

A study on the role of apoptotic human umbilical cord mesenchymal stem cells in bleomycin-induced acute lung injury in rat models

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Abstract. – OBJECTIVE: We sought to determine whether normal human umbilical cord mesenchymal stem cells and apoptotic human umbilical cord mesenchymal stem cells play any role in the lung repair following bleomycin-induced lung injury in rat models.

MATERIALS AND METHODS: Umbilical cord mesenchymal stem cells were obtained from the umbilical cord following caesarian section from healthy normal babies. Plasmin deprivation method was used for culture of human umbilical cord mesenchymal stem cells and flow cytometry was used to identify cell surface antigen and activity of stem cells and apoptosis. The animal model of acute lung injury was established by a one-off intratracheal instillation of bleomycin (BLM) (5 mg/kg) and then normal stem cells and apoptotic stem cells were separately injected. Alveolar lavage fluid and lung tissue were collected for further analysis prior to the injury and at days 3, 7, 14 after administration of BLM. The number of neutrophils in the broncho alveolar lavage fluid (BALF) was counted; Bicinchoninic Acid (BCA) method was used for estimation of total protein content in alveolar lavage fluid; biochemical assay was used for estimation of myeloperoxidase (MPO) activity; hematoxylin and eosin (HE) staining of lung tissue was used for histopathology analysis; reverse transcription-polymerase chain reaction (RT-PCR) assay was used for the determination of interferon-gamma (INF- γ) and mRNA changes of interleukin-4 (IL-4) in lung tissue. Enzyme-linked immunosorbent assay (ELISA) was used for the determination of cytokines TNF- α in the lung tissue.

RESULTS: Apoptotic human umbilical cord mesenchymal stem cells were more effective in reducing lung neutrophil infiltration and total protein leakage in rat models of acute lung injury (ALI). There was also an improvement in the degree of vascular permeability, reduction in the level of proinflammatory cytokines, INF- γ gene level and boost in anti-inflammatory cy-

tokine IL-4 levels which also helps in more effectively reducing the degree of injury in ALI.

CONCLUSIONS: Human umbilical cord mesenchymal stem cell transplantation may have a bright future in the clinical setting for the treatment of ALI/acute respiratory distress syndrome (ARDS). Apoptotic human umbilical cord mesenchymal stem cells may have more effective than normal human umbilical cord mesenchymal stem cells in the treatment of acute lung injury.

Key Words:

Apoptosis, Cytokines, Human umbilical cord mesenchymal stem cells, Bleomycin, Acute lung injury.

Abbreviations

ALI = acute lung injury; BLM = intratracheal instillation of bleomycin; RT-PCR = reverse transcription-polymerase chain reaction; INF- γ , interferon-gamma; ARDS = acute respiratory distress syndrome; HUMSCs = human umbilical cord mesenchymal stem cells; SD = Sprague Dawley; PS = phosphatidyl serine; FBS = fetal bovine serum; DMEM = Dulbecco's Modified Eagle Medium; BSA = bovine serum albumin

Introduction

Acute lung injury (ALI) is a type of acute lung inflammatory reaction and describes clinical syndromes of acute respiratory failure with substantial morbidity and mortality. Even in patients who survive ALI, there is evidence that their long-term quality of life is adversely affected^{1,2}. The acute inflammation in ALI causes disruption of the lung endothelial and epithelial barriers. Cellular characteristics of ALI include loss of alveolar-capillary membrane integrity, excessive transepithelial neutrophil migration, and release of pro-inflam-

matory cytokines^{3,4}. Biomarkers found in the epithelium and endothelium, and that are involved in the inflammatory and coagulation cascades predict morbidity and mortality in ALI. Recent advances have been made in the understanding epidemiology, pathogenesis, and treatment of this disease. Further, more progress and research is required to reduce the mortality and morbidity from ALI and ARDS. Therefore, in this study we sought to determine whether normal human umbilical cord mesenchymal stem cells and apoptotic human umbilical cord mesenchymal stem cells are involved in the lung repair following bleomycin-induced lung injury in rat models.

Mesenchymal stem cells (MSCs) have basic characteristics such as self-replication properties and multi-directional differentiation capacities. It is currently a hot topic in the study of stem cells transplantation. In recent years, MSCs have been introduced into the treatment of lung injury⁵. Studies have shown that MSCs implanted in animal models where bleomycin (BLM) has been used to induce acute lung injury can differentiate into type I alveolar cells, type II alveolar cells, endothelial cells and fibroblasts in the damaged lungs⁵. Furthermore, MSCs were also involved in the inhibition of inflammatory response, the triggering of repair-related growth factors and lessened to different extents the inflammatory process of the lung, which, thus, improved survival rates⁵.

Stem cell transplantation has brought a new ray of hope for the treatment of ALI/ARDS⁶⁻⁹. At present, MSCs is mainly obtained from bone marrow, but this source is relatively less. In addition to this, the acquisition of stem cells is done through an invasive and difficult procedure, with an increased risk of exposure, which has greatly restricted the clinical use of MSCs. Human umbilical cord mesenchymal stem cells (HUMSCs) is a type of MSCs, which is separated and extracted from discarded placenta. It is not only easy to obtain, with a rather wide variety of sources, but compared to bone marrow-derived MSCs, placenta-derived MSCs have lower immunogenicity¹⁰. Furthermore, it differentiates more easily *in vitro*, has less ethical constraints and is, thus, garnering more attention among researchers. Placenta-derived MSCs is becoming one of the most promising types of cells in the spheres of tissue engineering and regenerative medicine¹⁰.

Recent studies¹¹ have found that apoptotic stem cells have demonstrated unique anti-inflammatory properties and immunomodulatory effects. In acute myocardial infarction, apoptosis of

transplanted stem cells modulates local tissue reactions. Apoptotic cells affect immune reactivity by down-regulating innate and adaptive immunity, inhibiting the activation of macrophages and dendritic cells, and stimulating regulatory-T cells¹¹. This leads to reduced scar formation, repressed myocardial apoptosis, and improved cardiac outcome. Autologous blood, exposed *ex-vivo* to oxidative stress to induce cell apoptosis and administered intramuscularly decreases the production of inflammatory mediators, increases anti-inflammatory cytokines, and decreases cellular injury¹²⁻¹⁴. Pre-treatment of spontaneously hypertensive rats with apoptotic cells reduced severe renal ischemia-reperfusion injury¹⁵. Direct instillation of apoptotic cells enhanced the resolution of acute inflammation in the lung¹⁶. In this study, apoptotic cells with externally exposed PS-induced TGF-beta1 secretion, resulting in an accelerated resolution of inflammation. This *in vivo* study clearly demonstrates the proposed concept of anti-inflammatory actions of apoptotic cells.

