

Adipose-derived stem cell transplantation in treating monocrotaline-induced pulmonary arterial hypertension.

Youzhen Qin^{1,3*}, Mingsheng Tang^{1,2}, Huishen Wang¹, Yunquan Li¹, Shujuan Li¹, Yuese Lin¹

¹Department of Pediatrics Cardiology, the First Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong, PR China

²Postgraduate, Department of Pediatrics Cardiology, the First Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong, PR China

³Department of Cardiology, the First Affiliated Hospital of Sun Yat-sen University, Key Laboratory on Assisted Circulation, Guangzhou, Guangdong, PR China

Abstract

This study aims to investigate the roles of Adipose-Derived Stem Cells (ADSCs) transplantation in treating Monocrotaline (MCT) induced Pulmonary Arterial Hypertension (PAH). The adipose cells were obtained by collagenase digestion and the PAH model was obtained by intraperitoneal injection of MCT (50 mg/kg). On day 14, group ADSCs was injected with ADSCs, and the mean pulmonary artery pressure (mPAP), percentage of wall thickness to vascular diameter (WT%) and percentage of wall area to total vascular area (WA%) of tunica media in small artery, Right Ventricular Hypertrophy Index (RVHI%) and other indicators were detected. Results showed that, ADSCs were successfully cultured, and the MCT-induced PAH model was successfully established. After ADSCs transplantation, group ADSCs significantly improved mPAP and corresponding pulmonary pathological indicators than the group PAH. ADSCs significantly attenuated PAH as assessed by reductions in mPAP (32.5 ± 4.2 vs. 57.8 ± 4.4 , $P < 0.01$), the RVHI% (0.30 ± 0.01 vs. 0.40 ± 0.01 , $P < 0.01$), WT% (38.89 ± 2.12 vs. 49.62 ± 2.92 , $P < 0.01$) and WA% (54.73 ± 1.80 vs. 66.92 ± 2.86 , $P < 0.01$), the degree of non-muscular pulmonary arterial muscularization (0.32 ± 0.04 vs. 0.55 ± 0.07 , $P < 0.01$), infiltration of inflammatory cells around the small pulmonary arteries (2.52 ± 0.17 vs. 3.71 ± 0.20 , $P < 0.01$). ADSCs transplantation can reduce mPAP and pulmonary vascular pathological changes, and may have therapeutic effects on PAH.

Keywords: Adipose-derived stem cells, Monocrotaline, Pulmonary arterial hypertension, Stem cell transplantation.

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Introduction

Pulmonary Arterial Hypertension (PAH) is a refractory disease. Although the therapy of PAH has achieved great progress after decades of efforts, the current treatment can only delay the progression rate of this disease, and improve patients' quality of life [1,2], while the prognosis of patients with PAH is still not optimistic [3]. Currently, lung transplantation is the only clinical practice that can achieve radical treatment of PAH, while the donors of lung transplantation are seriously insufficient currently, and this treatment is also restricted by such difficulties as histocompatible antigen matching, surgical techniques and economics, etc. [4], so lung transplantation is still less carried out in the world. Currently, there are many animal species and models used for PAH researches, including hypoxia or Monocrotaline (MCT) induction, surgical lung lobe resection, surgery-induced left-to-right shunt, and inflammation-mediation, etc., among which MCT induction has been considered as the classic method for establishing

animal PAH model by its simple administration and good reproducibility, as well as it can cause lung vascular injury and remodeling under the premise of non-cardiopulmonary disease [5].

In recent years, a number of new pharmacological interventions have been proposed, focusing on fighting against various pro-growth factors, inhibiting the proliferation and migration of smooth muscle cells, inhibiting the activity of matrix protease, or inducing the apoptosis of endothelial cells [6-9]. These may have certain effects in treating and remit PAH, but they cannot promote the growth of new blood vessels, or radically expand the pulmonary vascular bed. Therefore, they cannot effectively reverse the pathophysiological basis of PAH. Stem cell transplantation is one of the new technologies in recent years [10,11]. Adipose-Derived Stem Cells (ADSCs) have 3-germ-layer differentiation ability, similar with the Bone Marrow Mesenchymal Stem Cells (BMSCs) [12-14] and Endothelial Progenitor Cells

(EPCs). Meanwhile, ADSCs has advantages such as not involving in ethics, abundant reserve, wide source range, easy sampling method, and rapid cell proliferation, etc. [15,16]. This study selected ADSCs as the seed cell to treat PAH, aiming to assess the effects of ADSCs in treating PAH.

