Original Article
Effect and functional mechanism of induced pluripotent stem cells on myocardial fibrosis after cardiac infarction

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Abstract: Acute myocardial infarction (AMI) has a high incidence and causes severe consequences. Induced pluripotent stem (iPS) cells derives from patient's own cells and has become one research focus for treating cardiovascular disease. The role and function of iPS cells on myocardial fibrosis after AMI, however, has not been illustrated. Healthy male Wistar rats were randomly divided into control, sham and AMI group. The AMI model was generated by occlusion of left anterior coronary artery. iPS cell transplantation group received mouse derived iPS cells after AMI model. M-type ultrasound was used to evaluate cardiac function of all rats. Immunohistochemistry staining was employed to evaluate the change of myocardial fibrosis. ELISA was adopted for measuring type I collagen content. Real time PCR and Western blotting were employed to quantify mRNA and protein levels of Bax or Bcl-2 in myocardial tissues, respectively. Left ventricular end-stage systolic dimeter (LVEDS), leaf ventricular end-stage diabolic diameter (LVEDD) and left ventricular mass index (LVMI) were all significantly increased in AMI model rats, which also showed elevated myocardial fibrosis, type I collagen content and Bax expression, along with decreased Bcl-2 mRNA or protein level (P<0.05 compared to sham group). iPS cell transplantation led to lower LVESD, LVEED and LVMI levels accompanied with fewer myocardial fibrosis, type I collage, Bax expression plus increased Bcl-2 expression (P<0.05 compared to AMI group). iPS cells can improve the condition of cardiac infarction and fibrosis via modulating apoptosis balance, protecting myocardial cells and suppressing type I collagen proliferation.

Keywords: Induced pluripotent stem cells, acute myocardial infarction, cardiac fibrosis, cell apoptosis

Introduction

Cardiovascular disease maintains a high incidence and has become the leading cause for mortality worldwide, thus severely affect the public healthy [1]. Acute myocardial infarction (AMI) is one common severe disease in clinics, and has now become the most popular cardiovascular disease which induces severe effects [2, 3]. With the transition of life style, diet habit and aging or mental factors, AMI incidence in China is now as high as 45~55 per 100,000 [4]. AMI can be caused by the rupture and coronary atherosclerosis plaque, followed by platelets activation, resulting in acute coronary artery occlusion [5]. The re-construction of myocardial tissues often occurs during AMI pathogenesis. Among these phenomena, the change of myocardial extracellular matrix (ECM) exerts an important role [6, 7]. ECM consists of various large molecules and structural proteins, including collage, glycoprotein, fibrous connecting protein, elastin and lammin proteoglycan. Among these, myocardial collagen is the major component of ECM [8, 9]. Therefore it is one critical treatment measure to alleviate myocardial fibrosis after infarction. Current treatment measures, including drugs, intervention and coronary artery bridging, cannot obtain satisfactory effects as re-infarction, heart failure or even death often occur [10]. Therefore, the establishment of treatment strategy for cardiac infarction has become one research focus.

As one special cell type, stem cell has self-renewal and unlimited proliferative potency and an differentiate into primordial cells including embryonic stem cell (ESC) and adult stem cell [11, 12]. Previous study showed the differentiation potency of stem cell into endothelial or myocardial cells during myocardial repair [13]. Similar to ESC, induced pluripotent stem (iPS)

cell is one pluripotent cell type with self-renewal and differentiation potency [14]. Deriving from various somatic cells of animals, iPS cells may differentiate into all kinds of body cells via re-programming and transformation, including myocardial cells with normal contraction and excitability [15]. As iPS can be derived from patient's own body, the immune rejection and ethical issue of xenograft ESC can be overcome, making it one new strategy for treating cardiovascular disease [16]. The effect and mechanism of iPS cells on myocardial fibrosis after cardiac infarction, however, has not been fully illustrated.

Materials and methods

Experimental animal

Healthy male Wistar rats (2 months old, SPF grade, body weight 250 ± 20 g) were purchased from Shandong animal experimental center and were kept in an SPF grade facility under fixed temperature (21 ± 1°C) and relative humidity (50~70%) with 12 h-12 h light/dark cycle.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Weifang People's Hospital.

