in liver fibrogenesis therapy.

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Introduction

It is estimated that over 100 million people suffer from liver fibrosis worldwide. Although liver fibrosis and the resulting cirrhosis are caused by a variety of etiologic agents, including chronic viral hepatitis, alcohol toxicity, autoimmune disease, and hereditary metabolic disorders, it is now generally accepted that a central pathologic mechanism underlying liver fibrosis is the generation and proliferation of smooth muscle $\alpha$-actin ($\alpha$-SMA) positive myofibroblasts of perportal and perisinusoidal origin that arise as a consequence of the activation of hepatic stellate cells (HSCs) and other cell types, such as perportal fibroblasts.1,2 As the main complication of chronic liver damage, liver fibrosis is a wound healing process characterized by the accumulation of extracellular matrix (ECM) proteins in the liver.3,4 HSCs are critically involved in the development of liver fibrosis because they are responsible for the excessive deposition of ECM proteins in the liver.3,4

Major progress has been made recently in the prevention, diagnosis, and treatment of liver fibrosis, including the application of liver transplantation and artificial livers.5 However, the increasing number of patients suffering from liver disease and the limited availability of suitable donor livers present substantial problems. As a result, the development of alternative, effective antifibrotic therapies to replace liver transplantation is urgently needed. Bone marrow mesenchymal stem cells (MSCs) are multipotential cells that reside within the bone marrow and can be induced to differentiate into various components of the marrow microenvironment, such as bone, adipose, and stromal tissues, depending on the conditions.6 Recent studies have shown that MSCs from bone marrow can alleviate fibrosis formation through the inhibition of HSC activation, suggesting that stem cell transplantation is a promising therapeutic approach for combating liver fibrosis.7–10

Unfortunately, the isolation of MSCs is a highly invasive and painful procedure, and the frequency of MSCs in bone marrow is relatively low. Therefore, alternative cell sources that overcome the disadvantages of bone marrow-derived stem cells are clearly needed. Adipose tissue-derived mesenchymal stem cells (ADSCs) are similar to MSCs in that they also have properties of stem cells, such as self renewal, extensive proliferation capacity, and the ability to differentiate into multiple cell lineages.11,12 More importantly, ADSCs have unique advantages compared to MSCs: they can be easily harvested from subcutaneous fat tissue using a safe and conventional liposuction procedure, the frequency of ADSCs in adipose tissue is much higher than that of MSCs in the bone marrow, and ADSCs proliferate markedly faster than MSCs.13,14 However, whether ADSCs can be exploited for cell transplantation to reduce liver fibrosis remains largely unknown. The apparent advantages of ADSCs led us to investigate whether they may be an ideal transplantable cell type to treat liver fibrosis. In the present study, we transplanted ADSCs into rats with carbon tetrachloride (CCl4)-induced hepatic cirrhosis and showed that ADSCs could ameliorate liver fibrogenesis by modulating HSCs.

Materials and methods

Animals

Six week-old inbred Sprague-Dawley rats with an initial body weight of 150–200 g were obtained from the Animal Experimental Center of Wenzhou Medical College and housed in a standard animal laboratory. They were kept at 25°C with a 12-hour light/dark cycle and allowed standard chow and water ad libitum until the time of the study. The study protocol and animal care were approved by the Animal Care and Use Committee of Wenzhou Medical College, and conformed to the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH Publication No. 80-23).

Culture of rat liver cells

Buffalo rat liver cells (BRLs) were obtained from the Longwan Experimental Center of Wenzhou Medical College and cultured in L-DMEM (HyClone; Thermo Scientific, Waltham, MA, USA) medium supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA) in a humidified atmosphere with 5% CO2 at 37°C. The medium was changed every 2–3 days.