Materials and Methods

Cell isolation and culture

According to the method proposed by Bunnell et al. 3-week-old SPF grade SD rats were selected, and the inguinal subcutaneous fat was isolated, followed by collagenase I digestion (Sigma, USA) to isolate the cells [15]. The mixture liquid was inoculated into cell culture flasks (Corning Co., USA) with the density as 10^5 - 10^6 cells/cm², then cultured in the cell incubator (20% O₂, 5% CO₂ at 37°C) (Corning Co., USA); when the cells covered 80-90% of the bottom, 0.25% trypsin (Wuhan Gino, China) was added for the digestion. When the cells, observed under light microscope (OLYMPUS Co., Japan), became round and wrinkled, immediately added the serum-containing culture medium (Gibco Co., California, USA) to terminate the digestion; inoculated the cells (P1 generation), with the density as 10^5 - 10^6 cells/cm², into the cell culture flasks for continuous culture, so the cells of P₂, P₃...P_n generation could be obtained. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the First Affiliated Hospital of Sun Yat-sen University.

The P3-generation ADSCs was prepared into single cell suspensions, then averaged into five tubes, among which one tube was added Marker antibody, while the other four were added CD29 (R and D Systems Co, Minnesota, USA), CD31 (Abcam, UK), CD44 (Abcam, UK), CD45 (R and D Systems Co, Minnesota, USA) antibodies according to the instructions; rinsed, centrifuged, re-suspended and washed off the excessive antibodies; the cells were then detected by flow cytometer (BD Company, New Jersey).

Induction, cryopreservation and recovery

i) Adipogenesis induction: P3-generation ADSCs were added the adipocyte medium for the induction, then switched to the maintenance medium for the continuous culture until the adipocytes appeared. When light microscopy (OLYMPUS Co., Japan) discovered the appearance of more fat droplets in the cytoplasm of the induction group, Oil Red O staining (Shanghai Baosheng Biological Engineering Co., China) was performed, and the results were photographed and recorded.

ii) ADSCs osteogenesis induction: P3-generation ADSCs were added into the osteoblast medium for the induction, and the changes of cell morphology were observed daily under light microscope (OLYMPUS Co., Japan). The cells cultured for 21 days were then sampled for climbing film experiment,

followed by the processing according to the instructions of the staining kit (Beijing Dingguo Biological Co., China); the cells were then observed and photographed under optical microscope (Olympus Co., Japan).

iii) Detection of ADSCs growth curve: ADSCs was seeded into 96-well plates (Corning, USA) with the density as 1×10^4 cells/ml, one plate was performed 4, 5-dimethyl-2-thiazolyl-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) every 24 h (Shanghai Baosheng Biological Engineering Co., China), and the absorbance of each well at 490 nm was measured for 7 consecutive days.

iv). Cryopreservation and recovery experiment of ADSCs: P3-generation ADSCs were suspended in cryopreservation liquid with the density as 10^6 - 10^7 cells/ml; the suspension liquid was then stood at 4°C for 0.5 h, followed by at -20°C for 1 h and at -80°C for 12 h, or moved into liquid nitrogen for the storage after overnight. After 2 month cryopreservation, took and thawed the cells. The suspension was then detected the survival rate; added the culture medium to suspend the cells, inoculated into the culture flasks and kept culturing, observed the cell morphology and growth conditions, as well as performed osteogenesis and adipogenesis inductive differentiation experiments.

Establishment of PAH model

Forty Specific Pathogen Free (SPF) male SD rats, weighed about 220 g, were randomly divided into four groups: the Control group (Ctr), the 7 day MCT treatment group (Sigma, USA, MCT-7 day), 14 day MCT treatment group (MCT-14 day), and 21 day MCT treatment group (MCT-21 day). The MCT groups were intraperitoneally injected with MCT solution (50 mg/kg), while group Ctr was injected with the same volume of saline.

