

Original Article

Ischemic preconditioning potentiates the protective effect of mesenchymal stem cells on endotoxin-induced acute lung injury in mice through secretion of exosome

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Abstract: Objective: To explore the effect of bone marrow mesenchymal stem cells (MSCs) on endotoxin-induced acute lung injury in mice and verify the role of exosome. Methods: Exosome was isolated from the culture supernatant of MSC. For ischemic preconditioning, MSCs were subjected to anoxia for 0 min (MSCs group), 30 min (MSCs^{IPC-30} group), 60 min (MSCs^{IPC-60} group) and 90 min (MSCs^{IPC-90} group), and then used to treat endotoxin-injured mice. The exosome from the optimal group was used to treat endotoxin-injured mice. In addition, the exosome from the optimal group was also used to treat the endotoxin-stimulated RAW 264.7 cells for 6 h and 12 h. Results: CD63 positive exosome were acquired through ExoQuick kits. Administration of MSCs, MSCs^{IPC-30}, MSCs^{IPC-60} and MSCs^{IPC-90} could reduce the level of white blood cells (WBC) and neutrophils into the bronchoalveolar lavage (BAL) fluid of endotoxin-injured mice, and the MSC^{IPC-60} group had the greatest reduction, which reduced WBC by 57% and neutrophils by 55%. Administration of MSCs^{IPC-60} exosome could also reduce the level of WBC, neutrophils, MIP-2 and penetration protein into the BAL fluid of endotoxin-injured mice, which had the same effect as MSCs^{IPC-60} and showed a dose dependent, compared to MSCs exosome. In addition, MSCs^{IPC-60} exosome were used to treat endotoxin-stimulated RAW 264.7 cells, and the level of TNF α at 6 h and 12 h was significantly reduced, while the level of IL-10 at 12 h increased. Conclusion: Ischemic preconditioning for 60 min can potentiates the protective effect of MSC on endotoxin-induced Acute Lung Injury through the secretion of Exosome.

Keywords: Ischemic preconditioning, bone marrow mesenchymal stem cells, exosome, and acute lung injury

Introduction

Acute lung injury (ALI) is a type of systemic inflammatory response syndrome induced by various non-cardiogenic factors, which characterizes refractory hypoxemia and respiratory distress. ALI is also one of major causes of respiratory failure for severe patients with common etiology of endotoxin-induced sepsis, trauma, shock and so on [1, 2]. Treating the primary diseases, proper fluid resuscitation, protective mechanical ventilation, fully lung recruitment, small dose corticosteroid, controlling blood glucose strictly, application of human activation protein C and nutritional support are common treatments [3, 4]. Although ALI gains great improvement in treatment and nursing, the mortality still maintains at around 40%. Recently, many studies have proved that MSCs could provide a novel treatment strategy for

endotoxin-induced ALI [5, 6]. Mechanism may be closely correlated with paracrine effect [7]. Paracrine soluble factors could stabilize the injured alveolar and epithelial cells, so as to reduce inflammatory and promote the recovery of pulmonary function. At present, many investigations focus on exosome, which is proved to be one of the critical mechanisms of mediating cell paracrine and can carry related mRNA, miRNAs and proteins to act on receptor cell to play regulative role [8, 9]. Zhu [10] found that exosome derived from MSCs can treat endotoxin-induced ALI and can achieve similar treatment efficacy with MSCs. In another word, MSCs took effect to treat ALI with exosome. Ischemic preconditioning can activate endogenous defense mechanism to protect cells and body from various kinds of acute attack damage [11]. Many studies demonstrated that ischemic preconditioning could potentiate the survivability and

