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Bone marrow mesenchymal stem cells against monocrotaline-induced pulmonary artery hypertension***

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Abstract

BACKGROUND: Stem cell transplantation has a certain effect in the treatment pulmonary arterial hypertension.

OBJECTIVE: To investigate the effect of bone marrow mesenchymal stem cells transplantation on the treatment of pulmonary arterial hypertension and to discuss the mechanism.

METHODS: Bone marrow mesenchymal stem cells were *in vitro* cultured, purified and amplified by density gradient centrifugation method, and labeled with the fluorescent dye for preparation. Pulmonary arterial hypertension model was established by subcutaneous injection of monocrotaline. One week after modeling, the rats were randomly divided into three groups. Rats in the stem cell transplantation group and pulmonary arterial hypertension group received subcutaneous injection of monocrotaline to establish the pulmonary arterial hypertension model. One week later, the rats in the stem cell transplantation group received sublingual vein injection of bone marrow mesenchymal stem cell solution, the rats in the pulmonary arterial hypertension group were injected with the culture medium without stem cells, and the rats in the control group were injected with the normal saline in the same dose.

RESULTS AND CONCLUSION: At 2 weeks after transplantation, compared with the mesenchymal-induced pulmonary arterial hypertension rats, the hemodynamic parameters and the ratio of right ventricular/body weight of the rats in the stem cell transplantation group were significantly improved ($P < 0.05$); the degree of pulmonary vascular remodeling was significantly reduced ($P < 0.05$). Fluorescence microscope observation showed that the transplanted bone marrow mesenchymal stem cells could alive at least 2 weeks in the stem cell transplantation group, and part of the stem cells could differentiate into pulmonary vascular endothelial cells. The results show that bone marrow mesenchymal stem cell transplantation can significantly improve the pulmonary vascular and right ventricular structural impairments in the rats with mesenchymal-induced pulmonary arterial hypertension.

Subject headings: monocrotaline; hypertension, pulmonary; cell transplantation; stem cell transplantation; stem cell research

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INTRODUCTION

Pulmonary arterial hypertension is a progressive disorder characterized by abnormally high blood pressure in the pulmonary artery. One of the most important mechanisms is to increase the pulmonary vascular resistance by functional and structural change in the pulmonary vasculature[1]. Many therapies have been proved useful in decreasing pulmonary arterial pressure, but an effective therapy of the long-term outcome in this disorder is lacking[2].

Bone marrow mesenchymal stem cells are multi-potential progenitor cells derived from the fetal bone marrow, which have the ability to differentiate into bone, cartilage,

muscle, bone marrow stroma, endothelial cells, and vascular smooth muscle cells and other connective tissues[3]. Studies also have showed that bone marrow mesenchymal stem cells can secrete a variety of growth factors, such as vascular endothelial growth factor. Recently, bone marrow mesenchymal stem cell transplantation has become a potential therapy for pulmonary arterial hypertension.

In our previous research, intravenous implantation of bone marrow mesenchymal stem cells improved the progression of right ventricular impairment caused by monocrotaline-induced pulmonary arterial hypertension[4]. The aim of this study was to further explore the effect of bone marrow mesenchymal stem cell transplantation on the model of pulmonary arterial hypertension induced by monocrotaline and discuss the mechanism.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment.

Time and setting

The experiment was conducted in the Second Hospital of Shandong University between January 2011 and April 2012.

Materials

Thirty-three SPF healthy Sprague-Dawley rats weighing 200–250 g were purchased from the Animal Experimental Center of Shandong University. Animal license was SCXK(Lu)20030004. During the experiment, the free drink, feeding feed and mixed feed were provided by Shandong University Animal Experiment Center, the room temperature was 18–25 ℃, relative humidity was 62%–72%. The study protocol was reviewed and approved by the Institutional Animal Care and Use Committee, the Second Hospital of Shandong University, and the experiments were conducted according to the Guidelines of the American Physiological Society.

