· Research Article ·

Therapeutic effect of human adipose stem cells derived exosomes on carbon tetrachloride induced liver fibrosis in rats

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Abstract

BACKGROUND: Liver fibrosis has higher morbidity and mortality. Activation and proliferation of hepatic stellate cells is a key link in the progression of liver fibrosis. At present, there are still no effective anti-fibrosis agents targeting single links or targets.

OBJECTIVE: To analyze the effect of human adipose stem cells derived exosomes on rats with liver fibrosis induced by carbon tetrachloride.

METHODS: Human adipose stem cells were obtained from healthy people by enzyme dissolution method. After *in vitro* culture, human adipose stem cells derived exosomes were obtained by multiple ultrafiltration. Different concentrations of exosomes were used to treat the hepatic stellate cells activated by transforming growth factor β1. The human adipose stem cells activated by transforming growth factor β1 were treated with different concentrations of exosomes. The expression of α-smooth actin in the cells was detected by quantitative PCR, and the growth and apoptosis of activated hepatic stellate cells were detected by CCK-8 and flow cytometry respectively. Rat models of liver fibrosis were established by intraperitoneal injection of carbon tetrachloride and treated by tail vein injection of exosomes. Rat liver function, serum levels of type III procollagen and type IV collagen, and Ishak score were determined. Semi-quantitative analysis of liver fibrosis was performed. The expression levels of tissue inhibitor of matrix metalloproteinase-1, matrix metalloproteinase 9 and α-smooth actin in liver tissue were measured by immunofluorescence method. The study protocol was approved by the Animal Ethics Committee and Medical Ethics Committee, Tongji University, China in January, 2017.

RESULTS AND CONCLUSION: Human adipose stem cells derived exosomes inhibited the proliferation of activated hepatic stellate cells. The possible mechanism is to inhibit the proliferation of activated macrophages, reduce the production of collagen fibers, α-smooth actin actin, and tissue inhibitor of matrix metalloproteinase-1, and to increase the expression of matrix metalloproteinase 9. These findings suggest that exosomes can be used to treat carbon tetrachloride induced liver fibrosis.

Key words: human adipose stem cells; exosomes; excessive extracellular matrix; liver fibrosis; hepatic stellate cells; carbon tetrachloride; transforming growth factor beta 1; α-smooth muscle actin; tissue inhibitor of metalloproteinase-1; matrix metalloproteinase 9

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INTRODUCTION

Liver fibrosis is a chronic liver injury characterized by excessive extracellular matrix (ECM) in liver tissue and caused by a variety of factors including viral hepatitis, drugs and autoimmune diseases[1]. All kinds of pathogenic factors can cause hepatocyte damage, necrosis and inflammatory reaction, activate Kupffer cells in the liver, and secrete tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β), interleukin (IL) 1β and IL-6. All cytokines can activate hepatic stellate cells (HSCs) toward myofibroblasts. Myofibroblasts accelerate the synthesis and deposition of ECM, eventually leading to liver fibrosis. ECM includes α-smooth muscle actin (α-SMA), tissue inhibitor of metalloproteinase-1 (TIMP-1), and matrix metalloproteinase-9 (MMP9)[2]. The activation of HSCs and inflammation are important factors contributing to the occurrence and development of liver fibrosis[3]. Therefore, promoting the apoptosis of activated HSCs and inhibiting the production of inflammatory factors are of great significance for the treatment of liver fibrosis.

Human adipose derived mesenchymal stem cells (hASCs), which have strong self-renewal capacity, multidirectional differentiation potential across the dermal layer and have advantages of relatively easy collection of sources and higher proliferation rate, are expected to treat liver fibrosis[4-7]. Multiple studies have shown that hASCs can alleviate liver fibrosis by promoting the apoptosis

of activated HSCs and reducing the production of fibers[2, 8]. hASCs can secrete exosomes, which have abundant proteins, lipids and RNA, into the extracellular space for intercellular communication.

Exosomes, which can be actively secreted by various cells, are lipid bilayer structure vesicles with the diameter of 30−150 nm[9]. Double membrane structure of exosomes can protect the substances from being degraded by various enzymes outside the cells. Exosomes play a therapeutic role by binding to the receptor expressed on the surface of the target cells[10-14]. At present, several research groups have established that the exosomes isolated from different stem cell conditioned medium can alleviate liver or kidney fibrosis in animals. Some scholars have found that HSCs in resting state contain relatively abundant miR-214, which can inhibit the expression of CCN2 protein by binding 3'UTR of CCN2 gene after being transferred to nearby HSCs or hepatocytes through exosomes. In addition, some studies have found that in the early stage of liver fibrosis, exosomes can mediate the activation of Toll-like receptor 3 (TLR3) of HSCs, promote the secretion of IL-17A, CCL20, IL-1beta, IL-23 and other factors by HSCs, and then promote liver fibrosis by increasing the production of IL-17A in γδT cells. It can be seen that exosomes can reduce the production of collagen I and III and alleviate the fibrosis by promoting the apoptosis of activated HSCs and decreasing the expression of inflammatory cytokines[15-18]. Also, exosomes can inhibit inflammation and immune response, and promote hepatocyte regeneration and angiogenesis in rats with liver failure[4, 14, 19-20]. However, there are few studies regarding the mechanism by which exosome exhibits effects on liver fibrosis. This study explored the effect of hASCs derived exosomes on rats with liver fibrosis induced by carbon tetrachloride (CCl_4). Indicators of liver fibrosis were tested, including MMP9, α-SMA, TIMP-1, type III precollagen (PCIII), type IV collage (IV-C) and the apoptosis of activated HSCs.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment.