Isolation and culture of adipose tissue-derived mesenchymal stem cells

One male rat was anesthetized with ether. The subcutaneous adipose tissue was harvested from the rat and carefully dissected. The tissue was digested using collagenase type IV (1 mg/mL; Sigma, St Louis, MO, USA) and then dissociated mechanically by shaking at 37°C for 1 hour. The suspension was then centrifuged at 200g for 5 minutes. The pellet cells were resuspended in DMEM supplemented with 10% FBS (Gibco) and cultured in a humidified atmosphere with 5% CO2 at 37°C. The medium was changed every 2–3 days. The cells were passaged when they grew to 80% confluence. The third to fifth generations of the passaged cells were collected and subjected to flow cytometry analysis using the ADSC-specific markers CD73, CD90, and CD45.

Isolation and culture of hepatic stellate cells (HSCs)

Rat HSCs were isolated and grown in primary culture as previously described.15 After isolation by density gradient centrifugation, cells were grown in L-DMEM supplemented with 10% FBS at 37°C in 5% CO2. The medium was changed after 48 hours. After 5–7 days, the HSCs grew rapidly and the medium was changed every 2–3 days thereafter.

Transwell co-culture of ADSCs and HSCs

ADSCs were cultured in apical compartments of transwells (transwell insert 0.4 µm; Millipore, Billerica, MA, USA) with HSCs grown in the basal compartment of a 6-well plate (Millipore). ADSCs were seeded onto the upper layer of transwells at a density of $2 \times 10^5$ cells/well and were not in...
direct contact with HSCs. HSCs of the primary culture or third generation culture were seeded onto the lower layer of transwells at a density of $2 \times 10^4$ cells/well. All cells were cultured in L-DMEM supplemented with 10% FBS at 37°C in 5% CO₂. In addition, BRLs were seeded instead of ADSCs onto the upper layer as a negative control, or no cells were seeded onto the upper layer as a blank control. After culturing the cells for 72 hours, the morphology of the cells grown in transwells was assessed under an inverted phase contrast microscope. The cell proliferation was analyzed using the CCK-8 kit (Dojindo, Kumamoto, Japan) following the manufacturer’s instructions. The absorption (A) was read at 450 nm using a spectrophotometer. The inhibition rate (%) of HSC proliferation was calculated as: 1 – (A of control – A of ADSCs)/(A of control – A of HSCs) × 100%. The experiments were performed in triplicate and repeated twice.

Establishment of rat model of liver fibrogenesis

To establish a model of liver fibrogenesis, male rats (weight 250–300 g) were selected and 1.5 mL/kg CCl₄ (diluted 1:1 in olive oil) was injected subcutaneously twice a week for 12 weeks. The hydroxyproline content in the liver was measured by chemical staining kit (Vector, Burlingame, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control.

Determination of the concentration of cytokines by enzyme-linked immunosorbent assay

ADSCs and BRLs were seeded in 6-well plates at a density of $2 \times 10^5$ cells/well. After culturing the cells in L-DMEM supplemented with 10% FBS for 72 hours, the medium was collected and the secretion of cytokines from ADSCs or BRLs into the medium was detected using enzyme-linked immunosorbent assay (ELISA) kits for hepatic growth factor (HGF), nerve growth factor (NGF), and interleukin-10 (IL-10; all kits obtained from R&D, Minneapolis, MN, USA) following the manufacturer’s instructions.

Immunocytochemical staining of α-SMA in HSCs

HSCs were cultured on coverslips and fixed with 4% paraformaldehyde. An α-SMA antibody (1:200 dilution; Boshide, Wuhan, China), biotin-labeled secondary antibody, horse-radish peroxidase-conjugated streptavidin, and diaminobenzidine were added sequentially according to the standard protocol. Brown staining indicated the expression of α-SMA.

Flow cytometry analysis of apoptosis

After co-culturing ADSCs and HSCs for 72 hours, the cells were collected by digestion with EDTA-free trypsin (Invitrogen, USA). The cell pellet was washed with PBS twice, and approximately $2 \times 10^5$ cells were resuspended in 200 μL Annexin V binding buffer (BD Biosciences, Franklin Lakes, NJ, USA). The cells were stained with 5 μL Annexin V FITC (BD Biosciences) for 20 minutes at 4°C in the dark. The reaction was stopped by the addition of 5 μL propidium iodide, and the samples were then incubated for 5 minutes at 4°C in the dark. The samples were subjected to flow cytometry analysis within 1 hour. The assay was performed in triplicate and repeated twice.

