Bone marrow mesenchymal stem cells attenuate lung inflammation of hyperoxic newborn rats


Abstract: BPD is a significant global health problem and currently lacks effective therapy. We established a neonatal rat model of BPD to investigate therapeutic potential of BMSCs in neonatal lung disease. BMSCs were isolated, identified, and transfected by lentiviral vector carrying GFP gene *in vitro*. Neonatal rats were injected intravenously with either BMSCs or PBS after they had been already exposed to high oxygen for seven days, and assessed on post-injection day 3, day 7, and day 14 for weight gaining, lung histology, radical alveolar counts, and lung cytokine level. BMSCs were positive for CD29, CD44, and CD90 whereas negative for CD34, CD45, CD11b and with differentiation potential into osteoblasts, adipocytes, and chondrocytes. BMSCs expressed GFP after transfected by lentivirus. After injection, BMSCs exert their therapeutic benefit of improving weight gaining, preventing alveolar growth arrest, and suppressing lung inflammation of neonatal rats. Intravenous delivery of BMSCs in newborn rats conferred protection from hyperoxia-induced lung injury, and one of the effects of BMSCs treatment is suppressing lung inflammation.

In the past two decades, the survival rate of VLBW infants has been reported to increase from <70% to over 80% and of ELBW infants from <40% to over 50% in developed country (1). Approximately 20% of infants with a birth weight <1500 g will have signs of BPD at 36 wk post-menstrual age (2). More recent studies (3, 4) report an even higher incidence of oxygen dependency at 36 wk among surviving infants born before 28 wk (53.2%), or with a birth weight of <1000 g (40%). BPD persists as one of the major complications in surviving very premature infants and has long-term respiratory and neurodevelopmental consequences that extend beyond childhood and result in increased health care costs (5, 6). However, therapies that can effectively attenuate lung injury and promote lung growth to lower the incidence and severity of BPD remain lacking. Further advances are required to prevent and repair of neonatal lung injury. Several lines of evidence in humans and experiments suggest that adult bone marrow–derived stem cells can reconstitute injured or defective alveolar epithelium. Recent studies indicate that BMSCs treatment can ameliorate bleomycin, monocrotaline, endotoxin, or LPS-induced lung injury in adult animals (7–13). In humans, lungs from bone marrow or lung transplant recipients demonstrate chimerism of epithelial and endothelial cells (14–16). Ringdén et al. (17) have shown that MSCs were used to treat toxicity, among other things pneumoniae unknown after

Abbreviations: BMSCs, bone marrow mesenchymal stem cells; BPD, bronchopulmonary dysplasia; DAPI, 4',6-diamino-2-phenylindole; ELBW, extremely low birth weight; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; HPF, high-power fields; IL-10, interleukin-10; LPS, lipopolysaccharide; MOI, multiplicity of infection; PBS, phosphate-buffered saline; RAC, radical alveolar counts; TGF-β1, transforming growth factor beta 1; TNF-α, tumor necrosis factor alpha; TU, transduction unit; VLBW, very low birth weight.
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hematopoietic stem cell transplantation. These exciting studies suggest that the administration of MSCs might be a possible candidate for the new therapeutic modality for lung injury. Potential mechanisms through which MSCs therapy improves lung structure include engraftment, differentiation into specific lung cell types, immunomodulatory functions, and antiapoptosis effects (8). However, little is known about its potential role in the setting of neonatal hyperoxia lung injury (18). Here, we established a neonatal rat model of BPD to investigate therapeutic potential of BMSCs in neonatal lung disease.

Material and methods
Obtaining and characterizing BMSCs
BMSCs were isolated from femurs and tibiae of 5–7-wk-old Sprague-Dawley rats as described (19–22). Briefly, BM from adult Sprague-Dawley rats was plated into tissue culture flasks. Adhered cells were allowed to grow to about 75% confluency and then trypsinized and reseeded at a density of $10^5$ cells/cm$^2$.