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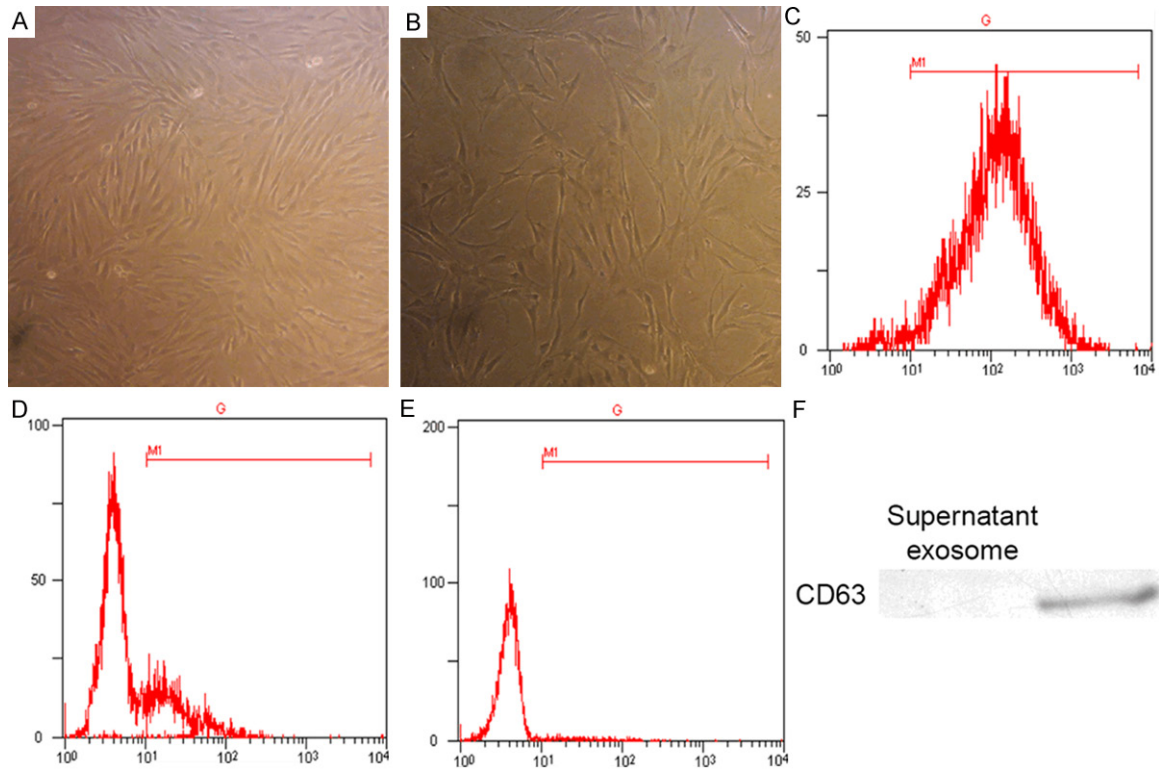


Figure 1. The isolation and identification of MSCs and exosome. A, B: Morphology of MSC (100 ×). A: MSCs of passaged 1; B: MSCs of passaged 2. C-E: Phenotypes of MSCs by flow cytometer. C: CD29, a MSCs marker; D: CD45, a pan-leukocyte marker; E: CD34, a hematopoietic progenitor marker. F: Western blotting for the exosome-enriched protein, CD63.

anti-apoptosis ability of MSCs, and transplanting ischemic preconditioning MSCs also suppressed related inflammatory factors and immune response, so as to promote function recovery of related organs [12]. However, there are no related reports about whether MSCs can further enhance the recovery of endotoxin-induced ALI or can perform treating effect with exosome.

This study aimed to compare the MSCs treatment effect on ALI at different time, screen the optimal ischemic preconditioning time, isolate exosome to treat ALI mice and intervene endotoxin-activated RAW264.7 cells. Then we observe the effect of exosome derived from the optimal ischemic preconditioning MSCs, in order to provide a novel strategy for ALI treatment.

Materials and methods

Isolation culture and identification of MSCs

Bone marrow was aspirated from the iliac crest bone of consenting healthy donors aged 20 to

40 years old. Adherent method was used to culture MSCs according to the literature [13]. Cells were digested to conventional passage or cryopreservation when the cells spread 80% to 90% of culture bottle bottom. Flow cytometry was used to identify their related markers CD29, CD34 and CD45.

Ischemic preconditioning of MSCs

MSCs inoculated on the cell culture plate by 5×10^5 cells/60 mm were through ischemic preconditioning in hypoxia incubator (Forma-1025 Anaerobic System) for 30 min, 60 min and 90 min respectively after starvation for a night.