Time and setting

The experiment was conducted in the Key Laboratory of Stem Cell Physiology and Disease Treatment Research Group, Department of Regenerative Medicine, Tongji University, Shanghai from January 2017 to June 2018.

Materials

Experimental animals: 4- to 6-week-old male specific-pathogen-free Sprague-Dawley rats (*n* = 36) were provided by Shanghai Bikai Experimental Animals Co., Ltd., and included in this study. All animals were allowed free access to food and water in the Laboratory Animal Center of Tongji University, China. Animal experiments had been approved by the Animal Ethics Committee of the 905 Hospital of PLA Navy, China.

Laboratory reagents: 0.1% collagenase I (Gibco, USA), F12/DMEM (Gibco, USA), fetal bovine serum (Gibco, USA), penicillin-streptomycin (Gibco, USA), anti-human CD44/CD90/CD73/CD19/CD34/CD45-FITC, CD105-PE antibodies (Bioscience, USA), adipogenic or osteogenic differentiation medium (Cyagen Biosciences, China), oil red (Sigma, USA), alizarin red (Sigma, USA), exosomes' surface molecule antibody chips (System Biscience, USA), TGF-1 factor (Pepro Tech, USA), TRIzol (Aidlab, beijing, China), RT reagent kit (Tiagen, Beijing, China), CCK8 (Dongren, Shanghai, China), apoptosis kit (BD, USA), 4% paraformaldehyde (Dingguo Changshen, Beijing, China), Sirius red and Masson trichrome stain (Solaibao, Beijing, China), ELISA kit (Solaibao), gradient alcohol (Tansoole, Shanghai, China), TIMP1/MMP9/SMA primary antibody (CST, USA), secondary antibody (SCT, USA), DAPI (Solarbio).

Experimental instrument: centrifuge (TD25-WS, Lu Xiangyi, China), incubator (Thermo, USA), flow cytometer (Caliber, BD), ultrafiltration enrichment centrifuge tube (Merk, Ireland), Nanosight granulometer (NS300, Britain), multifunctional enzyme marker (SoftMax Pro5, Molecular Devices, USA), automatic analyzer (Hitachi, Tokyo, Japan), microscopic examination (IX71, Olympus, Tokyo, Japan), Xylene (Merk, Ireland), and fluorescence microscope (IX71).

Methods

Separation and identification of hASCs and exosomes

Separation of hASCs: Harvesting of healthy human adipose tissue was approved by the Medical Ethics Committee of Tongji University, China in January, 2017. All patients provided written informed consent prior to the inception of the study. Healthy human adipose tissue was harvested from the subcutaneous portion of the neck provided by the 85th Hospital of Chinese PLA. After three PBS washes, human adipose tissue was chopped into small blocks with tissue scissors, digested by type I collagenase for 45−60 minutes, and centrifuged at 1 200 r/m for 5 minutes. The resultant cells were incubated in F12/DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin in an incubator at 37 °C with 5% CO_2. Cell density was 80% for these experiments.

Identification of hASCs: hASCs were prepared as single cell suspension in seven tubes. Anti-human CD44/CD90/ CD73/CD19/CD34/CD45-FITC, CD105-PE antibodies were added separately. Then the mixture was incubated at 37 °C for 30 minutes and flow cytometry was performed. hASCs were cultured in lipogenic or osteogenic differentiation medium for 3 weeks and stained with oil red and alizarin red, respectively.

Separation and identification of exosomes: When hASCs density reached 80%, the medium containing serum was removed and replaced with basal medium for 24 hours. The supernatant was collected and centrifuged at 4 000 × *g* at 4 °C for 40 minutes in ultrafiltration enrichment centrifuge tube. Then concentrate was collected and stored at −80 °C. Exosome size was

hASCs culture and identification	
Cell source	Healthy human adipose tissue derived from the subcutaneous portion of the neck
Primary culture method	Cultured with 0.1% collagenase I for 45−60 minutes and centrifuged at 1 200 x r/m for 5 minutes.
Basal medium	DMEM/F12
Added materials	10% Fetal bovine serum and 1% penicillin-streptomycin
Primary culture time	After refreshing culture medium, the cells were further cultured for 70 hours and then subcultured.
Cell passage:	Cells were digested with trypsin. After reaching 80−90% confluence, they were passaged by 1：3. There were three generations during a 72 hour period.
Cell identification:	Exosome size was measured by scanning electron microscope and Nanosight granulometer, Exosome's surface molecules were detected by antibody chips.
Approval by ethics committee:	Ethical approval had been obtained from the Medical Ethics Committee of the 905 Hospital of PLA Navy with the approval No. [2013] Ethical No.18.

measured using scanning electron microscope and Nanosight granulometer. Exosome surface molecules of FLOT1 (Flotillin 1), ICAM (intercellular adhesion), ALIX, CD81, CD63, EpCAM (epithelial cell adhesion molecule), ANXA5 (annexin A5) and TSG101 (tumor susceptibility gene 101) were detected by antibody chips.

Cell culture and treatments

Activation of HSCs: At 70−80% confluence, HSCs were inoculated in 6-well plates and treated with TGF-1 at a final concentration of 10 ng/mL or with PBS for 24 hours. Then, medium was removed and total RNA of activated HSC was extracted with TRIzol and then reverse transcribed to complementary DNA using a Primescript TM RT reagent kit. qPCR was then performed using SYBR Green qPCR Super Mix-UDG on a Step One Plus Real-Time PCR System. The relative abundance of the target genes was determined by the comparative cycle threshold Ct method ($2^{-\Delta\Delta Ct}$) and normalized to GAPDH.

