Original Research

Mesenchymal stem cells protects hyperoxia-induced lung injury in newborn rats via inhibiting receptor for advanced glycation end-products/nuclear factor κB signaling

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Abstract

Bone marrow-derived mesenchymal stem cells (BMSCs) have been shown recently to ameliorate hyperoxia-induced lung injury, but the underlying mechanism remains unclear. This study aimed to determine whether BMSCs attenuate hyperoxia-induced lung injury by down-modulating the inflammatory RAGE/NF-κB (receptor for advanced glycation end-products/nuclear factor-κB) signaling. Thirty Sprague-Dawley newborn rats were randomly divided into three groups ($n = 10$): sham control (C); hyperoxia-induced acute lung injury (ALI) (B) and ALI with BMSCs transplantation (A). Rats were sacrificed at three-day post-transplantation. RAGE and NF-κB expression in lung tissue was detected by reverse transcription polymerase chain reaction, Western blot and immunohistochemistry analysis. The levels of tumor necrosis factor α (TNF-α) and RAGE in bronchoalveolar lavage fluid (BALF) and in serum were detected by enzyme-linked immunosorbent assay. The lung damage was evaluated by histological examination. The results showed that RAGE and TNF-α concentrations in BALF were significantly lower in Group A than in Group B. Moreover, RAGE and NF-κB expression in lung tissue at mRNA and protein concentrations was significantly lower in Group A than in Group B. The lung damage score was significantly lower in Group A than in Group B. These data demonstrate that hyperoxia induces the inflammation and causes damage in the lung but BMSC transplantation could alleviate hyperoxia-induced lung injury by inhibiting the inflammatory process mediated by RAGE/NF-κB signaling.

Keywords: hyperoxia, acute lung injury, mesenchymal stem cells, RAGE, NF-κB

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Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung injury characterized by an arrest in alveolar development and increased apoptosis of alveolar epithelial cells (AECs), which are largely caused by mechanical ventilation and oxygen therapy.[1] Notably, children diagnosed with BPD have a high risk of developing chronic obstructive pulmonary disease later in life.[2] Although the molecular pathogenesis of BPD is not well understood, it is proposed that pathological mechanisms include fibrosis, apoptosis and abnormal cellular proliferation, which lead to arrested alveolar development and associated abnormal vascular growth.[3] Currently, no effective treatments for BPD have been developed in the clinic.

Recent evidence suggests that AECs play an important role in the pathological processes of the lung injury, such as the formation of pulmonary edema, alveolar and interstitial fibrin deposits, and affect the outcome and prognosis of acute lung injury (ALI) and acute respiratory distress syndrome.[4] Thus, further characterization of AECs may provide clues on the therapy of ALI induced by hyperoxia. The receptor for advanced glycation end-products (RAGE) is a member of the immunoglobulin superfamily of cell surface receptors which interacts with a wide range of ligands, such as High-Mobility Group Box-1 (HMGB-1), S100B, advanced glycation end-products (AGEs). RAGE and its isoforms play an essential role in the biology of the lung under both physiological and pathological conditions.[5] Recent studies show that RAGE is predominantly localized to alveolar type I cells in the lung, and considered as the marker of type I alveolar cell injury in ALI.[6] In addition, RAGE targeting could diminish hyperoxia-induced pulmonary injury.[7] Obviously, RAGE represents a new therapy target for newborn lung injury induced by hyperoxia.

A number of studies have demonstrated that bone marrow-derived mesenchymal stem cells (BMSCs) can

ameliorate hyperoxia-induced lung injury.[8–10] Mechanisms for this protection are not limited to tissue repair, such as engraftment and differentiation of BMSCs into specific lung cell types, but also include the suppression of lung inflammation in hyperoxic newborn rats reported recently.[11,12] These properties make BMSC treatment a promising approach for the repair of neonatal lung injury. In this study we tested our hypothesis that BMSCs may protect hyperoxia-induced lung injury via down-regulating the inflammatory signaling pathway of RAGE–NF-κB in AECs. Our results showed that hyperoxia upregulates RAGE/NF-κB signal pathway in lung tissue of newborn rats, and transplantation of BMSCs attenuates hyperoxia lung injury.

