



## Original Article

## Therapeutic effects of umbilical cord blood derived mesenchymal stem cell-conditioned medium on pulmonary arterial hypertension in rats

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## ABSTRACT

**Introduction:** Human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) may have multiple therapeutic applications cell based therapy with one being for treatment of pulmonary artery hypertension. As low survival rates and potential tumorigenicity of implanted cells could undermine the mesenchymal stem cell-based therapy, we chose to investigate the use of conditioned medium (CM) from a culture of MSC cells.

**Material and methods:** CM was prepared by culturing hUCB-MSCs in three-dimensional spheroids. In a rat model of PAH induced by monocrotaline, we infused CM or the control unconditioned culture media via tail-vein of 6-week-old Sprague-Dawley rats.

**Results:** Compared with the control unconditioned media, CM infusion reduced the ventricular pressure, the right ventricle/(left ventricle + interventricular septum) ratio, and maintained respiratory function in the treated animals. Also the number of IL-1 $\alpha$ , CCL5 and TIMP-1-positive cells increased in lung samples and the number of TUNEL-positive cells decreased significantly in the CM treated animals.

**Discussion:** From our in vivo data in the rat model, the observed decreases in the TUNEL staining point to a potential therapeutic benefit of the CM in ameliorating PAH-mediated lung tissue damage. Increased IL-1 $\alpha$ , CCL5 and TIMP-1 levels may play important roles in this regard.

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## 1. Introduction

Pulmonary artery hypertension (PAH) is a progressive chronic disease with a high mortality rate.<sup>1</sup> PAH has a complex disease mechanism, but its cardinal signs are an elevation of pulmonary artery pressure, right ventricular (RV) hypertrophy and arteriolar wall remodeling.<sup>2</sup> The pulmonary vascular is also remodeled with increased pulmonary vascular resistance and over-proliferation of pulmonary artery endothelial cells.<sup>3,4</sup> There is also damage to the pulmonary microvasculature impacting the flow blood from the

heart to the lungs.<sup>5</sup> Although current treatments may prolong and improve quality of life for the patients, the long-term prognosis for PAH is poor with a 2–3 year survival at the diagnosis.<sup>1</sup>

Autologous implantation of bone marrow mononuclear cells (BM-MNCs), known to be enriched in mesenchymal cells (MSCs), has demonstrated safety and effectiveness in therapeutic angiogenesis.<sup>6</sup> A number of studies have also indicated a therapeutic benefit from bone marrow derived MSCs in increasing respiratory function in animal models of PAH.<sup>7,8</sup> In separate studies, human umbilical cord blood-derived MSCs have also improved lung function in animal models of PAH and in addition in a number PAH patients tested.<sup>9,10</sup>

In previous studies, we demonstrated the neuroprotective potential of various conditioned media namely human adipose tissue-derived stem cell-conditioned medium (hADSC-CM) and human neural stem cell-conditioned media (hNSC-CM) to treat rats with stroke and Huntington's disease.<sup>11,12</sup> We also investigated

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gene expression changes by microarray analysis after injection of human umbilical-cord blood derived MSCs (hUCB-MSCs) into rats in an experimental model of PAH.<sup>13</sup> Based on our findings from that study, we undertook an investigation to assess the feasibility and safety of conditioned medium from hUCB-MSCs (hUCB-MSC-CM) in the same rat PAH model and to test the hypothesis that the conditioned media from these cells may lead to improved lung function in the affected rats. Here we elaborate on our results and demonstrate that the conditioned media provide a therapeutic benefit in the rat model of PAH tested. As there are certain advantages in using conditioned media in lieu of using autologous whole bone marrow or umbilical cord cells as sources for mesenchymal cells (MSCs), our data may provide a means of increasing the accessibility of MSCs to treat various disease including PAH.

## 2. Material and methods

### 2.1. Animals and pulmonary arterial hypertension (PAH) rat model

Six-week-old male Sprague-Dawley rats were used. All rats were housed in climate-controlled conditions with a 12-h light/12-h dark cycle, and had free access to food and water. The animals used in this experiment were treated according to the Principles of Laboratory Animal Care (NIH publication no. 86-23). PAH was induced by subcutaneous (sc) injection of 60 mg/kg monocrotaline (MCT) (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.5 N HCl. The rats were grouped into a control (C) group ( $n = 20$ ), injection of  $\alpha$ MEM followed by MCT group (M group) ( $n = 20$ ), and injection of MCT followed by hUCB-MSC-CM transfusion group (CM) ( $n = 20$ ).  $\alpha$ MEM and hUCB-MSC-CM (0.5  $\mu$ l/h) were transfused by tail-vein administered 7 days after MCT injection.