Chemicals and reagents are as follows:

Chemicals and reagents	Source
Monocrotaline, ficoll, penicillin, streptomycin, CD44, CD29, CD34, and CD90 antibody	Sigma-Aldrich, USA
Dulbecco's modified Eagle's medium/F-12, fetal bovine serum	Gibco-BRL, USA
Chloromethylbenzamido-1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate	Invitrogen, USA
Vascular endothelial growth factor, von Willebrand factor anti-body	Abcam, English

Methods

Establishment and evaluation of pulmonary arterial hypertension model

Monocrotaline was prepared as described and was dissolved in 1 mol/L HCl, neutralized to pH 7.4 with 0.5 mol/L NaOH, and diluted with saline just before injection[2]. One week after monocrotaline injection[5], the rats were anesthetized and inserted with a 3F-Miller microtip catheter *via* the right jugular vein into the right ventricle to obtain baseline measurements of hemodynamics, such as right ventricular systolic pressure, mean right ventricular pressure, and mean pulmonary arterial pressure. The rats were euthanized after hemodynamic measurements, the lung and heart were quickly harvested and fixed *in situ via* the trachea cannula with buffered 4% formaldehyde, and then were embedded in paraffin. The sections were cut into 4–5 μm slices and were stained with streptavidin peroxidase and hematoxylin-eosin. According to the results of hemodynamic parameters, right ventricular hypertrophy and pulmonary arterial pathological changes were used to evaluate if the model of pulmonary arterial hypertension was successful establishment.

Isolation, culture and labeling of bone marrow mesenchymal stem cells

Bone marrow cells were isolated by flushing the cavity of femurs and tibias and transferred to a tissue culture dish with 90 mm in diameter. The bone marrow mesenchymal stem cells and other cells were separated with the ficoll (1.077) density gradient centrifugation method as previously report[6].

Flow cytometry immunophenotyping was performed by using methods reported previously: 5×10^5 cells were suspended with trypsin and washed two times in PBS. After centrifugation, the cells were incubated with primary against human CD44, CD29, CD34, and CD90 antibodies for 30 minutes at 4 ℃.

Before implantation, the cells were labeled with the cross-linkable membrane dye

Cells were labeld according to the protocol of the supplier as previously described[7]. Briefly, $(1–5)\times10^5$ cells were incubated for 5 minutes at 37 ℃, and then for an additional 15 minutes at 4 ℃.

After labeling, 1×10^5 cells were being washed with PBS and resuspended in 100 μL saline and then kept on ice before transplantation, the labeling-efficiency reached more than 80%.

Groups of experimental animals

The male Sprague Dawley rats were randomly divided into three groups (n=10) as follows. Control group: animals received a sublingual vein injection of 0.9% saline instead of bone marrow mesenchymal stem cells. Pulmonary arterial hypertension group: animals received a subcutaneous injection of 50 mg/kg monocrotaline. Bone marrow mesenchymal stem cells group: animals received a sublingual vein injection of (1–5)×10^5 labeled bone marrow mesenchymal stem cells.

Evaluation of hemodynamic parameter and right ventricular impairment induced by monocrotaline

Two weeks after stem cell transplantation, the rats were anesthetized and inserted with a 3F-Miller microtip catheter *via* the right jugular vein into the right ventricle to obtain base line measurements of hemodynamics such as right ventricular systolic pressure, mean right ventricular pressure, and mean pulmonary arterial pressure[8]. The ratio of right ventricular to body weight was determined to measure the right ventricular hypertrophy[9].

Analysis of pulmonary vascular structural change

The morphometric analysis of pulmonary arteries was performed as described previously[10]. The structural changes in pulmonary vascular wall were observed by microscope, and the media thickness, extra-large diameter, pulmonary vessel area, and total vascular area were measured to calculate the ratio of media thickness/ extra-large diameter (%) and pulmonary vessel area/total vascular area (%). The ratio of right ventricular to body weight was determined to measure the right ventricular hypertrophy.

Analysis of cell differentiation by immunohistochemistry

Two weeks after cell transplantation, the rats were anesthetized and the lung was inflated with optimal cutting temperature compound, and then quickly frozen in liquid nitrogen and stored at –80 ℃. Sections were cut into 4 μm slices and fixed in acetone for 10 minutes at –20 ℃. Survival of mesenchymal stem cells was demonstrated by observing the presence of 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate(DiI)-labeled cells. Immunofluorescence was then carried out with goat anti-mouse monoclonal surfactant associated protein C (1:100) IgG antibody, rabbit anti-human von Willebrand factor (1:100) antibody and vascular endothelial growth factor (1:100) anti-body. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody was used as a secondary antibody. Two weeks after bone marrow mesenchymal stem cell implantation, the labeled red fluorescence-positive cells were observed by fluorescence microscope. The engraftment and integration of transplanted undifferentiated cells were identified through observing the distribution of red fluorescent-positive cells. The sections stained with von Willebrand factor, then the vascular endothelial growth factor and surfactant associated protein C presented as green fluorescence, and the merged images staining were yellow color.

