Hydrogen Peroxide Preconditioning Promotes Protective Effects of Umbilical Cord Vein Mesenchymal Stem Cells in Experimental Pulmonary Fibrosis

Tayebeh Mahmoudi
Kamal Abdolmohammadi
Hamed Bashiri
Mehdi Mohammadi
Mohammad Jafar Rezaie

See next page for additional authors
Authors
Tayebeh Mahmoudi, Kamal Abdolmohammadi, Hamed Bashiri, Mehdi Mohammadi, Mohammad Jafar Rezaie, Fardin Fathi, Shohreh Fakhari, Mohammad Ali Rezaee, Ali Jalili, Mohammad Reza Rahmani, and Lobat Tayebi
Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive inflammatory lung disorder with a not well-known etiology. IPF is identified by high deposition of extracellular matrix (ECM) proteins—mainly collagen—in lung tissue. Despite its obscure etiology, cellular damage and oxidative stress can activate inflammatory processes and recruit immune cells into damaged lung tissue. These uninterrupted inflammatory processes result in provoking lung tissue and unintentional expression of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-8. Along with reactive oxygen species, agents that facilitate apoptosis by inducing the activation of caspases—specifically caspase-3—have been produced.
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The umbilical cord, as a source of hUCV-MSCs by preconditioning, can increase their efficacy. Furthermore, pro-fibrotic cytokines, such as transforming growth factor beta 1 (TGF-β1) and alpha-smooth muscle actin (α-SMA), are overexpressed by differentiated myofibroblasts during fibrosis process in the lung of patients with PF and in animal models of experimentally-induced fibrosis. Bleomycin (BLM) is a chemotherapeutic medication, which can cause PF via regulation of TGF-β1. Therefore, BLM has been used to induce IPF in mice in many interventional studies.

Despite accomplished extensive studies completed to find a decisive treatment of IPF, no effective therapy has been found to cure pulmonary fibrosis (PF) to date. However, IPF is one of the main life-threatening diseases, as expected survival of these patients after initial diagnosis is approximately 3 years. Nowadays, cell therapy using mesenchymal stem cells (MSCs)—with regards to the immunomodulatory and regenerative effects of these cells—is a novel or promising opportunity for the treatment of diverse inflammatory, autoimmune and tissue damage diseases, specifically lung diseases. MSCs are recognized by their spindle-shaped morphology and expression of mesenchymal markers (such as CD44, CD73, CD90, and CD105) but no hematopoietic markers (CD14, CD34, and CD45). These cells have self-renewal capacity and differentiation potential to various cellular lineages, such as adipocytes, osteoblasts, and chondrocytes. Based on several studies, MSCs reduce tissue injury through the production of mediators with anti-apoptotic, anti-inflammatory and anti-fibrotic features. MSCs can be obtained from fetal tissues, such as umbilical cord blood and placenta, or adult tissues, like bone marrow and adipose tissue. Because they have a longer telomere, fetal tissue-isolated MSCs have higher proliferation capacity than that of adult tissue-MSCs. The umbilical cord, as a rich source of MSCs, is readily available and contains more MSCs than other tissues.

According to previous studies, anything that increases the migration ability and survival of MSCs results in a more effective treatment with stem cell therapy. Vital properties of transplanted stem cells—such as survival, migration, and tissue repair capacity—are influenced dramatically by in vitro preconditioning of MSCs. Widespread studies have been accomplished regarding in vitro preconditioning on MSCs, but there is little information provided that investigate the effects of preconditioning of these cells in experimental animal models. In the present study, the anti-inflammatory and anti-fibrotic effects of H₂O₂ preconditioned hUCV-MSCs were evaluated in a bleomycin-induced PF mouse model.

Materials and Methods

Animals

Twenty-eight male mice of the C57BL/6 strain were purchased from Pasture Institute of Iran (Tehran, Iran) and kept in an animal house under standardized conditions. In the time of experiments, mice were 6–8 weeks-old with average weight 20–30 g. The mice were transported to the laboratory for acclimation to the environment 72 h prior to the start of the experiment.