Proliferation of HSCs: Activated HSCs were inoculated in 96-well plates at 2 000 cells and treated with high concentration (10 μg/mL) or low concentration (1 μg/mL) exosomes. CCK8 was used to detect activated HSCs' proliferation according to the manufacturer's instructions and multifunctional enzyme marker was used to measure optical density value at 450 nm.

Apoptosis of HSCs: Activated HSCs were inoculated in a medium containing 0.01 μL/mL CCl4 for 24 hours and treated with different concentrations of exosomes (10 and 1 μg/mL). Apoptosis was detected according to the manufacturer's instructions (BD, USA).

Establishing rat models of liver fibrosis

Liver fibrosis was induced by intraperitoneal injection of CCl4 (reconstituted in olive oil at a ratio of 1：5 and administered at a dose of 2 mL/kg body weight) twice weekly for 4 consecutive weeks[21-22]. There are six groups in animal experiments (6 rats/group): A: normal animal; B: modeling for 4 weeks; C: modeling for 4 weeks + exosome therapy for 2 weeks (100 μg/rat); D: modeling for 4 weeks + PBS therapy for 2 weeks (100 μg/rat); E: modeling for 4 weeks + exosome therapy for 4 weeks (100 μg/rat); F: modeling for 4 weeks + PBS therapy for 4 weeks (100 μg/rat).

Exosomes treatment of liver fibrosis in rats

Exosomes (administered at a volume of 200 μL, 100 μg/rat) or PBS (administered at a dose of 200 μL) treatment was performed by tail vein twice weekly in rats 4 weeks after induction of liver fibrosis for 2 weeks or 4 weeks. Blood samples were centrifuged at 3 000×*g* for 10 minutes at 4 °C, and then serum was collected and stored at −80 °C until use. A section of hepatic tissue from the porta hepatis was fixed in 4% paraformaldehyde and the remaining hepatic tissue was preserved at −80 °C.

Serum liver enzyme detection

Serum total protein (TP), albumin (ALB), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed by an automatic analyzer.

Assessment of liver fibrosis

Paraformaldehyde-fixed samples were embedded in paraffin, sectioned (4-μm thickness), and stained with Sirius red and masson trichrome for microscopic examination. The liver fibrosis in rats was evaluated by Ishak score standard[23]. Image-pro Plus 6.0 software was used for semi-quantitative analysis of liver tissue images stained with Sirius red and masson trichrome. ELISA was performed to detect serum levels of PCIII and IV-C. Hepatic tissue embedded by paraffin was conventionally dewaxed by xylene and gradient alcohol. Then primary antibody was added after antigenic repair and serum closure. The sections were incubated overnight at 4 °C and then incubated with a suitable fluorescently-conjugated (FITC) secondary antibody. DAPI was used for nuclear counterstaining. TIMP-1, MMP-9 and α-SMA expression in rat liver tissue was evaluated under the fluorescence microscope. At the same time, Image-pro Plus 6.0 software was used for semi-quantitative analysis of immunohistochemical fluorescence images.

Main outcome measures

Expression of TIMP-1, MMP-9 and α-SMA in rat liver tissue, ISHAK score and serum levels of PCIII and IV-C.

Statistical analysis

All analyses were performed using Office Excel 2003. The variables were expressed as the mean ± standard deviation. The comparison between two independent groups was performed using Student's *t*-test. A *P*-value of 0.05 or less was considered statistically significant.

RESULTS

Separation and identification of hASCs and exosomes

Under the inverted microscope, hASCs were fibrous,

uniform in size and grew in an eddy-shaped manner. Flow cytometry showed that CD molecules on the surface of hASCs were positive for CD90, CD44, CD105 and CD73, but negative for CD34, CD19 and CD45. After cultured in lipid-induced medium, hASCs were stained with oil red O. Red lipid droplets were observed under optical fiber microscopy. After the differentiation of hASCs was induced by osteogenic induction medium, a large number of red nodules could be seen in intracellular calcium nodules stained with Alizarin red.

Under scanning electron microscopy, exosomes were observed as uniform round cup shapes with vesicular structures between 30 and 150 nm in size. The diameter of exosomes was 30–150 nm by Nanosight granulometer. The results of antibody microarray showed that FLOT1 (flotillin 1), ICAM1 (intercellular adhesion molecule 1), ALIX, CD81, CD63, EpCAM (epithelial cell adhesion molecule), ANXA5 (annexin A5), TSG101 (tumor susceptibility gene 101) were all positive, while GM130 (cis-Golgi matrix protein) was negative. Histogram statistical analysis showed that the gray values of FLOT1, ICAM, ALIX, CD81, CD63, EpCAM, ANXA5 and TSG101 were all higher than those of the blank group (data not shown).

Effect of exosomes on proliferation and apoptosis of activated HSCs

The expression level of α-SMA in HSCs stimulated by TGF-β1 was significantly higher than that of HSCs. It indicates that TGF-β1 could activate HSCs (**Figure 1A**). Compared with the PBS group, the proliferation of activated HSCs was inhibited by exosome, especially at 96 hours. However, there was no significant difference between the 10 μg/L and 1 μg/L exosome treatment groups. It indicates that exosome could inhibit the proliferation of activated HSC (**Figure 1B**). As shown in **Figure 3C**, the late apoptosis percentage of activated HSCs in the high or low concentration exosomes groups and control group was 26.5%, 21.4%, and 19.9% respectively, while the early apoptosis percentage was 10.6%, 5.56%, and 6.45% respectively. The early and late apoptosis rate of high concentration exosome treatment group was higher than that of the PBS group. The late apoptosis rate in the low concentration exosome treatment group was higher than that in the PBS group, while the early apoptosis rate in the low concentration exosome treatment group was lower than that of the PBS group (**Figure 1C**). Compared with PBS and low-concentration exosome treatment groups, the treatment of activated HSCs with high concentration exosomes can significantly promote later apoptosis. It indicates that the apoptosis of activated HSC was significantly promoted when the exosome concentration reached a certain level (**Figure 1D**).

