Cytotherapy, 2015;17(4):443-53

International Society for Cellular Therapy
ISCT®

Human peripheral blood CD34+ cells attenuate oleic acid–induced acute lung injury in rats

SOMIA H. ABD-ALLAH[1], SALLY M. SHALABY[1], EMAN ABD-ELBARY[2], AYMAN A. SALEH[3] & MOHAMMED ABU EL-MAGD[4]

Departments of [1]Biochemistry and Molecular Biology and [2]Pathology, Faculty of Medicine, Zagazig University, Zagazig, Egypt, and Departments of [3]Animal Wealth Development, Genetics and Genetic Engineering, and [4]Anatomy and Embryology, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafr El-Sheikh, Egypt

Abstract

Background aims. Adult stem cell–based therapy is a promising novel approach for treatment of acute lung injury (ALI). In this study, we evaluated the therapeutic effect of isolated human peripheral blood CD34+ progenitor cells in an ALI rat model, induced by oleic acid (OA) injection. *Methods.* Seventy-five adult female rats were used in this study. Group A, control without treatment, and group B, control injected with phosphate-buffered saline, comprised 15 rats each; the remaining 45 rats were injected with OA to induce ALI and were further subdivided into 3 groups: group C (ALI group, 15 rats), group D (ALI and fibroblast group, 15 rats) and group E (ALI and CD34+ cell group, 15 rats). *Results.* CD34+ cells transplantation in rats with OA-induced lung injury improves the arterial PaO_2 and wet/dry ratio, reduces infiltration of inflammatory cells and decreases lung vascular permeability as determined by reduced intra-alveolar and interstitial patchy congestion and hemorrhage as well as decreased interstitial edema. Additionally, lung inflammation determined by expression of the pro-inflammatory mediators intercellular adhesion molecule 1 and tumor necrosis factor-α was attenuated in CD34+ cell–treated rats at 6, 24 and 48 h post-OA challenge compared with non-treated rats. Moreover, the expression of anti-inflammatory molecule interleukin-10 was up-regulated in the lung of OA-induced ALI rats after administration of CD34+ cells. The important finding was that human TNF-α-induced protein 6 (*TSG-6*) gene expression was significantly up-regulated in rats treated with CD34+ cells. *Conclusions.* The freshly isolated human peripheral blood–derived CD34+ cells may be used as an important source of stem cells that improve ALI. The anti-inflammatory properties of CD34+ cells in the lung are explained, at least in part, by activation of CD34+ cells to express *TSG-6*.

Key Words: *acute lung injury, $CD34_+ve$,* ICAM-1, IL-10, TNF-α, *oleic acid,* TSG-6

Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are life-threatening conditions of acute respiratory failure that carry a 40% mortality in critically ill patients [1]. ALI/ARDS is initiated by direct lung injury or systemic inflammatory processes. Cellular characteristics of ALI include loss of alveolar-capillary membrane integrity, excessive trans-epithelial neutrophil migration and release of pro-inflammatory cytotoxic mediators [2].

Different animal models of experimental lung injury have been used to study ALI; most are based on reproducing known risk factors for ARDS, such as the administration of intratracheal lipopolysaccharide (LPS), intratracheal hydrogen chloride, repeated saline lavage, endotoxin infusion and intravenous injection of oleic acid (OA) [3]. OA-induced ALI is a well characterized and clinically relevant model of ALI/ARDS. In particular, it constitutes an excellent model of fat embolism syndrome–induced ALI, given that OA is a major component of the marrow-derived fat emboli released into the circulation after traumatic bone injury. The ALI produced by OA is relatively transient and resolves over 3 days [4,5].

No specific therapy for ALI/ARDS has been beneficial; instead, the patient is managed in terms of respiratory, circulatory and nutritional support. Recently, several new treatments, including inhaled nitrous oxide, instilled surfactant and interleukin

Correspondence: **Somia H. Abd-Allah**, PhD, Department of Biochemistry and Molecular Biology Zagazig University, Zagazig, Egypt. E-mail: dr.somiahassan@yahoo.com; **Mohammed Abu El-Magd**, MD, Department of Anatomy and Embryology, Faculty of Veterinary Medicine, Kafrelsheikh University, Kafr El-Sheikh, Egypt. E-mail: mohrizk73@yahoo.com

(Received 24 May 2014; accepted 6 November 2014)

http://dx.doi.org/10.1016/j.jcyt.2014.11.002
ISSN 1465-3249

(IL)-1 receptor antagonist have been attempted with ALI/ARDS and related disorders, but they have achieved little improvement in patient survival, and the mortality rate remains as high as 40% [6,7]. Thus, novel therapeutic strategies are needed to improve the outcome of this devastating disease. Recently, mesenchymal stem cells (MSCs) are shown to be protective in animal models of ALI induced by oleic acid (OA) [4], LPS [8] and live bacteria [9–11]. Other studies have suggested that human adult stem cells might have therapeutic effects on ALI/ARDS [12,13].

CD34 is a member of a family of single-pass transmembrane sialomucin proteins [14]. Cells expressing CD34 (CD34+ cells) are normally found in the umbilical cord blood and bone marrow as hematopoietic stem cells (HSCs), endothelial progenitor cells and activated endothelial cells of blood vessels [15]. A subpopulation of CD34+ mononuclear blood cells isolated from human peripheral blood induces angiogenesis [15]. Human CD34+ progenitor cells isolated from bone marrow, peripheral blood and cord blood have been tested in many preclinical models of ischemic vascular diseases. These cells are efficient to promote angiogenesis and provide beneficial effects on myocardial infarction [16], peripheral ischemia [17] and ischemic stroke [18]. More important, some clinical studies also confirmed the beneficial effects of human CD34+ progenitor cells on coronary heart and peripheral vascular diseases [19]. Injection of CD34+ HSCs has been clinically applied to treat other non-vascular diseases including spinal cord injury [20] and liver cirrhosis [21]. However, little is known about the effects of human CD34+ progenitor cells isolated from peripheral blood on ALI induced by OA. Only 1 recent published study has reported protective effects of umbilical cord–derived CD34+ cells on ALI induced by LPS challenge [22].

We hypothesized that intravenous injection of CD34+ cells could attenuate ALI induced by OA injection in a rat model, and, if so, the protective mechanism may be mediated by anti-inflammatory effects. We also demonstrated whether the anti-inflammatory properties of CD34+ cells were explained, at least in part, by activation of CD34+cells to express the anti-inflammatory factor TNF-α-induced protein 6 (TSG-6).

Methods

This study was performed in 3 collaborating laboratories in Egypt: the Clinical Chemistry and Stem Cell Research Laboratory, Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Zagazig University; the Molecular Biology and Biotechnology Research Unit, Animal Wealth Development Department, Genetics and Genetic Engineering, Faculty of Veterinary Medicine, Zagazig University; and the Biotechnology Laboratory, Faculty of Veterinary Medicine, Kafrelsheikh University.