Induction of PF and grouping

Experimental PF was induced by bleomycin under anesthesia, as previously mentioned. The mice were classified into four groups (n = 7). One group received sterile PBS intratracheal (IT) injection, which was considered as a control group (Ctrl). PF group was injected with bleomycin (4 U/kg, suspended in 50 µL sterile PBS, IT). The other treatment groups of mice include the PF+MSC group, which received 2x10⁴ hUCV-MSCs by intratracheal injection 1 week after bleomycin injection, while the PF+pMSC group received 2x10⁵ preconditioned hUCV-MSCs intratracheally.

Isolation, culture, and expansion of hUCV-MSCs

Before participating in the study, informed consent was completed by pregnant women. Umbilical cords were collected from cesarean operations within the healthy term (38–40 weeks) and held in Hanks balanced salt solution buffer containing 300 µg/mL streptomycin, 300 U/mL penicillin, and amphotericin B (1%) (Invitrogen Gibco); then UCs were transferred to the laboratory. The UC vein (UCV) was washed twice with Hanks balanced salt solution and loaded with collagenase IV (0.1%) (Invitrogen Gibco), then the ends of UCV were clamped and incubated (37°C, 5% CO₂, 20 min). The segregated cells were rinsed with Dulbecco’s Modified Eagle’s media with low glucose (DMEM-LG) (Invitrogen Gibco) and centrifuged at 1500 RPM for 15 min. The cell pellets were suspended and cultured in DMEM-LG containing 15% fetal bovine serum (FBS) (Gibco, USA), 100 U/mL of penicillin, and 100 µg/mL of streptomycin, then incubated (37°C and 5% CO₂). Initial media was replaced after 48 hours and non-adherent cells were omitted. Thereafter, this was performed every 48 or 72 hours. After achieving 80-90% confluence, MSCs were incubated with trypsin 0.05% (Sigma, USA) and 0.02% EDTA for new passage and were cultured until passage 4.

Characterization of hUCV-MSCs by flow cytometry analysis

MSCs at the fourth passage were trypsinized, washed using phosphate buffer saline (PBS) and resuspended in PBS containing FBS (1%). A 100 µL aliquot of suspended MSCs was incubated for 45 minutes at 4°C with one of the following anti-human monoclonal antibodies (mAb): phycocerythrin (PE)-conjugated CD105, CD34, and CD73, or fluorescein isothiocyanate (FITC)-conjugated CD45 (BioLegend, USA). In addition, the mouse isotypic antibodies, including PE-IgG1 and FITC-IgG1 (BioLegend, USA), were utilized as control. After labeling...
of cells, they were evaluated using BD FACS Calibur™ flow cytometer (BD, USA) and analyzed using Flow Jo 7.6 Software.

**Characterization of hUCV-MSCs by differentiation assay**

Human UCV-MSCs differentiation abilities into osteocyte and adipocyte lineages were examined at second passage.

**Osteogenic differentiation**

MSCs were cultured at a density of $1 \times 10^4$ cells/well in 24-well plates (SPL, Korea) and incubated at 37°C. After 24 hours, osteogenic differentiation media containing glycerol phosphate (10 mM), dexamethasone (100 mM) and ascorbic acid-2 phosphate (5 g/mL) were added to cells every 72 hours for 3 weeks. After fixing the cells with 4% paraformaldehyde, Alizarin Red S staining utilized for detecting the mineralization in these cells.

**Adipogenic differentiation**

$1.5 \times 10^4$ hUCV-MSCs/well were seeded in 24-well plates (SPL, Korea) and incubated at 37°C for 24 hours. Adipogenic differentiation media containing indomethacin (100 mM), 3-isobutyl-methylxanthine (0.5 mM), dexamethasone (250 mM) and insulin (5 mM) were added to cells every 3 days and incubated at 37°C for 2 weeks. After fixing the cells with 4% paraformaldehyde, Oil Red O staining utilized for determination of adipose vacuoles in these cells.

**Preconditioning of hUCV-MSCs with H$_2$O$_2$**

MSCs were preconditioned with 15 μmol H$_2$O$_2$ for 24 hours, as previously reported. This non-toxic concentration of hydrogen peroxide (15 μmol) induces a protective influence on MSCs against the lethal dose of H$_2$O$_2$ (300 μmol).