Isolation and identification of Exosome

Medium was collected and under centrifugal at 3000 r/min for 15 min, and then supernatant was transferred to an aseptic container and mixed with appropriate volume of ExoQuick Exosome Precipitation Solution (SBI) and through cold storage for 16 hours. The ExoQuick/supernatant mixer was under centrifugal at 3000 r/min for 30 min, and then under centrif-

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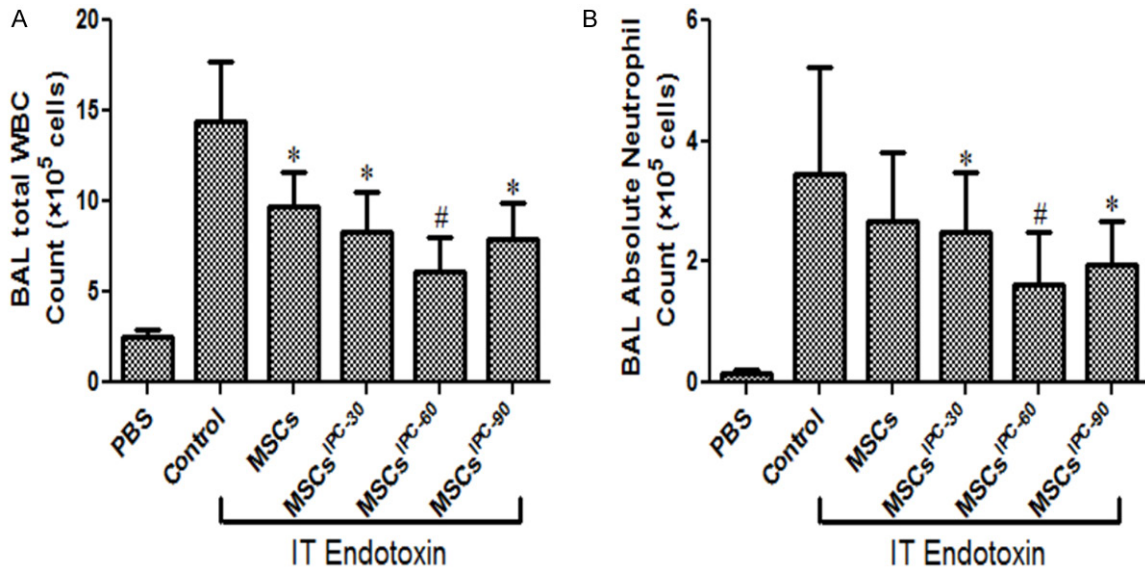


Figure 2. The effect of MSCs^{IPC} on endotoxin-induced acute lung injury (ALI) in mice. A, B: Administration of MSCs^{IPC} reduced the influx of WBC and neutrophils into the BAL fluid of endotoxin-injured mice. * $P < 0.05$ vs. Control group. # $P < 0.05$ vs. other groups.

ugal at 1500 r/min for 5 min to remove residual ExoQuick after discarding supernatant. The protein concentrations were detected by BCA kit and maker CD63 was identified by Western blot.

Model construction and treatment of endotoxin-induced ALI

Endotoxin from *E. coli* O111: B4 (Sigma-Aldrich, St. Louis, MO) was perfused to tracheas of mice by 4 mg/kg, and then the injury mice was treated through the tail vein by ischemic preconditioning MSCs or exosome. The treatment dose of MSCs and exosome were 5×10^4 cells/g and 1.5 $\mu\text{g/g}$ respectively.

Measurement of inflammatory cells, inflammatory factors and protein in BAL fluid

The BAL fluid in each group was collected after 48 h. Total cell number was counted by Z1 Coulter Particle Counter (Beckman Coulter). The differentiation of WBC was analyzed by Hemavet HV950FS. The level of MIP-2 was detected by ELISA kit. The protein concentration was detected by BCA.

Effect of exosome on endotoxin-activated RAW264.7 cells

RAW 264.7 was inoculated to a 24 well plate by 1×10^5 cells/well, then 500 ng/ml endotoxin

and 30 μg exosome were added simultaneously. Supernatant was collected after intervention for 6 h and 12 h respectively, and then the level of IL-10 and TNF α were detected by ELISA kit.

