Comparison of early and delayed transplantation of adipose tissue-derived mesenchymal stem cells on pulmonary arterial function in monocrotaline-induced pulmonary arterial hypertensive rats

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The aim of this article was to investigate the effects of early and delayed transplantation of adipose tissue-derived mesenchymal stem cells (ADMSCs) on pulmonary vasomotion in monocrotaline (MCT)-induced pulmonary arterial hypertensive (PAH) rats. Adipose tissue-derived mesenchymal stem cells were isolated from inguinal subcutaneous adipose tissue of Sprague-Dawley (SD) rats. Eighty male SD rats were divided into four groups (n = 20 per group): normal control (Ctr), PAH, early (at day 7, ADMSCs-7), and delayed (at day 14, ADMSCs-14) ADMSC intervention. PAH was induced by a single dose intraperitoneal injection of 40 mg/kg MCT. About 1 × 10⁶ ADMSCs were delivered via left external jugular vein. Mean pulmonary arterial pressure (MPAP) was measured by catheterization at days 21 and 28 after MCT injection. Pulmonary arteriolar endothelium-dependent relaxation (EDdR), endothelium-independent relaxation (EDiR), and vasoconstriction function were evaluated by the physiological vascular ring tension recording system. Mean pulmonary arterial pressure (mmHg) was increased on days 21 (26.88 ± 0.86 vs. 14.88 ± 0.98, P < 0.05) and 28 (32.22 ± 0.71 vs. 15.16 ± 0.95, P < 0.05) after MCT administration; EDdR and EDiR were lower in PAH rats at the 21st day (pD2:EDdR: 4.85 ± 0.29 vs. 8.12 ± 0.48 and EDiR: 6.28 ± 0.38 vs. 8.64 ± 0.35; all P < 0.05) and 28th day (pD2:EDdR: 3.22 ± 0.61 vs. 7.93 ± 0.5 and pD2:EDiR: 5.4 ± 0.55 vs. 8.58 ± 0.41; all P < 0.05). Mean pulmonary arterial pressure in ADMSCs-7 was decreased on the 21st day (16.37 ± 0.94 vs. 26.88 ± 0.86 mmHg, P < 0.05) and 28th day (18.26 ± 1.41 vs. 32.22 ± 0.71 mmHg, P < 0.05). Similarly, MPAP in ADMSCs-14 was also lowered on days 21 and 28. EDdR in ADMSCs-7 was increased on day 21 (pD2: 7.48 ± 0.31 vs. 4.85 ± 0.29, P < 0.05) and day 28 (6.29 ± 0.52 vs. 3.22 ± 0.61, P < 0.05). EDdR in ADMSCs-14 was also increased on day 21 (pD2: 8.08 ± 0.37 vs. 4.85 ± 0.29, P < 0.05) and day 28 (6.21 ± 0.67 vs. 3.22 ± 0.61, P < 0.05). EDdR in both ADMSCs-7 and ADMSCs-14 was increased on days 21 and 28. There was no significant difference in EDdR and EDiR between ADMSCs-EI and ADMSCs-DI. The impairment of EDdR and EDiR in PA was ameliorated, and PAH was reduced by both early and delayed transplantation of ADMSCs.
Introduction

Pulmonary arterial hypertension (PAH) is characterized by an increase in pulmonary arterial pressure, resulting in right ventricular failure and leading to death ultimately. Studies have shown that stem cell transplantation could reduce pulmonary arterial pressure and improve pulmonary vascular endothelial function and remodelling of the pulmonary arteries. The advantages of adipose tissue-derived mesenchymal stem cells (ADMSCs) include abundant autologous supply sources, higher proliferative activity, less immune rejection, potential multidirectional differentiation, and so on.

Our previous studies found that pulmonary arterial structural remodelling at day 14 after a single dose of 40 mg/kg monocrotaline (MCT) intraperitoneal (i.p.) injection was prior to the increase of pulmonary arterial pressure. Meanwhile, our recent studies suggested that ADMSC transplantation may ameliorate the remodelling of the pulmonary artery in MCT-treated rats. The transplanted ADMSCs could survive and colonize on the pulmonary arteries and differentiate into vascular endothelial-like cells in vivo in PAH rats.