Animal preparation

Seventy-five healthy adult female rats of matched weight (200 ± 15 g) were used in this study. The rats were housed under standard conditions of light-dark cycles with free access to food and tap water under controlled temperature (28–30°C). They were observed in this environment for 7 days before commencing treatment ensuring adequate adaptation. Animal housing conditions and all experimental procedures conformed to institutional guidelines and were in accordance with the National Institutes of Health guidelines on animal care.

Isolation of CD34+ cells from human peripheral blood

Human peripheral blood (1500 mL) was obtained from 15 healthy male volunteers aged 33.7 ± 1.5 years after strenuous physical exercise according to To *et al.* [23]. One hundred milliliters of blood was collected from each volunteer. Informed consent regarding the experimental use of the blood from the volunteers was obtained.

From the peripheral blood, the mononuclear cells (MNCs) were obtained by centrifugation over Ficoll-Paque (lymphocyte separation medium 1.077, Lonza Bioproducts). After washing in phospate-buffered saline (PBS), $\sim 4.5 \times 10^8$ MNCs were left from each 200 mL of blood. CD34+ cells were isolated from MNCs by the MACS CD34 progenitor isolation kit using immunomagnetic beads (CD34 MicroBead Kit, Miltenyi Biotec) as described previously [24]. The purified number of CD34+ cells is usually $\sim 5 \times 10^6$ when starting from 2×10^8 total cells (for 1 single isolation). The purified CD34+ cells were freshly injected. Human peripheral blood CD34+ cells were analyzed by flow cytometry. The following monoclonal anti-human antibodies were used to characterize the CD34 cell population: CD34-FITC (Becton, Dickinson and Company) and CD105-PE (Becton, Dickinson). Flow cytometric analysis demonstrated that CD34 cells did not express CD105 (0.2%) but did express CD34 (95%).

Activation of CD34+ cells to express TSG-6 in vitro

Human CD34+ cells and human fibroblasts (IMR-90) were plated at 50,000 cells/well in culture medium (α-minimum essential medium supplemented with 10% fetal bovine serum and 100 units/mL of

penicillin; 100 μg/mL of streptomycin) and containing 10 ng/mL of recombinant human TNF-α (R&D Systems) in 6-well plates and incubated for 0, 6, 24 and 48 h. Total RNA was extracted (RNeasy Mini Kit; Qiagen) for reverse transcriptase polymerase chain reaction (RT-PCR) assays. Human fibroblasts (IMR-90) were obtained from the American Type Culture Collection (ATCC) and cultured in α-minimum essential medium supplemented with 10% fetal bovine serum.

Establishment of the ALI model of rats

Forty-five adult female, pathogen-free rats were given OA solution (10 μL/kg body weight, OA was suspended in PBS [Lonza Bioproducts]) intravenously via the tail vein. Ten minutes after OA injection, rats were randomly assigned to 3 groups: group C (ALI, $n = 15$); group D ($n = 15$), which was intravenously injected with human fibroblasts IMR-90 (2×10^6 cells dissolved in 150 μL of PBS); and group E ($n = 15$), which was intravenously injected with CD 34+ve cells (2×10^6 cells dissolved in 150 μL of PBS). In addition, there were 2 control groups: control group A ($n = 15$), which did not receive any treatment, and control group B ($n = 15$), which was intravenously injected with PBS. Rats were sacrificed at 6, 24 and 48 h after OA administration. There were 5 rats in each group at each time point (6, 24 and 48 h after OA injection).

Blood and lung tissue sampling

Arterial blood samples (300 μL) were collected after scarification of the rats from the aortic artery via the opened chest into a heparinized syringe for measurement of arterial oxygen partial pressure (PaO_2) by blood gas analyzer ($n = 5$ per group) to assess hypoxia. Lungs were removed, and the right lung was weighed to examine the lung wet/dry weight ratio to detect edema. The left lung was divided into 2 parts: the first part was kept in formalin 10% to assess the lung injury and the response to CD 34+ve cell injection by histopathological examination with light microscope. The second part was stored in liquid nitrogen tank until total RNA and DNA extraction.

Lung wet/dry weight ratio

The wet weight of the immediate removed right lungs was recorded; lungs were then placed in an incubator at 60°C for 72 h, and the dry weight was detected. The wet lung mass divided by the dry lung mass represented the wet/dry lung ratio, which indicates the fraction of wet lung weight caused by water.

Light microscopic study

The specimens were fixed in 10% neutral-buffered formalin, dehydrated and embedded in paraffin; sections were then cut at 5 μm and stained with hematoxylin and eosin for routine histological examination [25].

PCR detection of human *β actin* gene

The presence of human *β actin* gene in the CD34+cells recipient rats was determined by PCR analysis according to Rajatileka *et al.* [26]. Amplification of the human *β-actin* gene; the housekeeping gene was performed by conventional PCR using forward primers: 5′-TGCCCATCTACGAGGGG TATG-3′ and reverse primer 5′-GAAATCGT GCGTGACATTAAGGAG-3′. Genomic DNA was extracted with phenol and chloroform from the rat lung. PCR was carried out in a final volume of 25 μL containing 2 μL (30 ng) of genomic DNA, 0.2 μmol/L of each primer and 10 μL of Taq PCR Master Mix (BIORON). The amplification protocol was as follows: 94°C for 10 min, then 35 cycles of 94°C for 1 min, 56°C for 45 s and 72°C for 45 s; then 72°C for 7 min, using heated lid thermal cycler. PCR products (325 bp) were separated with the use of 2% agarose gel electrophoresis.

Quantitative RT-PCR analysis for rat ICAM-1, TNF-α and IL-10

Total RNA was isolated from the lung tissue using an RNeasy Mini Kit including DNase I digestion following the manufacturer's protocol (Qiagen, #74104). The RNA was then reverse transcribed using Quantiscript reverse transcriptase. Following reverse transcription, quantitative real-time PCR analysis was performed with a (Rotor-Gene Q 2plex, Qiagen) with SYBR green master mix (Roche Diagnostics). The primers were designed by the Primer 3 web-based tool (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) based on the published rat sequence. The following primers were used for analysis: rat *ICAM-1*, 5′-AAACGG GAGATGAATGGTACCTAC-3′ and 5′-TGCAC GTCCCTGGTGATACTC-3′; rat *TNF-α*, 5′-CCA GGAGAAAGTCAGCCTCCT-3′ and 5′-TCATAC CAGGGCTTGAGCTCA-3′; rat *IL-10*, 5′-CCTTA CTGCAGGACTTTAAGGGTTA-3′ and 5′-TTTC TGGGCCATGGTTCTCT-3′; rat cyclophilin B, 5′-CAGGAGAGAAAGGATTTGGCTACA-3′, and 5′-TCCACCCTGGATCATGAAGTC-3′; human *TSG-6*, 5′-GGCCATCTCGCAACTTACA-3′ and 5′-CAGCACAGACATGAAATCCAA-3′. The rat gene expression was normalized to rat cyclophilin B.

