

## Original Article

# Mesenchymal stem cells attenuate oleic acid-induced acute lung injury may via keratinocyte growth factor

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**Abstract:** Background: Mesenchymal stem cells (MSCs) have been reported to treat acute lung injury (ALI) by modulating inflammation, maintaining lung structure, preserving lung function, and inhibiting epithelial cells apoptosis. The aim of the present study is to investigate whether MSCs enhance lung repair via keratinocyte growth factor (KGF) in Oleic acid (OA) induced ALI (OA-ALI). Methods: Rats were administered OA to induce ALI model. Then they were given intravenous injections of Phosphate Buffered Saline (PBS), dermal fibroblasts (DF), DF-CM, MSCs, and MSC-condition medium (MSC-CM) respectively. The extent of lung injury and lung function was assessed. A subsequent in vitro experiment was performed to examine the potential role of MSCs secretion in attenuating lung epithelial repairment using injured lung epithelial cells administered with involved MSCs, MSC-CM, KGF, HGF and TGF- $\beta$ . Results: Both MSCs and MSC-CM significantly improve lung function following OA-ALI. MSCs enhanced restoration of systemic oxygenation, reduced total lung water, decreased lung inflammation and histological lung injury and restored lung structure. These effects were not seen with dermal fibroblasts administration. In vitro experiment supported that, Keratinocyte growth factor improved lung epithelial repair. Conclusions: MSCs ameliorated lung repair by KGF in OA-induced ALI.

**Keywords:** Acute lung injury, stem cell, paracrine, keratinocyte growth factor

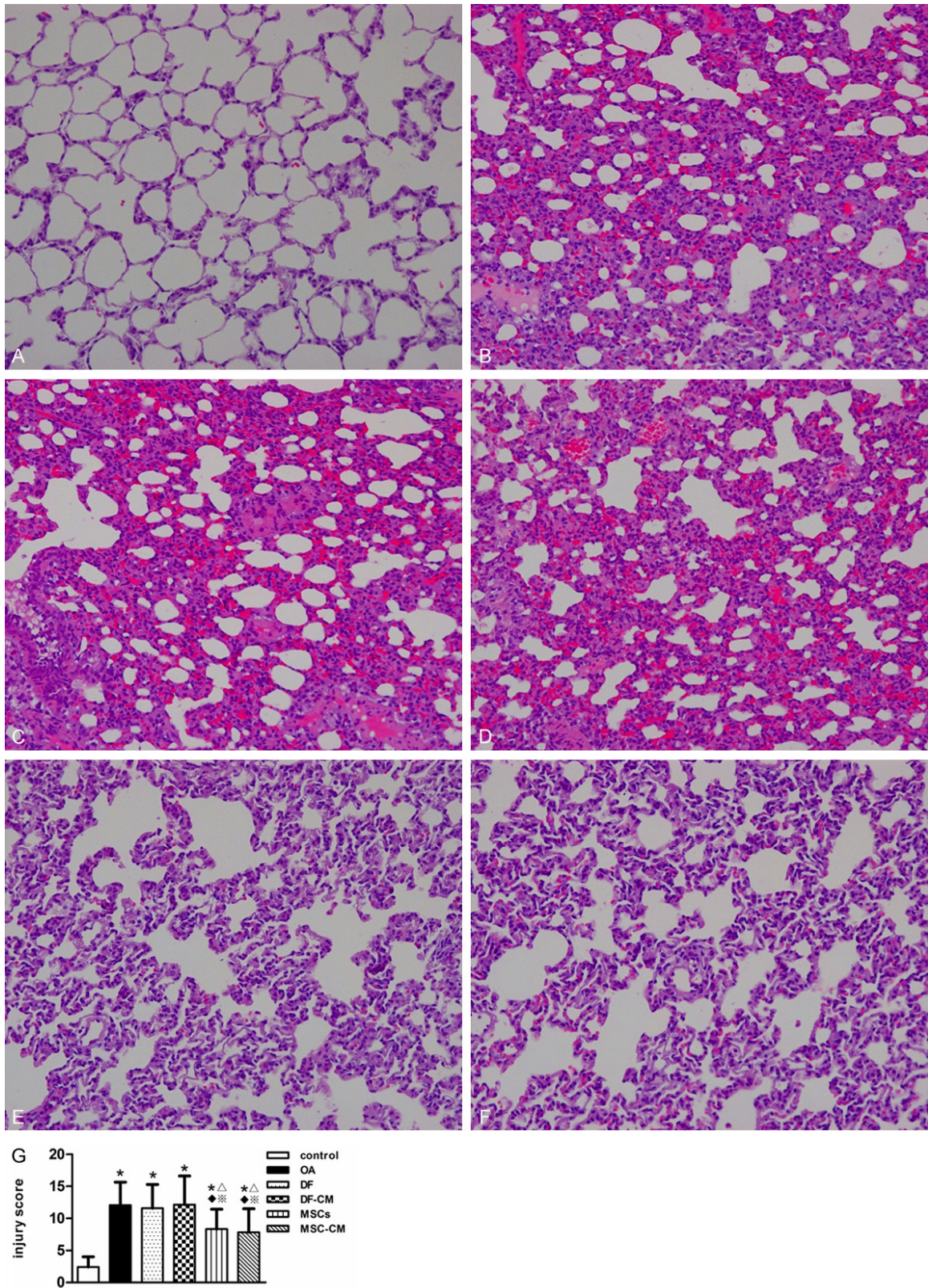
## Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) were firstly described in 1967 [1]. Despite advances in therapeutic principles, ALI/ARDS remains a leading cause of morbidity and mortality (up to 30 to 40%) in critically ill patients [2]. Mesenchymal stem cells (MSCs) is proved to be helpful in ALI model via decreasing lung inflammation and lung vascular permeability, this make it attractive to be a new treatment to deal with ALI [3]. Recent study has shown that MSCs can improve ALI via paracrine mechanism besides its differentiating into multiple cell types to augment or replace damaged tissues [4]. In paracrine mechanism, stem cell secretome is defined as the complex set of secreted molecules from stem cells that are crucial to many biological functions including cell growth, replication, differentiation, signaling, apoptosis, adhesion and angiogenesis. These secreted molecules are released by stem cells through classical