On the contrary, the effect of ADMSCs on the pulmonary vasomotion in MCT-treated rats and the optimal timing for ADMSC transplantation has not been determined. As a result, in this study, we administered ADMSCs before ('early' intervention on day 7 after MCT, ADMSCs-7) and after ('delayed' intervention on day 14 after MCT, ADMSCs-14) the onset of pulmonary arterial remodelling in order to investigate the efficacy and a proper 'therapeutic window' of ADMSC transplantation on the development of PAH and pulmonary arterial function.

Materials and methods

Experimental animals

Seven-week-old male Sprague-Dawley (SD) rats, weighed between 200 and 230 g, were purchased from Slaccas Inc. (Shanghai, China; Certificate No. SCXK 2007-0005). The housing room was kept at a 12 h light/dark cycle (07:00–19:00), and its temperature remained constant at 22 ± 2°C and humidity at 55 ± 5%. Rats were freely fed with standard rat chow and had access to food and water ad libitum. According to the guidelines of the Fujian Medical University, all procedures were performed and approved by the Institution Ethics Committee of First Affiliated Hospital of Fujian Medical University.

Experimental materials

The materials included Dulbecco’s modified Eagle's medium (DMEM)-F12 medium (Gibco Co., USA); trypsin (Gibco Co.); type I collagenase (Gibco Co.); bovine serum albumin (BSA) (Bovogen, Australia); foetal bovine serum (FBS) (Gibco Co.); fluorescein isothiocyanate (FITC)-tagged anti-rat CD45 (BioLegend Co., USA); FITC-labelled anti-mouse/rat CD29 (BioLegend Co.); PE/Cy7 anti-rat CD90 (BioLegend Co.); anti-CD34 (mouse anti-rat, Santa Cruz Co., USA); anti-CD31 (mouse anti-rat, Abcam Co., USA); FITC-labelled goat anti-mouse IgG secondary antibody (Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd); recombinant adenovirus vector Ad5-enhanced green fluorescent protein (EGFP) (Beijing Zhengyang Gene Co., Ltd); monocrotaline (Sigma Co.); acetylcholine (Ach) (Sigma Co.); NG-nitro-L-arginine methyl ester (LNAM) (Sigma Co.); sodium nitroprusside (SNP) (Yuanda Pharmaceutical Co., Ltd, China); and norepinephrine (NE) (Beijing Shuanghe Pharmaceutical Co., China).

Adipose tissue-derived mesenchymal stem cells culture and immunophenotype analysis

Adipose tissue-derived mesenchymal stem cells were isolated from the inguinal subcutaneous adipose tissue by enzymatic digestion and cultured, as described previously. Briefly, the adipose tissues were minced and incubated with type I collagenase prepared in phosphate-buffered saline (PBS) containing 1% BSA in water bath (37°C for 60 min) with intermittent shaking and pipetting to digest the tissue. Then, the digestion was terminated by adding equivalent volume of DMEM-F12 containing 15% FBS. The tissue debris was filtered with 80-mesh screen. After centrifugation (152 g) for 10 min, the mature adipose cells and the cell debris on the upper layer were discarded and the cells pellets of the stromal vascular fraction (SVF) were then cultured. Medium was changed 3 h later and cells were cultured, while maintained in a humidified incubator (37°C and 5% CO2), and medium was changed every 3 days. When attached cells were near confluent (defined as passage 0), they were subcultured.

In order to determine cell phenotype, the third passage of cultured cells was harvested and analysed for CD31, CD29, CD34, and CD90 by flow cytometry (BD FACSCalibur, USA). Briefly, 1 × 106 cells were suspended and washed in PBS. After centrifugation, the cells were incubated with primary antibodies against rat CD31 and CD34 and monoclonal antibodies conjugated with FITC or PE/Cy7 against rat CD29, CD45, and CD90 for 30 min at 4°C. The second polyclonal antibody conjugated with FITC against mouse IgG was added and incubated at 4°C for an additional 30 min in a dark room. The same isotype-irrelevant antibody of the same species was used as negative control. After washing, cells were resuspended in PBS for flow cytometry analysis.