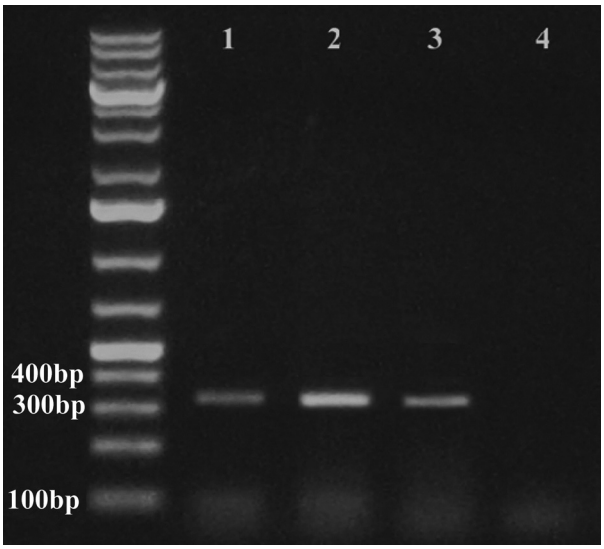

Figure 1. Agarose gel electrophoresis of PCR product (325 bp) of human β *actin* gene: lane 1: positive results for rats received human CD34+ cells (group E) at 6 h after OA challenge; lane 2: positive results for rats received human CD34+ (group E) at 24 h after OA challenge; lane 3: positive results for rats received human CD34+ (group E) at 48 h after OA challenge; lane 4: negative result for rat received oleic acid (group C).

The human *TSG-6* gene expression was normalized to human *GAPDH* 5′-GTCTTCACTACCATG GAGAAGG-3′ and 5′-TCATGGATGACCTTGG CCAG-3′. The following thermal cycling conditions were used for the 4 genes: initial denaturation at 94°C for 3 min, 35 cycles of amplification (DNA denaturation at 94°C for 40 s, annealing at 57°C for 1 min, extension at 72 °C for 1 min) and final extension at 72°C for 10 min.

Statistical analysis

Data were collected, arranged, summarized and then analyzed using SPSS/PC+ (SPSS). Statistical method used to estimate the difference between the groups was 1-way analysis of variance and least significant difference according to Snedecor and Cochran [27]. Data were presented as mean ± SEM and significance was declared at $P < 0.05$.

Results

Human CD34+ progenitor cells were isolated from human peripheral blood mononuclear cells (PBMCs) with purity greater than 95%. The human β *actin* gene was detected in the lungs of the female rats that injected by CD34+cells (group E) at 6, 24 and 48 h after OA challenge; however, the lung of the rats in other groups (A, B, C and D) lacked this gene (Figure 1). This indicates that the injected human CD34+cells had been successfully delivered into the lungs of the recipient rats.

Effect of CD34+ cells transplantation on arterial oxygen partial pressure and lung water content

There was no significant difference between control group A and control group B regarding arterial oxygen partial pressure (PaO_2; $P > 0.05$). In groups C and D, OA-induced ALI significantly decreased PaO_2 (more severe hypoxemia) compared with the control groups A and B at 6 and 24 h after OA challenge ($P < 0.05$; Figure 2). Treatment with CD34+ cells resulted in a significant increase of PaO_2 (decreased hypoxia) in group E compared with group C at 6 and 24 h ($P < 0.05$). There was no significant difference between all groups at 48 hours after OA challenge ($P > 0.05$; Figure 2).

There was no significant difference between control group A and control group B regarding wet/dry weight ratio ($P > 0.05$). In groups (C and D), OA induced ALI increased lung wet/dry weight ratio (which is an indicator for lung edema) when compared with other groups at all time points (6, 24 and 48 h) after OA challenge ($P < 0.05$; Figure 2). Treatment with CD34+ cells significantly decreased pulmonary edema in group E at all time points compared with other groups ($P < 0.05$; Figure 2).

Effect of CD34+ cells transplantation on lung histopathology

Histopathological assessment of lung sections was done by hematoxylin and eosin staining to evaluate pulmonary architecture, tissue edema formation, infiltration of the inflammatory cells, congestion and hemorrhage (Figure 3). Lung sections of the control groups A and B showed no obvious lesion in the lung tissues (normal pulmonary architecture), that is, normal thickness of the interalveolar septum, normal bronchioles and blood vessels. In groups C and D, the lung sections displayed extensive morphological damage, manifested by the severe infiltration of numerous polymorphonuclear leukocytes and macrophages in the interstitial spaces, intra-alveolar and interstitial patchy congestion and hemorrhage, interalveolar septal thickening and interstitial edema. These pathological changes were greatest at 6 h and gradually reduced at 24 and 48 h. The administration of CD 34+ve cells in group E reduced the severity of lung injury and improved the lung histopathology compared with OA group C and OA+ human fibroblast group D at all time points. Assessment of lung sections of group E revealed markedly reduced hemorrhage, congestion, neutrophilic inflammation and interstitial edema, with preservation of alveoli in the