and non-classical secretion mechanisms, including protein translocation, exocytosis, and vesicle or exosome encapsulation. One of the most important MSCs paracrine factors is called Keratinocyte growth factor (KGF), a 28-kDa heparin binding member of the Fibroblast Growth Factor (FGF) family [5]. KGF has been proved to be therapeutic in skin, lung, bladder, gastrointestinal tract, and Graft-versus-Host diseases through its ability of Mitogenicity, reepithelialization, Cytoprotection, Antiapoptotic Effects, Differentiation and Proliferation [6]. But whether KGF pathway is a mechanism that MSCs attenuate ALI is still unknown. The present study focuses on the novel critical role of KGF in MSCs treating OA-induced ALI.

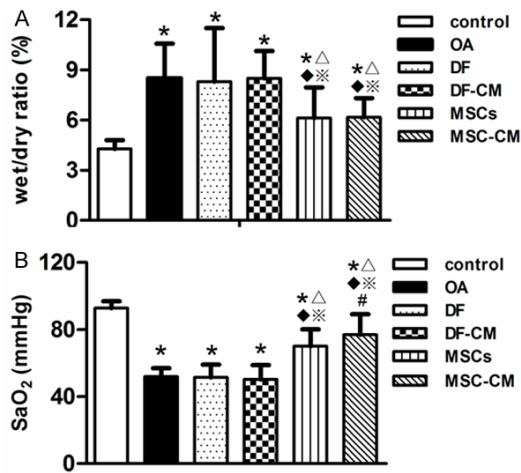
## Materials and methods

All animals received humane care in compliance with the Guide for the care and use of laboratory animals published by the National



**Figure 1.** MSCs and MSCs-CM restore lung structure following OA-induced ALI. Representative hematoxylin-eosin staining (HE) stained lung tissue from the following groups: control group (A); Oleic acid (OA) group (B); dermal fibroblasts (DF) group (C); dermal fibroblasts condition medium (DF-CM) group (D); Mesenchymal stem cells (MSCs) group (E) Mesenchymal stem cells condition medium (MSC-CM) group (F) (original magnification  $\times 100$ ); and injure

score of the groups above (G). \*Significantly different from control group.  $\Delta$ Significantly different from OA group.  $\blacklozenge$ Significantly different from DF group. \*Significantly different from DF-CM group. #Significantly different from MSCs groups ( $P < 0.05$  was considered as significant).