Based on the role and properties of MSCs and studies discussed above, we assumed that apoptotic umbilical cord mesenchymal stem cells when compared to normal umbilical cord mesenchymal stem cells, in the treatment of acute lung injury, would have a stronger anti-inflammatory, anti-oxidant, inflammation inhibition, neutrophil infiltration inhibition, inhibition of total protein leakage and improvement of vascular permeability role, thus leading to an improvement of acute lung injury. Therefore, we used a large rat model of bleomycin-induced acute lung injury. We infused normal or apoptotic umbilical cord stem cells and detected the ensuing changes in lung tissue. We measured the level of lung tissue inflammation and the level of other indicators to evaluate apoptotic umbilical cord stem cells and normal umbilical cord mesenchymal stem cells and the mechanisms of their protective effect in acute lung injury. We then attempted to provide a theoretical foundation for future clinical improvement in the use of stem cell transplantation in acute lung injury.

Materials and Methods

Experimental Animals

Clean male Sprague Dawley (SD) rats 120, weight (250 ± 20) g obtained from Animal Center of Chinese Academy of Sciences) were used in all experiments. They were randomized into various

groups, weighed and blood samples were collected. Animals were maintained in the animal care facility at the zoon of Zhongshan Hospital Affiliated to Fudan University, Shanghai, China. Approval of the experimental protocol by the Animal Center of Chinese Academy of Sciences was obtained before conducting the experiments.

Reagents

The various chemicals/reagents were purchased from different suppliers of the company. Bleomycin (Sigma-Aldrich, St. Louis, MO, USA); DMEM/F12 medium, fetal bovine serum (Gibco, Grand Island, NY, USA); PE anti-mouse CD45, CD 105, FITC anti-mouse CD29, CD34, CD44 (eBioscience, San Diego, CA, USA); BCA Protein Assay Reagent box, RIPA lysis buffer (Jiangsu Haimen City Yun Tian Biotechnology Research Institute); ACE2 antibody (Cell Signal Company, Danvers, MA, USA); MPO detection kit (Nanjing Jiancheng Bioengineering Institute); Trizol (Invitrogen, Carlsbad, CA, USA); reverse transcription kit (Promega Corporation, Madison, WI, USA); INF- γ , IL-4 primers (Shanghai Biological Engineering Company); cDNA synthesis kit (Promega Corporation, Madison, WI, USA); TNF- α ELISA kit (American R & D Company, Hercules, CO, USA).

Isolation and Culture of HUMSCs

Under sterile conditions, the umbilical cord of normal healthy full-term infants who were delivered by cesarean section was obtained from the First People's Hospital Affiliated to Shanghai Jiao-tong University, Shanghai, China. The specimens were placed into D-Hank's buffer containing antibiotic (100 U/mL penicillin and 100 U/mL streptomycin). The tissue pieces measuring about 1mm² were cut down after washing out the blood and the blood vessels removed. They were, then, seeded in an appropriate cell culture plate, con-

taining antibody and 10% FBS DMEM/F12 medium, cultured at 37°C in a saturated humidity and 5% CO₂ incubator. Fresh medium was added every three days. When colonies of fibroblasts-like cells were seen growing around the tissue blocks under a light microscope, the tissue blocks were removed, the culture medium was added and culture was continued. When the cells were grown to approximately 80% confluence, we used 37°C pre-warmed digestive juices containing 0.25% trypsin and 0.02% EDTA for digestion. The cells were, then, transferred to culture flasks at a density of 10⁴/cm².

Determination of Normal vs. Apoptotic Umbilical cord Mesenchymal Stem Cells

The 4th generation of cells was collected and the medium was aspirated out, 0.25% trypsin and 0.02% EDTA digestive juices was added for digestion purposes, and then an adequate amount of serum was used to terminate the digestion process. After centrifugation at 1000 rpm for 10 min, the supernatant was discarded, pre-cooled at 4°C. PBS was used to wash the cells three times and was, then, resuspended. PE anti-mouse CD86, CD45, CD105, FITC anti-mouse CD29, CD34, CD44 marker HUMSCs were separately added. After incubation at room temperature for 30 min, centrifugation at 1500 rpm for 10 min was performed and after that fixation was carried out. Analysis of HUMSCs phenotype was done with flow cytometry (Table I). The normal umbilical cord stem cells were cultured in normal culture medium with adequate nutritional supply. Serum deprivation of cells *in vitro* has been documented to induce apoptosis (36); hence, apoptotic umbilical cord mesenchymal stem cells were first cultured in normal culture medium followed by 96 hr of serum-free cell culture. The percentages of viable and dead cells were determined by flow cytometry using double staining

Table I. Flow cytometric analysis of normal umbilical cord mesenchymal stem cells and apoptotic umbilical cord mesenchymal stem cells surface markers following day-14 of cell culture.

Stem cell surface markers	Normal umbilical cord mesenchymal stem cells	Apoptotic umbilical cord mesenchymal stem cells additional 96 hr serum-deprived cell culture was performed	p-value
Early apoptosis (annexin V+/PI-)	4.1 ± 1.3	11.3 ± 1.9	< 0.001
Late apoptosis (annexin V+/PI+)	6.14 ± 1.23	19.1 ± 2.3	< 0.001

Data are expressed as percentage; n = 6 in each experimental study

of annexin V and propidium iodide (PI); this is a simple and popular method for the identification of apoptotic cells (i.e., early [annexin V+/PI-] and late [annexin V+/PI+] phases of apoptosis).

Bleomycin Administration

About 116 healthy adult male SD rats were used in this study. They were randomly divided into four groups, each group had 24 animals and the group were as follows: (1) BLM group (animal models with BLM-induced lung injury, injection of saline as control treatment), (2) normal umbilical cord mesenchymal stem cell group (animal models with BLM-induced lung injury, an injection of normal HUMSCs, was referred to as NHUMSCs group), (3) apoptotic umbilical cord mesenchymal stem cells group (animal models with BLM-induced lung injury, infusion of apoptotic HUMSCs, referred to as APHUMSCs group), (4) NS control group (normal animals).

