

Mesenchymal stem cell-derived exosomes improve diabetes mellitus-induced myocardial injury and fibrosis via inhibition of TGF- β 1/Smad2 signaling pathway

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Abstract: The aim of this study is to investigate the effect of mesenchymal stem cell (MSC)-derived exosomes on diabetes mellitus-induced myocardial injury, and the underlying mechanism. Thirty adult male Sprague Dawley rats were randomly assigned to three groups of ten rats each: normal control group, diabetic control group and MSC exosomes group. Exosomes were isolated from MSCs through gradient ultracentrifugation. With the exception of normal control, diabetes mellitus (DM) was induced in the rats with a single intraperitoneal injection of 30 mg/kg body weight streptozotocin (STZ) in 0.1 mol/L sodium citrate buffer. Rats in MSC exosomes group were intravenously injected with MSC-derived exosomes once a week for 12 weeks. Left ventricular collagen (LVC) level was measured using acid hydrolysis method. Fatty acid transporters (FATPs) and fatty acid beta oxidase (FA- β -oxidase) were determined using enzyme-linked immunosorbent assay (ELISA). Gene and protein expressions of TGF- β and Smad2 were determined using real-time quantitative polymerase chain reaction (qRT-PCR) and Western blotting. Flow cytometric analysis and Western blotting revealed positive expression of exosomal specific marker, CD63. The level of LVC was significantly higher in diabetic control group than in normal control group, but was significantly reduced after treatment with MSC-derived exosomes ($p < 0.05$). The levels of FATPs and FA- β -oxidase were significantly lower in diabetic control group than in normal control group ($p < 0.05$). However, treatment with MSC-derived exosomes significantly increased the levels of these proteins ($p < 0.05$). The levels of expression of TGF- β 1 and Smad2 mRNAs were significantly higher in the diabetic control group than in normal control group, but were significantly reduced after treatment with MSC-derived exosomes ($p < 0.05$). The expressions of TGF- β 1 and Smad2 proteins were also significantly upregulated in diabetic control group, when compared with normal control group ($p < 0.05$). However, treatment with MSC-derived exosomes significantly down-regulated the expression of these proteins ($p < 0.05$). The results obtained in this study indicate that MSC-derived exosomes improve DM-induced myocardial injury and fibrosis via inhibition of TGF- β 1/Smad2 signaling pathway.

Key words: Diabetes mellitus; Diabetic cardiomyopathy; Mesenchymal stem cell-derived exosomes; Fibrosis; Expression.

Introduction

Diabetic cardiomyopathy (DCM) is a common cardiovascular complication of DM characterized by cardiomyocyte hypertrophy, myocardial interstitial fibrosis and diastolic dysfunction. It may ultimately lead to congestive heart failure (CHF) if left untreated (1). Abnormally elevated blood glucose level leads to microvascular endothelial dysfunction, myocardial metabolic disorders and insulin signaling disorders, which are responsible for diabetic myocardial remodeling and cardiac dysfunction (1). The pathogenesis of DCM has not been fully elucidated and at present, there are no known therapeutic interventions. Exosomes are nano-sized membrane vesicles secreted by cells. They contain functional proteins, mRNA, microRNA and tRNA, and range from 30 to 100 nm in diameter. They play important roles in cell-to-cell communication (2). Exosomes affect the function of islet cells via activation of autoimmunity, increased beta cell apoptosis and regulation of cytokine levels, thus participating in the development and progression of DM (3). Although studies on the involvement of exosomes in diabetic nephropathy abound, reports on their roles in the pathogenesis of DCM are scanty (4). Studies have shown that rat bone marrow

MSC-derived exosomes inhibit hyperglycemia-induced expression of α -smooth muscle actin via reduction in Smad2/3 phosphorylation, inhibition of fibrillation and trans-differentiation of cells (5). The aim of this study was to investigate the effects of MSC-derived exosomes on DM-induced myocardial injury, and the underlying mechanism.

Materials and Methods

Materials

Rat bone marrow MSCs were obtained from Shenzhen Sanqi Biotechnology Co., Ltd. and Dulbecco's Modified Eagle/Ham's F12 (DMEM/F12) medium was purchased from Hangzhou Yinuo Biotechnology Co., Ltd. Bicinchoninic acid (BCA) protein quantitation kit was a product of Shanghai Bioengineering Co., Ltd. Streptozotocin was purchased from Shanghai Mengya Biotechnology Co., Ltd; hydroxyproline assay kit was obtained from KALANG (USA), while ELISA kits were purchased from Wuhan Doctor De Biology Co., Ltd. Smad2, TGF-beta 1 and β -actin primers were products of TakaRa Bioengineering Co., Ltd.