**Figure 2.** MSCs and MSC-CM treatments improved lung function. Mesenchymal stem cells (MSCs) and Mesenchymal stem cells condition medium (MSC-CM) therapy reduced wet to dry weight ratios of the injury lung (A) and enhanced lung ventilation function, which resulted in a higher systemic arterial oxygen pressure (SaO<sub>2</sub>) (B) following Oleic acid induced acute lung injury (OA-ALI) compared with other treatments and control group. \*Significantly different from control group.  $\Delta$ Significantly different from OA group.  $\blacklozenge$ Significantly different from dermal fibroblasts (DF) group. \*Significantly different from dermal fibroblasts condition medium (DF-CM) group. #Significantly different from MSCs groups ( $P < 0.05$  was considered as significant).

Institutes of Health (NIH publication 83-23, revised 1996). The study protocol was approved by the Laboratory Animal Ethics Committee of Capital Medical University.

#### MSCs culture and conditioned medium (CM) preparation

Frozen vial of passage 1 OriCell™ Sprague Dawley (SD) Rat MSCs with GFP (SD MSCs/GFP) and dermal fibroblasts (DF) was purchased from Cyagen Biosciences (CA, USA). The MSCs were thawed and expanded as previously described [7] and then generated using a protocol described. The MSCs constantly differentiated into bone, fat, and cartilage in culture and were negative for hematopoietic markers (CD34, CD36, CD117, and CD45) and positive for CD29 (95%), CD44 (>93%), CD49c (99%), CD49f (>70%), CD59 (>99%), positive for CD90

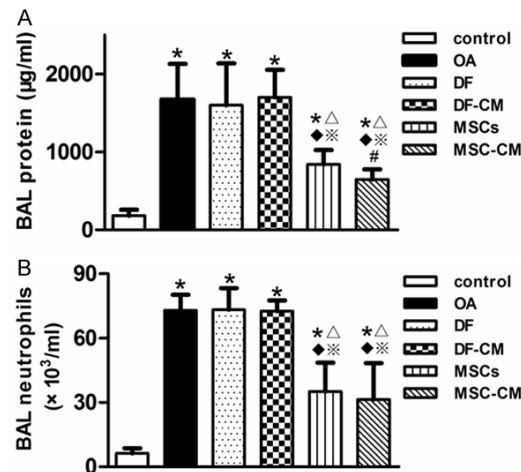
(>99%), CD105 (>99%), and CD166 (>99%). MSCs ( $2 \times 10^6$ ) and DF ( $2 \times 10^6$ ) were washed and cultured without serum for 24 h. The cells were again washed and the subsequent serum-free medium for the next 24 h was used as the conditioned medium (CM). For in vivo experiments, 15 ml of this medium was concentrated using a 3000 Da centrifugal concentrating filter (Amicon, Billerica, Massachusetts, USA) to give 500  $\mu$ l.

#### ALI model and grouping

Male SD rats weighing 180-225 g, 2-3 weeks old from the National Animal Center (Beijing China) were used in all experiments. 10 rats were randomly selected as control group, which received 500  $\mu$ l Phosphate Buffered Saline (PBS). OA and 95% ethanol were mixed at a solvent ratio of 1:1 and then injected after the left animals were anesthetized with 3% Pento-barbital through peritoneal cavity. When the model was established, the animal's cyanosis, breathed at 75-90/min, and delayed recovery. Then the model animals were randomly allocated to the groups as below ( $n=10$  each group): 1) OA group received an intravenous of 500  $\mu$ l PBS. 2) DF group received  $2 \times 10^6$  DF in 500  $\mu$ l PBS. 3) DF-CM group received 500  $\mu$ l DF-CM. 4) MSCs group received an intravenous injection of  $2 \times 10^6$  MSCs in 500  $\mu$ l PBS. 5) MSC-CM group received 500  $\mu$ l MSC-CM. The first injections were given immediately after the establishment of OA-ALI model and a second injection was administered 24 hours later.