**Transplantation of hUCV-MSCs to experimental models (treatment procedure)**

One week after bleomycin injection, the mice were euthanized, and hUCV-MSCs were prepared for administration to Mice. Both the hUCV-MSCs and H$_2$O$_2$ preconditioned hUCV-MSCs (p-MSC), at passage 4 with a concentration of $2 \times 10^5$ cell/mouse, were suspended in 50 µL sterile PBS and administered intratracheally. The control groups received only PBS in the treatment time. The survival rate and weight of mice were recorded every day, and mice were sacrificed 14 days after the treatment. Lung tissues of the mice were removed promptly, shared in several parts, fixed in formalin for histopathological and immunohistochemical (IHC) examinations or frozen at -70°C in order to investigate MPO enzyme activity. Design of study is demonstrated in Figure 1.

**Histopathological examination**

Formaldehyde-fixed (10% buffered formaldehyde) lung tissues were embedded in paraffin (5 μm) and stained with hematoxylin-eosin (H&E) to investigate the volume of alveolar space and connective tissue. In addition, Masson’s trichrome was applied to evaluate collagen deposition and connective tissue mass. The lung tissue sections were examined using the light microscopy at ×100 and ×400 magnifications. Our histologist colleague (MJ,R) was blinded to each group and performed the histopathological evaluation of lung sections. Moreover, in order to exactly investigate the histopathological changes, the volume of the alveolar space and connective tissue in all of the experimental groups were assessed applying morphometric analysis (graticule checkerboard 18×kpl-w12.5). 8,31

**IHC examination**

5-μm sections were obtained from paraffin-embedded
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Myeloperoxidase (MPO) enzyme activity
Neutrophil accumulation within the lung tissues was investigated by measuring the tissue MPO activity that was described previously.6,35 Frozen tissue samples were thawed at 4°C, then the tissues were homogenized in 20 mmol/L phosphate buffer (pH 7.4) and centrifuged (13,000 × g, 10 min, 4°C). The obtained pellet was resuspended with 0.5% hexadecyl trimethyl ammonium bromide (Sigma-Aldrich) in 50 mmol/L phosphate buffer (pH 6.0). Freeze-thaw cycles were done on the suspension four times, followed by sonication. The samples were recentrifuged (13,000 × g, 8 min, 4°C). MPO activity in the resulting supernatant was measured using TMB for ELISA at 405 nm. The results were reported as the absorbance per mg of lung tissue weight (A°/mg).

Statistical analysis
Data was analyzed applying Graph Pad Prism 5 software. All data was represented as a mean ± standard deviation from the total number of mice in this study. Normality of all data has been checked with Kolmogorov-Smirnov normality test and differences between groups were analyzed by one-way ANOVA and Tukey post test. P < 0.05 was demonstrated statistical significance.

Result and Discussion
Characterization of human UCV-MSCs by flow cytometry analysis and differentiation assay
MSCs at passages two were analyzed with regard to the high expression level of the mesenchymal markers (CD73 and CD105) and lack of expression of the hematopoietic markers (CD34 and CD45) (Figure 2). In addition, differentiation potency of these cells is demonstrated by staining osteocytes and adipocytes with Alizarin Red S and Oil Red O, respectively (Figure 3).

Survival and weight monitoring
The survival rate in all mice is similar between experimental groups (data not shown). All mice were sacrificed at the end of the experiment time (21 d) to perform histopathological and immunological investigations. In addition, the results of mice weight monitoring demonstrate no significant difference between PF and treated groups (PF+MSC, PF+pMSC) (data not shown).

Histopathological findings
The results of H & E staining at day 21 show a significant decrease of alveolar space and increase of connective tissue in mice receiving bleomycin (PF group), in comparison to the negative control group (Ctrl) (P<0.001). In addition, infiltration of inflammatory cells, fibroblasts, and consequent diffuse fibrosis confirmed the induction of PF using BLM. In the group treated with hUCV-MSCs (PF+MSC) and preconditioned MSCs (PF+pMSC), the alveolar space is significantly increased (P<0.001), while the connective tissue is significantly lower compared to the positive control group (PF) (P<0.001). Also, compared to the PF+MSC group, preconditioned MSCs transplantation results in significantly decreased connective tissue (P<0.005) and increased alveolar space (P<0.05) (Figure 4).