In the BLM control group, NHUMSCs group, APHUMSCs group, we used intratracheal instillation of BLM (5 mg/kg) to cause lung injury. A normal saline infusion was used for the control group. In the BLM control group, 3 ml saline was injected into the rats via the penile vein at different time intervals (30 minutes, 6 hours and 24 hours). In the NHUMSCs group, 1.2×10^6 normal umbilical cord vein mesenchymal stem cells were injected into the penile vein at 30 min, 6 hrs and 24 hrs. Similarly, in the APHUMSCs group, 1.2×10^6 apoptotic umbilical cord mesenchymal stem cells were injected into the penile vein at 30 min, 6 hrs and 24 hrs. Different samples and specimens were then collected for analysis at different time intervals i.e. before lung injury (D0), 3 days after lung injury (D3), 7 days after lung injury (D7) and 14 days after lung injury (D14).

Survival Analysis

The living conditions of each animal were separately recorded for all the 12 animals in each group. The number of days of each animal survived was recorded. The animals living conditions were observed during the 14-days study period. The survival rate was calculated and the survival curve plotted.

Bronchoalveolar Lavage Collection and Determination of Neutrophil Numbers and Total Protein

The lungs were weighed, and bronchoalveolar lavage fluid (BALF) was collected as described previously¹⁷. Total cell count in BALF was deter-

mined using a hemocytometer. The BALF was centrifuged immediately at 300 g for 20 min. The precipitate was stained with Wright-Giemsa dye, and differential cell count, based on morphologic criteria, was carried out on 200 consecutive cells. The number of polymorphonuclear neutrophils (PMNs) in BALF was calculated by multiplying the ratio of PMNs with the total cell count¹⁸. The protein concentration in the supernatant was determined by the method of Bradford¹⁹, with bovine serum albumin as the standard protein.

Histological Examination

The left lung specimens were fixed in the distended state by infusion of 10% formalin into the trachea and then kept for 1 week. The lung tissues were processed automatically using a Leica Microsystem tissue processor (ASP 300S, Weitzalar, Germany), then embedded in paraffin blocks, cut into 7 μ m slices using a Leica Microsystem microtome (Model RM 2265, Nussloch, Germany), and finally stained with hematoxylin and eosin using a Leica Microsystem auto strainer (XL, Germany). The slides were viewed under an Olympus microscope (4X-1, Tokyo, Japan), and evaluated by two different pathologists in a double-blind manner. The following four aspects: alveolar congestion, hemorrhage, infiltration or aggregation of neutrophils in air space or vessel wall, and thickness of alveolar wall/hyaline membrane formation were graded with five point scales: 0 = minimal damage, 1 = mild damage, 2 = moderate damage, 3 = severe damage, and 4 = maximal damage. A total lung injury score was calculated as the sum of the four items²⁰.

Lung Tissue Morphology Study and Lung injury Pathology Severity Score

Preparation of paraffin sections of lung tissues: dewaxing was done with dimethyl benzene and alcohol with low concentration. Hematoxylin staining done for 10 min, differentiation in 1% ethanol for 1s, 1% ammonia water back to blue, staining with 0.5% water-soluble dye eosin for 20-30s, dehydration with low concentration alcohol, made transparent with dimethyl benzene and mounted with neutral mounting medium. Light microscopy was used to observe the pathologic lesions of the lung tissues and changes in the neighboring tissues.

Lung injury severity scoring was carried out by an experienced pathologist in a double-blind fashion (Table II). He focused on signs of pulmonary

Table II. The scoring of the severity of the lung tissue injury during the disease process.

Group control group	BLM group	NHUMSCs group	APHUMSCs group	NS
D0	9.54±1.24	7.97±2.01*#	5.85±1.87*	4.12±1.47
D3	19.85±2.82	16.98±3.27*#	13.88±1.21*	6.01±2.33
D7	16.66±1.23	14.50±2.81*	11.24±2.46*	5.06±3.29
D14	12.85±2.23	9.09±2.84*#	7.83±1.56*	4.34±2.22

*When compared to the BLM experimental group over the same period of time, $p < 0.05$; #Comparison of NHUMSCs group and APHUMSCs group over the same period of time, $p < 0.05$.

congestion/pulmonary hemorrhage, alveolar exudation/alveolar and vessel wall neutrophil infiltration, degree of thickening of the alveolar walls and formation of hyaline membrane, four indexes which were used to score the severity of lung injury. The scoring was done as follows: 0 = no damage, 1 = mild damage, 2 = moderate damage, 3 = severe damage, 4 = very severe injury. Final scores were aggregated, out of which represents the degree of pulmonary injury.

Determination of Lung MPO Activity

Myeloperoxidase (MPO) activity was assayed as an index of neutrophil infiltration in rat lung that was subjected to bleomycin-induced ALI. Frozen lung tissues were thawed and immersed in 50 mM phosphate buffer containing 0.5% hexadecyl trimethyl ammonium bromide. Lung tissue samples were sonicated on ice using a homogenizer, and the homogenate was then centrifuged at 2,000 g for 15 min at 4°C. The MPO activity of homogenates was determined by adding them to phosphate buffer (pH 6.0) containing 0.167 mg/mL o-dianisidine hydrochloride and 0.0005% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO, USA). The light absorbance was monitored at 460 nm by a spectrometer for 5 min according to the kit instructions. MPO activity was calculated by comparing results with the standardized concentration curve derived from commercial human MPO (Sigma). Final values were all normalized to the corresponding protein concentration. A Bio-Rad assay kit (Hercules, CA, USA) was used to detect the protein content of every sample. Lung MPO activity was finally expressed in units per gram of tissue.

Determination of Lung Water Content

After removal of wet weight from fresh lung tissue, then oven roasted to constant weight, also referred to as dry weight. The lung tissue was

weighed at both times and the lung wet to dry ratio (W/D) was calculated and reflects the degree of pulmonary edema.

The water content of the lung was calculated as:

$$\text{water content} = \frac{\text{lung wet weight} - \text{lung dry weight}}{\text{lung wet weight}} \times 100(21).$$

Real Time-PCR

50 mg of lung tissue was used, TRIzol reagent was added and then total RNA was extracted. cDNA was synthesized according to the specification of the reverse transcription kit. IL-4 gene primer sequences were as follows: Forward primer: 5'-CTG ACG GCA CAG AGC TAT TGA-3', reverse primer: 5'-TAT GCG AAG CAC CTT GGA AGC-3'. INF- γ gene primer sequences were as follows: Forward primer: 5'-GCA TCT TGG CTT TGC AGC T-3', reverse primer: 5'-CCT TTT TCG CCT TGC TGT TG-3'. β -actin gene primer sequences were as follows: forward primer: 5'-AGC TGC GTT TTA CAC CCT TT-3', reverse primer: 5'-AAG CCA TGC CAA TGT TGT CT-3'. Real-time quantitative PCR reaction conditions were as follows: 12.5 μ l SYBR Green premix Ex TaqTM 1 $\times$, 1 μ l forward primer, 1 μ l reverse primer, cDNA template 2 μ l, 8.5 μ l dH₂O. Quantitative PCR amplification conditions set were as follows: 95°C denaturation 15s; 55°C annealing 15s; 72°C extension 15s; 40 cycles. We used the 2- $\Delta\Delta$ Ct method to analyze the data, this method is a relatively convenient method for gene expression analysis. Wherein, Δ Ct = Ct (target gene) - Ct (reference gene), $\Delta\Delta$ Ct = Δ Ct (sample) - Δ Ct (control).

