Study of Adipose Tissue-Derived Mesenchymal Stem Cells Transplantation for Rats with Dilated Cardiomyopathy

Liang Li, MS, and Yunfeng Xia, MS

Background: Increasing evidences indicated that adipose-derived mesenchymal stem cells (ADMSCs) can stay survive, then gradually proliferate and differentiate into myocardial cells after transplanted into damaged areas and improve function of heart.

Methods: In this article, ADMSCs were isolated from adipose tissue of Wistar rats and cultured. When treated with 5-azacytidine (5-aza), ADMSCs were differentiated into myocardial cells, then we implant these cells into myocardium of rats of DCM to observe cell population and differentiation and compare cardiac function and hemodynamics changes before and after transplantation.

Results: The expression of Cardiac-specific markers indicated that ADMSCs which were isolated from adipose tissue of Wistar rats can differentiate into various cell types. Meanwhile, the treatment group displayed a higher level of LVESP, left ventricular intraventricular pressure (+dP/dt max), left ventricular intraventricular pressure (−dP/dt max) and left ventricular EF (%) than the control group. Altogether, these results indicate that heart systolic and diastolic function of rats of DCM was significantly improved meanwhile ventricular dilatation remodeling was inhibited after ADMSCs transplantation.

Conclusions: Therefore, this research provides an experimental basis for further clinical application of ADMSCs transplantation for the treatment of DCM and non-ischemic HF.

Keywords: adipose-derived mesenchymal stem cells, Wistar rats, dilated cardiomyopathy, rats of DCM
Adipose tissue has been recently identified as a convenient source of MSCs. Gaustad reported that adipose-derived mesenchymal stem cells (ADMSCs) can be easily induced from the predicted developmental lineage to differentiate into myocardial cell in response to myocardial-specific induction factors in vitro. ADMSCs are also easily obtained by liposuction in the patient’s abdomen, which cause little harm to the patient’s body and health. In addition, ADMSCs can be cultured in vitro for shorter periods by a regular population doubling way. Furthermore, no distinct differentiation is found between ADSCs and MSCs on multilineage differentiation potential and surface markers. Additionally, this technique does not provocate any immunological rejection and has no involvement with ethics disputes. Therefore, we can infer that ADMSCs are ideal source for stem cells transplantation. However, until now few researches are concentrating on the treatment of dilated cardiomyopathy (DCM) by ADMSCs transplantation. But it is obviously that the breakthroughs in this technique would greatly relieve patients’ suffer, cut down medical expenses and bring much social benefits. In this article, by isolating ADMSCs from the adipose tissue of Wistar rats and then these ADMSCs were cultured and transplanted into myocardial cells, our group surveys the therapeutic effect of ADMSCs transplantation on dilated cardiomyopathy with congestive heart disease and provides experimental basis for the treatment of dilated cardiomyopathy.

Materials and Methods

Materials
Wistar rats were purchased from the Animal Center of Chinese Academy of Military Medical Sciences; Injection of doxorubicin hydrochloride and staining agent were purchased from Beijing Zhongshan Golden Bridge Biotechnology Company, Beijing, China. IMDM medium, type I collagenase and trypsin were purchased from Gibco; fetal bovine serum was purchased from Hyclone; penicillin and streptomycin, goat anti-rabbit horseradish peroxidase labeled secondary antibody and rabbit anti-monomonuclear cells of human leukocyte antigen DR was purchased from Sigma; peroxide of hydrogen solution and hematoxylin dyes were purchased from Beijing Chemical Factory, Beijing, China; rabbit anti-CD13, CD34 and CD44, CD105, CD45 and VWF polyclonal antibody, rabbit anti-cardiac troponin T antibody (cTnT) and rabbit anti-α-actin polyclonal antibody was purchased from Biomart Biological Engineering Company, Beijing, China; rabbit anti-viii polyclonal antibody was purchased from Beijing Boaosen Biotechnology Co., Ltd., Beijing, China.

ADMSCs isolation and culture
Adipose-derived mesenchymal stem cells (ADMSCs) were isolated from a single male Wister rat, then they were cultured and expanded for transplantation in all the experimental animals. Briefly, a male Wister rat (weighting 150 g) was injected intraperitoneally with 2% napental (2–3 mg/kg) under aseptic conditions. Liposaprate of the epididymis adipose tissue was performed. The adipose tissue added PBS (200 μ/ml penicillin and 200 μ/ml streptomycin) was cut into pieces and incubated in 3 ml of 0.25% trypsin for 30 min with sterile sealing. Then the tissue was incubated on a shaker at 37°C for 10 min. After removing the under-layer liquid, the tissue is incubated on a shaker at 140 rpm for 40–80 min at 37°C. Next, remove the upper-layer emulsified adipose cells, and the lower suspension was filtered through a 200-μm cell strainer. Cell pellets obtained after filtration was centrifuged in 10 ml centrifugal tube at 1500 rpm for 10 min and washed three times with IMEM containing FCS. Cells were cultured in culture flasks in 1 × 10⁵ and culture medium was changed every 2–3 days. Observe cells with inverted microscope.