Quantitative analysis of experimental animals

All rats were involved in the result analysis.

Effect of exosomes on liver fibrosis

CCl_4 was injected intraperitoneally for 4 weeks to induce liver fibrosis. Sirius red and Masson trichrome staining of liver tissue showed moderate to severe liver fibrosis. Continuous tail venous injection of exosomes alleviated liver fibrosis, with the best effect at 4 weeks compared with the other groups (**Figure 2A–L**). After liver staining, Ishak was scored and compared statistically (**Figure 2M**). After intraperitoneal injection of CCl4 in rats, Ishak score reached 3–4, indicating moderate and severe fibrosis has formed. Compared with the rat models of liver fibrosis without PBS or exosome therapy, the Ishak scores of PBS treatment at 2 or 4 weeks were insignificantly different, indicating that the establishment of liver fibrosis model in rats is relatively stable. After 4 weeks of treatment with exosomes, liver fibrosis was significantly reduced. Semi-quantitative fiber statistics showed that exosomes significantly reduced the degree of liver fibrosis after 4 weeks of treatment (**Figure 2N**). No animals died during the experiment.

Effects of exosomes on serum liver function, type PIII and IV-C collagen in rats with liver fibrosis

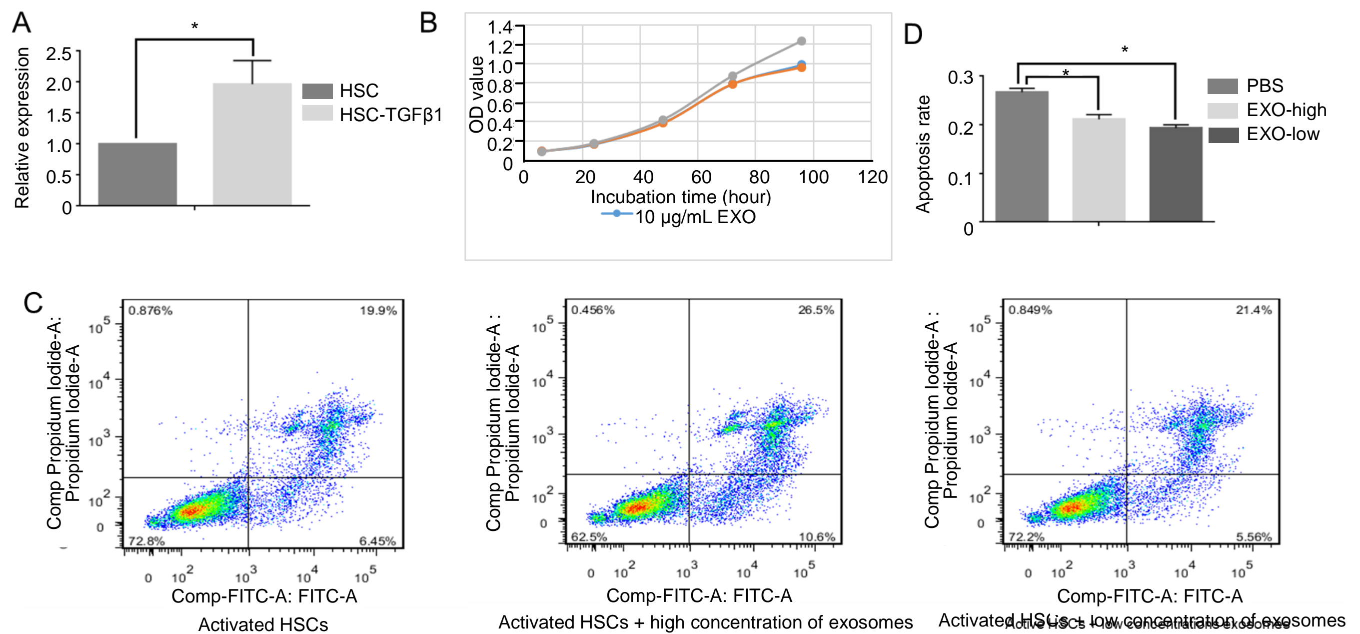
Compared with the exosome treatment group, serum ALT and AST levels were slightly higher, while serum TP and ALB levels were lower in the PBS group, indicating that exosome can improve the liver function. Serum TP level in the EXO-4W group was significantly higher than that in the PBS-4W group ($P < 0.05$). However, there was no significant difference in serum albumin level between exosomes and PBS groups (**Figure 3A–D**). It suggests that the effect of exosomes on the recovery of hepatic enzymes is not obvious in rats with liver fibrosis. Serum PIII and IV-C collagen levels in the exosome treatment group were lower than those in the PBS group, and there was statistical significance between EXO-4W and PBS-4W ($P < 0.05$) (**Figure 3E–F**). The results showed that exosomes decreased the expression of serum PIII/IV-C and alleviated liver fibrosis.