ELISA

The determination of the TNF- α level was done according to the instructions in the ELISA kit manual.

Table III. Comparison of the survival rate between the groups using Log Rank (Mantel-cox).

Group	BLM group	NHUMSCs Group	APHUMSCs group	NS control group
BLM group	-	0.087	0.037*	0.000**

*When compared to BLM group, $p < 0.05$ **When compared to BLM group, $p < 0.01$

Statistical Analysis

The experiment was repeated three times. We used SPSS13.0 (SPSS Inc., Chicago, IL, USA) for our statistical analysis. One-way ANOVA Bonferroni test was used for comparison between multiple samples and $p < 0.05$ was considered to be statistically significant.

Results

HUMSCs Histological Analysis

Under sterile conditions, HUMSCs were obtained and isolated at 37°C from the umbilical cord following a caesarian section of normal full-term healthy infants. After being cultured in 5% CO₂ incubator for 1-2 days, fusion form adherent cells were partly seen under the microscope, the morphology of the cells was relatively consistent after 7 days and long spindle cells formed which grew in colonies. After 14 days, 80% confluent monolayer of adherent cell was formed (Figure 1).

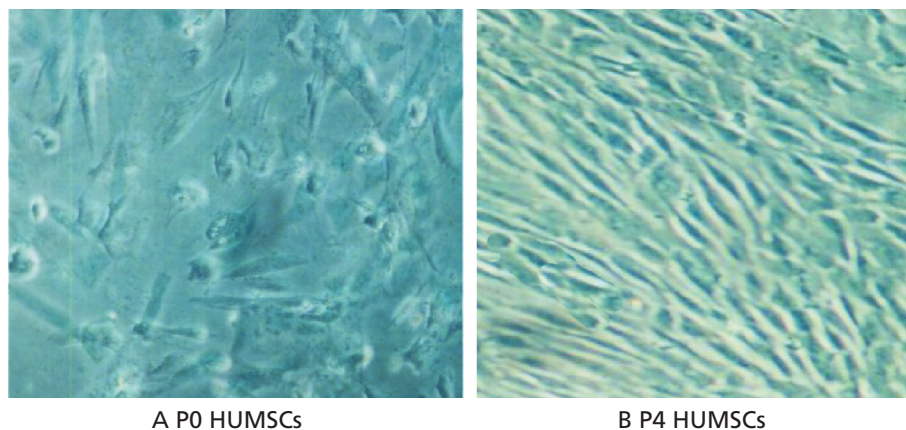
Survival Analysis

By drawing the Kaplan-Meier survival analysis graph, we found that the model animals were

more liable to dye between the day 3-day 7 interval, while the mortality rate was lower outside of this range (Figure 2). We used Log Rank (Mantel-cox) (as shown in Table III) to compare the survival rates between the intervention group and the BLM group. The results show that there was a significant difference in the APHUMSCs group, indicating that apoptotic umbilical cord mesenchymal stem cells (APHUMSCs) when used in lung injury has certain advantages.

Change in the Number of Neutrophils in BALF

Neutrophils are the index of choice for pulmonary vascular permeability. Under normal circumstances, the number of neutrophils in BALF is less. After BLM administration, each experimental group experienced an increase in the number of neutrophils in BALF, and there was a significant difference when compared to the NS control group ($p < 0.05$), demonstrating that there was an inflammatory response in the lung (Figure 3). The NHUMSCs group, BLM group and APHUMSCs group when compared over the same time period, the number of neutrophils at day 3, 7 and 14 when compared to the BLM group showed a time-de-

**Figure 1.** HUMSCs morphology ($\times 100$).

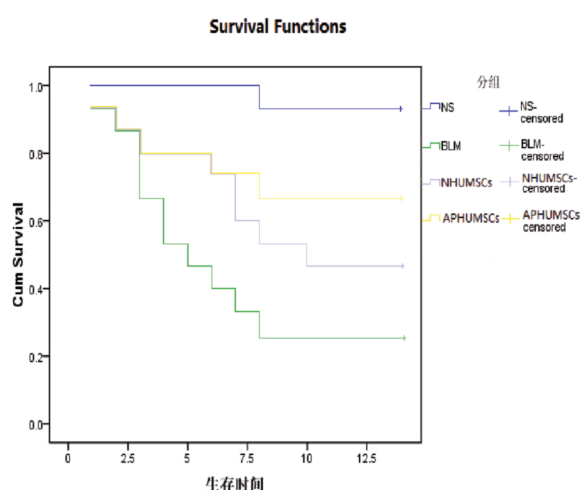


Figure 2. Survival analysis.

pendent decline, with the APHUMSCs group showing a very obvious reduction, the difference was statistically significant ($p < 0.05$).

Changes in Total Protein Content in BALF

An important indicator of pulmonary vascular permeability is the amount of total protein in BALF. Increased vascular permeability and increase in the amount of plasma protein extravasations is very suggestive of inflammation injury. BALF total protein analysis showed that following BLM administration, in each experimental group, the total protein content of BALF was increased. At day 3 of the experiment, the BALF total protein expression was maximum.

When the NHUMSCs group and APHUMSCs group were compared to the BLM group over the same time period, the total protein content of BALF was significantly lower in the APHUMSCs group. At day 3, 7 and 14, when compared over the same time period, the BLM group showed a time-dependent decrease pattern and this decrease was statistically significant ($p < 0.05$) (Figure 4).