Reagents and equipment

Mouse iPS cells were generated in-house. Leukocyte inhibitor factor was purchased from ESGRO (US). DMEM medium was purchased from Life (US). Pentobarbital sodium and lidocaine were purchased from Zhaohui Pharm (China). PVDF membrane was a product of Pall Life Sciences (UK). Western blot reagents were obtained from Beyotime (China). ECL reagent was a product of Amersham Biosciences (US). Rabbit anti-mouse Bcl-2 monoclonal antibody, rabbit anti-mouse Bax monoclonal antibody, anti-type I collagen antibody, and goat anti-rabbit horseradish peroxidase (HRP) labelled IgG secondary antibody were purchased from Cell signaling (US). ELISA kits form type I and type III collagen were purchased from R&D (US). RNA extraction kit and reverse transcription kit were produced by Axygen (US). Microscopic surgical instruments were obtained from Suzhou Medical Instrument (China). The microplate reader was produced by BD (US).

Other reagents were purchased from Sangon (China).

Animal grouping and treatment

A total of 45 healthy male Wistar rats were randomly divided into three groups (N=15 each), namely, control (sham) group, AMI group, in which rat AMI model was prepared by the occultation of left anterior descending branch of coronary artery, and iPS cell transplant group, which received transplantation of mouse iPS cells after AMI model generation.

Rat AMI model generation

After general anesthesia, rats were fixed in a supine position. After shaving and sterilization of surgical area, tracheal intubation was performed under visual assistance. The ventilation machine was connected for assisted respiration (tidal volume 4 ml/kg, respiration frequency 80 per min). An incision was made via the fourth rib on left chest, the heart was then exposed by blunting separation of subcutaneous tissues and muscle. The left coronary artery was ligated using 7-0 nylon suture via 2/3 myocardial layer on the bottom edge (1~2 mm) of left ventricular ear. Electrocardiogram was monitored in real time during the surgery. The model was generated when heart tissues turned white accompanied with continuous elevation of ST segment (>1/2 R wave) in a single peak. In sham group, the heart was exposed but without ligation.

Preparation and injection of iPS cells

iPS cells were resuscitated and passed. Before transplantation, iPS cells were digested by collagenase, and cultured for 3 days using DMEM medium containing 1000 IU/ml leukocyte inhibitory factor. Rats in iPS cell group were injected with $2×10^7$ per ml cell suspension in PBS into the infracted area during AMI model preparation. Penicillin was applied post-op to prevent infection.

Post-op evaluation of cardiac function

VEVO2100M ultrasound (Visual Sonic, Canada) was used to describe the change of cardiac function at 28 days post-op, including left ventricular quality index, ventricular systolic and diabolic diameter. In brief, rats were fixed in a

Table 1. Primer sequence

Target gene	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	ACCAGGTATCTGCTGGTTG	TAACCATGATGTCAGCGTGGT
Bcl-2	GACTTACATGTGACTGCCTG	TTCCGGTTCAACTCTCCTTA
Bax	TTACATGTGACTCTCCTTA	TGTGACTGCCTGGGTTCA

Table 2. Effect of iPS cell transplant on cardiac function of AMI rats

Group	LVEDD (mm)	LVESD (mm)	LVMI
Sham	0.37 ± 0.05	0.31 ± 0.03	2.11 ± 0.03
AMI	0.62 ± 0.06*	0.44 ± 0.07*	3.85 ± 0.17*
iPS	0.47 ± 0.02#	0.34 ± 0.05#	2.23 ± 0.18#

Note: *P<0.05 compared to sham group; #P<0.05 compared to AMI group.

supine position. Ultrasound probe (model 15-L8) was placed near the chest for a horizontal section of left ventricular short axis mammillary muscle. After obtaining clear 2D image, M-type ultrasound cardiac graphic was applied to measure left ventricular end-stage diabolic diameter (LVEDD) and left ventricular end-stage systolic diameter (LVESD). The left ventricular quality index was calculated based on the formula.