Statistical analysis

The software package SPSS 13.0 was conducted for statistical analysis. The data was demonstrated with means \pm standard deviation. One-way analysis of variance (ANOVA) was applied to analyze the difference among multiple groups. Least difference for significance was applied to analyze the difference between each two groups.

Results

Isolation and identification of MSCs and Exosome

The morphology of passage 1 MSCs showed spindle shape (**Figure 1A**), and the morphology of the MSCs had no significant change after continuous subcultivation. Passage 3 MSCs were more homogenous, but the light refraction of cytoplasm was better (**Figure 1B**). The flow cytometry showed that the positive rate of CD29, CD45 and CD34 were $98.09\% \pm 1.5\%$ (**Figure 1C**), $22.81\% \pm 1.8\%$ (**Figure 1D**) and $4.61\% \pm 0.5\%$ (**Figure 1E**), respectively. CD63

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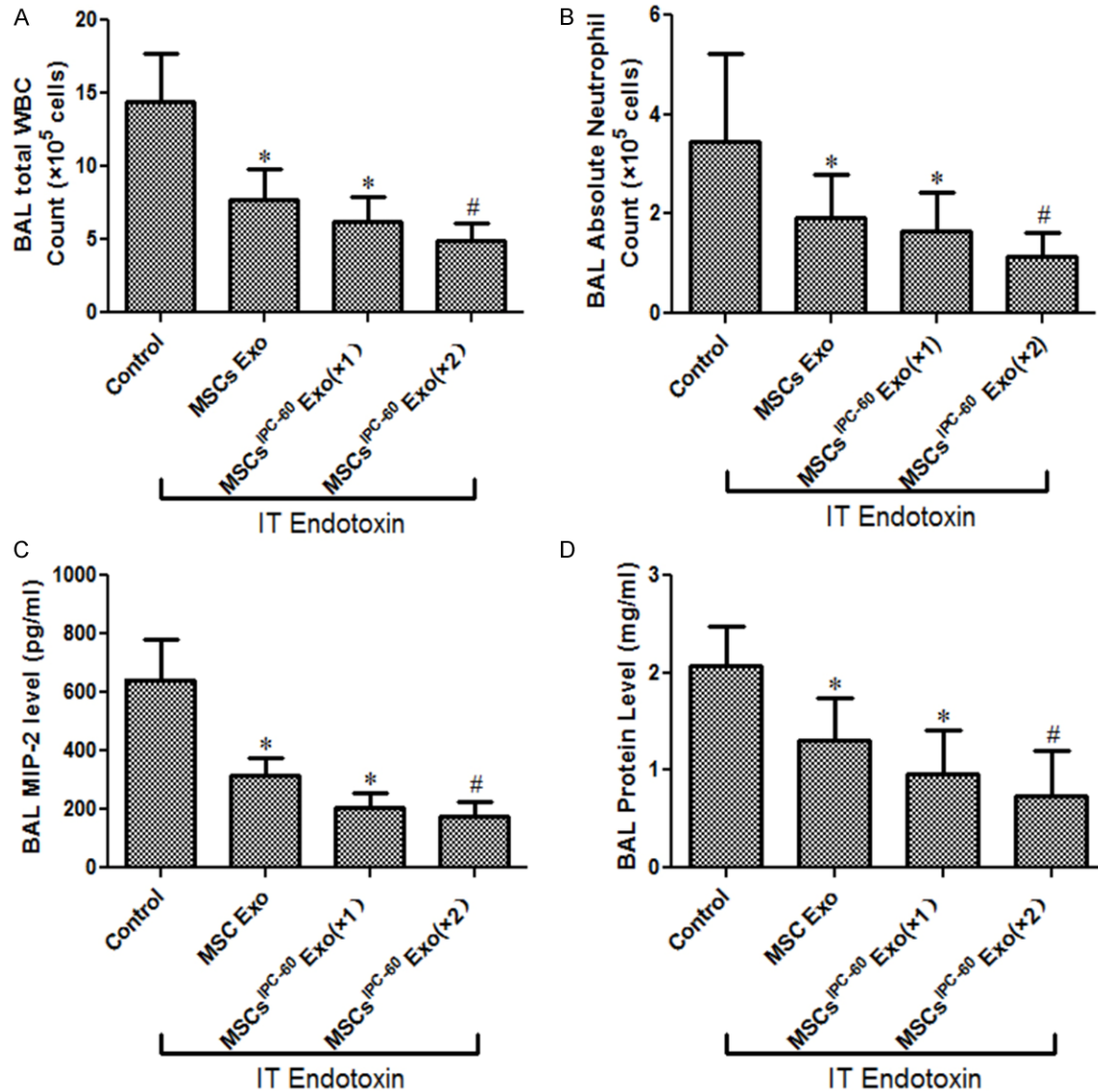