Monocrotaline induced pulmonary arterial hypertensive model and adipose tissue-derived mesenchymal stem cells transplantation

The PAH model was established by a single dose of 40 mg/kg, as used in previous studies. For cell implantation, rats were anaesthetized, and 1 mL of ADMSCs containing 1 × 10⁶ cells was intravenously injected via rat’s left external jugular vein. Eighty male SD rats were randomly assigned into four groups (n = 20 in each group) as follows: PAH, rats received an i.p. injection of 40 mg/kg of MCT; normal control (Ctr), rats received an i.p. injection of same volume of saline; early intervention of ADMSCs (ADMSCs-7), animals received ADMSC implantation 7 days after MCT injection; and delayed intervention of ADMSCs (ADMSCs-14), animals...
received ADMSC implantation 14 days after MCT injection. The time schedule of ADMSC implantation was shown in Figure 1.

On days 21 and 28 after MCT administration, mean pulmonary arterial pressure (MPAP) was measured by catheterization; and pulmonary arterial endothelium-dependent relaxation (EDdR), endothelium-independent relaxation (EDiR), and vasoconstrictive function (VCF) were also evaluated by the physiological vascular ring tension recording system.

**Measurement of mean pulmonary arterial pressure**

As described previously,7,11 the rats were anaesthetized. Linking to a pressure transducer (YPJ01; Instruments Factory, Chengdu, Sichuan, China), a right heart catheter (Department of Physiology, Peking Union Medical College, Tsinghua University) was inserted via the right external jugular vein into the main pulmonary artery to obtain the typical pulmonary arterial pressure wave pattern. Mean pulmonary arterial pressure was recorded by a computerized data acquisition system (Powerlab-ML221; AD Instruments Pty Ltd, Australia).

**Isolation and preparation of pulmonary arterial ring**

After the measurement of MPAP, all rats were euthanized by overdosing chloral hydrate. The chest was opened, and the lungs were excised and placed in oxygenated (95% O2/5% CO2) Krebs–Henseleit solution containing (in mmol/L): NaCl 118.3, KCl 4.7, CaCl2 2.52, KH2PO4 1.20, MgSO4 1.20, NaHCO3 24.2, and glucose 11.0 (pH 7.40). The seventh branch of the intralobar pulmonary artery (internal diameter, ≈ 200 μm) was carefully dissected to prevent injury of the endothelium and clean off connective tissue using small ophthalmic scissors, under a dissecting microscope, and then was cut into ring segments of 3 mm in length. The individual ring was mounted between two triangular tungsten wires (50 μm in diameter), of which one triangle was mounted to a stable hook, whereas the other one was attached to a tension transducer on a four-chamber in vitro tissue perfusion system (AD Instruments Pty Ltd) and suspended in a 10 mL organ bath filled with Krebs’ solution continuously bubbled with 95% O2 and 5% CO2 at 37 °C (pH 7.4). The changes in isometric force were recorded on a multichannel physiological recorder (Powerlab-ML221). The preparations were allowed to equilibrate under a resting tension of 0.5 g applied to the pulmonary arterial rings for up to 60 min, during which the bath fluid was replaced every 15 min with fresh solution.

**Tension measurements of isolated pulmonary arterial ring**

After the period of equilibrium, KCl (60 mM) was added to the bath to test tissue viability and then washed with Krebs’ solution. This contraction in response to 60 mM KCl was repeated once after 30 min. The tissues were then washed repeatedly over the next 30 min period, until identical contractions were obtained. Thereafter, the baths were washed repeatedly with Krebs’ solution until the preparations reached the balance, similar to the initial baseline tension. Then, pulmonary arterial rings were stimulated by NE in a concentration ranging from 10⁻¹⁰ to 10⁻⁵ mol/L. The cumulative concentration–response curves were constructed and VCF was measured. When the maximum response was reached, the washed preparations were allowed several times and allowed to recover for 60 min before establishing a new concentration–response curve.