Sample collection

Blood sample was collected from abdominal aorta from all rats using vacuum tubes. Blood samples were placed at room temperature for 30 min. After blood clotting, 10-min centrifugation was performed under 4°C for 3600 rpm. The supernatant was saved and stored at -20°C for further use. Myocardial tissues were collected from rats and stored at -80°C.

ELISA for serum type I collagen content

Rat serum samples were tested for type I collagen level following the manual instruction of ELISA kit. A linear regression function was generated based on concentrations and respective OD values of standard samples. The concentration in serum sample was then deduced on the regression model from OD values.

Real time PCR for Bcl-2 and bax mRNA expression in all groups

Trizol reagent was used to extract tissue RNA. DNA reverse transcription synthesis was performed based on the manual instruction of test

kit. Primers were designed based on target gene sequence in Primer 6.0 software, and were synthesized by Invitrogen (China). Sequences of all primers were shown in **Table 1**. Real-time PCR was employed to test target gene expression. The reaction conditions were: 55°C for 1 min, followed by 35 cycles each containing 92°C 30 s, 58°C 45 s and 72°C 35 s. Data were collected by PC cycler and the build-in software. Using GAPDH as the internal reference, fluorescent value was used to calculate CT values of all standards and samples. The standard curve was firstly plotted using CT value as the reference, followed by semi-quantitative analysis by $2^{-\Delta Ct}$ method.

Western blotting for Bcl-2 and Bax protein expressions

Total tissue proteins were extracted from myocardial tissues. In brief, cells were lysed on ice for 15~30 min, with ultrasound treatment (5 s, 4 times). After centrifugation at 10 000 g for 15 min (4°C), the supernatant was saved, quantified and stored at -20°C for Western blot assay. Proteins were separated in 10% SDS-PAGE, and were transferred to PVDF membrane by semi-dry method under an electrical field (110 mA, 1.5 h). Non-specific binding sites were removed by 5% defatted milk powder for 2 h. Anti-Bcl-2 (1:1000) or anti-Bax monoclonal antibody (1:2000) was added for 4°C overnight incubation. After PBST washing, goat anti-rabbit secondary antibody (1:2000) was added for 30 min incubation at room temperature. ECL reagent was then added for developing the membrane for 1 min, followed by X-ray exposure. The result was obtained by protein imaging system and Quantity One software (Gene, US) for measuring band density. Each experiment was replicated for four times (N=4) for statistical analysis.

Evaluation of myocardial fibrosis in all rats

Immunohistochemistry (IHC) staining was used to reveal the condition of myocardial fibrosis. Cardiac tissues were collected, fixed in 10% neutral buffered paraformaldehyde, dehydrated in gradient ethanol and xylene, embedded in paraffin, and prepared in serial sections. Xylene and gradient ethanol were then sequentially used to de-wax and rehydrate slices, which were stained in hematoxylin-eosin for 3 min. IHC staining was performed based
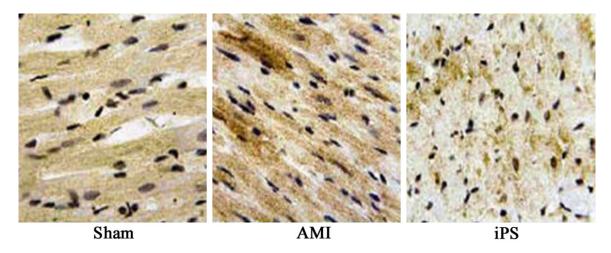
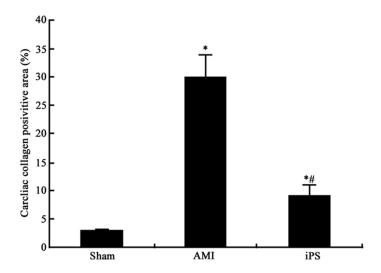**Figure 1.** Expression of myocardial type I collagen fiber (×400).