Immunohistochemical staining

After HSC transplantation, rat liver tissues were fixed in 40 g/L paraformaldehyde and then embedded in paraffin wax. Subsequently, 4-μm thick serial sections of the tissues were cut. The sections were washed carefully with 0.01 M phosphate buffered saline (PBS) three times (10 minutes each), and then blocked with 2% goat serum in 0.01 M PBS containing 0.3% Triton X-100 (PBS-X) for 1 hour at room temperature. The sections were incubated at 4°C overnight with an anti-desmin rabbit antibody (1:200 dilution). The slides were then processed using an immunohistochemical staining kit (Vector, Burlingame, CA, USA). After visualizing the reaction with the DAB chromogen, the slides were counterstained with hematoxylin and covered with a...
glycerin gel. In negative control experiments, the primary antibodies were replaced with PBS.

**TUNEL assay**

After ADSC transplantation, rat liver tissues were fixed in 40 g/L paraformaldehyde and then embedded in paraffin wax. Subsequently, 4-μm thick serial sections of the tissues were cut and the apoptosis of HSCs was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using a kit (Roche, Grenzach-Wyhlen, Germany) following the manufacturer’s protocol.

**Statistical analysis**

The experiments’ data were expressed as mean ± standard deviation. The differences between different groups were analyzed by one way ANOVA using SPSS16.0 software (SPSS, Inc., Chicago, IL, USA) and \( p < 0.05 \) was considered significant.

**Results**

**Isolation and characterization of ADSCs**

Approximately \( 1 \times 10^6 \) ADSCs were harvested from 1–2 mg of rat adipose tissue. The proportion of freshly isolated living ADSCs was >95%, as defined by trypan blue staining. ADSCs adhered to the plate wall after 4–6 hours and developed a spin phenotype after 48 h. The ADSCs grew very fast after 5–6 days and were passaged when they grew to 80% confluence (Fig. 1). In addition, we performed flow cytometry analysis on the isolated ADSCs to detect the expression of ADSCs markers. The results showed that the isolated ADSCs exhibited high expression of CD73 and CD90 but low expression of CD45, consistent with recent report.16

**Isolation and characterization of HSCs**

Approximately \( 1–2 \times 10^7 \) HSCs were harvested from each rat. The proportion of freshly isolated living HSCs was more than 95%, as defined by trypan blue staining. Under an inverted phase-contrast microscope, the freshly isolated cells were round and full of lipid droplets in the cytoplasm. When stimulated at 328 nm wavelength, most HSCs had blue or green intrinsic autofluorescence due to the presence of vitamin A-rich lipid droplets (Fig. 2A). After culturing the cells for 2–3 days, the cells adhered to the wall of the plate, and after 5–7 d of growing the cells in culture, they extended and presented an asteroid phenotype (Fig. 2).

**ADSCs co-culture inhibits the expression of \( \alpha \)-SMA in HSCs**

After co-culturing HSCs with ADSCs for 72 hours, the number of HSCs expressing \( \alpha \)-SMA was much lower compared to HSCs co-cultured with BRLs, as detected by immunocytochemistry (Fig. 2B). These results were confirmed by western blot analysis. The results showed that the level of \( \alpha \)-SMA was lower in HSCs co-cultured with ADSCs than in HSCs co-cultured with BRLs (Fig. 2C).