Experimental rats

Thirty adult male Sprague Dawley rats weighing 300 – 320 g (mean weight = 310 ± 10 g) were obtained from Chengdu Dashuo Laboratory Animal Co., Ltd. and used for this study. The rats were housed in plastic cages under optimum conditions: 12 h day/night cycle, 21 °C and 50 - 55 % humidity. They were allowed free access to standard rat feed and clean water.

Isolation and identification of MSC-derived exosomes

Rat bone marrow MSCs were cultured in DMEM-F12 medium at 37 °C in a humidified atmosphere containing 5 % CO₂ and 95 % air. Cells in logarithmic growth phase (1×10^7 cells) were selected and used for this study. Exosomes were isolated from MSCs using gradient ultracentrifugation method as previously reported (6). Exosomal protein was quantified using BCA protein quantitation kit, and CD63, the marker of exosomes, was identified using flow cytometry and Western blotting.

Induction of DM and grouping

The rats were randomly assigned to three groups of ten rats each: normal control group, diabetic control group and MSC exosomes group. Rats in the normal control group were fed basal diet.

High-fat diet was used to induce insulin resistance in diabetic rats. Diabetes was induced with intraperitoneal injection of STZ (30 mg/kg body weight) in 0.1 mol/L sodium citrate buffer for six weeks, which resulted in partial disruption of islet β cell function. After 72 h, rats with blood glucose concentration ≥ 11.1 mmol/L were considered diabetic (7). After the induction of DM, 200 μ L of phosphate-buffered saline (PBS) containing 100 μ g MSC-derived exosomes was intravenously injected into rats in the MSC exosomes group, while the diabetic control group received 200 μ L of pure PBS solution via their tail vein. The treatment regimen was performed once a week and lasted 12 weeks.

Determination of level of LVC

Acid hydrolysis was used for determination of level of collagen in left ventricle. Left ventricular tissue (0.1 g) was taken from the rats after sacrifice (8). The assay was carried out strictly according to the instructions of hydroxyproline assay kit and results expressed in mg/g.

Determination of lipid metabolism-related enzymes in myocardium

Myocardial homogenate (20 %) was prepared with PBS buffer. The levels of FATPs and FA- β -oxidase in rat myocardium were determined using their respective ELISA kits.

qRT-PCR

Quantitative real-time PCR was used to determine the effect of MSC-derived exosomes on the levels of expression of TGF- β 1 and Smad2 mRNAs. The upstream of TGF- β 1 primer sequence was 5'-CCTT-CACTTTCAGGTCGAG-3', while the downstream was 3'-CAGTTTGGGACCTTACA-5'. The Smad2 primer sequence upstream was 5'-CATGCTGTCCCGTGCA-GA-3', while the downstream sequence was 3'-AGG-

TAACGCCAGGAATGCTA-5'. The upstream sequence of the internal reference (actin) was 5'-CCCATCTA-TGAGGTTACGC-3', while its downstream sequence was 3'-TTTAGTCACGCACGATTTC-5'. Trizol RNA extraction reagent was used to extract total RNA from the cells. The reaction conditions of RT-PCR were: pre-denaturation at 95°C for 5 sec, and PCR at 95 °C for 5 sec and 60°C for 30 sec, and a total of 40 cycles. The mean threshold value for each cycle was normalized to the expression of actin. The relative expression of RNA was calculated using 2^{- $\Delta\Delta$ Ct} method.

Western blotting

The expressions of TGF- β 1 and Smad2 protein were determined using Western blotting. The cells were washed twice with PBS and ice-cold radio-immunoprecipitation assay buffer (RIPA) containing protease inhibitor was used to lyse them. The resultant lysate was centrifuged at 15,000 rpm for 15 min at 4 °C, and the protein concentration of the supernatant was determined using BCA assay kit. A portion of total cell protein (10 μ g) from each sample was separated on a 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 °C for 120 min. Subsequently, non-fat milk powder (5 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C and incubated to block non-specific binding of the blot. Incubation of the blots was performed overnight at 4 °C with primary antibodies of rabbit polyclonal anti-TGF- β 1, Smad2 and actin at a dilution of 1 : 1000. Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 2 h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using Enhanced chemiluminescence (ECL). Respective protein expression levels were normalized to that of actin which was used as a standard.

Statistical analysis

Data are expressed as mean \pm SEM, and statistical analysis was performed using SPSS (19.0). Groups were compared using Student *t*-test. Values of $p < 0.05$ were considered statistically significant.

Results

Identification of MSC-derived exosomes

As shown in Figure 1, flow cytometric analysis and Western blotting revealed positive expression of exosomal specific marker, CD63.