Immnohistochemical staining
The ADMSCs were cultured with 10⁴/cm² in 24-well plates. Abandon the medium after 24 hours. After washing three times, the cells were fixed with 4% paraformaldehyde for 60 minutes. Endogenous peroxidase activity was blocked with 0.3% H₂O₂ (30 min). Then, cells were washed in PBS three times. Nonspecific binding was blocked by incubating cells with 10% normal goat serum for 20 minutes, cells were incubated overnight (4°C) with antibody (1:200 diluted). After washing 3 times with PBS, goat anti-rabbit secondary antibody was added, and then cells were incubated for 30 min (37°C). The immune reactivity was visualized by the Liquid DAB-Black Substrate Kit (15–30 min). After washing with H₂O, the specimens were mounted with resin, observed and taken pictures.

ADMSCs transplantation
Before transplantation, the cells were labeled with 1,1’-dioctadecyl-3,3,3’,3’-testramethylindocarbocyanine perchlorate (DiI) according to the previous report.10)
Prepare 3 to 6 generations ADMSCs 100 μl of cell suspension at density of 2.0 × 10^7/ml, place them in refrigerator at 4°C. The 50 DCM rats were equally divided into two groups after fed for 6 weeks. Successively intraperitoneal anaesthetize them with 1% amyl barbital sodium (in accordance with dose of 0.15 ml/100 g) Connect them to a small animal ventilator (respiratory rate of 30 times/min, tidal volume of 20 ml/s) with trachea cannula. Make longitudinal incision between the 3rd, 4th intercostal space about 0.5 cm away from the left side of sternum. Open the pericardium and gently fixed heart with a cotton swab. Transplanted cells were injected into the myocardium of treated rats by specially designed syringe at three points, which were near left ventricular apical, near the mitral valve of left ventricular free wall and the central of left ventricular free wall. Each point injected 100 μl (2.0 × 10^6 cells). The control group was injected with the same amount IMEM without serum medium.

Hematoxylin-eosin staining
Hematoxylin-eosin staining was performed according to standard histological procedures on myocardial tissue paraffin sections. Observe the inflammatory response of myocardial tissue.

Echocardiographic detection
The left ventricular end systolic volume (LVESV), left ventricular end-diastolic volume (LVEDV) and left ventricular ejection fraction (LVEF) of anesthetized rats with 30 mg/kg pentobarbital were measured with single-blind line echocardiography (the probe frequency 1.7–3.4 MHz, the scanning speed 100 mm/s two-dimensional ultrasound imaging by conventional operation) before the cell transplantation, 1 week and 4 weeks after transplantation.

Hemodynamics
The rats were anesthetized with 30 mg/kg pentobarbital after 4 weeks of transplantation. Fixed its limbs and connected them to RM6240 physiological experimental system. Make neck midline incision in order to separate the left common carotid artery. And then record left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP) and left ventricular pressure rise and the rate of decline (±dP/dt max) with multi-channel physiologic recorder after intravenous injection of heparin (2 mg/ml). We selected three areas for each curve to measure and average as the final measurement result.

Statistical analysis
Data are expressed as means ± SEM (± s) which used single factor analysis of variance was used to compare each observation point in the group. p <0.05 indicated a significant difference, p <0.01 indicated that there was a very significant difference. All the data used SPSS12.0 software for statistic.
Results

ADMSCs surface markers identification

We initially identify the cells that isolated from lipoaspirates of Wistar rats and then cultured in vitro. The cells from third to eighth generation express surface markers that represent characteristic of MSCs are CD13, CD44 and CD105. In our results, there was a strong expression of CD13 on the cell surface, meanwhile CD45, HLA-DR, VIII, VWF were not expressed (Fig. 1). However, when the isolated cells were recovered, they expressed surface markers CD13, CD44 and CD55, strongly suggesting that those cell that we isolated from lipoaspirates of Wistar rats and cultured in vitro were ATSCs.

Multilineage differentiation capacity of ADMSCs

Because the multilineage differentiation capacity of ADMSCs is the fundamental premise for this research, so identification of ADMSCs’ multilineage differentiation capacity is conducted. Cells were cultured in medium with lineage-specific induction factors for 2 weeks. Then, they were stained by α-actin (Fig. 2A), rathonum red (Fig. 2B) and kaline phosphatase (Fig. 2C). The observation results were all positive, the results show that the ADMSCs we obtain possess the multilineage differentiation capacity.