Lung tissue Morphology and Pathological Lung Injury Severity Score

HE staining of lung tissues showed that the lung tissue damage was most severe at day 3 and day 7. There was pulmonary edema with increased inflammatory cell infiltration, severe pulmonary fibrosis, and these findings were consistent with the time distribution of animal deaths. The severity of lung injury was reduced; after that, alveolar inflammation improved. There was a small amount of lymphocytic infiltration and a small amount of fibroblast proliferation. At day 7, the HE staining results were as follows: the NS control group showed normal lung tissue structure, alveoli showed structural integrity and were smooth, no inflammatory cell infiltration was noticed (Figure 5A). In the BLM group, there was lung edema and a small amount of bleeding. Light microscopy investigation showed a large amount of neutrophil infiltration, alveolar septal thickening and alveolar wall destruction (Figure 5B). In the NHUMSCs group and APN-HUMSCs group, there was decreased infiltration of inflammatory cells and the degree of lung injury, when compared to the model group, was significantly reduced. APHUMSCs group

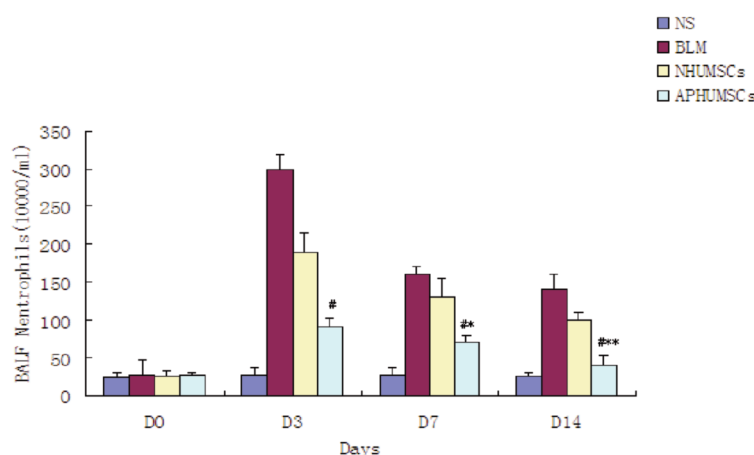


Figure 3. BALF neutrophil count

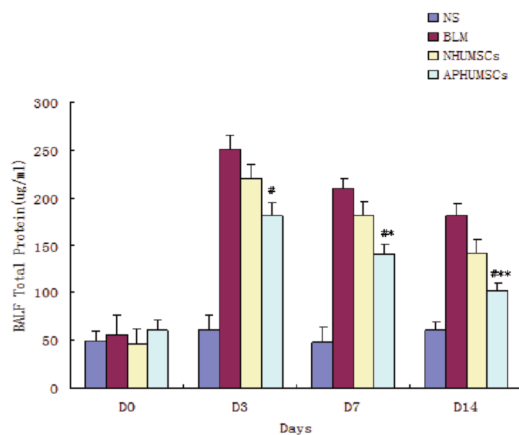


Figure 4. The changes in total protein content in BALF. *When compared to the BLM group over the same period of time, $p < 0.05$. **When compared to the D3 APHUMSCs group, $p < 0.05$. ***When compared to the D3 APHUMSCs group, $p < 0.01$.

showed a larger degree of improvement than the NHUMSCs group (Figure 5C and D).

Pathological severity scoring of lung injury showed that, after intervention with NHUMSCs and APHUMSCs, lung tissue damage improved to varying degrees, especially in the APHUMSCs group. Table II shows that, after induction with BLM, all experimental groups experienced lung tissue damage, with the worst change being no-

ticed at day 3. When NHUMSCs group and APHUMSCs group are compared to the BLM experimental group, the pathological score at day 3, 7 and 14 for NHUMSCs and APHUMSCs groups were lower than that of the BLM group and the difference was statistically significant, $p < 0.05$. When the APHUMSCs group is compared to the NHUMSCs group over the same period of time, the APHUMSCs group had a lower score and the difference was statistically significant, $p < 0.05$.

Measurement of Lung Tissue MPO Activity

Lung tissue MPO activity is an excellent indicator of the degree of infiltration of neutrophils in lung tissue and it can indirectly reflect the degree of lung inflammation and lung injury. As shown in Figure 6, after stimulation with BLM, MPO activity was significantly higher in all the experimental groups, peaking at day 3, and falling between day 7 and day 14. When compared to the NS control group, the difference was statistically significant ($p < 0.05$). NHUMSCs group and APHUMSCs group when compared to the BLM group over the same time period, showed lower MPO activity. APHUMSCs group had a significantly lower MPO activity. Compared over the same period of time, on day 3, 7 and 14, the BLM control group showed a time-dependent decrease in MPO activity and the difference was statistically significant ($p < 0.05$). On

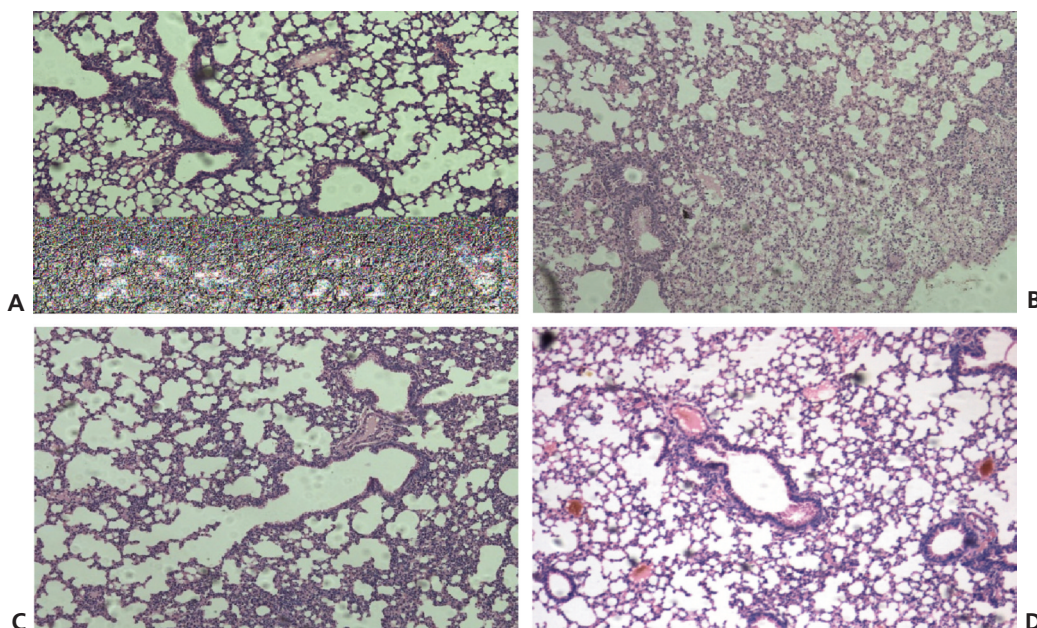


Figure 5. D7 HE staining of lung tissue ($\times 200$). **A**, NS group. **B**, BLM group. **C**, NHUMSCs group. **D**, APHUMSCs group.