Detection of mPAP and sampling

Each group was performed Ultrasound Cardiogram (UCG) (GE-6000, Fairfield, Conn., USA), cardiac catheter manometry and sampled. The sampling was performed before the heart stopped beating, meanwhile, the right lung low lobe was sampled for paraformaldehyde fixation (Guangzhou Chemical Reagent Factory, China), dehydration, paraffin embedding and slicing, followed by Haematoxylin and Eosin (HE) staining and immunohistochemical detection. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Sun Yat-sen University.

Pulmonary pathological indicators

Detection of percentage of wall area to total vascular area (WT %), percentage of wall thickness to vascular diameter (WA%), Non-Muscular Pulmonary Arterial Muscularization (NMPAM) degree (NMPAM degree=(complete muscularization+partial muscularized vessels)/number of the observed blood vessels ×

100%), lung perivascular inflammation score (depending on the infiltration degrees of perivascular inflammatory cells: i) no infiltration: 0 point; ii) mild infiltration: 1 point, the infiltration areas of perivascular inflammatory cells did not exceed 25%; iii) moderate infiltration: 2 points, covered by 25-50%; iv) severe infiltration: 3 points, covered by 50-75%; v) very severe infiltration: 4 points, the infiltration areas >75%), Right Ventricular Hypertrophy Index (RVHI)% (weight of free right ventricular wall (RV)/weight of Left Ventricle+Septum (LV+IVS)).

Cells transplantation

Thirty 8 week old SPF SD male rats, weighed about 220 g, were randomly divided into three groups: group Ctr, group PAH and ADSCs treatment group. Group PAH and group ADSCs were intraperitoneally injected MCT to induce PAH, while group Ctr was injected the same amount of saline. On week 2, group ADSCs was administered 10^6 - 10^7 cells through the external jugular vein for the intervention, while group Ctr and group PAH were given the same amount of culture medium. Each group was performed UCG on week 4 (namely day 28), and observed the changes of right ventricular cavity size, Right Ventricular Systolic Pressure (RVSP) and pulmonary flow. mPAP was detected on week 5 (namely day 35) by right heart catheter, such specimens as blood, heart and lung tissues were kept for further detections. The methods were the same as those in the second part.

Statistical analysis

Experimental data were presented as the mean \pm SD, while the SD for the larger variation, switch to mean \pm SEM. Baseline characteristics were compared between the groups by using Wilcoxon's rank sum test for non-normally distributed variables while independent-samples t-test for normally distributed variables. Rats weight, mPAP, RVHI%, muscularization of pulmonary arterioles degree were evaluated by a one-way ANOVA (GraphPad In Stat) and a significant difference determined by the Student-Newman-Keuls Multiple Comparisons post-hoc test. $P < 0.05$ was considered as statistically significant.

Results

Morphology and identification of ADSCs

The primary ADSCs that were just wall-adherent was small and round, then enlarged and appeared short spindle or polygonal, elongated fibroblast-like on day 7, and gradually merged and formed a single layer on day 9. After passage, the cells were spindle, with shape and size basically the same, while the processes were reduced, and the growth rate was accelerated; the cells were arranged in bundles or swirling, then gradually merged into a single layer. After passage for 20 generations, the cells' morphology and proliferation ability did not exhibited significant change. Flow cytometry showed that ADSCs highly expressed CD29 and CD44, while rarely expressed CD31 and CD45. The P3-generation ADSCs

exhibited the strongest proliferation ability. After adipogenesis induction, light microscopy found that the cells in the induction group turned rounder, with grape-bunch-like fat droplets gradually increased inside the cytoplasm. Oil Red O staining revealed red particles inside the cytoplasm, and partial red articles integrated into fat particles. After performed osteogenesis induction, light microscopy revealed that the cells changed from spindle to polygonal, with the cell bodied enlarged and the processes increased, and the nuclei became rounded, with black fine particles visible in the cytoplasm. As for the results of climbing film assay, light microscopy revealed that the wall-adherent cells exhibited positive to alkaline phosphatase, the blue-black particles in the cytoplasm of partial cells merged into net form. Von kassa staining: black mass like phosphates deposited in the extracellular matrix, namely the so-called mineralized nodules.

P3 and P5-generation ADSCs were taken for resuscitation experiment after 2 month cryopreservation, and the survival rates of these two generations were more than 75%, the cells were continued culture and subculture after resuscitation, and it was found that the cells might have 1 to 2 days stagnant growth period when just resuscitated, after that the cells' growth would be accelerated, the growth state was good, and showed no difference with the original generation isolated and cultured.