EDdR in response to Ach was measured on the basis of pre-contraction caused by 10⁻⁵ mol/L NE. After a 60 min washout period, the rings were pre-contracted again with 10⁻⁵ mol/L NE. Then EDIR to SNP was examined in the presence of L-NAME (10⁻⁴ mol/L, given 30 min in advance). Different concentrations of ACh and SNP ranging from 10⁻¹⁰ to 10⁻⁴ mol/L were applied into organ bath in a cumulative manner. These concentration–response curves were further analysed by commercial computer software (Chart 5; AD Instruments), and the potency of vascular contraction and relaxation was expressed as pD2, which is the negative logarithm of the effective concentration producing 50% of the maximum response.

**Figure 1 Time schedule of ADMSC implantation.**

**Figure 2** Morphological characteristics and fluorescent marker of cultured rat ADMSCs (×100). (A) Phase-contrast microscopic images showed ADMSCs at growth phase. (B) EGFP-labelled ADMSCs were detected under a fluorescence microscope in the same field as (A). The transduction efficiency at MOI 100 was >90%.

**Detection of adipose tissue-derived mesenchymal stem cell distribution in pulmonary tissue of rats**

To investigate whether the infused ADMSCs were localized in pulmonary tissue of rats or not, the middle lobe of the left lung of rats from ADMSCs-EI and ADMSCs-DI groups was taken for frozen...
section on days 21 and 28, and the distribution of EGFP-labelled ADMSCs was detected under a fluorescence microscope.

**Statistical analysis**

Data were expressed as means ± SD. Analysis of variance was used to test the difference between groups. The software spss13.0 was used for statistical analysis. $P < 0.05$ indicates statistically significant difference.

**Results**

**Morphological characterization of adipose tissue-derived mesenchymal stem cells**

The cells appeared as small polygonal or short fusiform with large oval-shaped nucleus in primary culture and tightly attached to dishes and proliferated rapidly. Subcultured for 3 days, the cells showed a homogeneous fibroblast-like
morphology and were spindle-shaped (Figure 2A). Cells at 70–80% confluences were used for all experiments. The surface markers of the third passage ADMSCs were identified with flow cytometry, as reported previously. Higher expression of mesenchymal stem cell–related surface markers, CD29 and CD90 (86.6 and 97.9%, respectively), and lower expression of CD31 (0.9%), CD34 (37.9%), and CD45 (6.2%), representative of the surface markers of hematopoietic and endothelial cells, were detected.

Adipose tissue-derived mesenchymal stem cells labelled with enhanced green fluorescent protein

The green fluorescence was observed under a fluorescence microscope after ADMSCs being transfected with Ad5-EGFP at 100 MOI for 72 h with transfection efficiency >90% (Figure 2B).

Distribution of adipose tissue-derived mesenchymal stem cells in the lung tissue of rats

The green fluorescence was detected in frozen sections of lung tissue after ADMSC transplantation under a fluorescence microscope. On days 21 and 28, EGFP-labelled green fluorescence-positive cells surrounding and engrafting at the blood vessel walls of lung were observed in rats from ADMSCs-EI and ADMSCs-DI groups (Figure 3). These results suggested that the transplanted ADMSCs should migrate and colonize in the lung tissue of rats.

Effect of early or delayed adipose tissue-derived mesenchymal stem cell transplantation on mean pulmonary arterial pressure in pulmonary hypertensive rats

Two rats in the PAH group died because of right heart failure before the end of the experiment and were excluded. As shown in Table 1, on days 21 and 28 after MCT administration, MPAP elevated in PAH rats when compared with Ctr rats ($P < 0.05$), whereas MPAP was lower either in ADMSCs-7 or in ADMSCs-14 rats compared with PAH rats ($P < 0.05$). There was no difference in MPAP between ADMSCs-EI and ADMSCs-DI groups ($P > 0.05$).