Effect of exosomes on the ECM protein expression in rats with liver fibrosis

Immunohistochemical staining showed that the expression of α-SMA and TIMP1 was high in liver tissue of rats with liver fibrosis, while the expression of MMP9 was low. With the prolongation of exosome therapy time, the expression of α-SMA and TIMP1 in liver tissue decreased gradually, and the expression of MMP9 in ECM increased gradually. There was significant difference in protein expression in ECM between PBS-4W and EXO-4W. It indicates that exosomes could inhibit activated HSCs to produce α-SMA and TIMP1, and promote HSCs to produce MMP9.

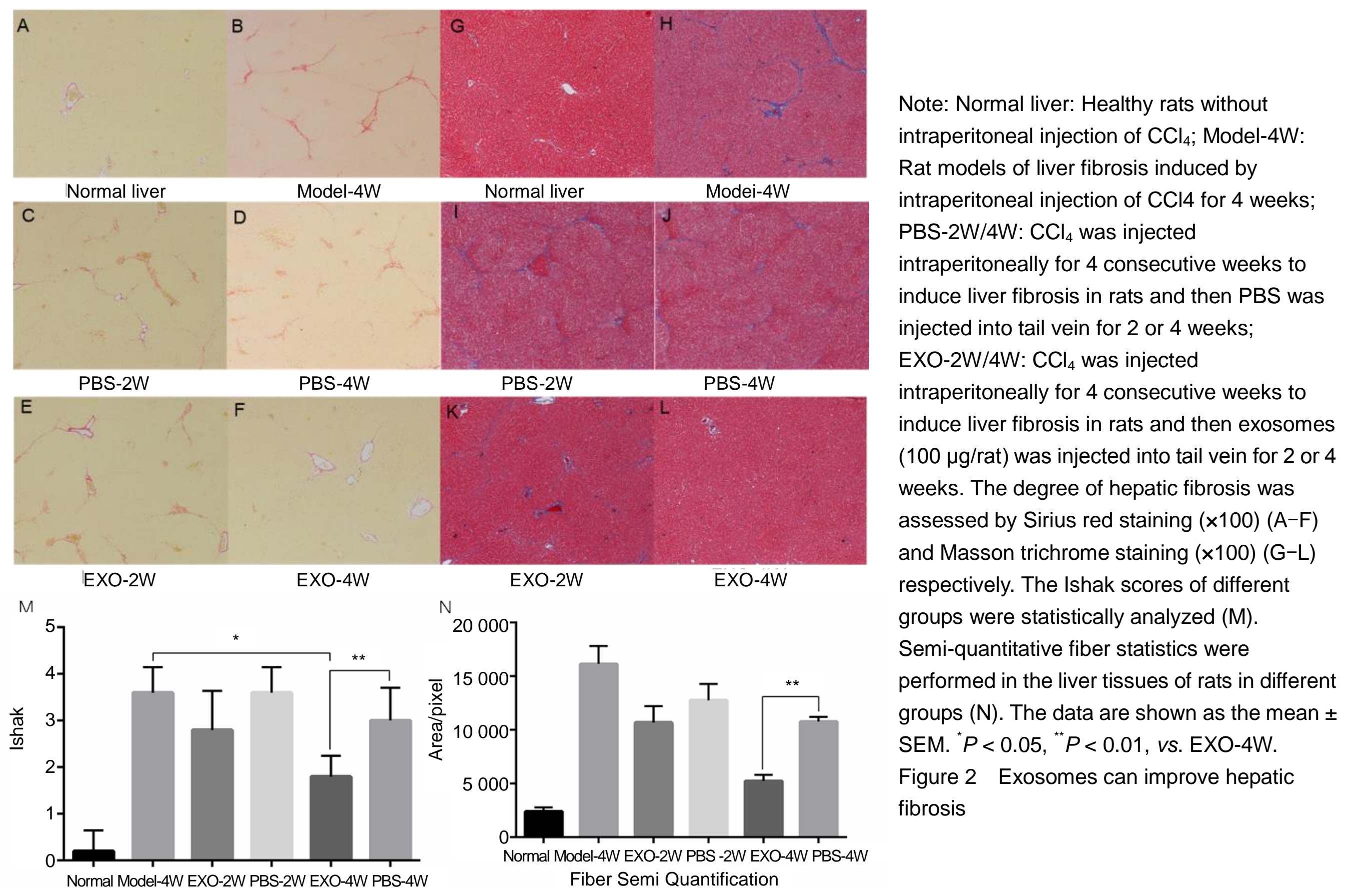
DISCUSSION

Liver fibrosis is the liver's response to chronic inflammation, necrosis, or other damages. Liver failure or liver cancer caused by liver fibrosis is the leading cause of death. The continuous activation of HSCs is a key link in the development of liver fibrosis. The proliferation, adhesion, and migration of activated HSCs will increase the expression of type I and III collagen and reduce



Note: (A) The expression of α-SMA in HSCs and HSCs stimulated by TGF-β1 was detected by qPCR. (B) CCK-8 was used to detect the proliferation of activated HSC treated with PBS, 10 μg/mL or 1 μg/mL exosomes. (C) The apoptosis rate of activated HSCs treated with PBS or different concentrations of exosomes was determined by flow analysis. (D) Statistical analysis was performed on the apoptosis rate of activated HSCs. The data are shown as the mean ± SEM. $^{*}P < 0.05$. α-SMA: α-smooth muscle actin; HSCs: hepatic stellate cells; TGF-β1: transforming growth factor beta1; qPCR: quantitative polymerase chain reaction.