Identification of rats modeling of DCM

To establish the rats model of DCM, we randomly select five living rats and apply doxorubicin induction to them. 2 weeks later, these rats were killed and analyzed. The pathological examination of heart anatomy reported that rat hearts were spherical, the wall is thin, and the heart chambers were large. Hematoxylin-eosin staining (HE) revealed that Myocardial tissue were damaged, and there were cardiomyopathy-like changes. Part of myocardial cells displayed edema and vacuolar degeneration. Steatosis and small focal necrosis were also observed in myocardial cells. In addition, muscle source of fiber was dissolute, Myocardial fiber fractured with Myocardial cell gap enlarging, and there was Neutrophils and monocytes appeared, more than that, lymphocyte infiltration and Telangiectasia increased in mesenchyme (Fig. 3A). After 3 weeks of modeling, these rats had a higher level of LVEDV and LVESV and displayed a lower level of EF in left ventricle and other reduced cardiac function phenotype (Table 1 and Fig. 3B–3E). Altogether, these results suggested that the rat model of DCM was successfully established.

ADMSCs survival and differentiation detection after transplantation

We determined whether ADMSCs were still survival and had cardiac-specific differentiation after ADMSCs transplantation 4 weeks later. The myocardial slices from treatment group were stained with antibody against cTnT (Fig. 4A), then they were observed under fluorescent microscope. As shown in Fig. 4B, DiI labeled cells were detected in myocardial slice, indicating the engraftment and survival of transplanted cells in myocardium;
Fig. 3  Dilated cardiomyopathy (DCM) rats model identification. 80 healthy male Wistar rats randomly selected were assigned and recorded their weight (200 ± 20) g. The rats were intra-peritoneally injected with doxorubicin hydrochloride (use water for injection 2 mg/ml, 1.25 ml/kg bw) three times a week, with a total of 3 weeks. After that, identify DCM rat model. (A) The DCM rat myocardial biopsy (HE × 100); (B) Rats LVESV, LVEDV and left ventricular stroke volume comparisons before and after modeling; (C) Heart EF before and after the modeling; (D) Left ventricular systolic pressure before and after the modeling; (E) Rat left ventricular pressure rise and fall speed comparison before and after the modeling. LVESV: left ventricular end systolic volume; LVEDV: left ventricular end-diastolic volume; LVP: left ventricular pulse.

The nuclear was stained with DAPI (Blue, Fig. 4C); when the green (cTnT +), red (DiI +) and blue (DAPI) fluorescence from the same field were merged, the co-localization of cTnT expression (Green) and DiI (Red) was observed (Yellow), indicating that transplanted cells have differentiated toward cardiomyocytes and expressed cTnT.
Fig. 4  Survival and differentiation of transplanted cells in heart. (A) myocardial section was immunostained with antibody against cTnT; (B) Dil labeled cells were detected 4 weeks after myocardial transplantation, indicating engraftment and survival of transplanted cells in myocardium; (C) the nuclear was stained with DAPI; (D) the co-localization of cTnT expression (Green) and Dil (Red) was observed (Yellow), indicating that transplanted cells have differentiated toward cardiomyocytes and expressed cTnT (Bar = 25 μm).

Fig. 5  Wistar rats echocardiography. (A) control rats echocardiography; (B) treated rats echocardiography.
Enhanced Therapy of MSC for Dilated Cardiomyopathy

We continue to detect the biological effects of ADMSCs transplantation on heart function of Wistar rats. After 4 weeks of ADMSCs transplantation, the levels of LVEDV, LVESV, left ventricular systolic pressure in treatment groups were significantly lower than control groups, while left ventricular EF, LVESP, left ventricular end-systolic volume and systolic pressure were significantly higher than control groups.