Figure 3. The effect of MSCs^{IPC-60} on endotoxin-induced acute lung injury (ALI) in mice. A-D: Administration of MSCs^{IPC-60} reduced the level of WBC, neutrophils, MIP-2 and penetration protein into the BAL fluid of endotoxin-injured mice. * $P < 0.05$ vs. Control group. # $P < 0.05$ vs. other groups.

positive exosome were acquired through Exo-Quick kits (Figure 1F).

Effect of MSCs^{IPC} on endotoxin-induced acute lung injury (ALI) in mice

Relatively strong inflammatory reaction appeared in alveoli of mice with the level of the WBC and neutrophils increase after perfusing endotoxin into mouse trachea for 48 h. 100 μ L MSCs, MSCs^{IPC-30}, MSCs^{IPC-60}, and MSCs^{IPC-90} were injected through the tail vein, and the endotoxin was perfused at the same time.

The level of the WBC and neutrophils decreased at different degree after 48 h. The levels of WBC and neutrophils in MSCs^{IPC-60} decreased by 57% and 55% respectively, which indicated MSCs^{IPC-60} had the best treatment effect on ALI (Figure 2).

Effect of MSCs^{IPC-60} on endotoxin-induced acute lung injury (ALI) in mice

30 μ L MSCs exosome and MSCs^{IPC-60} exosome were injected through tail vein respectively when endotoxin was injected. After 48 h, the