Figure 1 TGF-β1 can activate HSCs and exosomes can inhibit the proliferation of activated HSCs and promote their apoptosis

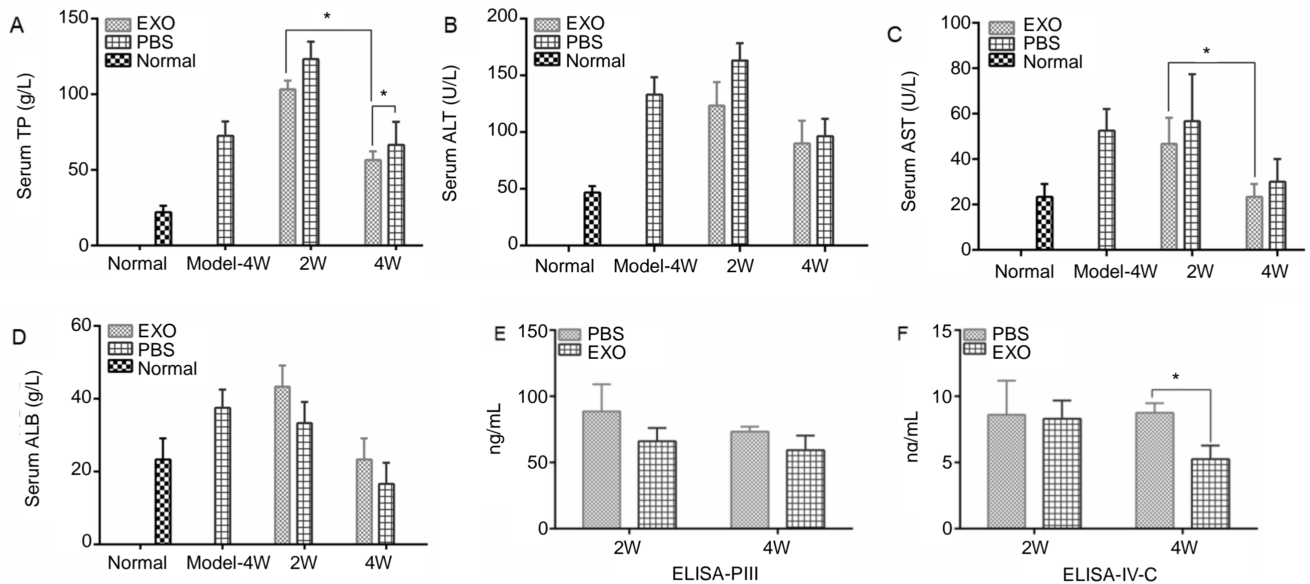


Note: Normal liver: Healthy rats without intraperitoneal injection of CCl_4; Model-4W: Rat models of liver fibrosis induced by intraperitoneal injection of CCl4 for 4 weeks; PBS-2W/4W: CCl_4 was injected intraperitoneally for 4 consecutive weeks to induce liver fibrosis in rats and then PBS was injected into tail vein for 2 or 4 weeks; EXO-2W/4W: CCl_4 was injected intraperitoneally for 4 consecutive weeks to induce liver fibrosis in rats and then exosomes (100 μg/rat) was injected into tail vein for 2 or 4 weeks. The degree of hepatic fibrosis was assessed by Sirius red staining (×100) (A–F) and Masson trichrome staining (×100) (G–L) respectively. The Ishak scores of different groups were statistically analyzed (M). Semi-quantitative fiber statistics were performed in the liver tissues of rats in different groups (N). The data are shown as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, *vs.* EXO-4W.

Figure 2 Exosomes can improve hepatic fibrosis

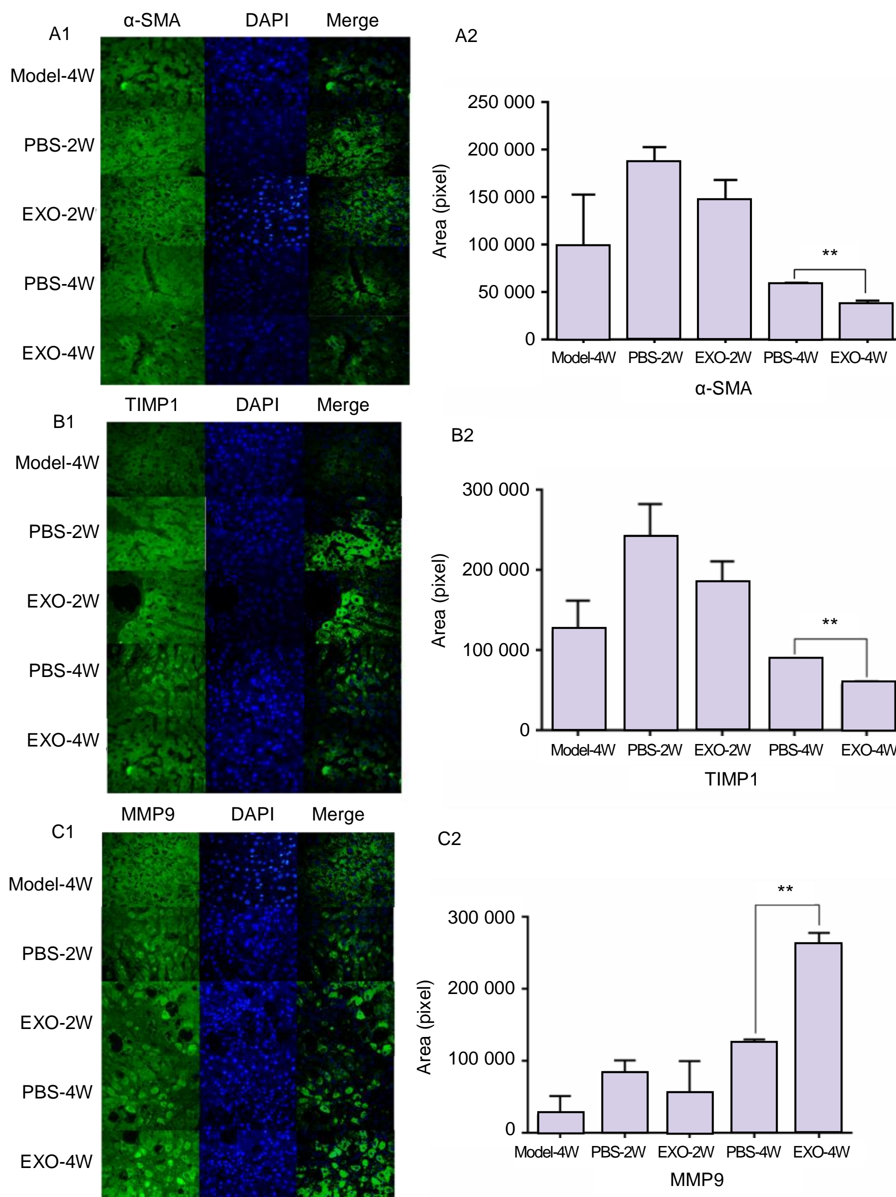
degradation in EMS. Increasing the apoptosis and collagen degradation of activated HSCs can significantly improve or reverse liver fibrosis[24].