Establishment of MCT-induced PAH model

The weights of group MCT were 259.7 ± 3.8 g, 295.7 ± 7.2 g and 315.5 ± 8.9 g on day 7, 14 and 21, respectively, while those of group Ctr were 273.7 ± 6.9 g ($P < 0.05$), 329.2 ± 10.8 g and 383.3 ± 11.9 g ($P < 0.01$) correspondingly, the weight increasing in group MCT was more slowly than group Ctr. The rats in group MCT exhibited rough and dull fur on day 14, and partial rats exhibited accelerated breathing; on day 21, the phenomenon of breathing acceleration was more obvious, and some exhibited shortness of breath, with aggravated mouth nasal cyanosis, fluffy and easily-falling-off fur. Group MCT exhibited slight thickening of vascular tunica media on day 7, and on day 14, the pulmonary artery wall was thickened obviously and showed concentricity and narrowed luminae; on day 21, the thickening of vascular tunica media was much more serious, even some luminae were completely occluded.

Table 1. Comparisons of mPAP and RVHI between group MCT and group Ctr.

Index	Control	MCT		
		Day 7	Day 14	Day 21
mPAP (mmHg)	22.6 ± 1.8	31.5 ± 1.7^a	42 ± 1.8^b	50.4 ± 5.3^b
RVHI (%)	0.25 ± 0.01	0.29 ± 0.01^b	0.33 ± 0.01^b	0.36 ± 0.01^b

^a $P < 0.05$ compared with group Ctr; ^b $P < 0.01$ compared with group Ctr; mPAP, mean pulmonary artery pressure; RVHI: Right Ventricular Hypertrophy Index; MCT: Monocrotaline; Ctr: Control.

mPAP in group MCT was 31.5 ± 1.7 mmHg on day 7, which was a little higher than that in group Ctr (22.6 ± 1.8 mmHg,

$P < 0.05$), and mPAP on day 14 and 21 were 42 ± 1.8 mmHg and 50.4 ± 5.3 mmHg, which were increased much more significantly ($P < 0.01$). RVHI% in group MCT on day 7, 14 and 21 were 0.29 ± 0.01 , 0.33 ± 0.01 and 0.36 ± 0.01 , respectively, exhibiting significant changes than group Ctr (0.25 ± 0.01 , $P < 0.05$, $P < 0.01$, $P < 0.01$) (Table 1).

WT% in group MCT were 28.21 ± 2.60 , 40.28 ± 4.02 and 46.02 ± 3.28 , on day 7, 14 and 21, respectively, exhibiting significant changes than group Ctr (22.81 ± 1.85 , $P < 0.05$, $P < 0.01$, $P < 0.01$); and WA% in group MCT were 48.34 ± 4.71 , 55.56 ± 3.50 and 69.61 ± 3.19 , on day 7, 14 and 21, respectively, exhibiting significant change when compared with group Ctr (37.05 ± 2.04 , $P < 0.05$, $P < 0.01$, $P < 0.01$). The NMPAM degrees in group MCT were 0.37 ± 0.07 , 0.46 ± 0.11 and 0.53 ± 0.05 on day 7, 14 and 21, respectively, which were much more severe than that in group Ctr (0.29 ± 0.03 , $P < 0.05$, $P < 0.01$, $P < 0.01$) (Table 2).

Table 2. Comparisons of WT (%) and RVHI between group MCT and group Ctr.

Index	Control	MCT		
		Day 7	Day 14	Day 21
WT%	22.6 ± 1.8	22.81 ± 1.85^a	40.28 ± 4.02^b	46.02 ± 3.28^b
WA%	37.05 ± 2.04	48.34 ± 4.7^a	55.56 ± 3.50^b	69.61 ± 3.19^b
NMPAM degree	0.29 ± 0.03	0.37 ± 0.07^a	0.46 ± 0.11^b	0.53 ± 0.05^b

^a $P < 0.05$ compared with group Ctr; ^b $P < 0.01$ compared with group Ctr. WT (%): Percentage of Wall Thickness to Vascular Diameter; WA (%): Percentage of Wall Area to Total Vascular Area; NMPAM: Non-Muscular Pulmonary Arterial Muscularization; MCT: Monocrotaline; Ctr: Control.