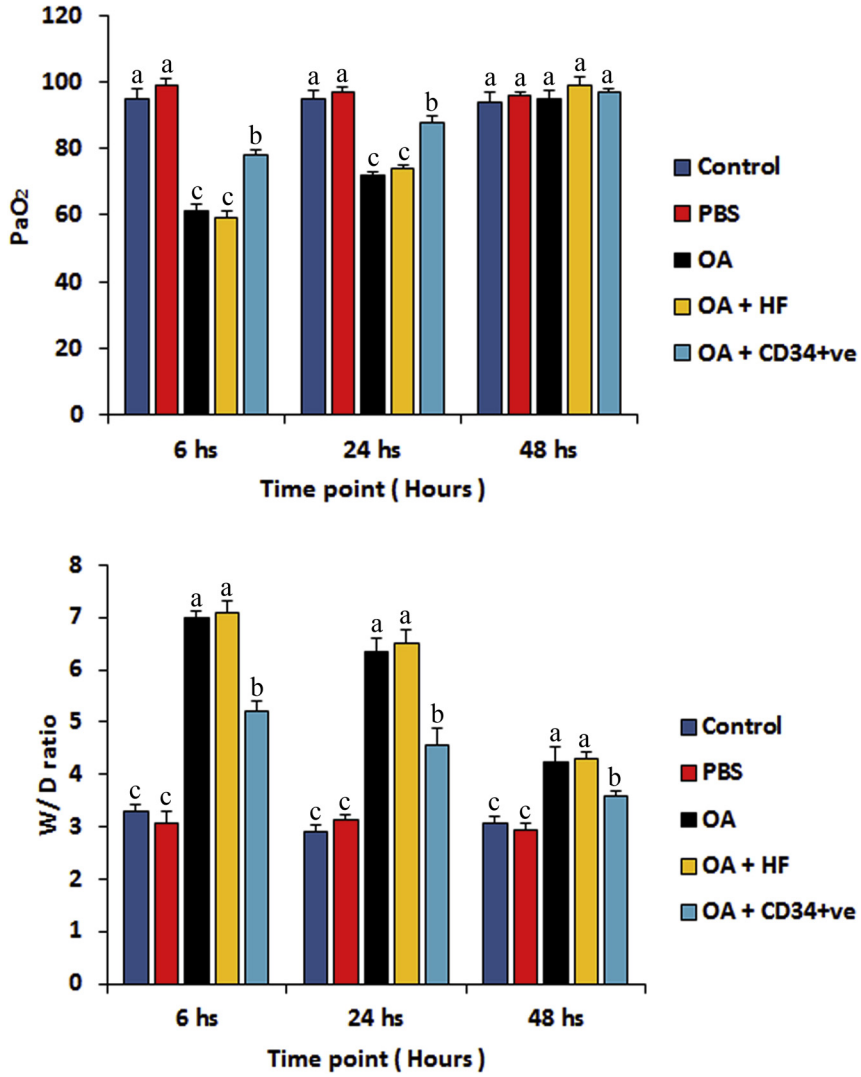

Figure 2. Effect of CD34+ cells engraftment on arterial oxygen partial pressure (PaO_2) and wet/dry ratio for all groups at different time points after OA challenge. CD34+ cells increased PaO_2 and reduced lung W/D ratio, severity of lung injury and pulmonary edema. Results expressed as mean ± SEM. $^{a-c}$Means carrying different superscripts at the same time point are significantly different at $P \leq 0.05$ based on least significant difference. HF, human fibroblast.

lung tissue and the destruction of lung tissue was lighter compared with OA group C and OA+ human fibroblast group D. These findings are suggesting that ALI induced by OA injection was attenuated by transplantation of CD 34+ve cells.

CD34+ cells are activated to express TSG-6 in vitro and in vivo

There was significant increase of expression of TSG-6 from CD34+ cells incubated with TNF-α for 48 h than for 0, 6 and 24 h ($P < 0.05$). Interestingly, the response of CD34+ cells to TNF-α far exceeded the response of human fibroblasts ($P < 0.05$; Figure 4A). Real-time RT-PCR assays demonstrated that human *TSG-6* mRNA in rat lung was increased at 6 h and further increased at 24 and 48 h after injection of CD34+ cells (Figure 4B). Surprisingly, *TSG6* mRNA expression has not been investigated in lungs of rats received human fibroblasts.

Effect of CD34+ cells transplantation on ICAM1, TNF-α and IL-10 gene expression

Quantitative RT-PCR analysis revealed that there was a significant increase in the expression of pro-inflammatory factors such as *ICAM*-1 and *TNF-α* gene in the lungs of rats at 6, 24, and 48 h post-OA challenge (Figure 5). CD34+ cell treatment resulted in a drastic decrease of expression of these pro-inflammatory molecules. This result indicated that CD34+ transplantation mediates a reduction in pro-inflammatory activity in the OA-induced ALI rat.