Materials and methods

Cell preparation

This study was approved by Animal Care and Use Committee of Nanjing Medical University. BMSCs were isolated and cultured from healthy donors who gave informed consent as previously reported.[13] The cells were shown to express CD91 and CD73 (99.6% and 96.3%, respectively) but not CD34, CD45 or CD14 (0.1%, 0.2% and 0.1%, respectively). The cells were positive for HLA-AB (96.8%) but not HLA-DR (0.1%). In this study, fifth passage human MSCs from a single donor were used for the transplantation.

Animal model

Newborn Sprague-Dawley rats were purchased from the laboratory central of Jiangsu province (Nanjing, China) and housed in individual cages with free access to water and laboratory chow. Animals were divided into three groups: sham control (C, $n = 10$), hyperoxia-induced ALI control (B, $n = 10$) and ALI with human MSCs transplantation (A, $n = 10$). To induce ALI, the rats from the Groups A and B were placed in a sealed Plexiglas chamber with a minimal in-and-out flow, providing 6–7 exchanges per hour of the chamber volume with O_2 concentration maintained above 95%, while the rats in the Group C were only exposed to air.

For cell transplantation, 5×10^4 MSCs in 0.05 mL phosphate-buffered saline (PBS) were administered intravenously after seven-day hyperoxia exposure. The rats in Groups B and C received the same volume of PBS intravenously. After transplantation, the rats were allowed to recover and subsequently returned to their dams under air. Rats were sacrificed at three-day post-transplantation.

Tissue preparation

Rats were weighed at age 10 d when they received intraperitoneal injection of 10% chloral hydrate (8 mL/kg). Blood samples (1–2 mL) were taken for assay of tumor necrosis factor α (TNF-α) concentration. The abdominal aortas were cut and the trachea in middle of the neck was exposed. The specimens from bronchoalveolar lavage fluid (BALF) were used for the detection of TNF-α and RAGE concentrations. The left upper lung tissues were homogenized in lysis buffer (20 mmol/L Tris/HCl, 100 mmol/L NaCl). The lysates were centrifuged at 100,000 *g* for 15 min and used for Western blot analysis. The right upper lung tissue was fixed by 4% paraformaldehyde, embedded with paraffin and cut into 6 μm sections for immunohistochemical and histological examinations.

Enzyme-linked immunosorbent assay

TNF-α and RAGE concentrations in BAL and blood were measured using specific ELISA kits according to the manufacturer's protocol (Abcam, Cambridge, MA, USA).

Reverse transcription polymerase chain reaction

Total RNA (3 μg) was extracted from lung tissue by using TRIzol (Invitrogen, Paisley, UK) and used to synthesize cDNA with reverse transcription system kits (Promega, Madison, WI, USA). Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using the following primers: RAGE 5′ GGTGCTGG TTCTTGCTC 3′, 5′ TCCCTCGCCTGTTAGTT 3′, amplicon 235 bp; NF-κB 5′ GAAGAAGCGAGACCTGGAG 3′, 5′ TCCGGAACACAATGGCCAC 3′, amplicon 398 bp; β-actin 5′ GATGACAAGC AGCCCTAT 3′, 5′ TCCATGCCAATTT-ACAAC 3′, amplicon 450 bp. PCR products were separated by 1.5% agarose gel electrophoresis and quantified by in gel imaging system. The absorbance ratio of RAGE mRNA and NF-κB mRNA to β-actin mRNA was calculated, respectively.