Current treatments for liver fibrosis include etiological therapy, antifibrotic drugs, orthotopic liver transplantation, cell-based therapy and traditional Chinese medicine. These treatments mainly focus on etiological treatment, inhibition of inflammation, regulation of HSCs, regulation of other liver immune cells, regulation of cell receptor-ligand interactions related to liver fibrosis, correction of the imbalance between ECM production and



Note: Serum levels of total protein (TP, A), alanine aminotransferase (ALT, B), AST (glutamic oxaloacetic transaminase, C) and ALB (albumin, D) were detected by a biochemical analyzer. Serum PIII (E) and IV-C (F) levels were detected by ELISA. The data are presented as the mean ± SEM. $^{*}P$ < 0.05, *vs.* EXO-4W.

Figure 3 After 4 weeks of exosome therapy, serum TP and PIII/IV-C levels were significantly improved



Note: The expression levels of α-SMA (A1), TIMP1 (B1) and MMP9 (C1) in rat liver tissues were detected by immunohistochemistry (×400). The data are expressed as the mean ± SEM. $^{**}P$ < 0.01. α-SMA: α-Smooth muscle actin; TIMP-1: tissue inhibitor of metalloproteinase-1; MMP9: matrix metalloproteinase-9.

Figure 4 Exosome reduced the expression of TIMP and α-SMA in liver tissues and increased the expression of MMP9

degradation and probiotics[25-27].

However, due to the complexity of the etiology and pathogenesis of liver fibrosis, the limitations of anti-etiology and fibrosis drugs, the lack of liver source and the potential of liver injury of Chinese herbal medicine, cell therapy has been widely studied. At present, cell therapy is mainly based on the ability of MSCs to differentiate into multiple tissues. However, the immune rejection of MSCs derived from various tissues remains a major problem when transplanted into damaged organisms, which limits the use of cell therapy due to safety and other problems. MSCs play a therapeutic role by producing some bioactive factors, and the exosome is one of the most important substances[28-30].

In this study, multiple ultrafiltration concentration method was used to obtain hASCs derived exosomes for treatment (Ultrafiltration method of extracting exosomes with 100 KD filter membrane at 4 000 × *g* per min was invented by our research group. It conforms to the characteristics of exosomes and has applied for a patent. The patent number is CN201710447410.4). Exosomes contain rich RNA, microRNA and protein components, which can exchange information with target cells, promote cell proliferation, migration or inhibit cell apoptosis, and thus promote tissue damage repair. For example, stem cell-derived exosomes inhibit the release of CX3CL1 macrophage chemoattractant protein and increase the expression of IL-10 to repair damage[31-32].

In the previous experiments, our research group carried out hemolytic test, vascular stimulation test, muscle stimulation test, active systemic allergy test, passive skin allergy test, and other safety tests on hASCs derived exosomes. The results showed that hASCs derived exosomes obtained by ultrafiltration concentration method were safe for *in vivo* injection[9, 33-37].

TGF-β1, which is the most abundant member of TGF-β1 family in cells and tissues, can activate HSCs. Activated HSCs expressing α-SMA, vimentin and desmin transformed into myofibroid mother cells[36, 38-43]. Activated HSCs can promote mass synthesis of ECM and accelerate the progression of liver fibrosis[44-45]. Inhibiting the proliferation or promoting the apoptosis of activated HSCs can inhibit the progression of liver fibrosis and even alleviate liver fibrosis. *In vitro*, we found that the activated HSCs treated by high concentration of exosomes could significantly inhibit cell proliferation at 96 hours, and promote early and late apoptosis of activated HSCs. In summary, exosomes can promote the apoptosis of activated HSCs and alleviate liver fibrosis.

Serum type III and IV collagen is one of the commonly used non-invasive detection indicators reflecting liver fibrosis. It is mainly synthesized and released by HSCs, and it is the main component in the matrix of liver connective tissue. It is considered as a more sensitive detection indicator to judge the degree of liver fibrosis[46]. In this study, serum PIII and IV-C level of rats with hepatic fibrosis was detected after 2 or 4 weeks of treatment with exosome or PBS. Serum PIII and IV-C expression of EXO-4W rats was decreased by exosomes in the same cycle treatment group, and serum PIII/IV-C expression in EXO-4W rats was significantly different from that of PBS-4W rats. In addition, serum PIII/IV-C expression decreased gradually with the increase of exosome treatment time. The results suggest that exosomes can reduce the production of collagen fibers and inhibit the progression of liver fibrosis.