#### Injured epithelial recovery experiment

An in vitro model of alveolar epithelial repair was used, as used in prior studies [8]. SD rat type II epithelial cells (Cyagen Biosciences, CA, USA) were wounded in confluent A549 monolayers in 24 well plates with a 1000  $\mu$ l pipette tip. The wounds were exposed to nothing, KGF, hepatocyte growth factor (HGF) and transforming growth factor  $\beta$  (TGF- $\beta$ ) (Abcam, Cambridge, UK) (as the concentration as that in MSCs-CM), respectively. At 48 h the extent of epithelial restitution was determined (Photoshop v8.0, Adobe Systems, San Jose, California, USA).



**Figure 3.** MSCs and MSC-CM decreased alveolar fluid protein concentrations and Neutrophils count. Both Mesenchymal stem cells (MSCs) and MSCs-conditioned medium (MSC-CM) (A) decreased bronchoalveolar lavage (BAL) neutrophil counts, (B) decreased BAL protein concentrations compared with other groups (n=10 animals per group). \*Significantly different from control group. ΔSignificantly different from oleic acid (OA) group. ♦Significantly different from dermal fibroblasts (DF) group. #Significantly different from dermal fibroblasts condition medium (DF-CM) group. #Significantly different from MSCs groups (P<0.05 was considered as significant).

#### Histological assessment of lung injury

Animals were killed at 48 hours after OA-ALI model was established and arterial blood gases were analyzed by blood gas analyzer. Postmortem, bronchoalveolar lavage (BAL) differential cell counts, protein concentration and cytokine levels were determined. Wet to dry lung weight ratios were measured and histological lung damage was assessed. Lung injury score = [(alveolar hemorrhage points/no. of fields) + 2 × (alveolar infiltrate points/no. of fields) + 3 × (fibrin points/no. of fields) + (alveolar septal congestion/no. of fields)]/total number of alveoli counted [3].

#### Measurements of soluble mediator concentrations in MSCs supernatants

MSCs between passages 2 and 5 were cultured on sixwell plates until 85-90% confluence was achieved. After exposure to experimental conditions, the supernatants were harvested and immediately centrifuged. The concentrations of soluble mediators, HGF, TGF-β, and KGF were measured by ELISA (R&D Systems) according to the manufacturer's instructions [9].

#### Bronchoalveolar lavage (BAL)

Rats were euthanized with 3% Pentobarbital through peritoneal cavity. Following surgical visualization of the trachea, BAL was performed by insertion of a 0.18 gauge angiocatheter and flushing of the lungs with 0.5 ml ice-cold PBS until a total volume of 3 ml was obtained. BAL fluid was strained (40-µm) and centrifuged. The cellular fraction was suspended in 1 ml PBS and total cell counts were determined using a hemocytometer. Differential cell counts were done following cytopspin and staining with a Diff-Quick Staining Kit (IMEB Inc.); >300 cells were counted in three separate fields for each animal. Vascular leakage in BAL fluid was assessed using a rat albumin ELISA Quantitation Set (Bethyl Laboratories, Inc., Montgomery, TX). Levels of Tumor Necrosis Factor (TNF-α), Interleukin (IL)-6, IL-10, in BAL fluid were performed using a Rats Inflammatory Cytometric Bead Array (BD Biosciences).

#### Statistical analysis

Data were analyzed using SPSS 16.0 (Chicago, USA). The distribution of all data was tested for normality using Kolmogorov-Smirnov tests. Data were analyzed by one-way ANOVA. Comparisons between two groups were performed using unpaired two-tailed Student t tests or a Mann-Whitney U test. A two-tailed P value of <0.05 was considered significant.

#### Results

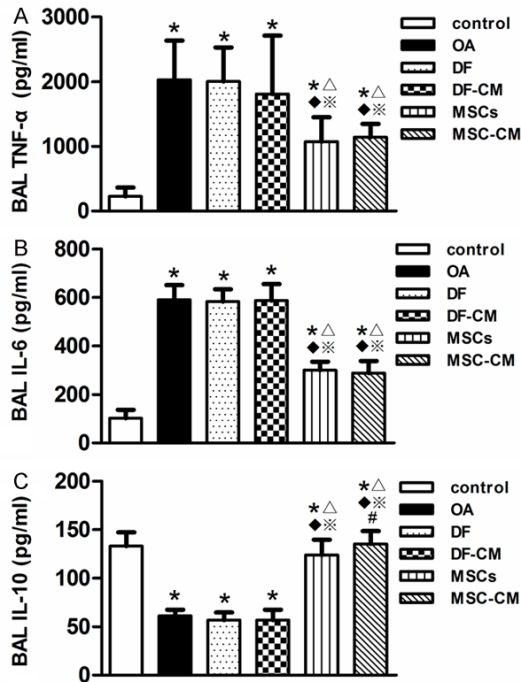
There were no differences between the groups at baseline with regard to animal weight.