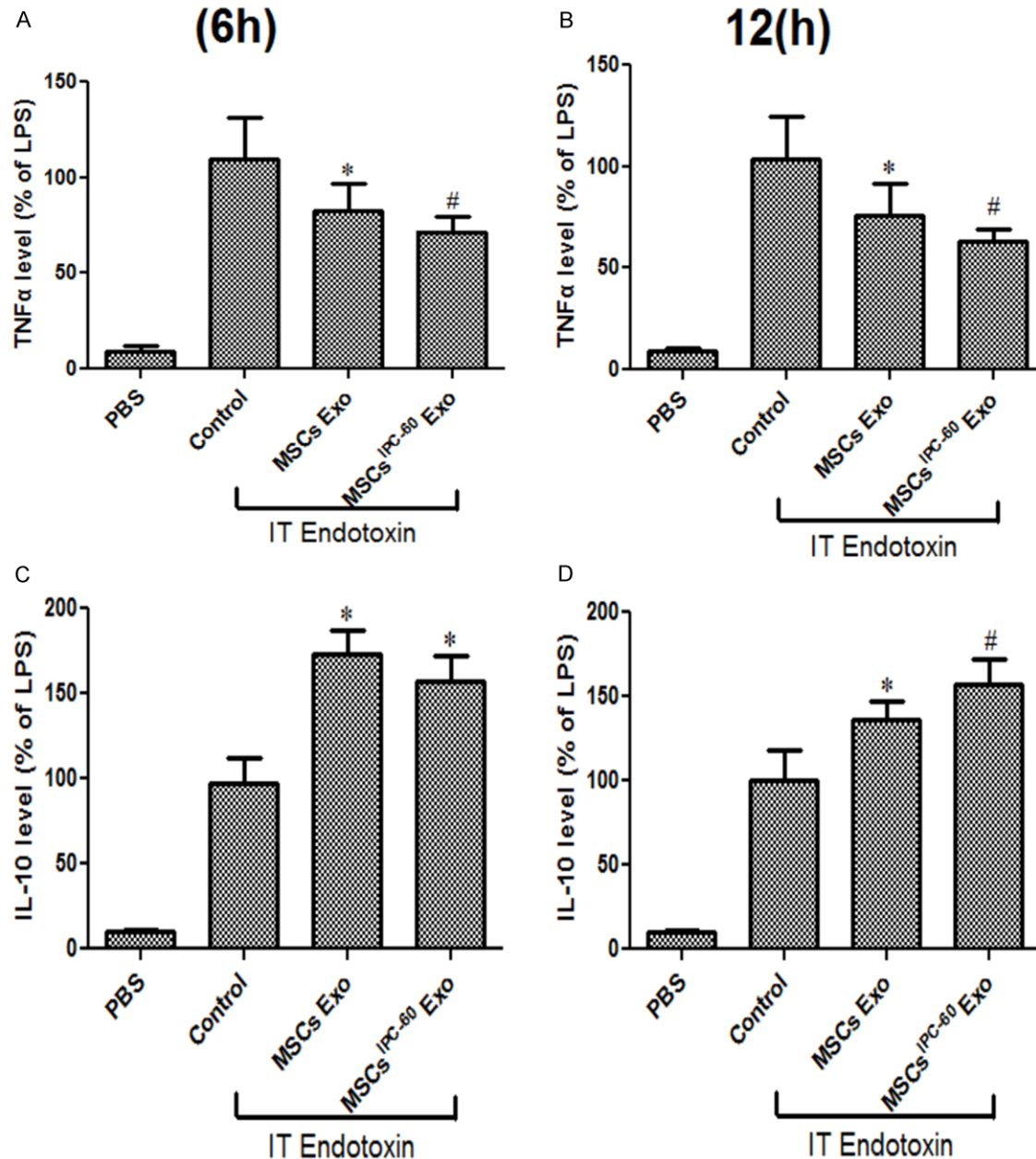


Figure 4. The effect of MSCs^{IPC-60} exosome on endotoxin-induced RAW 264.7 cells. A, B: MSCs^{IPC-60} exosome significantly reduced the levels of TNF α at 6 h and 12 h; C, D: MSCs^{IPC-60} exosome increased the levels of IL-10 at 6 h and 12 h. * $P < 0.05$ vs. Control group. # $P < 0.05$ vs. other groups.

level of WBC, neutrophils, inflammatory factors MIP-2 and osmotic protein decreased. The level in MSCs^{IPC-60} exosome group having most significant decrease were 60%, 56%, 72% and 55% respectively, which proved the effect was similar with injecting MSCs^{IPC-60}. Besides, the level of WBC, neutrophils, inflammatory factors MIP-2 and osmotic protein decreased more significantly when the dose of MSCs^{IPC-60} exosome

was doubled, which proved that MSCs^{IPC-60} exosome in the ALI treatment was dose-dependent (Figure 3).

Effect of MSCs^{IPC-60} exosome on endotoxin-induced RAW 264.7 cells

30 μ L MSCs exosome and MSCs^{IPC-60} exosome were added at the same time after RAW 264.7

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cell was activated by endotoxin. The supernatant was collected and the level of TNF α and IL-10 were detected after 6 h and 12 h. The level of TNF α both decreased at 6 h and 12 h, and there had more significant decrease in MSCs^{IPC-60} exosome group. In contrast, the level of IL-10 increased, and the MSCs^{IPC-60} exosome at 12 h were much more significantly increased (Figure 4).

Discussion

Acute lung injury is one of the major causes for respiratory failure in critical patients and threatens the life of patients severely. Thus, a novel treatment of ALI is of great significant. Our study showed that ischemic preconditioning for 60min could potentiate the treatment effect of MSCs on ALI significantly. We found that the treatment effect of MSCs^{IPC-60} exosome was similar to MSCs^{IPC-60} and was dose-dependent when we treated ALI with MSCs^{IPC-60} exosome further. As a result, the treatment effect of MSCs^{IPC-60} on ALI model was mainly through exosome related way.