Figure 2. Positive expression of collagen fiber in myocardia tissues. *, P<0.05 compared to sham group; #, P<0.05 compared to AMI group.
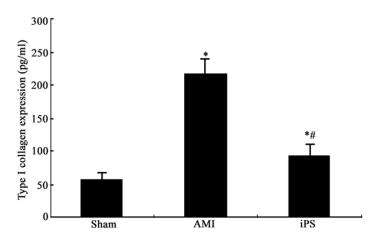

Figure 3. Effects of iPS cell transplant on secretory type I collagen in serum of AMI rats. *, P<0.05 compared to sham group; #, P<0.05 compared to AMI group.

on the instruction of SABC kit. In brief, anti-type I collagen antibody (1:1000) was added for 1 h incubation at 37°C. After PBS rinsing for three times, biotin-labelled secondary anti-IgG antibody was added. A computer assisted analysis was performed to semi-quantitatively analyze positive expression in rat myocardial tissues. The result was expressed as the percentage of positive cell area against total area. Those samples with positive arear larger than 50%, between 25% and 50%, and less than 25% were defined as strong positive, positive and weak positive, respectively.

Statistical analysis

SPSS 19.0 software was used to process all data, of which measurement data were expressed as mean ± standard deviation (SD). The comparison of means among multiple groups was performed using one-way analysis of variance (ANOVA). A statistical significance was defined as P<0.05.

Results

Effect of iPS cell transplantation of myocardial function in AMI rats

We used M-type ultrasound to compare the effect of iPS cell transplantation on the cardiac function
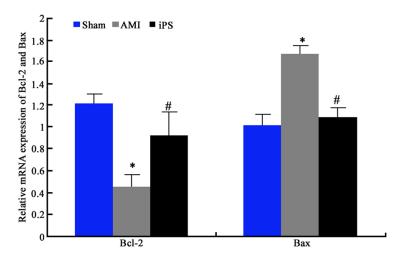
Figure 4. Bcl-2 and Bax mRNA expression in AMI rat myocardial tissues and the effect of iPS cell transplantation. *, P<0.05 compared to sham group; #, P<0.05 compared to AMI group.

change in AMI model. Results showed significant change of cardiac function in AMI rats, which had higher LVESD and LVEDD (P<0.05 compared to sham group). The transfection of iPS cells into AMI rats decreased LVESD and LVEDD (P<0.05 compared to AMI group). LVMI showed similar pattern as those for LVESD and LVEDD, indicating compromised cardiac function after AMI, which can be remarkably improved by iPS cell transplantation (**Table 2**).

Effect of iPS cell transplantation on myocardial fibrosis of AMI rats

We further analyzed the expression of type I collagen 28 d after surgery by IHC staining. Results showed significantly increased type I collagen fiber after AMI model, as suggested by elevated positive rate for collagen fiber (P<0.05 compared to sham group). The transplantation of iPS cells into AMI rats significantly decreased the positive expression rate of type I collagen (P<0.05 compared to AMI group, **Figures 1** and **2**). Therefore, type I collagen fiber was up-regulated after AMI, and was suppressed by iPS cell transplant.

Effects of iPS cell transplantation on secreted type I collagen in rat serum

ELISA approach was employed to detect the secretion level of type I collagen fiber in rat serum samples. Results showed significantly elevated secretion of type I collagen in AMI model rat's serum samples (P<0.05 compared

to sham group). The transfection of iPS cells into AMI rats significantly decreased serum level of type I collagen fiber (P<0.05 compared to AMI group, **Figure 3**). These results collectively suggested the suppression of type I collagen fiber expression of iPS cell transplantation.

Effects of iPS cell transplantation on mRNA expression of Bcl-2 and Bax in myocardial tissues of AMI model rats

Real time PCr was used to detect the expressional change of Bcl-2 and Bax mRNA in myocardial tissues of AMI rats. Results showed decreased Bcl-2 and increased Bax mRNA levels in AMI rat cardiac tissues (P<0.05 compared to sham group). When iPS cells were transplanted into AMI rats, it was found that Bcl-2 mRNA was up-regulated while Bax mRNA was depressed (P<0.05 compared to AMI group, **Figure 4**).

Expressions of Bcl-2 and Bax proteins and effects of iPS cell transplantation

Western blotting was employed to describe the expressional profile of Bcl-2 and Bax proteins in myocardial tissues from all groups of rats. Results revealed similar patterns as those in mRNA: Bcl-2 protein was down-regulated while Bax protein was up-regulated in myocardial tissues of AMI rats, causing lower Bcl-2/Bax ratio (P<0.05 compared to sham group). When iPS cells were transplanted onto AMI rats, Bcl-2 protein was potentiated while Bax protein was suppressed, thus elevating Bcl-2/Bax ratio (P<0.05 compared to AMI group, **Figures 5** and **6**).