Immunohistochemistry

The sections from rat lungs were washed in 0.1 mol/L PBS for several times and placed in 3% H_2O_2 to quench endogenous peroxidase for 15 min. Then the section were preincubated with 10% normal goat serum for 30 min at 37°C to block non-specific binding, and incubated overnight at 4°C with rabbit RAGE antibody (Millipore, Billerica, MA, USA). Next, the sections were washed in PBS and incubated with biotinylated anti-rabbit IgG (Boster, Wuhan, China) for one hour at room temperature. After washing, the sections were incubated for 30 min with streptavidin-biotin and horseradish peroxidase, then developed with 3,3′-diaminobenzidine. For negative controls, the primary antibodies were replaced with PBS.

Western blot analysis

Total protein was extracted from homogenized lung tissues using lysis buffer (Pierce, Rockford, IL, USA) and quantified using the Bradford method. Then, 80 μg of protein were separated with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore). The membranes were incubated overnight at 4°C with antibody against RAGE, NF-κB or β-actin (Millipore). After incubation with peroxidase-coupled IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 37°C for two hours, the membranes were

developed using ECL (Pierce) and detected using BioImaging Systems (UVP Inc., Upland, CA, USA). The relative protein concentrations were calculated based on β-actin protein as loading control.

Evaluation of lung injury

The morphological changes of lung tissue were evaluated by staining the sections with hematoxylin and eosin. Lung injury was scored according to the following four categories: alveolar congestion, hemorrhage, neutrophil infiltration into the airspace or vessel wall, and alveolar wall thickness/hyaline membrane formation. Each category was graded on a five point scale: 0 = minimal injury, 1 = injury up to 25% of the field, 2 = injury up to 50% of the field, 3 = injury up to 75% of the field and 4 = diffuse injury. Each slide was evaluated by two investigators in a blinded manner. To generate the lung injury score, a total of 300 alveoli were counted on each slide at $\times 400$ magnification. Within each field, points were assigned according to predetermined criteria.

Statistical analysis

All data were expressed as means $\pm$ standard deviations (mean $\pm$ SD) and analyzed by using SSPS 11.5 (SPSS Inc., Chicago, IL, USA). Differences among multigroups were analyzed with one-way analysis of variance, *post hoc* multiple comparisons was assumed by least significant difference. Differences between two groups were analyzed with the Student's *t*-test. P value <0.05 was considered statistically significant.

Results

TNF-α and RAGE concentrations in serum and BALF of different groups of rats

As shown in Figure 1, by enzyme-linked immunosorbent assay we detected no significant difference among three groups in the levels of TNF-α and RAGE in serum ($F = 0.194$, $P = 0.86$; $F = 0.85$, $P = 0.08$). However, we detected significant differences among the three groups in the levels of both TNF-α and RAGE in BALF ($F = 53.72$, $P = 0.0000$; $F = 4.804$, $P = 0.0191$). Compared with control, RAGE and TNF-α concentrations increased obviously in both Groups A and B ($P < 0.05$). Moreover, RAGE and TNF-α concentrations were lower in Group A than in Group B. These results suggest that hyperoxia promotes the production of TNF-α and RAGE in BALF but transplantation of BMSCs could antagonize it.

Expression of RAGE and NF-κB in lung tissues of different groups of rats

High levels of TNF-α and RAGE in BALF of hyperoxia treated rats indicate the inflammation in the lung tissues. Thus we detected the expression of RAGE and NF-κB in the lung tissues. RT-PCR analysis showed that there were significant differences among three groups in RAGE mRNA and NF-κB mRNA levels ($F = 37.21$, $P = 0.000$; $F = 5.695$, $P = 0.011$). Compared with control, RAGE and NF-κB mRNA concentrations increased obviously in both Groups A and B ($P < 0.05$). Moreover, RAGE and NF-κB mRNA levels were significantly lower in Group A than in Group B ($P < 0.05$, Figure 2).