In addition, Masson’s trichrome was applied to evaluate the ECM macromolecules, such as collagen deposition. The results of trichrome staining at day 21 show a considerable increase of the collagen deposition (blue color) in the PF group in compared to the Ctrl group. In the
groups treated by hUCV-MSCs (PF+MSC), and especially preconditioned MSCs (PF+pMSC), a remarkable decrease of collagen deposition compared to the positive control group (PF) is observed (Figure 5).

Immunohistochemical analysis of TGF-β1 and α-SMA expression

According to IHC staining results, there is a high expression of the TGF-β1 and α-SMA protein in the parenchyma areas of lung tissues from PF group compared to the negative control (Ctrl) group. However, the group administered with hUCV-MSCs (PF+MSC) and preconditioned MSCs (PF+pMSC) show a remarkable decrease of the TGF-β1 and α-SMA protein expression compared to the positive control group. In addition, α-SMA expression is mainly localized in the pulmonary vessel walls in the negative control (Ctrl) group, whereas in the PF group was localized in the parenchyma of fibrotic areas (Figures 6 and 7).

MPO enzyme activity assay

MPO has been known as a valuable biomarker of lung inflammation and injury. MPO enzyme activity confirms...


the oxidative stress following BLM exposure and extent/activation of inflammatory cells, such as neutrophils or monocyte/macrophages in lung tissue. Accordingly, Figure 8 demonstrates a five-fold increase in MPO activities following BLM injection (PF group) as compared to the negative control (Ctrl) group \((P < 0.001)\). HUCV-MSCs and preconditioned MSCs significantly decrease MPO activities in the treated groups (PF+MSC \((P < 0.01)\) and PF+pMSC \((P < 0.001)\)) as compared to the PF group. Yet, no significant difference is observed between the two treated groups.

PF is a progressive inflammatory and fibrotic lung disorder, distinguished by overexpression of pro-inflammatory cytokines, reactive oxygen species and pro-fibrotic cytokines, such as TGF-β1. Deposition of ECM proteins, like collagen, aggravates the lung injury. Moreover, the connective tissue will reduce the alveolar space of the lung. To date, there is no useful cure for PF. The only possible treatment approach for PF patients at the end stage of the disease is lung transplantation. However, PF is a life-threatening disease. Regarding fibrosis immunopathogenesis, many previous studies have revealed that MSCs, because of anti-inflammatory and anti-fibrotic effects, could be potentially helpful for the treatment of PF. Nevertheless, the inappropriate environment of inflammatory and fibrotic tissue with large amounts of pro-inflammatory cytokines, reactive oxygen species, and many other harsh factors will diminish the potency of cell therapy after transplantation.

Some studies reported that in vitro preconditioning of MSCs could promote essential features, such as survival, migration and tissue repair capacity of transplanted stem cells against harsh microenvironment. Regarding the previous studies and the necessity of conducting in vivo study, our work is among the few studies ever done in this field on experimental animal models.

In the current study, we are the first to evaluate the anti-inflammatory and anti-fibrotic effects of H2O2.
Nevertheless, local study, administration of there are a few studies to preconditioning of MSCs with 20 μM H$_2$O$_2$ (12 h) improved cell resistance against lethal microenvironment via HIF-1α overexpression. Also, Bashiri et al$^{27}$ demonstrated that preconditioning with 15 μM (24 hours) has better protective effects than other concentrations of H$_2$O$_2$. For this reason, we used 15 μM (24 hours) for preconditioning of the hUCV-MSCs in the present study.

**Conclusion**

The results of this study reveal that in vitro preconditioning of MSCs with a sub-lethal concentration of H$_2$O$_2$ can improve the therapeutic potency of MSC therapy in IPF and diminish inflammatory and fibrotic factors in bleomycin-induced PF. Nevertheless, further examinations should be conducted to evaluate the anti-inflammatory and anti-fibrotic effects of H$_2$O$_2$ preconditioned MSCs in other experimental models of diseases. In addition, future studies are required to investigate the cytoprotective mechanisms involved during the in vitro preconditioning.

**Conflict of Interest**

The authors declare that they have no conflict of interest.

**Ethical Issues**

All of the animal studies were performed under approval of the Ethics Committee of the Kurdistan University of Medical Sciences, Sanandaj, Iran (Project number: 1392.106, Approval date: Feb 16, 2014).

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