### 2.2. Cell preparation and culture of hUCB-MSCs

hUCB-MSCs were obtained from Biomedical Research Institute (Seoul, Korea). Isolated human MSCs were expanded in culture as previously described.<sup>5</sup>

### 2.3. Preparation of hUCB-MSC-CM

To generate hUCB-MSC-CM spheroids,<sup>13,14</sup> 30  $\mu$ l of cell suspension ( $1 \times 10^6$  cells/ml) was applied to the lid of a Petri dish containing phosphate buffered saline (PBS). After 24 h incubation, spheroids formed in the drops were retrieved. For the three-dimensional bioreactor culture, hUCB-MSC spheroids ( $4.2 \times 10^7$  cells) were cultured in a siliconized spinner flask (Bellco, Vineland, NJ, USA) containing 70 ml  $\alpha$ MEM with stirring at 70 rpm. To obtain CM, the medium was changed to  $\alpha$ MEM without serum, and the cells were cultured for 2 days.<sup>11</sup>

### 2.4. Determination of the organ weights and right hypertrophy index

The rats were weighed and observed for general appearance during the study period. The animals were sacrificed at the scheduled time. The wet weights of excised right ventricle (RV), left ventricle (LV) plus interventricular septum (IVS) (LV + IVS) were measured.

### 2.5. Pulmonary hemodynamics

A catheter filled with heparin saline was rapidly inserted along the incision and slowly advanced for about 5 cm to enter the pulmonary artery. The standard of pulmonary hypertension was defined as systolic pulmonary pressure (SPAP)  $>50$  mmHg.<sup>15</sup>

### 2.6. Immunohistochemistry and Western blot analysis

Briefly, immunohistochemistry and Western blot analysis were done according to the instructions of the manufacturer (Dako, Kyoto, Japan).

### 2.7. Cytokine array and gene expression in lung tissues

The lung samples were collected at termination (4 days after hUCB-MSC-CM injection) and quickly frozen in liquid nitrogen. A rat cytokine array (ARY008; R&D Systems, Minneapolis, MN, USA) was used to screen the lung homogenates according to manufacturer's instructions.

### 2.8. In situ TUNEL assay for lung cell apoptosis

Apoptotic cells in the tissue sections were detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling technique (TUNEL) using a commercial apoptosis kit (TACS TM TdT Kit; R&D Systems, Minneapolis, MN, USA) according to the supplier's instructions.

### 2.9. Statistical analyses

Results were expressed as the mean  $\pm$  standard deviation. An unpaired two-tailed *t*-test and Mann-Whitney test were used, and a *p* value  $<0.05$  was considered statistically significant. SPSS 14.0 for windows (SPSS, Chicago, IL, USA) was used for all statistical analyses.

## 3. Results

### 3.1. Changes in body and organ weights

There was a significant decrease in body weight at 14, 21 and 28 days in the monocrotaline group (M) compared to the control (C) group. However, body weight increased at 21 and 28 days in the conditioned media treated (CM) group compared to the M group. The M group also showed increased weights of the right ventricle (RV) at 21 and 28 days. The sum weight of left ventricle (LV) + interventricular septum (IVS) (LV + IVS) was not significantly different between the C, M and the CM groups at the time point tested. Ratio of RV to LV + IVS, namely RV/LV + IVS, was significantly higher at 14, 21 and 28 days in the M group compared with the C group. However, the RV/LV + IVS ratio was significantly decreased at 28 days in the CM group compared with the M group. Also LV + IVS was significantly lower in both M and CM groups compared to the C group at 14, 21 and 28 days. The lung weight was significantly increased in the M group compared with the C group at 21 and 28 days. However, the lung weight was significantly decreased in the CM group compared to the M group at 28 days (Table 1).

### 3.2. Cytokine profile in the lung tissues

A profile of the cytokine in the lung homogenates was made to investigate potential changes from hUCB-MSC-CM treatment (Fig. 1). CINC-1, CINC-2a/b, CX3CL1, LIX, LECAM-1, TIMP-1 and VEGF were lower in the M and CM groups, whereas TIMP-1, IL-1 $\alpha$  and CCL5 were higher in the CM group compared to the C and M groups. CCL7 was higher in the M group, whereas CCL7 was lower in the CM group compared to the M group (Fig. 1).

### 3.3. Immunohistochemistry analysis

Immunohistochemistry (IHC) staining of the lung tissue revealed that TIMP-1-, IL-1 $\alpha$ - and CCL5-positive cells were more

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