![Primary ADSCs](image)

**Figure 1**  (A) The phenotype of adipose-derived mesenchymal stem cells isolated from the rat adipose tissue (on the first day). Magnification 200×. (B) The expression of CD45, CD73 and CD90 in isolated adipose-derived mesenchymal stem cells.
Co-culturing ADSCs with HSCs inhibits the proliferation and promotes apoptosis of HSCs

The CCK-8 assay showed that after HSCs and ADSCs were co-cultured for 72 hours, the proliferation of HSCs was significantly inhibited (1.209\(\pm\)0.117) compared to HSCs co-cultured with BRLs (1.424\(\pm\)0.013) or HSCs cultured alone (2.172\(\pm\)0.107; Fig. 3A). Flow cytometry analysis showed that the apoptosis rate of HSCs was significantly higher in the ADSCs\(\oplus\)HSCs group (4.600\(\pm\)0.794\%) than in the BRLs\(\oplus\)HSCs (2.817\(\pm\)0.225\%) and HSCs groups (2.496\(\pm\)0.115\%; Fig. 3B). Taken together, these results demonstrate that co-culturing ADSCs and HSCs inhibits the proliferation and promotes apoptosis of HSCs.

ADSCs transplantation alleviates rat liver fibrogenesis in vivo

CCL\(_4\) induced liver fibrogenesis in rats, as determined by H&E and Masson staining (Fig. 4A–C). After transplantation of ADSCs into CCL\(_4\)-injured rats, the hepatic engraftment rate was approximately 20\%, and the mortality rate of rats receiving ADSC injection was approximately 30\%. Transplantation of ADSCs into the liver reduced the fibrotic area. The transplantation of ADSCs alleviated rat liver fibrogenesis, as confirmed by the expression of collagen I and \(\alpha\)-SMA, which are two proteins that are highly expressed during liver fibrogenesis. Western blot analysis showed that the transplantation of ADSCs reduced the expression of collagen I and \(\alpha\)-SMA in the liver (Fig. 4D). In addition, the transplantation of ADSCs led to a reduced hydroxyproline level in the liver and reduced collagen III and hyaluronic acid levels in the serum of CCL\(_4\)-induced rats, although the levels of these indicators of liver fibrogenesis were still higher in rats transplanted with ADSCs than in normal rats (Table 1). Collectively, these data suggest that the transplantation of ADSCs can ameliorate the formation of liver fibrosis in rats exposed to CCL\(_4\).

In addition, immunohistochemical staining and a TUNEL assay detected the proliferation and apoptosis of HSCs in vivo, respectively. The results showed that in the control group, the number of desmin positive and TUNEL positive HSCs was 45\(\pm\)11 and 11\(\pm\)3, respectively; however, in the treatment group, the HSC numbers were 31\(\pm\)7 and 17\(\pm\)5, respectively (Fig. 5). These data suggest that transplantation of ADSCs into rat liver inhibits proliferation and promotes apoptosis of HSCs, which is consistent with the in vitro results.

Cytokines secreted by ADSCs and HSCs

Since ADSCs prevent the proliferation and promote the apoptosis of HSCs without direct contact to HSCs, it was assumed that the cytokines secreted by ADSCs may help mediate the effects observed. Therefore, the concentration of cytokines secreted into the medium was examined by ELISA. The results showed that ADSCs secreted high levels of HGF and low levels of NGF and TGF\(\beta\)1 compared to BRLs (Table 2).
An ELISA was performed to detect the serum levels of HGF, NGF, TGFβ1, and IL-10 in the rats after ADSC transplantation. The results showed that, compared to the control group, HGF levels were higher while NGF and TGFβ1 levels were lower in the treatment group (p < 0.05; Table 3). The IL-10 levels were not significantly different between control and treatment groups. These results are consistent with the in vitro secretion of cytokines by ADSCs.