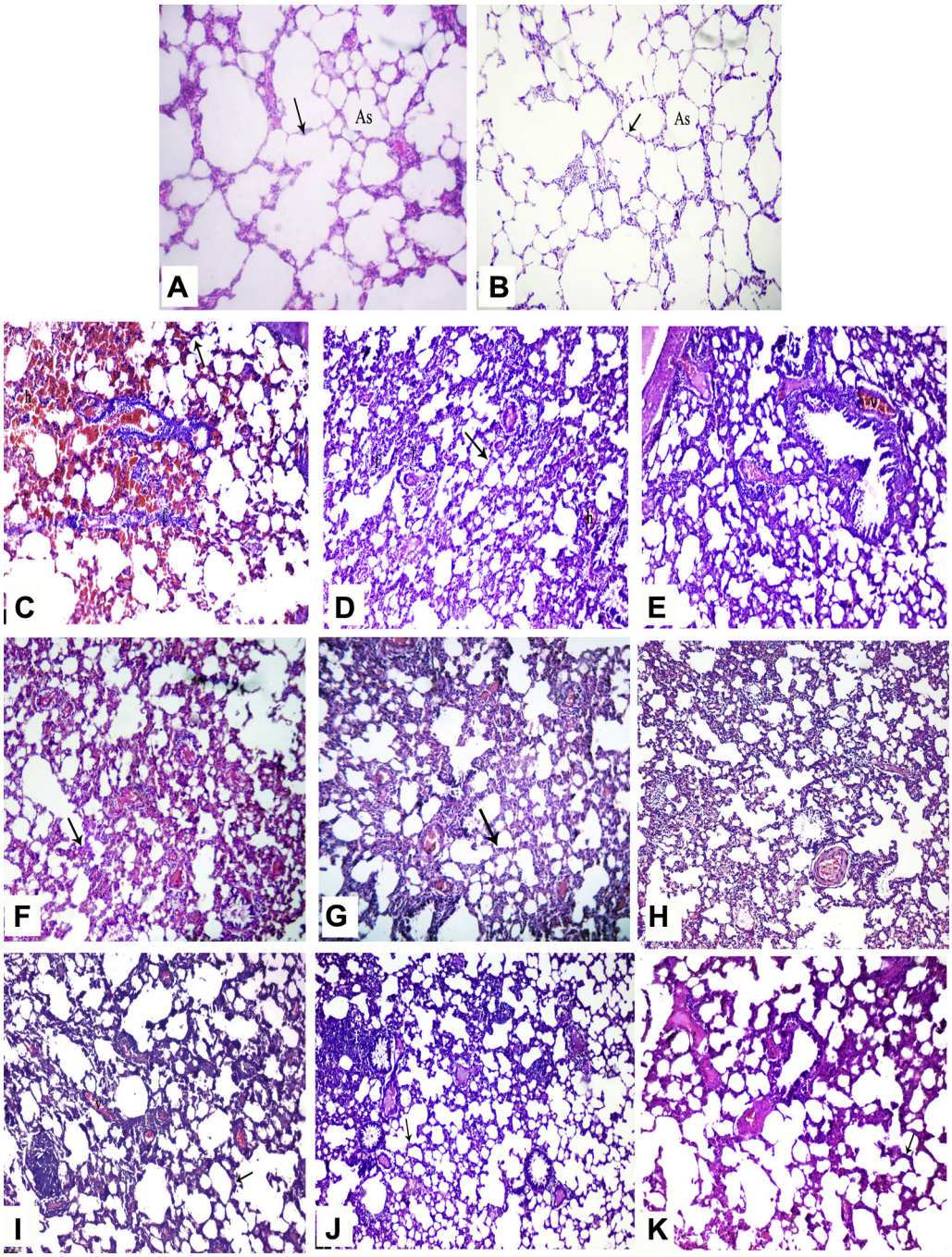

Figure 3. Representative photomicrographs images of hematoxylin and eosin (H-E)-stained lung sections from the 5 experimental groups (original magnification, ×100). (A, B) H-E staining of lung sections of groups A and B, respectively, showing no obvious lesion in the lung tissues, alveolar sac (As), alveolar septum (arrow). (C, F) H-E staining of lung sections of groups C and D at 6 h showing severely reduced pulmonary alveolus, interstitial edema, cellular infiltration, thickened alveolar septum (arrow) and hemorrhage. (D, G) H-E staining of lung sections of groups C and D at 24 h showing moderate histopathological changes of cellular infiltration, thickened alveolar septum (arrow) and congested blood vessels. (E, H) H-E staining of lung sections of groups C and D at 48 h showing mild histopathological changes. (I, J, K) H-E staining of lung sections of group E at 6, 24 and 48 h, respectively, demonstrating the reduced severity of lung injury and improvement the lung histopathology, in comparison to groups C and D at the 3 time points.

We investigated levels of *IL-10*, a well-established anti-inflammatory factor, in the lung of OA-induced ALI rat collected in the presence or absence of CD34+ transplantation. Quantitative RT-PCR analysis revealed a significant increase in the expression of *IL-10* in the lung of OA-induced ALI rat after CD34+ cells transplantation on various time points (6, 24 and 48 h) compared with other groups (Figure 5).

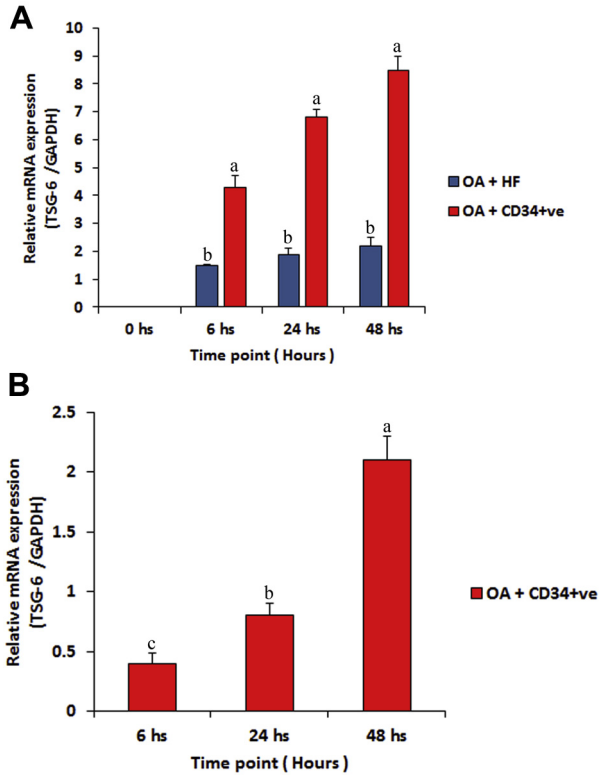

Figure 4. Graphical presentation of real-time quantitative PCR analysis of the expression of human *TSG-6* in (A) culture medium and (B) lung of ALI rats received CD34+ cells at various time points. The human *GAPDH* was used as an internal reference for normalization. *t*-test was used to compare between each 2 groups in panel A. Expression level of *TSG-6* gene in control rat (time point 6 h) was considered the baseline in panel B. Data expressed as mean ± SEM ($n = 3$ in triplicate). $^{a-c}$Means carrying different superscripts at the same time point are significantly differed at $P \leq 0.05$ based on least significant difference.

Discussion

In this study, we have studied the effects of transplantation of freshly isolated human peripheral blood CD34+ cells on OA-induced ALI rats and found that these cells alleviate lung injury and inflammation following OA challenge. The flow cytometric analysis verifies that the collected HSCs are CD34+. In addition, detection of human *β actin* gene within the lung tissue of rats included in group E confirms that human CD34+ cells were delivered to the damaged lung tissue. The mechanisms by which intravenously administered CD34+ cells or MSCs reach to the lung remain to be elucidated. It is possible that most of the intravenously injected CD34+ cells or MSCs initially migrate to the lungs because the lungs are the first major capillary bed, and damage of lung tissues can increase retention of these cells in the lung [28].

In the majority of ALI studies, HSCs were originated from the same lab animal as the ALI model. However, this lacks accuracy and resulted in disease, including cancer. Murine HSCs, but not human HSCs, differentiated into osteosarcomas when injected into the lung [29]. Therefore, we used adult human HSCs, the CD34+ cells, in this study. We also isolated these cells from peripheral blood to avoid problems and limitations associated with bone marrow collection. Harvesting a patient's bone marrow to isolate and culture autologous CD34+ cells cannot be achieved quickly enough to provide emergency treatment for acute disease such as ALI. Therefore, human peripheral blood may be an ideal and practical source for HSCs (such as CD34+ cells and MSCs) because of its accessibility (they do not require invasive bone marrow biopsies), lower risk of viral contamination, immunosuppressive properties and homing to sites of tissue injury [9].

The promising cell-based therapeutic effect of human MSCs is limited because of a decrease in their regenerative potential with increasing donor age and the need for *in vitro* expansion before treatment [22]. *In vitro* cultivation may affect the character and effects of human adult stem cells because it can reduce MSC adhesion to laminin and endothelium [30] and cause MSCs to progressively lose their progenitor characteristics [31]. Thus, fresh isolation without *in vitro* expansion excludes the potential artificial effects from cultivation or other supplements in the medium. This prompted us to use freshly collected human peripheral blood CD34+ cells in this study.

One of the major early drawbacks to the therapeutic use of CD34+ cells was their relatively low basal density in the circulation. Fortunately, the discovery of agents that help to mobilize these cells from their resident bone marrow niche into the systemic circulation (where they can be collected and purified) considerably strengthened the field. The increase of circulating stem cells can be induced by administration of dextran sulfate or adrenocorticotrophin and by strenuous physical exercise [23]. A similar technique, strenuous physical exercise, was adopted in this study to ensure progenitor cell mobilization to blood.