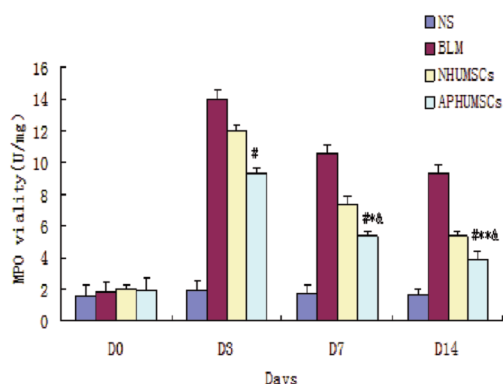


Figure 6. MPO activity in lung tissue. #When compared to the NS group over the same period of time, $p < 0.05$. *When compared to the D3APHUMSCs group, $p < 0.05$. **When compared to the D3APHUMSCs group, $p < 0.01$. &When compared to the HUMSCs group over the same period of time, $p < 0.05$.

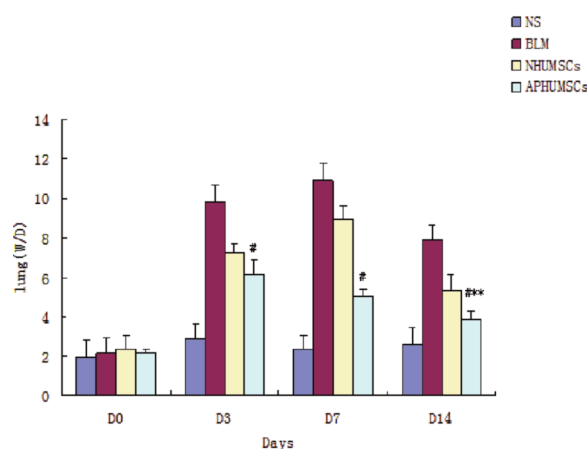


Figure 7. Water content of lungs. #When compared to the BLM group over the same time period, $p < 0.05$. **When compared to D3 APHUMSCs group, $p < 0.01$.

day 3, APHUMSCs group had a lower MPO activity than that of the NHUMSCs group, but the difference was not statistically significant. At day 7 and day 14, APHUMSCs group had a significantly lower MPO activity than that of NHUMSCs group, which goes to show that apoptotic umbilical cord mesenchymal stem cells is more effective in reducing the MPO activity of lung tissue than normal umbilical cord mesenchymal stem cells (Figure 6).

Determination of Lung Water Content

Pulmonary edema was measured by lung wet and dry ratio method. A significant increase in wet dry ratio was noticed after BLM intervention as compared to the NS group. The APNHUMSCs group began to decrease after reaching a peak on

day 3. NHUMSCs group peaked on day 7. NHUMSCs group and APNHUMSCs when compared to the BLM group over the same time period, the lung wet-dry ratio were decreased, with the most noticeable decrease being in the APNHUMSCs. These points to the fact that apoptotic umbilical cord mesenchymal stem cells are better than normal umbilical cord mesenchymal stem cells at reducing pulmonary edema (Figure 7).

RT-PCR Assay to Determine the Changes in mRNA Expression of Proinflammatory cytokine $INF-\gamma$ and Anti-inflammatory cytokine $IL-4$

Figure 8A shows the expression of $INF-\gamma$ mRNA in the lung tissue and Figure 8B shows the relative expression of $INF-\gamma$ mRNA in lung tis-

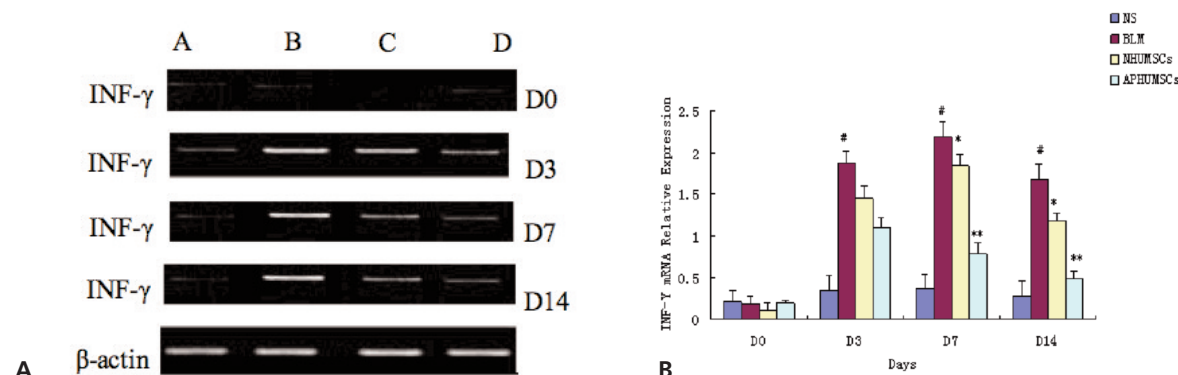


Figure 8. A, The expression of $INF-\gamma$ mRNA in lung tissue. B, The expression of $INF-\gamma$ mRNA in lung tissue.