Discussion

The incidence of hepatitis is high in China. Hepatitis can develop into liver cirrhosis, which can further cause liver failure or liver cancer, and is the leading cause of death in these patients. Therefore, the early detection and prevention of hepatic fibrosis is very important. The current clinical treatment for liver fibrosis is surgery, which cannot reverse the process of liver fibrogenesis. Furthermore, liver transplantation has many disadvantages, such as a shortage of donors, high cost, and trauma during operation. In recent years, a large number of basic experimental studies and clinical research have demonstrated that stem cell transplantation is a promising approach for the treatment of liver fibrosis.16–20

HSCs are mesenchymal cells that are critically involved in liver fibrosis. In pathological conditions, such as liver

Figure 3  Co-culturing adipose-derived mesenchymal stem cells with hepatic stellate cells (HSCs) inhibits proliferation and promotes apoptosis in HSCs. (A) After co-culturing HSCs with buffalo rat liver cells (BRLs) or HSCs for 72 hours, the proliferation of HSCs was examined using the CCK-8 assay. The proliferation of HSCs was significantly inhibited compared to HSCs co-cultured with BRLs or HSCs cultured alone (1.209 ± 0.117 vs. 1.424 ± 0.013 vs. 2.172 ± 0.107, p < 0.05). (B) The apoptosis of HSCs was detected by Annexin V-PI double staining and flow cytometry analysis. The apoptosis rate of HSCs was significantly higher in the adipose-derived mesenchymal stem cells + HSCs group than in the BRLs + HSCs group or HSCs group. All data were expressed as mean ± standard deviation derived from three independent experiments performed in triplicate.
injury by physical, chemical, or biological factors, HSCs are activated and proliferate rapidly. The secretion of components of the ECM from HSCs to the liver is an important factor of liver fibrosis. MSCs have been shown to inhibit the activation of HSCs and contribute to the recovery of liver fibrogenesis in animal models. However, the application of MSCs is limited by the availability of MSCs. Since ADSCs were first isolated in 2001, an increasing body of evidence has shown that ADSCs have many advantages compared to MSCs and are better suited for applications in tissue engineering.

Most of the previous studies on stem cell transplantation for the treatment of liver fibrogenesis have concentrated on the differentiation of stem cells into liver cells, which may improve liver function. Given that the activation and proliferation of HSCs are important for the development of liver fibrosis, we wondered whether ADSCs inhibit liver fibrosis by regulating the proliferation and activation of HSCs. Therefore, we co-cultured ADSCs and HSCs in 6-well plates through a transwell insert and found that ADSCs could inhibit the activation and proliferation of HSCs, suggesting that these effects were mediated not via direct cell-to-cell contact, but rather through the factors secreted into the medium. Indeed, the ELISA results showed that ADSCs secreted large amounts of HGF but very low levels of TGF-β into the medium. These results are consistent with a previous study reporting that high HGF and low TGF-β exhibited beneficial effects on the recovery from liver cirrhosis.

Table 1  Mean ± standard deviation hepatic hydroxyproline levels and serum laminin and hyaluronic acid levels in different groups of rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Hyaluronic acid (µg/L)</th>
<th>Collagen III (µg/L)</th>
<th>Hydroxyproline (µg/L)</th>
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<tr>
<td>Normal</td>
<td>81.23 ± 5.97</td>
<td>9.33 ± 1.18</td>
<td>212.8 ± 9.4</td>
</tr>
<tr>
<td>Treatment</td>
<td>178.8 ± 28.2</td>
<td>21.74 ± 3.30</td>
<td>325.8 ± 28.2</td>
</tr>
<tr>
<td>Control</td>
<td>282.3 ± 18.7</td>
<td>35.28 ± 3.31</td>
<td>458.4 ± 38.1</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
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Figure 4  Transplantation of ADSCs alleviates rat liver fibrogenesis in vivo. (A–C) Pathological examination of liver tissues from different groups of experimental rats: (A) hematoxylin and eosin (H&E) staining of liver sections in rats fed with a normal diet for 12 weeks; B. H&E staining and Masson Staining of liver sections in control group (rats fed with a CCl₄ diet for 12 weeks); (C) H&E staining and Masson Staining of liver sections in treatment group (rats fed with a CCl₄ diet + ADSCs for 12 weeks). (D) Western blots showing the protein levels of α-SMA and collagen I in the liver tissues (p < 0.05). Representative blots were shown from three independent experiments with similar results. Glyceraldehyde 3-phosphate dehydrogenase served as a loading control.
In addition, the flow cytometry results demonstrated that ADSCs promote the apoptosis of HSCs. Taken together, these data suggest that the cytokines secreted by ADSCs may modulate the activation, proliferation, and apoptosis of HSCs, which ultimately leads to the reduction of HSCs and the recovery of liver fibrogenesis.