Levels of collagen in left ventricle of rats

The level of LVC was significantly higher in diabetic control group than in normal control group, but was significantly reduced after treatment with MSC-derived exosomes ($p < 0.05$). These results are shown in Figure 2.

Effect of MSC-derived exosomes on levels lipid metabolism-related enzymes in myocardial tissue

The levels of FATPs and FA- β -oxidase were signi-

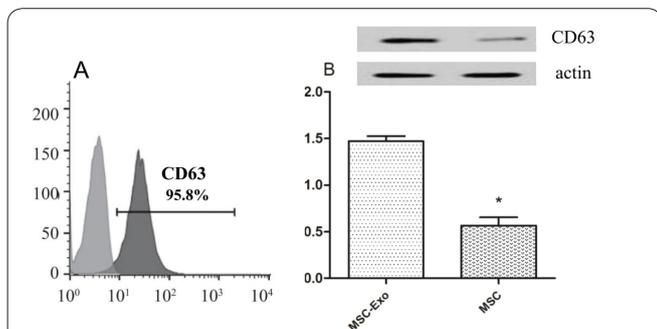


Figure 1. Identification of MSC-derived exosomes. (A): CD63 levels as determined using flow cytometry, and (B): CD63 levels as determined using Western blotting. * $p < 0.05$, when compared with MSC exosomes group.

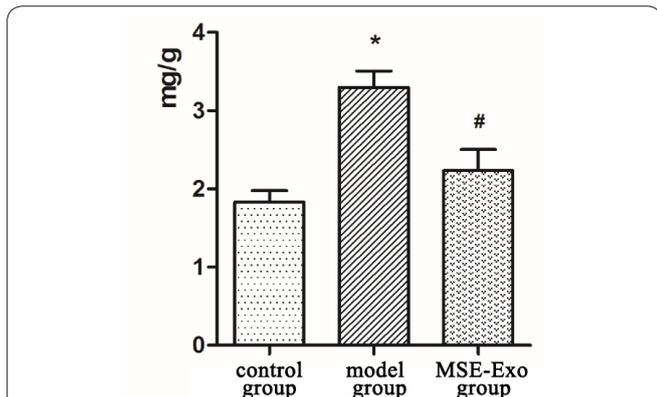


Figure 2. Effect of MSC-derived exosomes on the level of LVC. * $p < 0.05$, when compared with the normal control group; # $p < 0.05$ when compared with diabetic control group.

ificantly lower in diabetic control group than in normal control group, but were significantly increased after treatment with MSC-derived exosomes ($p < 0.05$; Figure 3).

Levels of expression of TGF- β 1 and Smad2 mRNAs

The levels of expression of TGF- β 1 and Smad2 mRNAs were significantly higher in the diabetic control group than in normal control group ($p < 0.05$). However, treatment with MSC-derived exosomes significantly reduced the expression levels of these mRNAs ($p < 0.05$). These results are shown in Figure 4.

Effect of MSC-derived exosomes on expression of TGF- β 1 and Smad2 proteins

The expression of TGF- β 1 and Smad2 proteins were significantly upregulated in diabetic control group, when compared with normal control group ($p < 0.05$). However, treatment with MSC-derived exosomes significantly down-regulated the expression of these proteins ($p < 0.05$; Figure 5).

Discussion

Diabetic cardiomyopathy (DCM) is a common cardiovascular complication of DM, and oxidative stress, apoptosis, inflammation, fibrosis and disorders of energy metabolism play important roles in its development and progression (1, 9). Myocardial biopsies of diabetic patients reveal cardiac hypertrophy, myocardial hypertrophy, decreased myofibrils, interstitial fibrosis, increased arterial wall matrix, and myocardial microvascular disease. In diabetic patients without heart di-

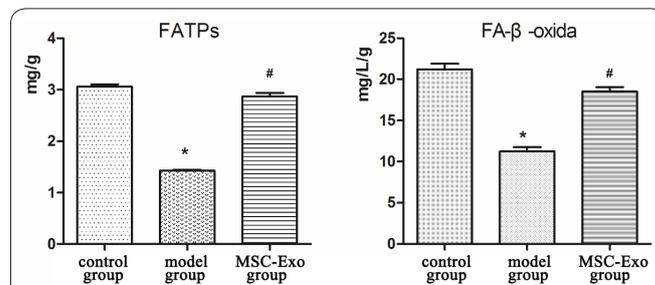


Figure 3. Effect of MSC-derived exosomes on lipid metabolism-related enzymes in myocardial tissue. * $p < 0.05$, when compared with the normal control group; # $p < 0.05$, when compared with diabetic control group.