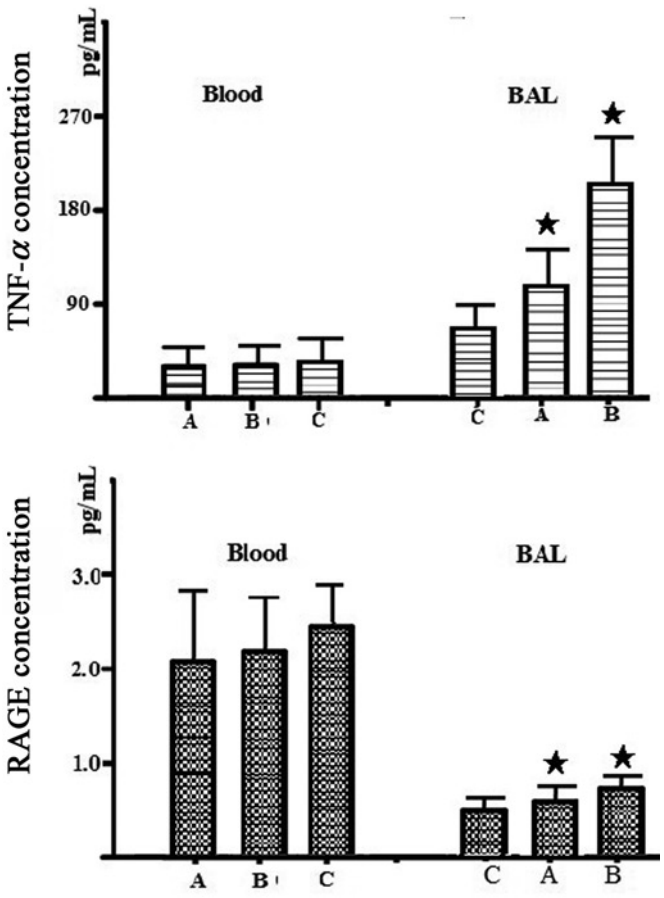

Figure 1 The levels of RAGE and TNF-α in BAL and blood of different groups of rats. A: ALI with bone marrow-derived mesenchymal stem cells transplantation; B: hyperoix-induced ALI; C: sham control. $n = 10$. $^{\star}P < 0.05$ versus C. RAGEl, receptor for advanced glycation end-products; TNF-α, tumor necrosis factor α; ALI, acute lung injury; BAL, bronchoalveolar lavage

To confirm our observation, next we detected the protein concentrations of RAGE and NF-κB in the lung tissues. Western blot analysis showed that there were significant differences among three groups in RAGE mRNA and NF-κB protein concentrations ($F = 15.88$, $P = 0.000$; $F = 4.223$, $P = 0.0288$). Compared with control, RAGE and NF-κB protein concentrations increased obviously in both Groups A and B ($P < 0.05$). Moreover, RAGE and NF-κB protein concentrations were significantly lower in Group A than in Group B ($P < 0.05$, Figure 3). Taken together, these data provide evidence that hyperoxia induces the inflammation in the lung but transplantation of BMSCs could antagonize it.

BMSCs transplantation alleviates hyperoxia-induced lung injury

By immunohistochemistry staining we observed strong RAGE expression in AECs exposed to hyperoxia (Figure 4a).