Group MCT exhibited the infiltration of monocytes and lymphocytes on day 7, which partially spread to pulmonary alveoli and interstitial tissues, and much more obvious on day 14 and 21, the inflammatory cells infiltrated more than 75% of perivascular areas. The pulmonary perivascular inflammation scores in group MCT were 1.28 ± 0.15 , 2.85 ± 0.28 and 3.66 ± 0.12 on day 7, 14 and 21, respectively, significantly higher than that in group Ctr (0.38 ± 0.11 , $P < 0.05$, $P < 0.01$, $P < 0.01$). Group MCT began to appear right ventricular enlargement and tricuspid regurgitation on day 14, and became more obvious on day 21, with RVSP estimated as 39.5 ± 3.9 and 45.2 ± 3.9 mmHg on day 14 and 21, respectively.

ADSCs treatment outcome towards PAH

Group PAH and group ADSCs exhibited slower weight growth rate than group Ctr; the rats injected with ADSCs gradually increased their appetite, and their weight gains were accelerated, while still exhibited significant gap than group Ctr. The rats in group PAH exhibited tarnish fur on day 14, which became rough and easily-falling-off on day 24.

mPAP in group PAH was 57.8 ± 4.4 mmHg on day 35, which was significantly higher than 20.9 ± 2.3 mmHg in group Ctr ($P < 0.01$). mPAP in group ADSCs was 32.5 ± 4.2 mmHg,

significantly lower than group PAH (57.8 ± 4.4 , $P < 0.01$), but with no significant difference with group Ctr ($P > 0.01$). RVHI% in group PAH was 0.40 ± 0.01 on day 35, which was significantly higher than 0.24 ± 0.01 in group Ctr ($P < 0.01$). RVHI% in group ADSCs was 0.30 ± 0.01 , significantly lower than group PAH ($P < 0.05$), but with no significant difference with group Ctr ($P > 0.01$) (Table 3).

Table 3. Comparisons of mPAP and RVHI among group ADSCs, group PAH and group Ctr.

Index	Group Ctr	Group PAH	Group ADSCs
mPAP (mmHg)	20.9 ± 2.3	57.8 ± 4.4^a	32.5 ± 4.2^b
RVHI (%)	0.24 ± 0.01	0.40 ± 0.01^a	0.30 ± 0.01^c

^a $P < 0.01$ compared with group Ctr; ^b $P < 0.01$ compared with group PAH; ^c $P < 0.05$ compared with group PAH; mPAP: Mean Pulmonary Artery Pressure; RVHI: Right Ventricular Hypertrophy Index; ADSCs: Adipose-Derived Stem Cells; PAH: Pulmonary Arterial Hypertension; Ctr: Control.

WT% in group Ctr, group PAH and group ADSCs on day 35 was 26.57 ± 2.15 , 49.62 ± 2.92 and 38.89 ± 2.12 , respectively. WA% in group Ctr, group PAH and group ADSCs on day 35 was 41.35 ± 2.51 , 66.92 ± 2.86 and 54.73 ± 1.80 , respectively. WT% and WA% in group PAH were significantly higher than group Ctr ($P < 0.01$), and those in group ADSCs was significantly lower than group PAH ($P < 0.01$). There was no significant difference of WT% or WA% between group ADSCs and group Ctr ($P > 0.05$). NMPAM degrees in group Ctr, group PAH and group ADSCs were 0.24 ± 0.04 , 0.55 ± 0.07 and 0.42 ± 0.04 , respectively. NMPAM degree in group PAH was significantly higher than group Ctr ($P < 0.01$), and that in group ADSCs was significantly lower than group PAH ($P < 0.05$). There was no significant difference between group ADSCs and group Ctr ($P > 0.05$) (Table 4).

Table 4. Comparisons of WT (%) and WA (%) among group ADSCs, group PAH and group Ctr.

Index	Group Ctr	Group PAH	Group ADSCs
WT%	26.57 ± 2.15	49.62 ± 2.92^a	38.89 ± 2.12^b
WA%	41.35 ± 2.51	66.92 ± 2.86^a	54.73 ± 1.80^b
NMPAM	0.24 ± 0.04	0.55 ± 0.07^a	0.32 ± 0.04^c

^a $P < 0.01$ compared with group Ctr; ^b $P < 0.05$ compared with group PAH; ^c $P < 0.01$ compared with group PAH. WT (%): Percentage of Wall Thickness to Vascular Diameter; WA (%): Percentage of Wall Area to Total Vascular Area; NMPAM: Non-Muscular Pulmonary Arterial Muscularization; ADSCs: Adipose-Derived Stem Cells; PAH: Pulmonary Arterial Hypertension; Ctr: Control.