#### MSCs and MSCs-CM restored lung structure following OA-induced ALI

MSCs decreased alveolar thickening as evidenced by reduced alveolar tissue volume fraction while MSCs increased recovery of airspace volume as evidenced by increased alveolar airspace volume fraction. Representative histological sections of lung demonstrate the greater degree of resolution of injury and alveolar infiltrates in each group (Figure 1A-F). The injury score of each group was shown (Figure 1G).

#### The administration of MSCs and MSC-CM enhanced injured lung function

Both MSCs and MSC-CM enhanced the restoration of lung micro vascular permeability, as evi-



**Figure 4.** MSCs and MSC-CM balanced inflammatory cytokines. Mesenchymal stem cells (MSCs) exert benefits via a paracrine mechanism. Both MSCs and MSCs conditioned medium (MSC-CM) (A) decreased BAL tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) concentrations, (B) reduced alveolar interleukin 6 (IL-6), and increased interleukin 10 (IL-10) compared with other groups (n=10 per group). \*P<0.05 vs fibroblast CM and fibroblast groups. \*Significantly different from control group.  $\Delta$ Significantly different from oleic acid (OA) group.  $\diamond$ Significantly different from dermal fibroblasts (DF) group. \*Significantly different from dermal fibroblasts condition medium (DF-CM) group. \*Significantly different from MSCs groups (P<0.05 was considered as significant).

denced by a decrease in lung wet to dry weight ratios (Figure 2A). MSCs and MSC-CM enhanced oxygenation following ALI (Figure 2B).

#### MSCs and MSC-CM decreased alveolar fluid protein concentrations and neutrophils count

Both MSCs and MSC-CM decreased BAL protein concentration (Figure 3A) and neutrophils count (Figure 3B) compared with groups that received fibroblasts or fibroblast conditioned medium.

#### Both MSCs and MSC-CM balanced the inflammatory response

Both MSCs and MSC-CM decreased BAL tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) concentrations (Figure 4A), reduced alveolar interleukin 6 (IL-

6) concentrations (Figure 4B) and increased alveolar interleukin 11 (IL-11) concentrations (Figure 4C) compared with groups that received fibroblasts or fibroblast conditioned medium (n=10 animals per group).

#### KGF enhances pulmonary epithelial wound repair

Through our ELISA detection, the KGF, HGF and TGF- $\beta$  concentration in MSCs-CM is 307 pg/ml, 13.7 ng/ml and 0.2 ng/ml respectively.

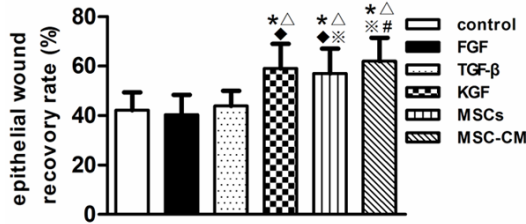
Incubation of MSCs, MSC-CM or KGF appeared to have beneficial effects on epithelial wound repair. In contrast, incubation with HGF and TGF- $\beta$  did not alter wound repair (Figure 5). Measurement of KGF concentrations in the MSCs and fibroblast CM showed that MSCs produced substantially greater amounts of KGF (310 $\pm$ 90 vs. 68 $\pm$ 13 pg/ml, P<0.0001).

#### Discussion

The present study, which investigated the therapeutic impact of MSCs, and MSCs-CM on OA-ALI, provided striking implication. Both MSCs and MSCs-CM attenuated OA-induced ALI. KGF enhanced pulmonary epithelial wound repair. Moreover, our data proved that MSCs secreted KGF in CM. Therefore, MSCs may alleviate OA-induced ALI via secreting KGF, which suggests KGF might be a new target for ALI treatment.

The results showed that, the injured score in MSCs group is significantly lower than that in OA-ALI group, which was accordance with previous study. Some study has shown that the MSCs improve ALI through its Homing to injured cells and transaction to function cells. So, in the present study, the MSCs-CM which was free with MSCs was involved, and we found that MSCs-CM which was not with can also be therapeutic to OA-ALI, which indicates the paracrine mechanism may be one of its therapeutic pathway.