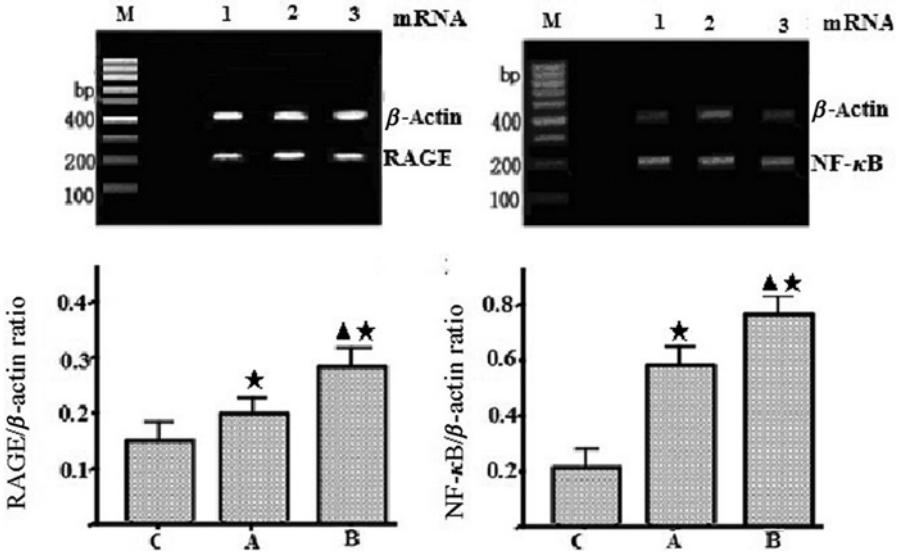

Figure 2 The expression of RAGE and NF-κB at mRNA level in lung tissues of different groups of rats. The relative mRNA levels were expressed as the ratio to β-actin. A: ALI with bone marrow-derived mesenchymal stem cells transplantation; B: hyperoix-induced ALI; C: sham control. $n = 10$. Representative pictures were shown on the upper panel and statistical analysis was shown in the corresponding columns on the lower panel. $^{\star}P < 0.05$ versus C. $^{\blacktriangle}P < 0.05$ versus A. RAGE, receptor for advanced glycation end-products; NF-κB, nuclear factor κB; ALI, acute lung injury

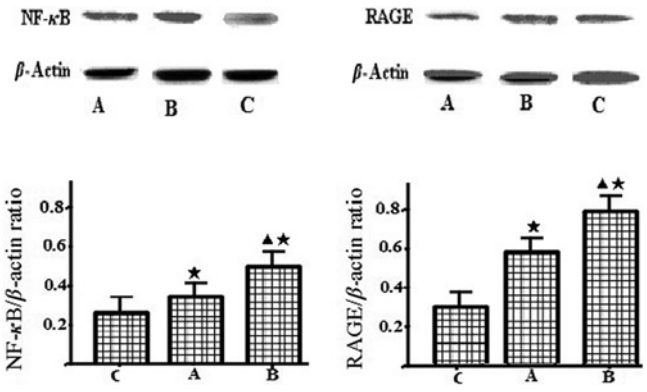

Figure 3 Expression of RAGE and NF-κB at protein concentration in lung tissues of different groups of rats. The relative protein concentrations were expressed as the ratio to β-actin. A: ALI with bone marrow-derived mesenchymal stem cells transplantation; B: hyperoix-induced ALI; C: sham control. $n = 10$. Representative pictures were shown on the upper panel and statistical analysis was shown in the corresponding columns on the lower panel. $^{\star}P < 0.05$ versus C. $^{\blacktriangle}P < 0.05$ versus A. RAGE, receptor for advanced glycation end-products; NF-κB, nuclear factor κB; ALI, acute lung injury

In comparison, RAGE expression was weaker in AECs exposed to hyperoxia but with BMSCs transplantation (Figure 4b), and was the weakest in normal AECs (Figure 4c). These data are consistent with the results of RT-PCR and Western blot analysis.

By histological analysis, we observed severe damage in the lung exposed to hyperoxia (Figure 4d), minor damage in the lung exposed to hyperoxia but with BMSCs transplantation (Figure 4e) and no damage in the lung exposed to air (Figure 4f). The lung damage score was significantly different among three groups ($F = 51.59$, $P = 0.000$). The scores of Groups A and B were significantly higher than that of Group C and the score of Group A was significantly lower than that of Group B ($P < 0.05$, Figure 4g). These results indicate that transplantation of BMSCs alleviates hyperoxia-induced lung injury.