To our knowledge, this is the first study to indicate that freshly isolated human peripheral blood–derived CD34+ cells can be used as a novel and promising therapeutic and/or protective approach for OA-induced ALI. OA and LPS-exposed rodents displayed ALI presentation similar to that of human ALI, including induced pulmonary edema (which is indicated by an increased lung wet/dry weight ratio, hypoxemia and increased capillary permeability), and alveolar infiltration of inflammatory cells [8,32–34]. Our results also showed a reduction in the wet/dry ratio and edema after CD34+ cells transplantation in OA-induced lung injury rats. Thus, treatment with CD34+ cells significantly decreased the pulmonary edema and improved the

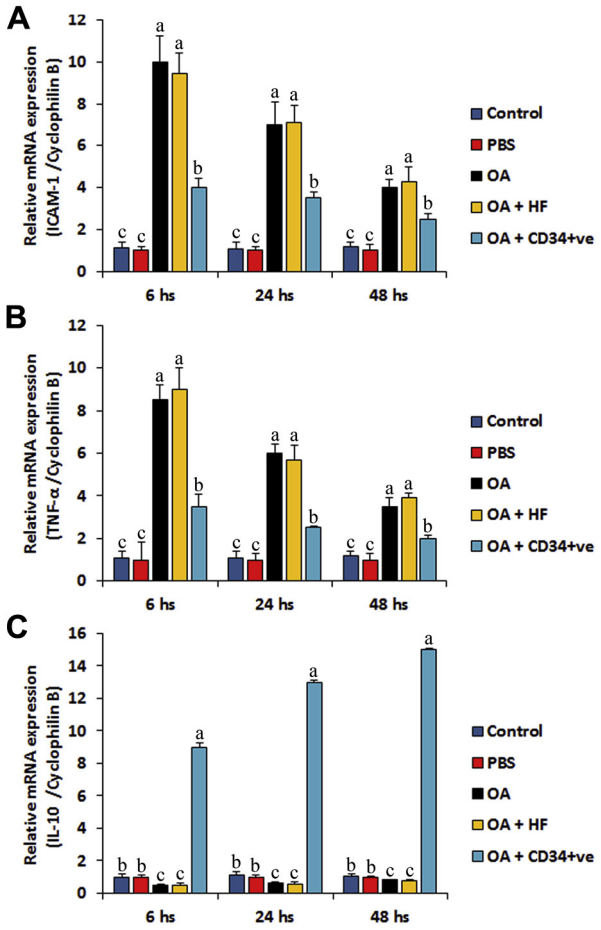

Figure 5. Graphical presentation of real-time quantitative PCR analysis of the expression of pro-inflammatory genes such as *TNF-α* and *ICAM1*, as well as anti-inflammatory gene *IL-10*, in OA-induced ALI rats with or without CD34+ cell therapy at various time points (6, 24 and 48 h). The housekeeping gene encoding cyclophilin B was used as an internal reference for normalization. Data expressed as mean ± SEM ($n = 3$ in triplicate). The expression level of each target gene in control rat (group A) at various time points was considered the baseline. $^{a-c}$Means carrying different superscripts at the same time point are significantly differed at $P \leq 0.05$ based on least significant difference.

lung wet/dry ratio. Likewise, pulmonary edema is relieved after administration of MSCs derived from bone marrow [4,9] or adipose tissue [35]. We also found that CD34+ cells transplantation reduces hypoxemia at 6 and 24 h after OA challenge. Chang *et al.* [36] also observed a remarkable increase in PaO_2 in a patient with ARDS after intratracheal administration of umbilical cord blood–derived MSCs. On the other hand, our results showed no significant difference of PaO_2 values between all groups at 48 h after OA challenge. These results were consistent with those of Xu *et al.* [4] and Kennedy *et al.* [5]. The improvement of PaO_2 at 48 h in the injured group may be due to hypoxic pulmonary vasoconstriction (HPV) through which the lung preserves oxygenation to the injured region [37,38].

Persistently increased lung microvascular permeability resulting in protein-rich lung edema is a hallmark of ALI/ARDS [39]. Thus, targeting microvascular leakiness to restore lung fluid homeostasis is a potential therapeutic approach for the prevention and treatment of ALI/ARDS. Histopathological analysis revealed that ALI rats (groups C and D) show intra-alveolar and interstitial patchy congestion and hemorrhage and interstitial edema. However, CD34+ treated rats (group E) show evidence of vascular improvement, reduction of hemorrhage and edema. Similar results have been observed by Huang *et al.* [22], who found that the freshly collected human umbilical cord derived CD34+ cells can restore vascular integrity in LPS-induced ALI mice. Another previous study has shown that these cells promote regeneration of injured alveolar epithelium in models of neonatal lung injury/bronchopulmonary dysplasia [40]. Other studies with human MSCs have shown decreased lung edema formation and lung vascular and epithelial barrier permeability in various animal models of ALI [8,41]. These data suggest that human CD34+ cells exhibit similar vascular protective effects as other adult stem cells. In contrast, Mao *et al.* [42] showed that *in vitro* expanded cord blood–derived CD34+ cells, but not freshly isolated CD34+ cells, induce lung growth and alveolarization in injured newborn lungs. This difference may be attributed to different animal models and different source of CD34+. This suggests that inhibition of inflammatory lung injury is mediated by mechanisms different from lung growth and alveolarization.

The mechanisms through which CD34+ cells exert their action is still unknown; however, it may be similar to other HSCs, such as MSCs, through paracrine and immunomodulatory mechanisms. Secretion of the paracrine-soluble factor Ang1 by human MSCs mainly restores epithelial protein permeability across primary cultures of human alveolar epithelial type II cell monolayer after cytomix-induced injury [43]. MSCs seem to induce macrophages to produce more IL-10 through the release of prostaglandin E2 [44]. MSCs could down-regulate the inflammatory process by decreasing the pro-inflammatory cytokine expression such as TNF-α and secreting anti-inflammatory agents such as IL-10, IL-1RA, IL-13, angiopoietin-1 and keratinocyte growth factor [43,45]. Recently, Huang *et al.* [22] demonstrated that freshly isolated human cord blood–derived CD34+ cells were protective against inflammatory lung injury induced by LPS challenge. They also observed drastic decrease of expression of proinflammatory molecules including *TNF-α*, *IL-6*, *ICAM-1* and *iNOS* in CD34+ treated lungs [22]. Consistent with this, our data showed that the

improvement in lung injury was associated with a decrease of expression of proinflammatory molecules including *TNF-α* and *ICAM-1*, as well as an increase of the expression of anti-inflammatory molecule *IL-10*.

The anti-inflammatory effects of CD34+ cells in ALI model may be attributed to their activation to secrete TSG-6. We found significant increase of the expression of *TSG-6* after activation of CD34+ cells *in vitro*. Also, human *TSG-6* gene was significantly upregulated in rats treated with CD34+ cells *in vivo*. The upregulation of the *TSG-6* was of special interest because of the known anti-inflammatory effects of the protein [46,47]. TSG-6 is a 35-kDa secreted protein produced by many cell types in response to TNF-α and IL-1β [46,47]. In transgenic mice, inactivation of the *TSG-6* gene increased inflammatory responses [48], and overexpression of the gene decreased inflammatory responses [49].