Main outcome measures

The analysis of animal determination of hemodynamic data; right ventricular weigh and pulmonary vascular structural induced by monocrotaline; characterization of cultured bone marrow mesenchymal stem cells; the identification of the transplanted bone marrow mesenchymal stem cells; effect of bone marrow mesenchymal stem cells on the hemodynamic data and right ventricular impairment; the effect of bone marrow mesenchymal stem cells on pulmonary artery wall.

Statistical analysis

Statistical analysis was performed with one-way analysis of variance followed by Bonferroni test or t-test when appropriate by using SPSS 13.0 statistical software. Differences were considered significant at $P < 0.05$, and presented as mean±SD unless otherwise stated.

RESULTS

Characterization of cultured bone marrow mesenchymal stem cells

After being primary cultured for 24 hours, the bone marrow mesenchymal stem cells appeared as colonies of spindle-like cells. Three days after being sub-cultured, the cells were attached to the culture dish tightly and proliferated rapidly in the culture medium.

Effect of bone marrow mesenchymal stem cells on hemodynamic data and right ventricular impairment

At 2 weeks after bone marrow mesenchymal stem cell administration, right ventricular systolic pressure, mean right ventricular pressure, mean pulmonary arterial pressure and the ratio of right ventricular to body weight were significantly lower in stem cell transplantation group when compared with pulmonary arterial hypertension group ($P < 0.05$; Table1).

Table 1 Effect of bone marrow mesenchymal stem cells on hemodynamic data and right ventricular weight ($\bar{x}$±s, n=10)

Item	Control	PAH	BMSCs
RVSP (mm Hg)	35.17±1.86	56.84±1.54[c]	43.83±2.13[d]
MPAP (mm Hg)	20.36±2.13	41.37±2.24[c]	26.82±3.42[d]
MRVP (mm Hg)	19.74±5.23	41.68±6.17[c]	28.34±1.98[d]
RV/BW	0.47±0.034	0.61±0.07[a]	0.55±0.04[b]

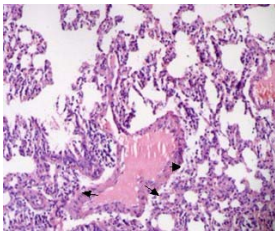
[a]P < 0.05, [c]P < 0.01, *vs*.control group; [b]P < 0.05, [d]P < 0.01, *vs*. PAH group. RVSP: right ventricular systolic pressure; MRVP: mean right ventricular pressure; MPAP: mean pulmonary arterial pressure; RV/RW: ratio of right ventricular to body weight; PAH: pulmonary arterial hypertension; BMSCs: bone marrow mesenchymal stem cells; 1 mm Hg=0.133 kPa.

Effect of bone marrow mesenchymal stem cells on pulmonary artery structure (Table 2, Figure 1)

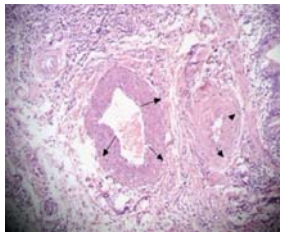
Table 2 Effect of bone marrow mesenchymal stem cells on pulmonary artery wall ($\bar{x}$±s, n=10, %)

Item	Control	PAH	BMSCs
MT	12.08±1.30	45.21±4.37[a]	20.83±5.49[b]
VA	42.31±4.39	20.36±6.81[a]	38.27±3.48[b]

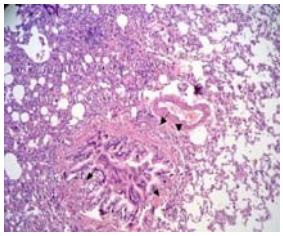
[a]P < 0.01, *vs*. control group; [b]P < 0.05, *vs*. PAH group. MT: media thickness; ED: extra-large diameter; VA: pulmonary vessel area; TAA: total vascular area; MT%(=MT/ED); VA%(=VA/TAA; PAH: pulmonary arterial hypertension; BMSCs: bone marrow mesenchymal stem cells.