To validate these in vitro data, we employed a CCl4-induced liver cirrhosis rat model and transplanted ADSCs into the liver. The results showed that the liver transplantation group had mild fibrosis compared to the control group. Measurement of biochemical parameters also demonstrated that ADSCs ameliorated the fibrosis. In addition, we performed immunohistochemical staining and a TUNEL assay to detect the proliferation and apoptosis of HSCs in vivo, respectively. The results showed that transplantation of ADSCs into rat liver inhibited the proliferation and promotes apoptosis of HSCs. In addition, an ELISA assay showed that compared to the control group, HGF levels were higher, while NGF and TGF-β1 levels were lower in the treatment group, which is consistent with the in vitro secretion of cytokines by ADSCs. Collectively, these in vivo data complement the in vitro data and provide evidence that ADSCs help in the recovery of liver fibrogenesis through indirect modulation of HSCs, instead of direct differentiation into functional hepatocytes. The positive effects of ADSCs subsequently promote liver regeneration.

Although we have shown that ADSCs can markedly inhibit liver cirrhosis in a rat model, it is important to point out the limitations of the present study. We only examined the effects of ADSC transplantation on liver fibrogenesis 4 weeks after the transplantation. Future studies with multiple points of time and over a longer period of time are required to determine the dosage and for how long the transplanted ADSCs can inhibit liver fibrogenesis. Moreover, future studies should determine the time point at which the transplanted ADSCs exhibit optimal therapeutic effects. In addition, the tumorigenicity and allograft rejection of the transplanted ADSCs need be addressed in further studies to pave the way for the clinical application of ADSCs in the treatment of liver fibrogenesis.

<table>
<thead>
<tr>
<th>Cells</th>
<th>HGF (ng/L)</th>
<th>NGF (ng/L)</th>
<th>TGFβ1 (ng/L)</th>
<th>IL-10 (ng/l)</th>
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<tr>
<td>ADSCs</td>
<td>74.93 ± 2.54</td>
<td>5.46 ± 0.45</td>
<td>10.65 ± 0.46</td>
<td>23.82 ± 0.92</td>
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<td>BRLs</td>
<td>58.08 ± 2.44</td>
<td>14.68 ± 0.95</td>
<td>136.06 ± 1.51</td>
<td>27.73 ± 0.22</td>
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<td>p</td>
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ADSCs = adipose-derived mesenchymal stem cells; BRLs = buffalo rat liver cells.
Support statement

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References


Table 3 Mean ± standard deviation concentrations of hepatic growth factor (HGF), nerve growth factor (NGF), transforming growth factor (TGFβ1), and interleukin-10 (IL-10) in rat serum.

<table>
<thead>
<tr>
<th>Group</th>
<th>HGF (ng/L)</th>
<th>NGF (ng/L)</th>
<th>TGFβ1 (ng/L)</th>
<th>IL-10 (ng/L)</th>
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<tbody>
<tr>
<td>Control</td>
<td>71.8 ± 5.4</td>
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<td>195.3 ± 14.8</td>
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<tr>
<td>Treatment</td>
<td>193.5 ± 17.5</td>
<td>43.4 ± 3.3</td>
<td>73.1 ± 3.6</td>
<td>52.7 ± 3.5</td>
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<tr>
<td>Normal</td>
<td>21.7 ± 3.6</td>
<td>34.7 ± 2.0</td>
<td>39.5 ± 2.0</td>
<td>24.7 ± 1.6</td>
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<tr>
<td>p</td>
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