**Table 1** Effect of early or delayed ADMSC transplantation on MPAP in pulmonary hypertensive rats ($\bar{x} \pm s$, mmHg)

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctr</td>
<td>14.88 ± 0.98 (n = 10)</td>
<td>15.16 ± 0.95 (n = 10)</td>
</tr>
<tr>
<td>PAH</td>
<td>26.88 ± 0.86a (n = 10)</td>
<td>32.22 ± 0.71a (n = 8)</td>
</tr>
<tr>
<td>ADMSCs-7</td>
<td>16.37 ± 0.94b (n = 10)</td>
<td>18.26 ± 1.41b (n = 10)</td>
</tr>
<tr>
<td>ADMSCs-14</td>
<td>17.74 ± 1.07c,d (n = 10)</td>
<td>21.87 ± 0.78c,d (n = 10)</td>
</tr>
</tbody>
</table>

*$P < 0.05$ vs. Ctr of the corresponding age of rats.
*$P < 0.05$ vs. PAH.
*$P < 0.05$ vs. PAH.
*$P > 0.05$ vs. ADMSCs-EI.

Discussion

In this study, it was demonstrated that MCT induced an increase in MPAP and impairment in pulmonary arterial EDdR and EDiR in a time-dependent manner, whereas no obvious changes in VCF in rats were seen. On the one hand, the impairment of EDdR and EDiR of pulmonary arteries was ameliorated; on the other hand, the increased MPAP in MCT-induced PAH rats was attenuated in both early and delayed transplantation of ADMSCs. There was no difference in reducing MPAP and improving pulmonary arterial EDdR and EDiR between early and delayed treatment.

Similarly, several groups\textsuperscript{[11–13]} reported that there was a decrease in pulmonary arterial reactivity in response to Ach in MCT-induced PAH rats and that the impairment in pulmonary arterial EDdR and EDiR was improved in rats treated with ADMSC transplantation.
Figure 4  Effect of early or delayed ADMSC transplantation on pulmonary arterial EDdR in pulmonary hypertensive rats at days 21 and 28 after MCT administration. (A1 and A2): the pD2 value of EDdR and (B1 and B2): the % relaxation of EDdR. *P < 0.05 vs. Ctr; **P < 0.05 vs. PAH; ***P > 0.05 vs. ADMSCs-7.

Figure 5  Effect of early or delayed ADMSC transplantation on pulmonary arterial EDIR in pulmonary hypertensive rats at days 21 and 28 after MCT administration. (A1 and A2): the pD2 value of EDIR and (B1 and B2): the % relaxation of EDIR. *P < 0.05 vs. Ctr; **P < 0.05 vs. PAH; ***P > 0.05 vs. ADMSCs-7.
endothelium function resulted in decreased NO synthesis and secretion and reduced endothelium-dependent vasodilatation capacity. Maruyama and Maruyama\textsuperscript{14} demonstrated that there was a decreased vasodilatation of pulmonary arterial ring in response to SNP and that the downstream effect of NO-mediated vasodilatation contributed to the impairment in pulmonary vasodilatation function in MCT-induced PAH rats. Different from our results, Khan \textit{et al.}\textsuperscript{15} found that there was a weakened vasoconstriction reactivity in response to NE in the extralobar pulmonary artery ring after 3 weeks of treatment with MCT, in a PAH rat model induced by an i.p. injection of MCT 60 mg/kg, and this blunted response could be related to the Rho kinase activation coupling barrier. However, our results did not show obvious change in vascular contraction function. Maybe, different parts of pulmonary arteries used would contribute to different results.

In this study, it was showed that ADMSCs labelled with GFP may survive and incorporate into the vascular wall of pulmonary tissue of MCT-induced PAH rats from 14 to 21 days after early transplantation or from 7 to 14 days after delayed transplantation. The early or delayed intervention of ADMSCs may reduce impaired pulmonary arterial EDdR and EDIR and effectively attenuate PAH induced by MCT in rats. The ADMSCs showed a strong capacity of self-multiplication. It may be of potential to differentiate into many kinds of differentiated cell lines\textsuperscript{5,6} under specific conditions. Meanwhile, it was reported that the ADMSCs play physiological roles via paracrine-producing vascular endothelial growth factor (VEGF), hepatocyte growth factor, and other factors related to angiogenesis and neovascularization, resulting in the repairment of injured endothelium.\textsuperscript{16,17} Recently, it had been reported by our group\textsuperscript{18,19} that the transplantation of adipose SVF resulted in the improvement of cardiac function and myocardial remodelling in the rat models of heart failure, partially through the promotion of myocardial tissue neovascularization. Furthermore, our previous research\textsuperscript{10} demonstrated that the reduction of pulmonary arterial pressure by ADMSC transplantation in MCT-induced pulmonary hypertensive rats may be related to the changes of expression of calcium channels, including an increased expression of Cav\textsubscript{a}1c, TRPC1, and TRPC6 at mRNA and protein levels and a decreased mRNA and protein level of SERCA-2a and IP3R-1.