A: Control group



B: Pulmonary arterial hypertension group



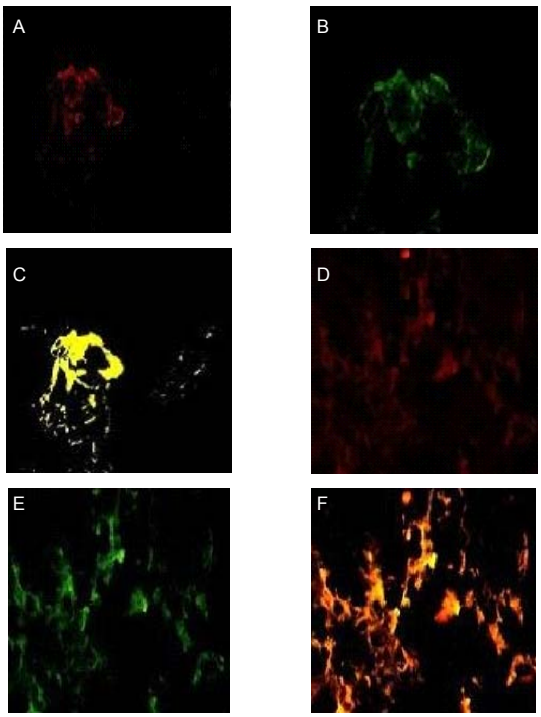
C: Stem cell transplantation group

The intima thickening and luminal stenosis in stem cell transplantation group were significantly lighter than those in the pulmonary arterial hypertension group (arrows)

Figure 1 Effect of bone marrow mesenchymal stem cells on pulmonary artery wall (Hematoxylin-eosin staining, ×100)

Hematoxylin-eosin staining results demonstrated that the intima thickening and luminal stenosis were significantly decreased in the stem cell transplantation group when compared with the pulmonary arterial hypertension group. The ratios of media thickness/extra-large diameter (%) and pulmonary vessel area/total vascular area (%) of muscular arteries was significantly improved in the stem cell transplantation group as compared with pulmonary arterial hypertension group, respectively (P < 0.05).

Differentiation of transplanted bone marrow mesenchymal stem cells (Figure 2)



Transplanted BMSCs showed red fluorescence (A, D); sections stained with vascular endothelial growth factor (VEGF) (B) and von Willebrand factor (vWF) (E) showed green fluorescence; merged image of DiI with VEGF (C) and vWF (F) staining respectively showed yellow fluorescence

Figure 2 Differentiation of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled bone marrow mesenchymal stem cells (BMSCs) by fluorescence microscope (×400)

Two weeks after cells transplantation, DiI-labeled positive cells were observed at the transplanted area in bone marrow mesenchymal stem cell group. In many regions, the red fluorescence cells were observed coincident with the green fluorescence spots of vascular endothelial growth factor and vWiFanti-body, but not surfactant associated protein C anti-body, which suggested that the intravenous implantation of bone marrow mesenchymal stem cells could differentiate

into vascular endothelial cells *in vivo* although they did not actively survive as lung cells. There was no evidence of red and green fluorescence in the control and pulmonary arterial hypertension groups.

DISCUSSION

The structural changes in pulmonary vascular wall were observed by microscope, and media thickness, extra-large diameter, pulmonary vessel area, and total vascular area were measured to calculate ratios of media thickness/extra-large diameter (%) and vessel area/total vascular area (%). Pulmonary artery hypertension is a progressive disorder characterized by progressive increase of pulmonary arterial pressure and resistance, eventually leading to right heart failure and death in patients with refractory disease[11].

Although in the past ten years, the treatment of PAH had apparent progress, but the prognosis is still poor. Therefore, for further experimental studies, looking for reasonable and safe method of treatment of pulmonary arterial hypertension has become an urgent problem to be solved.