Discussion

Although multiple treatment approaches including drug, surgery and intervention exist for AMI, the clinical treatment efficacy is still unsatisfactory with high recurrent rate, poor prognosis, both of which cause shorter survival span of AMI patients and lower life quality, brining heavy burdens for the public health. Therefore
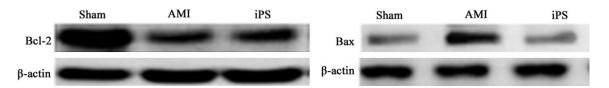
Figure 5. Effects of iPS cell transplantation on Bcl-2 (left) and Bax (right) protein expression in myocardial tissues of AMI rats.
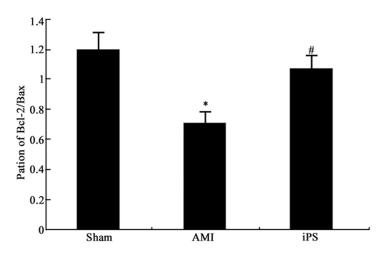

Figure 6. Bcl-2/Bax protein expression ratio in AMI model rats and after iPS cell transplantation. *, P<0.05 compared to sham group; #, P<0.05 compared to AMI group.

the establishment of effective stem cell treatment is of critical importance for improving AMI treatment efficacy [17].

Various stem cell therapy including ESC, bone marrow mesenchymal stem cell, cardiac stem cell, skeletal muscle stem cell, adipocyte originated stem cells, and bone marrow derived mononuclear cell have been employed to treat ischemia cardiac diseases [18, 19]. However, other cell types except ESC had inherent weakness such as difficulty of *in vitro* proliferation to meet the transplanting requirement, poor targeted differentiation *in vivo*, inability for long-term survival or differentiation after transplantation, or even the induction of malignant cardiac failure by the coupling of transplanted cells to host cells [20, 21]. ESC has unlimited proliferative potency and pluripotent differentiation abilities toward multiple tissues, both of which bring promising insights. The transplantation of ESC and derived myocardial cells can effectively integrate with existing tissues and improved cardiac function. Certain ethical issues and tumorigenic problems, however, existed for ESC transplantation [22].

iPS cells had similar morphology and growing features as those of ESCs. Both of them had pluripotent differentiated abilities. iPS can be generated from somatic cells of various species including human, pig and monkey, and from various cell types such as hepatocytes, dermal fibroblasts, gastric cells and neural progenitors [23]. The role of iPS in AMI and related myocardial fibrosis, however, has not been illustrated. This study established an AMI model in rat, on which mouse-derived iPS cells were transplanted. Our results confirmed that the intervention using iPS cells significantly improved cardiac function of Ami rats, suppressed myocardial fibrosis and decreased type I collagen fiber contents. Among cardiac collagen fiber, type I occupie more than 90%. It has important roles in formation and maintaining of ventricular wall tension due to its rigidity, lower elasticity and extension. It has been shown that increased level of type I collagen fiber elevated stiffness [24]. Bcl-2 and Bax have been widely accepted as important members regulating cell apoptosis. The over-expression of pro-apoptotic protein Bax plus suppression of anti-apoptotic protein Bcl-2 are closely correlated with the imbalance between apoptosis and anti-apoptosis in AMI pathology [25]. The transplantation of mouse-derived iPS cells to AMI rats revealed elevated mRNA and protein levels of Bcl-2, plus decreased Bax gene expression. Such increase of Bcl-2/Bax ratio suggested the involvement of iPS cell intervention in regulating apoptosis/anti-apoptosis homeostasis.

Conclusion

iPS cell can improve myocardial fibrosis via regulating apoptosis/anti-apoptosis homeostasis, protecting cardiomyocytes, and decreasing pro-

liferation of type I and type III collagen fiber. It thus can be used as one novel choice for treating AMI, and may decrease morbidity and mortality related with cardiac infarction.

Disclosure of conflict of interest

None.

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