Discussion

Chronic lung diseases are characterized by loss of lung tissue, inflammation and fibrosis and represent a major global health burden. Recent studies have shown that mesenchymal stem cells can protect lung tissue from acute or chronic lung injury, suggesting that cellular therapy would be a promising approach for the treatment of chronic lung diseases. Notably, the protective effects of mesenchymal stem cells may be not due to the replacement of damage lung cells by stem cells, but rather may be mediated by paracrine mechanism through anti-inflammatory.

In the present study we found that BMSCs transplantation reduced hyperoxia-induced lung injury and this is correlated with reduced levels of RAGE, TNF-α and NF-κB in lung tissues. These data indicate that BMSCs play a protective role in hyperoxia-induced lung injury by inhibiting hyperoxia-induced inflammation.

Numerous studies have identified that in the majority of healthy adult tissues, RAGE is expressed at a low basal level. The up-regulation of RAGE has been associated with a diverse range of pathological events, such as diabetes, atherosclerosis, cancer, inflammation and Alzheimer's disease.[14,15] Unlike other tissues, pulmonary tissues express remarkably high basal level of RAGE.[16] Indeed, studies have illustrated that RAGE plays a number of important physiological roles in the lung including alveolar gas exchange, modulation of cell spreading, adhesion to ECM components and proliferation. Under pathological conditions, RAGE binds the corresponding ligands to activate downstream signal pathways, among which NF-κB is one of the most important. The activation of RAGE–NF-κB