**Table 1** Cardiac function 4 weeks after cell transplantation ($\bar{x} \pm s$)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (n = 18)</th>
<th>ADMSCs transplantation (n = 17)</th>
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<tbody>
<tr>
<td>LVESV (ml)</td>
<td>1.175 ± 0.251</td>
<td>0.910 ± 0.228$^b$</td>
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<tr>
<td>LVEDV (ml)</td>
<td>1.700 ± 0.264</td>
<td>1.455 ± 0.197$^b$</td>
</tr>
<tr>
<td>LVP (ml)</td>
<td>0.799 ± 0.164</td>
<td>0.920 ± 0.220$^b$</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>41.78 ± 12.69</td>
<td>55.51 ± 9.56$^b$</td>
</tr>
<tr>
<td>LVSP (mmHg)</td>
<td>122.36 ± 11.63</td>
<td>131.26 ± 9.75$^a$</td>
</tr>
<tr>
<td>$+dP/dt$ max (mmHg/s)</td>
<td>3647.7 ± 267.5</td>
<td>3996.0 ± 378.2$^b$</td>
</tr>
<tr>
<td>$-dP/dt$ max (mmHg/s)</td>
<td>2712.5 ± 209.8</td>
<td>2989.7 ± 283.6$^b$</td>
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ADMSCs: adipose-derived mesenchymal stem cells; LVESV: left ventricular end systolic volume; LVEDV: left ventricular end-diastolic volume; LVP: left ventricular pulse; LVEF: left ventricular ejection fraction; LVSP: left ventricular systolic pressure; $+dP/dt$ max: maximum rising speed of left ventricle; $-dP/dt$ max: maximum lowering speed of left ventricle.

a: p <0.01; b: p <0.05 compared with control.

**Fig. 6** Identification of efficacy of ADMSCs transplantation for DCM rats. (A) The comparison of two groups in LVESV, LVEDV and left ventricular stroke volume when transplant for 4 weeks; (B) Two groups of rats EF value; (C) left ventricular systolic pressure of two groups (D) Left ventricular pressure rise and fall speed comparison of two groups after 4 weeks of transplantation. ADMSCs: adipose-derived mesenchymal stem cells; DCM: dilated cardiomyopathy; LVESV: left ventricular end systolic volume; LVEDV: left ventricular end-diastolic volume; LVP: left ventricular pulse.
ventricular pressure the rate of increase (+dP/dt max) and left ventricular pressure maximal rate of decline (–dP/dt max) of treatment groups were significantly increased, which indicate that ADMSCs transplantation could significantly improve the heart function of rats (Figs. 5 and 6, Table 1).

**Discussion**

DCM is one of most common cardiomyopathy with the occurrence rates high up to 90% and mortality rates reaching 45%. In past decades heart transplantation has been accepted and achieved great clinical success. However, its clinical application still progress slowly because of the deficiency of donators. Therefore, the discovery of a new and alternative treatment method is of great importance and urgency. More and more evidence indicates that stem cell transplantation can increase myocardial cells, and ADMSCs can be induced to differentiate into myocardial cells and cultured in vitro with a regular doubling population. In this study, mesenchyme stem cells were isolated from adipose tissue and Induced by 10 μmol/15-aza for 24 h, the properties of myocardial cells were identified with α-actin, cTnT markers on myocardial cells. And animal models of DCM were described below:

1. Doxorubicin induction. Either intravenous or intraperitoneal injection or coronary artery local injection can be adopted; 2. Overdrive-pacing; 3. Viral infection; 4. Furazolidone induction; 5. High salt diet in order to raise blood pressure, cardiac preload and afterload, which contribute to heart enlargement and heart failure. In this article, rats models of chronic congestive heart failure were established by intraperitoneal injection, and it was identified that rats possess cardiomyopathy and chronic congestive heart failure properties through the index of pathological and hemodynamic detection. In addition, the pathological, hemodynamic and hormone changes of DCM rats induced by Doxorubicin are similar to those reported in human. Therefore, rats models for chronic congestive heart failure induced by doxorubicin developed in this experiment was proved critically important and offer a simple, accessible and reliable modeling approach for the study of DMC pathogenesis mechanism and the advancement of clinical therapy.

In our results, the LVESP, internal pressure of left ventricular (+dP/dt max) and right ventricular (–dP/dt max), and left ventricular EF of treatment groups after 4-week cell transplantation were higher than those of control group, however, the LVEDP, LVEDV and LVESV were evidently lower. These changes hint us that implanting ADMSCs into myocardium of DCM rats can prevent DCM ventricular dilatation remodeling and effectively improve Cardiac Systolic and Diastolic function. In addition, we also prove that ADMSCs transplanted into myocardium can differentiate into myocardial cells and vascular endothelial cells even without 5-aza induction. Above all, stem cell transplantation can effectively improve Cardiac Systolic and Diastolic function of part or whole heart, furthermore, no malignant ventricular arrhythmias, infection and Immune rejection have been found after transplantation. There were also no osteoblasts, tumor cells and vascular tumor-like change reported in histological examination. These results demonstrate that ADMSCs transplantation is an effective and safety method of treatment for congestive heart failure. However, the molecular mechanisms that how transplanted cells can improve cardiac function remain largely unknown and a further investigation is still needed.

**Disclosure Statement**

No conflict of interest exists.

**References**

7) Campard D, Lysy PA, Najimi M, et al. Native umbilical cord matrix stem cells express hepatic markers and