Both Sirius red and Masson's trichrome staining of liver tissues indicate that liver fibrosis in rats treated with exosomes was milder than that in the PBS group. The Ishak score of liver tissues and semi-quantitative analysis of stained tissues by Image-pro Plus 6.0 software further confirmed that exosomes could alleviate liver fibrosis, and there was significant difference between EXO-4W and PBS-4W rats. Longer exosome treatment indicates stronger effects on alleviating liver fibrosis.

Immunohistochemical staining showed that exosome inhibits TIMP1/SMA expression in liver tissue and increases MMP9 expression. The results suggest that exosomes can alleviate the progression of liver fibrosis by reducing the production of ECM. In addition, exosomes can alleviate liver fibrosis by increasing the expression of collagen degradation-related enzymes in ECM. The degradation of ECM in liver tissue mainly depends on the regulation of endogenous MMPs and TIMPs. HSCs can secrete a variety of collagenase and matrix metalloproteinases to degrade various ECM, while activated HSCs promote the development of liver fibrosis by promoting their own mitosis through autocrine TIMP-1. Meanwhile, TIMPs bind to specific active collagenase MMP9, which mainly degrades type IV collagen and denatured collagen, to inhibit the degradation of ECM.

Combined with the experimental results *in vivo* and *in vitro*, we speculate that exosome inhibits the progression of live fibrosis or treats liver fibrosis through inhibiting the proliferation and promoting the apoptosis of activated HSCs, decreasing the number of activated HSCs in liver tissue, and lowering TIMP1 expression in ECM. The expression of MMP9, which can degrade collagen fibers produced by inactivated HSCs, is gradually enhanced, and the production and degradation of collagen fibers in ECM of liver tissue are gradually restored to equilibrium. However, in this experiment, the degradation of TIMP1, MMP9 and α-SMA mRNA in liver tissues by exosomes was not studied intuitively, which will be further confirmed in subsequent experiments.

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人脂肪干细胞来源外泌体对四氯化碳诱导肝纤维化模型大鼠的治疗作用

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文题释义：

脂肪干细胞：是指从脂肪组织中分离得到的一种间充质干细胞，不但具有跨胚层多向分化潜能，在不同培养条件下可以分化成肌肉、软骨、脂肪组织、神经组织或肝脏组织，而且具备取材方便、来源广阔、增殖能力强、免疫原性低等优点，近年来成为干细胞治疗的热点。

外泌体：是一种细胞主动分泌的大小均一、直径为50-150 nm的脂质双分子层结构囊泡，可由树突细胞、淋巴细胞、成纤维细胞、间充质干细胞和肿瘤细胞等多种不同细胞类型释放。

摘要

背景：肝纤维化具有较高的发病率和死亡率，肝星状细胞的活化和增殖是肝纤维化进程中的关键环节。目前还没有针对单一环节或靶点的有效抗纤维化药物。

目的：分析人脂肪干细胞来源外泌体对四氯化碳诱导的大鼠肝纤维化的影响。

方法：①通过酶溶解法获取健康人群来源脂肪中干细胞，体外培养获取一定数量细胞后通过多重超滤法获取外泌体。体外培养的肝星状细胞经转化生长因子β1活化后利用不同浓度外泌体进行处理，通过定量PCR检测细胞内α-平滑肌动蛋白的表达明确其活化程度，以及分别使用CCK-8及流式细胞术检测各组外泌体处理后活化肝星状细胞的生长率及凋亡率。②通过腹腔注射四氯化碳构建肝纤维化大鼠动物模型，尾静脉注射外泌体进行治疗。检测各组动物的肝功能及血清Ⅲ型前胶原、Ⅳ型胶原，肝组织Ishak评分及肝纤维化半定量，以及通过免疫荧光法检测肝组织内基质金属蛋白酶组织抑制剂1、基质金属蛋白酶9及α-平滑肌动蛋白的表达。实验方案于2017年1月经同济大学动物实验伦理委员会以及医学伦理学委员会批准。

结果与结论：人脂肪干细胞来源外泌体可抑制活化的肝星状细胞增殖，其可能的机制为抑制活化巨噬细胞的增殖，减少胶原纤维、α-平滑肌动蛋白及基质金属蛋白酶组织抑制剂1的表达，并促进基质金属蛋白酶9的表达。提示外泌体可治疗四氯化碳诱导肝纤维化。

关键词：

人脂肪干细胞；外泌体；细胞外分泌物质；肝纤维化；肝星状细胞；四氯化碳；转化生长因子β1；α-平滑肌蛋白；基质金属蛋白酶组织抑制物1；基质金属蛋白酶

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机构伦理问题：实验方案于2017年1月经海军第905医院动物实验伦理委员会以及医学伦理学委员会批准。其中动物实验过程遵循了国际兽医学编辑协会《关于动物伦理与福利的作者指南共识》和本地及国家法规。实验动物在戊巴比妥钠麻醉下进行所有的手术，并尽一切努力最大限度地减少其疼痛、痛苦和死亡。人体组织取得过程符合《赫尔辛基宣言》的规定。

知情同意问题：参与实验的患者为自愿参加，所有患者对试验过程完全知情同意，在充分了解试验方案的前提下签署了“知情同意书”。

写作指南：该研究遵守国际医学期刊编辑委员会《学术研究实验与报告和医学期刊编辑与发表的推荐规范》。

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