Cell surface markers
Phycoerythrin labeling for rat monoclonal antibodies against CD29 (BioLegend Company, San Diego, CA, USA), CD44 (Abd Serotec Company, Kidlington, England), CD90 (BioLegend Company), CD34 (BioLegend Company), CD45 (BioLegend Company), CD44 (Abd Serotec Company, Kidlington, England), CD45 (BioLegend Company), and CD11b (BioLegend Company) was used according to the manufacturer's protocol and was selected in accordance with the position statement for the minimal criteria to define an MSCs, from the International Society for Cellular Therapy (22). Cells were analyzed with a FACScan (Becton Dickinson).

Stem cell lineage differentiation assay
The lineage differentiation assay was performed in accordance with the position statement for the minimal criteria to define an MSC, from the International Society for Cellular Therapy (22). Osteogenic, adipogenic and chondrogenic induction was performed on passage 4 of BMSCs following published protocols (19, 20, 23).

Stem cell labeling
BMSCs were transfected by lentivirus carrying GFP gene (lentiviral vector: pLV/EX2D-Neo-EF1A-eGFP; virus titre: $1 \times 10^5$ TU/mL) (Cyagen Biosciences, Guangzhou, China) at different MOI (8, 12, 16) in vitro according to the manufacturer's protocol. The infection efficiency of BMSCs was assessed under fluorescence microscope after transfected by lentivirus at 48 h. For the assessment of growth characteristics of BMSCs transfected by lentivirus, 2 × $10^5$ cells of passage 3 of normal BMSCs and BMSCs transfected by lentivirus were seeded per well in 24-well plate and counted every day for one wk.

Animals and hyperoxia exposure
Experimental hyperoxia lung injury was induced as published protocol (24). Sprague-Dawley rats were obtained from Laboratory Animal Center, Guangdong, China, and raised in the Laboratory Animal Center of Sun Yat-sen University. Newborn rat pups exposed to normoxia (21% O$_2$) or hyperoxia (95% O$_2$, BPD model) from post-natal day 3 to post-natal day 10 in sealed Plexiglas chambers with continuous O$_2$ monitoring (CYES, O$_2$, and CO$_2$ recorder). Newborn rat pups from four different litters were randomized to four groups: (i) hyperoxia plus BMSCs, (ii) hyperoxia plus PBS (95% O$_2$, BPD group), (iii) normoxia plus BMSCs, and (iv) normoxia plus PBS (21% O$_2$, control group).

BMSCs treatment
A suspension of $1 \times 10^5$ BMSCs in 100 µL PBS was injected via the superficial femoral vein after the animals had been already exposed to high oxygen for seven days (post-natal day 10). This procedure was resulting in a total delivery of $1 \times 10^5$ BMSCs per pup. After BMSCs injection, all newborn rat pups were maintained under normoxia conditions (21% O$_2$).

Samples collection and test methods
On days 1, 3, 7, and 14 of post-injection, eight rats from each group were selected randomly and sacrificed after anesthesia. Left lung was fixed in 4% polyformaldehyde followed by embedding for frozen tissue section and paraffin section. Right lung specimens were placed into Eppendorf tubes and stored in an −80 centigrade refrigerator for enzyme-linked immunosorbent assay of cytokines levels.

Assessment of BMSCs engraftment
Frozen sections were processed for DAPI immunofluorescent staining. The fluorescent events of frozen sections were analyzed under fluorescence microscope and determined by the intensity per HPF ($\times400$) field. The number of GFP-positive cells with DAPI nuclear staining and all DAPI nuclear staining cells retained in recipient lungs were manually counted in 10 random fields throughout the lung under fluorescence microscope.

RACs
RAC refers to the number of alveoli transected by a perpendicular line drawn from the center of the most peripheral bronchiolo (recognizable by not being completely covered by epithelium) to the pleura or the nearest interlobular septum. This is an important index to evaluate the stage of lung development (8). Five hematoxylin and eosin staining sections of each time point in each rat were randomly selected. Five fields for each section were examined under a light microscope ($\times100$) to estimate RAC and calculate the mean value.

Cytokine levels in lung tissue homogenate
The enzyme-linked immunosorbent technique was used. The contents of TNF-$\alpha$, TGF-$\beta1$, and IL-10 were detected following the instructions of Kits (R&D Systems, Inc, Minneapolis, MN, USA). The results were expressed by the contents of TNF-$\alpha$, TGF-$\beta1$, and IL-10 per mL lung specimen (pg/mL).