Danchuk *et al.* [8] found that hMSCs in the LPS-exposed lung upregulated their *TSG-6* mRNA expression more than 900-fold and suggested that the anti-inflammatory properties of hMSCs in the lung are explained by activation of hMSCs to secrete TSG-6. Lee *et al.* [50] indicated that the hMSCs that were trapped in the lung were activated to secrete TSG-6, and the TSG-6 suppressed the excessive inflammatory response in murine myocardial infarction model. We first studied the activation of CD34+ cells to express TSG-6; however, our results did not exclude the possibility that the CD34+ cells secreted other anti-inflammatory factors in addition to TSG-6. A previous study by Majka *et al.* [51] reported that human CD34+ cells release numerous growth factors and cytokines. Krenning *et al.* [52] reported that CD34+ cells modulate proliferation and endothelial differentiation of CD14+ cells by releasing hepatocyte growth factor, IL-8 and monocyte chemoattractant protein 1 (MCP-1). Recently, CD34+ cells were shown to secrete interleukins, growth factors and chemokines that are capable of accelerating vascular network formation *in vivo* and enhancing healing of ischemic ulcers in diabetic mice [53]. Culture-derived progenitor cells release IL-8, producing a mitogenic effect on vascular endothelial cells [54].

Conclusions

Our study has demonstrated that intravenous administration of CD34+ cells freshly isolated from human peripheral blood attenuates OA-induced lung injury in rat model primarily by down-modulating the inflammatory process. TSG-6 protein secreted from CD34+ cells could involve in modulating the inflammation. Given the easy availability without defects from *in vitro* culture, CD34+ cells may be an important source of stem cells for the treatment of ALI/ARDS. However, further investigations are needed to determine the optimal dose and safety of CD34+ cells and their ability to prevent ALI/ARDS in high-risk groups so that improved clinical outcomes can be ascertained.

Acknowledgments

This work was funded by support of academic research in Zagazig University Projects, Zagazig University Postgraduate & Research Affairs.

Disclosure of interest: The authors have no commercial, proprietary, or financial interest in the products or companies described in this article.

References

[1] Matthay MA, Thompson BT, Read EJ, McKenna DH Jr, Liu KD, Calfee CS, et al. Therapeutic potential of mesenchymal stem cells for severe acute lung injury. Chest 2010; 138:965–72.

[2] Matthay MA, Zimmerman GA. Acute lung injury and the acute respiratory distress syndrome: four decades of inquiry into pathogenesis and rational management. Am J Respir Cell Mol Biol 2005;33:319–27.

[3] McGuigan C, Mullenix C, Norlund M, Ward M, Walts M, Azarow L. Acute lung injury using oleic acid in the laboratory rat: establishment of a working model and evidence against free radicals in the acute phase. Curr Surg 2003;60:412–7.

[4] Xu Y, Liu Y, Wang Q, Li G, Lu X, Kong B. Intravenous transplantation of mesenchymal stem cells attenuates oleic acid induced acute lung injury in rats. Chin Med J 2012;125: 2012–8.

[5] Kennedy MT, Higgins BD, Costello JF, Curtin WA, Laffey JG. Hypertonic saline reduces inflammation and enhances the resolution of oleic acid induced acute lung injury. BMC Pulmon Med 2008;8:9.

[6] Ware LB, Matthay MA. The acute respiratory distress syndrome. New Engl J Med 2000;342:1334–49.

[7] Weiner RE, Sasso DE, Gionfriddo MA, Thrall RS, Syrbu S, Smilowitz HM, et al. Early detection of oleic acid-induced lung injury in rats using (111)in-labeled anti-rat intercellular adhesion molecule-1. J Nucl Med 2001;42:1109–15.

[8] Danchuk S, Ylostalo JH, Hossain F, Sorge R, Ramsey A, Bonvillain RW, et al. Human multipotent stromal cells attenuate lipopolysaccharide-induced acute lung injury in mice via secretion of tumor necrosis factor-alpha-induced protein 6. Stem Cell Res Ther 2011;2:27.

[9] Shalaby SM, El-Shal AS, Abd-Allah SH, Selim AO, Selim SA, Gouda ZA, et al. Mesenchymal stem cell injection protects against oxidative stress in *Escherichia coli*-induced acute lung injury in mice. Cytotherapy 2014;16:764–75.

[10] Popov BV, Serikov VB, Petrov NS, Izusova TV, Gupta N, Matthay MA. Lung epithelial cells induce endodermal differentiation in mouse mesenchymal bone marrow stem cells by paracrine mechanism. Tissue Eng 2007;13:2441–50.

[11] Lee JW, Fang X, Gupta N, Serikov V, Matthay MA. Allogeneic human mesenchymal stem cells for treatment of *E. coli* endotoxin-induced acute lung injury in the ex vivo perfused human lung. Proc Natl Acad Sci U S A 2009;106: 16357–62.