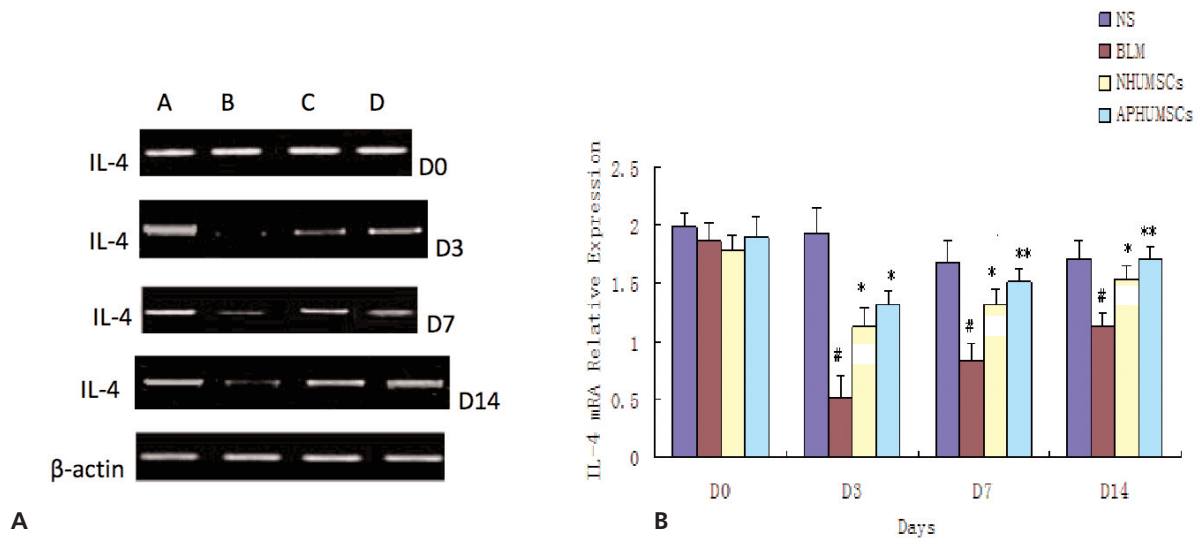


Figure 9. **A**, The expression of IL-4 mRNA in lung tissue. **B**, The expression of IL-4 mRNA in lung tissue. A: NS control group; B, BLM group C: ACE2 group; D: HUMSCs group; E: HUMSCs-ACE2 group. #When compared to NS group, $p < 0.05$. *When compared to BLM group, $p < 0.05$. **When compared to BLM group, $p < 0.01$.

sue. In the BLM group and the NHUMSCs group, the expression of IFN- γ mRNA was maintained at relatively high levels, peaking on day 7, and then declining over time. In the APNHUMSCs group, the expression of IFN- γ mRNA peaked at day 3, and declined at day 7 and day 14. When compared to the BLM group over the same time period, the NHUMSCs group and APNHUMSCs group showed reduced level of IFN- γ mRNA, with the APNHUMSCs showing a more significant reduction. At day 3, 7, 14, when compared to the BLM group over the same time period, there is a time-dependent decrease trend which was statistically significant ($p < 0.05$). Figure 9A shows the expression of IL-4 mRNA in lung tissue and Figure 9B shows the relative expression of IL-4 mRNA in lung tissue. After BLM stimulation, all groups experienced a decrease in the expression of IL-4 mRNA, reaching maximum on day 3, followed by an increase on day 7 and 14. When compared to the NS group, the difference was statistically significant ($p < 0.05$). When compared to the BLM group over the same period of time, the NHUMSCs group and APHUMSCs group both showed an increase in IL-4 mRNA expression, with the APHUMSCs group showing the most increase. When compared to the BLM group at day 3, 7, 14, there was a time-dependent increase in the tendency of IL-4 mRNA expression which was statistically significant ($p < 0.05$). This shows

that both normal and apoptotic umbilical cord mesenchymal stem cells dynamically adjust pro-inflammatory and anti-inflammatory cytokine levels so as to reduce pulmonary inflammation. Apoptotic umbilical cord stem cells have a better anti-inflammatory effect than normal umbilical cord stem cells.

ELISA for Determination of Lung Tissue Cytokines TNF- α

ELISA was used for the determination of the expression of TNF- α in lung tissue and the results are shown in Figure 10. In the BLM group, the TNF- α content was consistently at relatively high levels. It was highest at day 3 and between day 7 and day 14, it declined in a time-dependent manner. NHUMSCs group and APHUMSCs group when compared to the BLM group over the same period of time, the expression of TNF- α was less with the APHUMSCs group having a significantly lower level. At day 3, 7 and 14, when compared to BLM control group over the same time period, TNF- α showed a decrease in trend, and the difference was statistically significant ($p < 0.05$). At day 3, the expression of TNF- α in the APHUMSCs group was higher than that of the NHUMSCs group, but at day 7 and 14, the expression of TNF- α in the APHUMSCs group was significantly lower than in the NHUMSCs group.

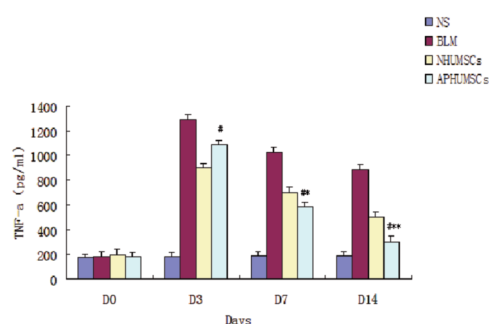


Figure 10. The expression of cytokine TNF- α in lung tissue. [#]When compared to NS group over the same time period, $p < 0.05$. ^{*}When compared to D3 APHUMSCs group, $p < 0.05$. ^{**}When compared to D3APHUMSCs group, $p < 0.01$.

Discussion

Bleomycin is one of the most extensively studied and reproducible models for lung fibrosis in mice. When bleomycin is given into the airway, it produces lung epithelial injury, followed by an inflammatory response over several days that is followed by lung fibrosis that eventually resolves²². Ortiz et al²³ delivered purified BMDMSC from bleomycin-resistant mice to susceptible mice after bleomycin administration. They found that the donor cells homed to the injured lung and adopted epithelial phenotypes, including that of type II alveolar epithelial cells. Because the numbers of donor-derived cells engrafting the lung did not appear sufficient to account for the therapeutic response, they suggest that donor stem cells may have other local effects. The quantitative importance of this process is not known, and the source of signals that are responsible for mobilization and homing of endogenous stem cells remains to be defined. The effects of stem cell transplant other than provision of pluripotent cells to the area of injury that may contribute to the apparent therapeutic effect also remain to be determined.

Stem cells research is currently one of the hottest topics now a days. Stem cells can play the role of vectors in *in vivo* gene therapy and are widely used in breast cancer²⁴, in the treatment of pulmonary fibrosis²⁵, Parkinson's disease²⁶ and in other diseases. Stem cells are at present the most promising types of "seed cells"²⁷. Mesenchymal stem cells have recently been introduced in the treatment of lung injury such that under appropriate conditions, MSCs can differentiate into lung cells, capillary endothelial cells or other tissue

cells²⁷. Because of their special biological properties, MSCs are now gradually being used in tissue engineering and clinical regenerative medicine. As of now, the main source of MSCs is still bone marrow, but the source is limited and invasive operations have to be done for the collection of stem cells. In addition to this, the absolute number of MSCs and the proliferation and differentiation capacities decline with increasing donor age. Thus, it is a top priority to find a new alternative method for the collection of stem cells. HUMSCs are isolated and extracted from discarded placenta, which is not only a rich source, readily available, easy to isolate and expand but also exhibit lower transplantation immune response. Umbilical cord MSCs also might not be as controversial with respect to ethics²⁸.