More recent work suggests a complex role of stem cells in functional recovery since many studies of stem cells in animal models suggest that MSCs are relatively short-lived after delivery and do not engraft and differentiate to form new permanent tissues [10-13]. Additionally, stem cells delivered into animal models have



**Figure 5.** Mesenchymal stem cells (MSCs) may enhance pulmonary epithelial wound closure via a KGF-dependent mechanism. In pulmonary epithelial layers, both Mesenchymal stem cells (MSCs) and MSCs conditioned medium (MSC-CM) enhanced wound closure. Incubation with keratinocyte growth factor (KGF), but not hepatocyte growth factor (HGF) or transforming growth factor  $\beta$  (TGF- $\beta$ ), can lead to a similar result. \*Significantly different from control group.  $\Delta$ Significantly different from FGF group.  $\blacklozenge$ Significantly different from TGF- $\beta$  group.  $\circ$ Significantly different from KGF group. #Significantly different from MSCs groups ( $P < 0.05$  was considered as significant).

been found to exert therapeutic benefits despite engraftment distant from the site of actual injury. Contemporary studies have demonstrated that, in addition to differentiating into target tissue types, stem cells likely exert a therapeutic effect via the secretion of bioactive factors that have antiapoptotic, antiscarring, and neovascularization effects, as well as immunomodulatory properties [14]. The investigation of this protein milieu or “secretome” is a subject of growing interest with the increasing recognition of the paracrine/autocrine role of cell secretions in the regulation of many physiological processes and their potential for therapeutic application.

The exact mechanism of action of exogenous KGF remains unclear. It imitates the actions of intrinsic KGF and binds to the FGF receptor 2b (FGFR2b) which is a tyrosine kinase receptor. FGFR2b is present in the epidermis, oral mucosa, GI mucosa and urothelium. KGF, through stimulation of this receptor, increases the regenerative capacity of these tissues. It is responsible for various biological actions and its effects can be divided into three categories [15]. These include cytoprotective effects, modulation of cytokine profile and trophic or regenerative effects. The cytoprotective effects are caused by upregulation of detoxifying enzymes such as glutathione peroxidase and glutathione S-transferase. These enzymes protect against injury from ROS [16]. This may be

mediated by transcription factors such as nuclear factor erythroid-derived 2 (NF-E2)-related factor (Nrf-2) and Nrf-3. There is also activation or upregulation of DNA polymerases, protein kinase C and tyrosine kinases. These enzymes prevent damage to the DNA strand [17]. Prevention of cellular damage is also caused by inhibition of epithelial cell apoptosis by modulation of apoptotic and antiapoptotic factors [18]. It has also been shown in vitro to stimulate the extracellular-signal-regulated kinase and cause upregulation of the v-akt murine thymoma viral oncogene homolog (Akt) pathway [19]. Akt is an antiapoptotic pathway and its stimulation leads to increased survival of cells. Cytokines play an important role in the development of mucositis. KGF downregulates the T helper type 1 (Th-1) proinflammatory cytokines, (TNF- $\alpha$ ) and IFN- $\gamma$  and cause upregulation of Th-2 cytokines IL-4 and IL-13 [20, 21]. KGF has trophic or regenerative effects on the oral and intestinal mucosa. It has been known to decrease the atrophy and ulcer formation in oral epithelium in animal models. It enhances the survival and proliferation of crypt cells and goblet cells in the intestine after chemoradiation [22]. It also stimulates EGF receptors and upregulates proteases involved in remodeling of tissues [23].

In our study, the inflammatory cytokine level of MSC-CM group was lower and the Anti-inflammatory factor level was higher than that in MSCs group; the  $PO_2$  is higher and the wet/dry ratio is lower than that in MSCs group, which indicated a better therapeutic effect in MSC-CM.

The possible reason may be that the therapeutic paracrine factor like KGF in the MSC-CM is much higher than that in MSCs group, and at the 24 h after the ALI model was made, a second dose was given, which may further increase the concentration of KGF.

In conclusion, one of MSCs secret factors called KGF, may contribute to injured lung epithelial recovery and result a better lung function.

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## Disclosure of conflict of interest

None.

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