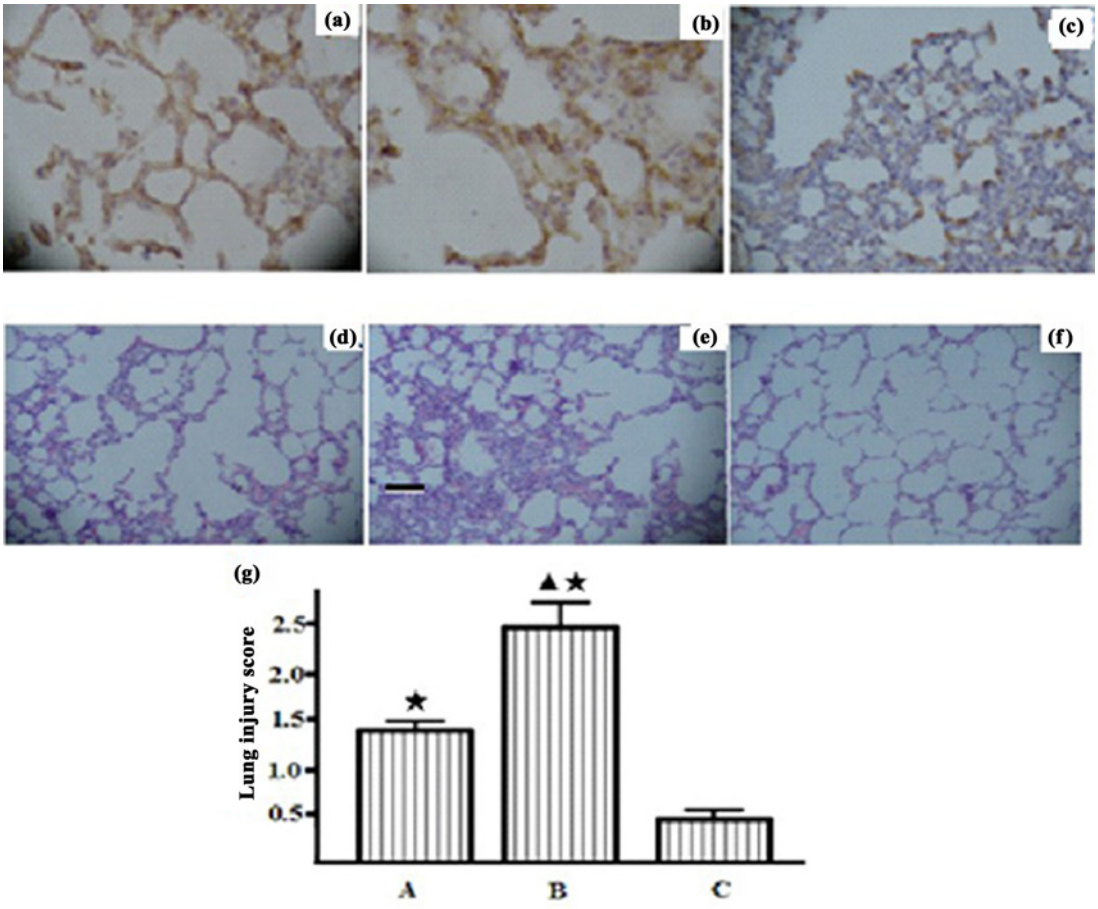

Figure 4 Histological analysis of lung tissues of different groups of rats. Immunohistochemistry staining of RAGE expression in the lungs from rats exposed to hyperoxia and transplanted with BMSCs (a), rats exposed to hyperoxia (b) and rats exposed to normoxia as control (c). Hematoxylin and eosin staining of the lungs from rats exposed to hyperoxia and transplanted with BMSCs (d), rats exposed to hyperoxia (e) and rats exposed to normoxia as control (f). (g) Quantization of lung injury score. A: ALI with BMSCs transplantation; B: hyperoix-induced ALI; C: sham control. $n = 10$. $^{\star}P < 0.05$ versus C. $^{\blacktriangle}P < 0.05$ versus A. BMSC, bone marrow-derived mesenchymal stem cell; RAGE, receptor for advanced glycation end-products; ALI, acute lung injury. (A color version of this figure is available in the online journal)

pathway promotes the transcription of the inflammation factors and forms a positive feedback loop by inducing RAGE expression, which eventually lead to histocyte damage.[17,18]

In this study we found that after the transplantation of BMSCs, RAGE signal pathway in lung tissue was obviously down-regulated because the levels of RAGE and NF-κB at both mRNA and protein concentrations were reduced. These data indicate that BMSCs inhibit the positive inflammatory feedback loop in AECs exposed to hyperoxia, therefore provide protection against lung injury. This corresponds with previous report that the inhibition of RAGE signaling alleviated lung damage.[19]

In summary, in this study for the first time we reported that hyperoxia upregulates RAGE/NF-κB signal pathway in lung tissue of newborn rats, and transplantation of BMSCs attenuates hyperoxia lung injury, which may be mediated by the anti-inflammatory effects. Our results suggest that BMSCs transplantation is a promising therapeutic approach for hyperoxia-induced ALI.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; ZT designed the study and wrote the manuscript; YL, PJ and SZ conducted the experiments; HC performed statistical analysis. ZT and YL contributed equally to this work.

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REFERENCES

1 Hayes D Jr, Feola DJ, Murphy BS, Shook LA, Ballard HO. Pathogenesis of bronchopulmonary dysplasia. *Respiration* 2010;**79**:425–36
2 Broström EB, Thunqvist P, Adenfelt G, Borling E, Katz-Salamon M. Obstructive lung disease in children with mild to severe BPD. *Respir Med* 2010;**104**:362–70
3 Abman SH, Groothius JR. Pathophysiology and treatment of bronchopulmonary dysplasia. Current issues. *Pediatr Clin North Am* 1994;**41**:277–315
4 Whitsett JA, Wert SE, Weaver TE. Alveolar surfactant homeostasis and the pathogenesis of pulmonary disease. *Annu Rev Med* 2010;**61**:105–19
5 Buckley ST, Ehrhardt C. The receptor for advanced glycation end products (RAGE) and the lung. *J Biomed Biotechnol* 2010;**2010**:917108
6 Uchida T, Shirasawa M, Ware LB, Kojima K, Hata Y, Makita K, Mednick G, Matthay ZA, Matthay MA. Receptor for advanced