In the present study, we found that apoptotic stem cells have demonstrated unique anti-inflammatory properties and immunomodulatory effects. In acute myocardial infarction, apoptosis of transplanted stem cells modulates local tissue reactions. Apoptotic cells impact immune reactivity by down-regulating innate and adaptive immunity, inhibiting macrophages and dendritic cells activities, and stimulating regulatory T cells¹¹. This leads to reduced scar formation, repressed myocardial apoptosis, and improved cardiac outcome. Autologous blood exposed *ex vivo* to oxidative stress to induce cell apoptosis and administered intramuscularly decreases the production of inflammatory mediators, increases anti-inflammatory cytokines, and decreases cellular injury¹²⁻¹⁴. Pre-treatment of spontaneously hypertensive rats with apoptotic cells reduced severe renal ischemia-reperfusion injury¹⁵. Direct instillation of apoptotic cells enhanced the resolution of acute inflammation in the lung¹⁶. In some study, apoptotic cells with externally exposed PS-induced TGF- β 1 secretion, resulting in an accelerated resolution of inflammation. This *in vivo* study nicely demonstrates the proposed concept of anti-inflammatory actions of apoptotic cells²⁹.

Based on the above study, we assumed that apoptotic umbilical cord mesenchymal stem cells when compared to normal umbilical cord mesenchymal stem cells have a stronger anti-inflammatory, anti-oxidant, inhibit the inflammatory reaction, inhibiting neutrophil infiltration and total protein leakage, improving vascular permeability and having the function of improving acute lung injury. Therefore, we used bleomycin to induce lung injury in large rats model and we then used

an infusion of either normal or apoptotic umbilical cord stem cells. Comparison of pathological changes in the lung tissue, lung tissue inflammation levels and other indicators were used to evaluate the difference between apoptotic umbilical cord mesenchymal stem cells and normal umbilical cord MSCs. Our study showed that apoptotic umbilical cord stem cells offered better results in cases of ALI.

Pathological analysis and the scoring of the severity of lung injury showed that lung tissue damage was most severe on the third and seventh day and this was consistent with the time distribution of animal deaths. The degree of injury then followed a decreasing trend. After an intervention with either NHUMSCs or APHUMSCs, lung histopathology and lung tissue damage was improved to varying degrees. These improvements were especially evident in the APHUMSCs group.

Neutrophils injury highlights the key factor in the pathogenesis of pulmonary edema. Myeloperoxidase is a peroxidase released by the azurophilic granules of neutrophils and the level is proportional to the number of neutrophils. The excessive production of both of these can cause tissue damage. By measuring lung tissue MPO activity and the number of BALF neutrophils at day 3, the MPO activity of each group and the number of neutrophils in BALF was at its highest level. When the APHUMSCs group of animals were compared to the NHUMSCs group, MPO activity and the number of neutrophils in BALF after day 3, there was a decline for both of these measurements, which indicates an improvement and suggests that apoptotic umbilical cord stem cells are more efficient in the treatment of lung injury as compared to normal umbilical cord mesenchymal stem cell.

The level of cytokines and their changes is an important manifestation in the course of the disease in lung injury. According to the type of inflammatory reaction, cytokines can be divided into pro-inflammatory cytokines and anti-inflammatory cytokines. Pro-inflammatory cytokines mainly consist of INF- γ , TNF- α , IL-1 or IL-6 and anti-inflammatory factors mainly consist of IL-4 and IL-10. Currently, there are many hypotheses about the anti-inflammatory role of apoptotic cells, and experiments have been done to verify some of these theories which have showed that; apoptotic cell ingestion by macrophages induces expression of anti-inflammatory cytokines, such as IL-10 and TGF- β . The increased expression of anti-inflammatory cy-

tokines by macrophages, phagocytosing apoptotic cells might suppress synthesis of pro-inflammatory mediators such as TNF- α , IL-1 β , and IL-6. This leads to accelerated resolution of the inflammatory response. Apoptotic cells are also recognized by immature dendritic cells, which are present in all tissues³⁰. This might additionally lead to an enhanced tissue-specific production of anti-inflammatory cytokines and down-regulation of inflammatory mediators.

In this work, we evaluated pro-inflammatory cytokines INF- γ and TNF- α and anti-inflammatory cytokines, mainly IL-4, as indicators for investigation. Their expression levels were detected by the use of ELISA and RT-PCR. The results showed that after BLM administration, each group had an increased expression of pro-inflammatory cytokines INF- γ and TNF- α , attaining a peak on the third day. The expression of anti-inflammatory cytokine, i.e. IL-4, was decreased and the lowest value was recorded on the third day. On day 7 and day 14, the expression of pro-inflammatory cytokines INF- γ and TNF- α decreased with time and the expression of anti-inflammatory cytokine IL-4 mRNA levels increased over time. This change was most evident in the APHUMSCs group, suggesting that apoptotic umbilical cord stem cells are more efficient than normal umbilical cord stem cells in the inhibition of acute lung inflammation. Here, we have showed that apoptotic stem cells have a stronger anti-inflammatory effects in acute lung injury than normal stem cells. Another study has shown that B-Glucan has a therapeutic effect on Bleomycin-induced lung injury which can be another avenue in terms of treatment³¹.

Conclusions

Experimental results have showed that apoptotic umbilical cord mesenchymal stem cells can more effectively reduce the inflammatory exudate and extent of vascular permeability in ALI rat lung than normal umbilical cord mesenchymal stem cells. It can also more effectively reduce the level of pro-inflammatory factors gene, and can more effectively improve the expression of anti-inflammatory cytokines IL-4, which in turn is able to reduce the degree of pathological injury due to ALI in rats. This suggests that apoptotic umbilical cord mesenchymal stem cells can repair damaged lungs, and this ability is better than that of normal mesenchymal stem cells.

Currently, the research for the use of umbilical cord mesenchymal stem cell transplantation in the treatment of ALI/ARDS is still at the stage of animal experimentation and there are still several challenges before it can be applied in the clinical settings. However, with the in-depth study of the repair mechanism of umbilical cord stem cells in lung tissue, we believe that umbilical cord mesenchymal stem cell transplantation will have a bright future in the clinical setting for the treatment of ALI/ARDS. We conclude that apoptotic umbilical cord MSCs may be beneficial for the treatment of ALI/ARDS. However, extensive clinical research is required before its uses.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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