In addition, our previous study also found that the transplantation of ADMSCs at day 14 after MCT administration when pulmonary arterial remodelling occurred only partially ameliorated MCT-induced pulmonary arterial remodelling in rats.\textsuperscript{8} Interestingly, some investigators also reported that the benefits brought by stem cell transplantation may be reduced if the treatment was given at later stages of PAH. Zhao \textit{et al.}\textsuperscript{21} transplanted bone marrow-derived endothelial-like progenitor cells (ELPCs) to syngeneic rats 3 days after MCT injection and found that the increase in right ventricular pressure was successfully prevented after 3 weeks compared with that of MCT-alone injection rats. However, delayed transplantation of ELPCs 3 weeks after MCT injection only partly prevented the further progression of PAH over the subsequent 2-week interval. In other study, Campbell \textit{et al.}\textsuperscript{22} tested the effects of transplanting PASMCs transfected with the gene encoding
VEGF-A to rats simultaneously with MCT injection. After 4 weeks, the PAH, right ventricular hypertrophy, and medial hypertrophy of pulmonary arterioles in the VEGF-treated rats were obviously reduced, compared with control rats. Even when the transplantation of PASMCs with expression of VEGF was delayed till the occurrence of PAH, a significant decrease in PAH and right ventricular hypertrophy was also seen. The works of Mathew et al. demonstrated that pulmonary arterial remodeling existed at day 14 after MCT administration. The endothelial injury was slight soon after MCT injection. Therefore, transplanted ADMSCs may play a role earlier to prevent the further progression of PAH. In this study, the 7th day and the 14th day after MCT injection served as cut-off point for early and delayed intervention of ADMSCs in order to investigate the effect of timing in ADMSC treatment of PAH by examining two different durations corresponding to different stages and severity of disease. It was shown that there were no statistical differences in rats’ MPAP and pulmonary arterial EDdR and EDiR between early and delayed intervention of ADMSCs. Based on our previous study, it was suggested that MPAP was markedly increased, whereas EDdR and EDiR were obviously decreased at weeks 2 and 3 after delayed transplantation of ADMSCs, when compared with those at week 1 after delayed transplantation of ADMSCs. Similar results were found in the early intervention group in our laboratory. In addition, it was reported that green fluorescence could still be traced in lungs and kidneys at day 28 after a single dose transplantation of ADMSCs, but the cells with fluorescence were hardly seen, suggesting a deceasing trend of functioning of transplanted cells. Therefore, we speculated that intervention of ADMSCs only postponed the progress of pulmonary hypertension and the impairment of pulmonary arterial function induced by MCT. As time goes by, the effect of a single dose transplantation of ADMSCs may fade out. Therefore, it is practical to adjust therapeutic time window of single dose transplantation of ADMSCs may fade out. Therefor, it is practical to adjust therapeutic time window of single dose transplantation of ADMSCs may fade out. Therefor, it is practical to adjust therapeutic time window of single dose transplantation of ADMSCs may fade out. Therefore, we speculated that intervention of ADMSCs only postponed the progress of pulmonary hypertension and the impairment of pulmonary arterial function induced by MCT. As time goes by, the effect of a single dose transplantation of ADMSCs may fade out. Therefore, it is practical to adjust therapeutic time window of single dose transplantation of ADMSCs may fade out. Therefore, we speculated that intervention of ADMSCs only postponed the progress of pulmonary hypertension and the impairment of pulmonary arterial function induced by MCT. 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