Bone marrow mesenchymal stem cells can secrete a variety of growth factors to promote angiogenesis, such as vascular endothelial growth factor. Transplantation of endothelial progenitor cells into the monocrotaline-injured lung could improve the impairment, but the treatment effect is not ideal. The reports about bone marrow mesenchymal stem cell transplantation for the treatment of pulmonary hypertension are very small. In our previous research, intravenous implantation of Bone marrow mesenchymal stem cells could improve the progression of right ventricular impairment caused by monocrotaline-induced pulmonary arterial hypertension. In the present study, we also found that 2 weeks after sublingual vein administration of bone marrow mesenchymal stem cells to pulmonary arterial hypertension rats, the pulmonary arterial pressure was significantly lower in the stem cell transplantation group when compared with the non-treated pulmonary arterial hypertension group. Right ventricular systolic pressure, mean right ventricular pressure and mean pulmonary arterial pressurefluorescein isothiocyanate were significantly lower in the stem cell transplantation group when compared with pulmonary arterial hypertension group, the ratio of right ventricular to body weight was significantly lower in the stem cell transplantation group when compared with pulmonary arterial hypertension group. On the other hand, 2 weeks after injection, hematoxylin-eosin staining results demonstrated that the intima thickening and luminal stenosis in the stem cell transplantation group were significantly improved than that in the pulmonary arterial hypertension group. The ratio of media thickness/extra-large diameter (%) muscular arteries was significantly decreased and the ratio of vessel area/total vascular area (%) was significantly increased in the stem cell transplantation group as compared with pulmonary arterial hypertension group, respectively.

Although the underlying mechanism are complicated and not yet determined, several factors are expected to be contributed, such as the role of stem cell differentiation, para-secretion effects, and so on[12-13]. Our experiments also demonstrated that the red fluorescence-positive cells were observed coincident with the green fluorescence spots of vascular endothelial growth factor and vWF anti-body but not surfactant associated protein C anti-body in many regions. These results suggested that the intravenous implantation of mesenchymal stem cells have the ability to differentiate into vascular endothelial cells *in vivo* but not lung cells. Therefore, transplantation of bone marrow mesenchymal stem cells by homing to the lung and transforming into vascular endothelial cells may create a wide range of collateral circulation, increase the total area of pulmonary vascular bed, improve pulmonary blood supply and effectively reduce the pulmonary hypertension.

In conclusion, our results showed that intravenous implantation of mesenchymal stem cells may improve the lung and the heart impairment caused by monocrotaline-induced pulmonary hypertension.

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移植骨髓间充质干细胞预防野百合碱诱导的肺动脉高压***

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文章亮点：

采用骨髓间充质干细胞移植治疗野百合碱诱导的大鼠肺动脉高压肺血管重构模型，对肺组织进行显微及超微结构分析，结合血流动力学指标，认识骨髓间充质干细胞移植逆转肺血管重构的机制。发现静脉移植骨髓间充质干细胞能明显改善野百合碱造成的肺动脉高压大鼠肺血管和右心室结构的损伤。

关键词：
干细胞；干细胞移植；骨髓间充质干细胞；肺动脉高压；移植；野百合碱；右心室损伤；血流动力学参数；血管重构；转化；省级基金；干细胞图片文章

主题词：
野百合碱；高血压，肺性；细胞移植；干细胞移植；干细胞研究

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摘要

背景：干细胞移植治疗肺动脉高压有一定疗效。

目的：观察骨髓间充质干细胞移植治疗肺动脉高压的效果及并探讨其作用机制。

方法：采用密度梯度离心法体外培养、纯化、扩增获得大鼠骨髓间充质干细胞，经荧光染料标记后备用。大鼠皮下注射野百合碱建立肺动脉高压模型，建模后 1 周将大鼠随机分为 3 组，干细胞移植组和肺动脉高压组大鼠皮下注射野百合碱建立肺动脉高压模型，1 周后干细胞组大鼠经舌下静脉注射骨髓间充质干细胞悬液，肺动脉高压组注射等量不含干细胞的培养液，对照组皮下注射等量生理盐水。

结果与结论：移植后 2 周，与野百合碱诱导的肺动脉高压大鼠相比干细胞移植组血流动力学参数及右心室与体质量之比明显改善($P < 0.05$)；肺血管重构程度减轻($P < 0.05$)。荧光显微镜下发现干细胞组移植的骨髓间充质干细胞在大鼠体内能存活至少 2 周，部分干细胞能转化为血管平滑肌细胞。说明静脉移植骨髓间充质干细胞能明显改善野百合碱造成的肺动脉高压大鼠肺血管和右心室结构的损伤。

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*利益冲突：*课题未涉及任何厂家及相关雇主或其他经济组织直接或间接的经济或利益的赞助。

*伦理要求：*实验对动物的处理方法符合中华人民共和国科学技术部颁发的《关于善待实验动物的指导性意见》。

*学术术语：*肺动脉高压是各种原因引起的静息状态下右心导管测得的肺动脉平均压≥25 mm Hg(1 mm Hg=0.133 kPa)的一组临床病理生理综合征。

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