With the advantage of isolation, purification, proliferation easily and less involved in ethical issue, MSCs provide a novel strategy for ALI treatment [14]. The mechanism may be related with the followings: 1) MSCs can migrate to specific tissue to take effect locally. Liang [15] found that the density of exogenous MSCs in the region of lung injury increased when they treated ALI model with MSCs after transplantation for 1 d. 2) MSCs have the function of anti-inflammatory and immune regulation. This study found that the level of WBC and neutrophils in BAL fluid decreased significantly compared with control group, which was consistent with previous reports. However, the homing and anti-inflammatory of MSCs were both closely with the paracrine function of MSCs. Many studies in recent years found that MSCs could improve the main conditions of ALI through secreting growth factors, anti-inflammatory factors and kinds of soluble factors [7]. Exosome is an important material to mediate cell paracrine, which can mediate mRNA, miRNA and protein to receptor cells, and then change cell function. Some studies demonstrated that exosome played an important roles in many biological processes, e.g. tumor metastasis, artery sclerosis and antigen presentation [16, 17]. Zhu [10] found that exosome derived from

MSCs could treat endotoxin-induced ALI, and MSCs played roles through KGF carried by exosome. The present study found that compared with control group, exosome derived from MSCs could reduce the level of WBC and neutrophils in BAL liquid in the ALI mice. That was to say, it was of great importance to investigate MSCs mechanism from the aspect of exosome.

Although many scholars in the domestic and overseas have proved that stem cell transplantation could improve the function of injury organs in animal experiments and clinical trials. In the practice, however, the treatment effect of stem cells was not ideal because of low survival rate of stem cells [18, 19]. In order to guarantee the treatment effect of the stem cells, improving the survival ability and anti-apoptosis was very important. Therefore, scholars adopted various kinds of ways, in which ischemic preconditioning was an important way to improve the survival rate of stem cells. Endogenous defense mechanism was activated by ischemic preconditioning, so as to protect stem cells from various kinds of acute attacking damages [11]. The present study compared the treatment effect of MSCs on the ALI model after ischemic conditioning for 30 min, 60 min and 90 min, which indicated that the treatment effect of MSCs had the best efficacy after ischemic preconditioning for 60 min. Feng [20] found that MSCs^{IPC-60} had a reasonable effect on treating myocardial infarction, which was consistent with our findings. Besides, Feng [20] also found that the survival ability and anti-apoptosis ability had a great improvement when ischemic preconditioning MSCs and myocardial cells were cultured together, which took effects by the miR-22 mediated by exosome. Hence, the treatment effect of ischemic preconditioning MSCs not only improved the survival rate and anti-apoptosis ability of MSCs by ischemic preconditioning, but also improved the survival rate and anti-apoptosis ability of surrounding cells through exosome ways. Therefore, exosome derived from MSCs^{IPC-60} was used to treat ALI mice in this study, which showed a similar treat effect with MSCs^{IPC-60} and was dose-dependent.

Moreover, exosome derived from MSCs^{IPC-60} was used to intervene the endotoxin-activated RAW 264.7 cells in our study, which showed that compared with exosome derived from MSCs, the level of TNF α decreased significantly

after 6 h and 12 h and the level of IL-10 increased significantly after 12 h. TNF α is a kind of promotion inflammation cytokine, the level of which decrease means that the level of inflammation drops. IL-10 is a kind of anti-inflammatory cytokine and was reported to play an important role in the processes of MSCs treating sepsis [5]. Therefore, it is demonstrated that MSCs^{IPC-60} treat endotoxin-induced ALI model through the exosome way at the cellular level.

However, there were some limitations in our study. For example, transplanted MSCs and exosome were not marked, and the contents of exosome derived from MSCs^{IPC-60} were not identified. Thus, we need to mark MSCs and exosome to trace on the homing of MSCs and exosome. The difference between the contents of MSCs^{IPC-60} exosome and MSCs exosome need to be compared by proteomics technology and miRNA array, so as to find molecule that have important effect to further explain the mechanism of MSCs. In summary, this study identified that MSCs^{IPC-60} treated endotoxin-induced ALI through exosome related ways and provided a novel strategy for ALI treatment.

Disclosure of conflict of interest

None.

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