glycation end-products is a marker of type I cell injury in acute lung injury. *Am J Respir Crit Care Med* 2006;**173**:1008–15
7 Reynolds PR, Schmitt RE, Kasteler SD, Sturrock A, Sanders K, Bierhaus A, Nawroth PP, Paine R III, Hoidal JR. Receptors for advanced glycation end-products targeting protect against hyperoxia-induced lung injury in mice. *Am J Respir Cell Mol Biol* 2010;**42**:545–51
8 Rojas M, Xu J, Woods CR, Mora AL, Spears W, Roman J, Brigham KL. Bone marrow-derived mesenchymal stem cells in repair of the injured lung. *Am J Respir Cell Mol Biol* 2005;**33**:145–52
9 Aslam M, Baveja R, Liang OD, Fernandez-Gonzalez A, Lee C, Mitsialis SA, Kourembanas S. Bone marrow stromal cells attenuate lung injury in a murine model of neonatal chronic lung disease. *Am J Respir Crit Care Med* 2009;**180**:1122–30
10 van Haaften T, Byrne R, Bonnet S, Rochefort GY, Akabutu J, Bouchentouf M, Rey-Parra GJ, Galipeau J, Haromy A, Eaton F, Chen M, Hashimoto K, Abley D, Korbutt G, Archer SL, Thébaud B. Airway delivery of mesenchymal stem cells prevents arrested alveolar growth in neonatal lung injury in rats. *Am J Respir Crit Care Med* 2009;**180**:1131–42
11 Zhang H, Fang J, Su H, Yang M, Lai W, Mai Y, Wu Y. Bone marrow mesenchymal stem cells attenuate lung inflammation of hyperoxic newborn rats. *Pediatr Transplant* 2012;**16**:589–98
12 Ou-Yang HF, Huang Y, Hu XB, Wu CG. Suppression of allergic airway inflammation in a mouse model of asthma by exogenous mesenchymal stem cells. *Exp Biol Med (Maywood)* 2011;**236**:1461–7
13 Tian ZF, Du J, Fu XM, Wang B, Hong XY, Feng ZC. Influence of human bone marrow-derived mesenchymal stem cells on the lung of newborn rats damaged by hyperoxia. *Zhonghua Er Ke Za Zhi* 2008;**46**:4–8
14 Ramasamy R, Vannucci SJ, Yan SS, Herold K, Yan SF, Schmidt AM. Advanced glycation end products and RAGE: a common thread in aging, diabetes, neurodegeneration, and inflammation. *Glycobiology* 2005;**15**:16R–28R
15 Zhang L, Postina R, Wang Y. Ectodomain shedding of the receptor for advanced glycation end products: a novel therapeutic target for Alzheimer's disease. *Cell Mol Life Sci* 2009;**66**:3923–35
16 Shirasawa M, Fujiwara N, Hirabayashi S, Ohno H, Iida J, Makita K, Hata Y. Receptor for advanced glycation end-products is a marker of type I lung alveolar cells. *Genes Cells* 2004;**9**:165–74
17 Reynolds PR, Wasley KM, Allison CH. Diesel particulate matter induces RAGE expression in pulmonary epithelial cells and RAGE signaling influences NF-kB-mediated inflammation. *Environ Health Perspect* 2011;**119**:332–6
18 Reynolds PR, Kasteler SD, Schmitt RE, Hoidal JR. RAGE signals through Ras during tobacco smoke-induced pulmonary inflammation. *Am J Respir Cell Mol Biol* 2011;**45**:411–8
19 Zhang H, Tasaka S, Shiraishi Y, Fukunaga K, Yamada W, Seki H, Ogawa Y, Miyamoto K, Nakano Y, Hasegawa N, Miyasho T, Maruyama I, Ishizaka A. Role of soluble receptor for advanced glycation end products on endotoxin-induced lung injury. *Am J Respir Crit Care Med* 2008;**178**:356–62

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