Group PAH exhibited the infiltration of a large number of inflammatory cells, such as monocytes and lymphocytes, around small pulmonary arteries; the inflammatory cell infiltration score in group ADSCs was 2.52 ± 0.17 , significantly reduced than group PAH (3.71 ± 0.20 , $P < 0.01$). All rats in group PAH exhibited right ventricular enlargement and tricuspid regurgitation; partial rats in group ADSCs exhibited right ventricular enlargement and tricuspid regurgitation, with RVSP estimated as 32.9 ± 3.5 mmHg,

significantly lower than group PAH (48.2 ± 4.9 mmHg, $P < 0.01$).

Discussion

It was reported that the application of MCT (50-60 mg/kg) for 3-4 weeks could produce PAH model [5]. The results of this study also showed that, on day 7 of MCT administration, mPAP, RVHI%, WT% and WA%, which might prompt the thickening of tunica media and pulmonary artery remodeling, all exhibited slight changes, and on day 14, the changes of mPAP and RVHI% were much more obvious, and the thickening of tunica media and the infiltration of inflammatory cells around small arteries were more obvious; on day 21, mPAP could reach middle to severe pressure value, and RVHI% also exhibited significant changes, and the degrees of inflammatory cell infiltration towards pulmonary artery, pulmonary arterial thickening, and non-muscle vascular muscularization were much more serious, some blood vessels were even completely occluded, indicating the intraperitoneal injection of MCT could successfully induce PAH model.

ADSCs have become a hotspot of researches in recent years for its advantages as rich source, easy sampling and stable content, etc. [12,15,16]. Referring to the method developed by Bunnell, ADSCs was successfully isolated and cultured in this study. ADSCs could highly express CD29 and CD44, while hardly express CD31 and CD45, consistent with the results of Takamiya [15]. The experiments of osteogenesis and adipogenesis induction towards the isolated ADSCs also obtained success, proving the obtained cells were stem cells. The cryopreservation and resuscitation experiments showed that ADSCs still had strong survival rate, and kept differentiation ability after passage, so ADSCs could be used as ideal stem cells.

ADSCs might be one kind of much more desirable seed stem cells than EPCs and BMSCs. ADSCs and BMSCs belong to adult stem cells, with the potential of multi-differentiation. ADSCs had already been reported the applications in treating myocardial infarction and vascular occlusive diseases [17,18], and achieved better therapeutic effects. In this study, ADSCs was injected through the jugular vein to treat MCT-induced PAH rats, and the results suggested that ADSCs could reduce mPAP and RVHI% in PAH rats, meanwhile, it could improve the reconstruction of pulmonary micro-arteries and reduce the infiltration of inflammatory cells around the pulmonary arteries, therefore, it might have therapeutic effects towards PAH. And this was similar to the roles of BMSCs towards PAH [13].

In this study, ADSCs were injected through the external jugular vein to minimize the impacts caused by the filtration of liver and spleen, so it might be used as the preferred injection approach in treating PAH [19,20]. Some scholars [21] considered that on week 1, the cells would migrate and colonize to the damaged parts, and on week 2, these stem cells would differentiate into specific cells and secrete large amounts of vasoactive factors to repair the damaged blood

vessels, even formed new blood vessels to replace the damaged vascular bed [22]. The results of the second part of this study suggested that MCT induced PAH model exhibited certain changes on week 1, which would be much more obvious on week 2, so it would be more appropriate to perform cell intervention on week 2 (day 14) of MCT administration; Zhao et al. administrated BMSCs on day 3 and 21 of MCT induction to treat PAH rats, and found that pulmonary hemodynamics and cardiopulmonary histopathology were also significant improved, and no significant difference existed [23]. Therefore, the best time for ADSCs transplantation in treating PAH still remained to be studied.

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*Correspondence to

Youzhen Qin

Department of Pediatrics Cardiology

The First Affiliated Hospital of Sun Yat-sen University

China

E-mail: qyouzhen@163.com