[12] Krause DS. Bone marrow–derived cells and stem cells in lung repair. Proc Am Thorac Soc 2008;5:323.
[13] Rojas M, Xu J, Woods CR, Mora AL, Spears W, Roman J, et al. Bone marrow–derived mesenchymal stem cells in repair of the injured lung. Am J Respir Cell Mol Biol 2005; 33:145.
[14] Simmons DL, Satterthwaite AB, Tenen DG, Seed B. Molecular cloning of a cDNA encoding CD34, a sialomucin of human hematopoietic stem cells. J Immunol 1992;148: 267–71.
[15] Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964–7.
[16] Kawamoto A, Tkebuchava T, Yamaguchi J, Nishimura H, Yoon YS, Milliken C, et al. Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia. Circulation 2003; 107:461–8.
[17] Murohara T, Ikeda H, Duan J, Shintani S, Sasaki K, Eguchi H, et al. Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. J Clin Invest 2000;105:1527–36.
[18] Taguchi A, Soma T, Tanaka H, Kanda T, Nishimura H, Yoshikawa H, et al. Administration of CD34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. J Clin Invest 2004;114:330–8.
[19] Subrammaniyan R, Amalorpavanathan J, Shankar R, Rajkumar M, Baskar S, Manjunath SR, et al. Application of autologous bone marrow mononuclear cells in six patients with advanced chronic critical limb ischemia as a result of diabetes: our experience. Cytotherapy 2011;13:993–9.
[20] Srivastava A, Bapat M, Ranade S, Srinivasan V, Murugan P, Manjunath S, et al. Multiple injections of in vitro expanded autologous bone marrow stem cells for cervical level spinal cord injury—a case report. J Stem Cell Regen Med 2010;6:175–6.
[21] Terai S, Ishikawa T, Omori K, Aoyama K, Marumoto Y, Urata Y, et al. Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy. Stem Cell 2006;24:2292–8.
[22] Huang X, Sun K, Zhao YD, Vogel SM, Song Y, Mahmud N, et al. Human CD34+ progenitor cells freshly isolated from umbilical cord blood attenuate inflammatory lung injury following LPS challenge. PloS One 2014;9:e88814.
[23] To LB, Haylock DN, Simmons PJ, Juttner CA. The biology and clinical uses of blood stem cells. Blood 1997;89: 2233–58.
[24] Araki H, Yoshinaga K, Boccuni P, Zhao Y, Hoffman R, Mahmud N. Chromatin-modifying agents permit human hematopoietic stem cells to undergo multiple cell divisions while retaining their repopulating potential. Blood 2007;109: 3570–8.
[25] Bancroft JD, Gamble M. Theory and practice of histological techniques. 6th ed. New York/London: Churchill Livingstone; 2008. p. 165–75.
[26] Rajatileka S, Luyt K, El-Bokle M, Williams M, Kemp H, Molnar E, et al. Isolation of human genomic DNA for genetic analysis from premature neonates: a comparison between newborn dried blood spots, whole blood and umbilical cord tissue. BMC Genet 2013;14:105.
[27] Snedecor G, Cochran W. Statistical methods. Ames: Iowa State University Press; 1980.
[28] dos Santos CC, Maron-Gutierrez T, Rocco PRM. Cell therapy in acute lung injury. Pulmão RJ 2011;20:64–8.
[29] Abdel Aziz MT, El-Asmar MF, Haidara M, Atta HM, Roshdy NK, Rashed LA, et al. Effect of bone marrow-derived mesenchymal stem cells on cardiovascular complications in diabetic rats. Med Sci Monit 2008;14: 249–55.
[30] Dreher L, Elvers-Hornung S, Brinkmann I, Huck V, Henschler R, Gloe T, et al. Cultivation in human serum reduces adipose tissue-derived mesenchymal stromal cell adhesion to laminin and endothelium and reduces capillary entrapment. Stem Cells Dev 2013;22:791–803.
[31] Geissler S, Textor M, Kuhnisch J, Konnig D, Klein O, Ode A, et al. Functional comparison of chronological and in vitro aging: differential role of the cytoskeleton and mitochondria in mesenchymal stromal cells. PloS One 2012; 7:e52700.
[32] Song L, Xu J, Qu J, Sai Y, Chen C, Yu L, et al. A therapeutic role for mesenchymal stem cells in acute lung injury independent of hypoxia-induced mitogenic factor. J Cell Mol Med 2012;16:376–85.
[33] Mei S, Yao W, Zhu Y, Zhao J. Protection of pirfenidone against an early phase of oleic acid-induced acute lung injury in rats. J Pharmacol Exp Ther 2005;313:379–88.
[34] Smith DB, Janmey PA, Lind SE. Circulating actin-gelsolin complexes following oleic acid-induced lung injury. Am J Pathol 1988;130:261–7.
[35] Gao J, Zhan Y, Chen J, Wang L, Yang J. Triptolide ameliorates lipopolysaccharide-induced acute lung injury in rats. Eur J Med Res 2013;18:1–7.
[36] Chang Y, Park SH, Huh J-W, Lim C-M, Koh Y, Hong S-B. Intratracheal administration of umbilical cord blood-derived mesenchymal stem cells in a patient with acute respiratory distress syndrome. J Korean Med Sci 2014;29:438–40.
[37] Naeije R, Brimioulle S. Physiology in medicine: importance of hypoxic pulmonary vasoconstriction in maintaining arterial oxygenation during acute respiratory failure. Crit Care 2001;5:67.
[38] Easley RB, Fuld MK, Fernandez-Bustamante A, Hoffman EA, Simon BA. Mechanism of hypoxemia in acute lung injury evaluated by multidetector-row CT. Acad Radiol 2006;13:916–21.
[39] Matthay MA, Ware LB, Zimmerman GA. The acute respiratory distress syndrome. J Clin Invest 2012;122:2731–40.
[40] De Paepe ME, Mao Q, Ghanta S, Hovanesian V, Padbury JF. Alveolar epithelial cell therapy with human cord blood–derived hematopoietic progenitor cells. Am J Pathol 2011;178:1329–39.
[41] Gonzalez-Rey E, Anderson P, Gonzalez MA, Rico L, Buscher D, Delgado M. Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. Gut 2009;58:929–39.
[42] Mao Q, Chu S, Ghanta S, Padbury JF, De Paepe ME. *Ex vivo* expanded human cord blood-derived hematopoietic progenitor cells induce lung growth and alveolarization in injured newborn lungs. Respir Res 2013;14:37.
[43] Fang X, Neyrinck AP, Matthay MA, Lee JW. Allogeneic human mesenchymal stem cells restore epithelial protein permeability in cultured human alveolar type II cells by secretion of angiopoietin-1. J Biol Chem 2010;285: 26211–22.
[44] Schinköthe T, Bloch W, Schmidt A. *In vitro* secreting profile of human mesenchymal stem cells. Stem Cells Dev 2008;17: 199–206.
[45] Lee JW, Fang X, Krasnodembskaya A, Howard JP, Matthay MA. Concise review: mesenchymal stem cells for acute lung injury: role of paracrine soluble factors. Stem Cells 2011;29:913–9.
[46] Milner CM, Higman VA, Day AJ. TSG-6: a pluripotent inflammatory mediator? Biochem Soc Trans 2006;34:446–50.
[47] Wisniewski HG, Vilcek J. Cytokine-induced gene expression at the crossroads of innate immunity, inflammation and

fertility: TSG-6 and PTX3/TSG-14. Cytokine Growth Factor Rev 2004;15:129–46.

[48] Szanto S, Bardos T, Gal I, Glant TT, Mikecz K. Enhanced neutrophil extravasation and rapid progression of proteoglycan-induced arthritis in TSG-6-knockout mice. Arthritis Rheum 2004;50:3012–22.

[49] Mindrescu C, Dias AA, Olszewski RJ, Klein MJ, Reis LF, Wisniewski HG. Reduced susceptibility to collagen-induced arthritis in DBA/1J mice expressing the TSG-6 transgene. Arthritis Rheum 2002;46:2453–64.

[50] Lee RH, Pulin AA, Seo MJ, Kota DJ, Ylostalo J, Larson BL, et al. Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. Cell Stem Cell 2009; 5:54–63.

[51] Majka M, Janowska-Wieczorek A, Ratajczak J, Ehrenman K, Pietrzkowski Z, Kowalska MA, et al. Numerous growth factors, cytokines, and chemokines are secreted by human CD34(+) cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner. Blood 2001;97:3075–85.

[52] Krenning G, van der Strate BW, Schipper M, van Seijen XJ, Fernandes BC, van Luyn MJ, et al. CD34+ cells augment endothelial cell differentiation of CD14+ endothelial progenitor cells in vitro. J Cell Mol Med 2009;13: 2521–33.

[53] Barcelos LS, Duplaa C, Krankel N, Graiani G, Invernici G, Katare R, et al. Human CD133+ progenitor cells promote the healing of diabetic ischemic ulcers by paracrine stimulation of angiogenesis and activation of Wnt signaling. Circ Res 2009;104:1095–102.

[54] He T, Peterson TE, Katusic ZS. Paracrine mitogenic effect of human endothelial progenitor cells: role of interleukin-8. Am J Physiol Heart Circ Physiol 2005;289:H968–972.