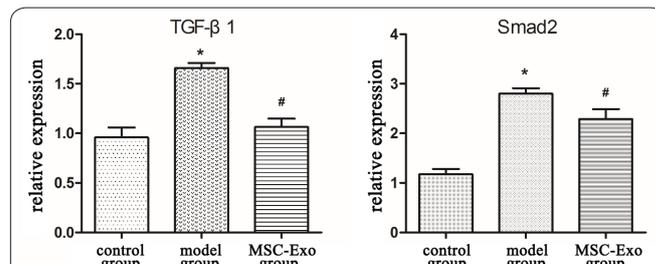


Figure 4. Effect of MSC-derived exosomes on TGF- β 1 and Smad2 mRNA expressions. * $p < 0.05$, when compared with normal control group; # $p < 0.05$, when compared with diabetic control group.

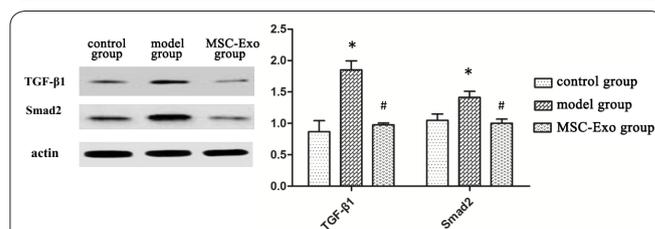


Figure 5. Effect of MSC-derived exosomes on TGF- β 1 and Smad2 proteins expression. * $p < 0.05$, when compared with normal control group; # $p < 0.05$, when compared with diabetic control group.

sease, myocardial fibrosis occurs in the early stage of the disease as left ventricular dysfunction characterized by increased level of myocardial collagen fiber. Proliferation of collagen fiber leads to increased myocardial tension, which is an important cause of cardiac systolic dysfunction (10, 11). The present study investigated the effect of MSC-derived exosomes on DM-induced myocardial injury, and the underlying mechanism.

The results showed that LVC level in the diabetic control group was significantly increased, while myocardial tissue lipid metabolism-related enzymes were significantly decreased. However, treatment with MSC-derived exosomes significantly reversed these changes.

Studies have shown that the effect of MSCs on myocardial regeneration is due principally to paracrine effect of cells, rather than cell differentiation (12). It has also been reported that the therapeutic effects of MSCs are due largely to the formation of exosomes (13-15).

Compared with cell therapy, exosome therapy produces low immune rejection which makes it suitable for allo-transplantation. The secretion of MSCs from human umbilical cord has been shown to alleviate liver fibrosis (16). Myocardial infarction leads to myocardial cell necrosis and cardiac fibrosis. It has been reported that exosomes formed by human umbilical cord MSCs alleviate injury caused by myocardial infarction and cardiac fibrosis (17). In this study, the MSC-derived

exosomes significantly ameliorated myocardial injury in diabetic rats, reduced LVC level and significantly increased the levels of lipid metabolism-related enzymes in myocardial tissue. MSC-derived exosomes inhibit hyperglycemia-induced fibroblast trans-differentiation *in vitro*, and may be effective in the treatment of DCM and fibrosis.

As a fibrogenic cytokine, TGF- β regulates the proliferation, differentiation and migration of fibroblasts. Three subtypes of TGF- β bind to types I and II TGF- β receptors on the cell membrane, and this results in the phosphorylation of Smad in the cytoplasm. On entering the nucleus, phosphorylated Smad binds to target gene response elements and activates the expression of the gene (18). Overexpression of TGF- β results in increased synthesis of collagen fibers type I and III (19). In this study, the relative expressions of TGF- β 1 and smad2 were significantly upregulated in diabetic control group than in the normal control group, suggesting that myocardial injury and fibrosis induced by DM may be linked to the TGF- β 1/Smad2 pathway. These results are in agreement with those reported in previous studies (20). The results of this study also suggest that MSC-derived exosomes may reduce the expression of genes and proteins of the TGF- β 1/Smad2 pathway. It has been reported that MSC-derived exosomes inhibit the expression of alpha-smooth muscle actin and differentiation of fibroblasts induced by hyperglycemia via reduction in the phosphorylation of Smad2/3 (5). This suggests that the effect of MSC-derived exosomes on DCM may be connected with the TGF- β 1/Smad2 pathway.

The results obtained in this study show that MSC-derived exosomes improve DM-induced myocardial injury and fibrosis via inhibition of TGF- β 1/Smad2 signaling pathway.

Acknowledgements

None.

Conflict of Interest

There is no conflict of interest in this study.

Author's contribution

All work was done by the author s named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Yuan Lin; Yuan Lin, Fang Zhang, Xiao-fen Lian, Wei-qun Peng, Chao-yao Yin collected and analyzed the data; Yuan Lin wrote the text and all authors have read and approved the text prior to publication.

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