Statistical analysis
All values were expressed as mean ± s.d. Comparison of results between different groups was made by one-way analysis of variance (including post hoc analysis), as appropriate, using Statistic Package for Social Science
p-value < 0.05 was considered significant.

Animal experimentation was performed according to the Helsinki Declaration. Experiments were also conducted in accordance with the guidelines for breeding and experiments on animals set forth by the Ministry of Social Justice and Empowerment, Government of China, and the formalized ethical guidelines of our institution.

Results

Isolation culture, identification, and labeling rat BMSCs in vitro

MSCs extracted from the bone marrow formed a homogeneous population of cells and displayed the fibro-like phenotype (Fig. 1a). The analysis of cell surface phenotype indicated that MSC population was positive for CD29, CD44, and CD90 whereas negative for CD34, CD45, and CD11b (Fig. 2). BMSCs differentiated into three mesenchymal lineages (osteoblasts, adipocytes, and chondrocytes) when grown in specific medium for each lineage (Fig. 1b–d).

BMSCs were labeled with GFP after transfected by lentivirus in vitro (Fig. 3a,b). These cells grew well. The efficiency of infection for 48 h at MOI 12 was more than 95%. Growth curve of BMSCs showed that lentivirus transfection had little effect on proliferation of BMSCs (Fig. 3c).

Assessment of BMSCs engraftment

GFP-positive cells were found in both lungs of hyperoxic or normoxic rats within 24 h after BMSCs injection which indicates that BMSCs first home to the lung after i.v. infusion and confirms previous experimental reports (25, 26). GFP-positive cells are found in the lung sections from BMSCs-treated hyperoxic group in fluorescence microscope (Fig. 4a, x400); frozen section of rat lung tissue is stained with DAPI for nuclear chromatin in a same field (Fig. 4b, x400); GFP-positive cells with DAPI for nuclear chromatin were found in a same field of fluorescence microscopy.

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Fig. 1. Isolation and differentiation of BMSCs of rats in vitro. BMSCs cultures were prepared from bone marrow of SD rats by adherent culture and displayed the fibro-like phenotype. (a) The fourth passage of BMSCs (x40). After cultured in differentiation medium (osteogenic, adipogenic, and chondrogenic), BMSCs were stained for alizarin red (b: Os staining, x100), oil red O (c: Ad staining, x200), and alcian (d: Ch staining, x400).
microscope (Fig. 4c, ×400). No GFP-positive cells were seen in the lungs of respective PBS control groups. The ratio of GFP-positive cells with DAPI nuclear staining to all DAPI nuclear staining cells in lung of hyperoxic rats was higher than that of normoxic group, p < 0.05. GFP-positive cells in both groups of BMSCs treatment declined as time went on (Table 1).
Effect of BMSCs treatment on hyperoxia-induced lung injury

Oxygen induced the growth retardation of rats. BMSCs treatment improved weight gaining of rats after oxygen exposure. The animal body mass of rat of BMSCs-treated hyperoxic group was higher than that of rat of PBS-treated hyperoxic group, \( p < 0.05 \); although less than that of rat of PBS-treated normoxic group on post-injection day 14, \( p < 0.05 \) (Fig. 5a). RAC revealed a significant decrease in alveolar number in lungs from the hyperoxia group compared with normoxia, \( p < 0.05 \). BMSCs treatment resulted in a significant increase in alveolar number in the hyperoxia group compared with the respective PBS controls on post-injection day 7, \( p < 0.05 \), although less than that of rat of PBS-treated normoxic group on post-injection day 14, \( p < 0.05 \) (Fig. 5b).

Representative histology images of lungs from treated animals are shown in Fig. 6. Lungs from animals in the normoxia group who had received PBS or BMSCs appeared normal with numerous small alveoli and thin alveolar septa (Fig. 6a,c). Compared with lungs from normoxic animals, lungs from animals of oxygen exposure for seven days demonstrated enlarged simplified alveoli and lung inflammation (Fig. 6b), which were ameliorated by BMSCs treatment (Fig. 6d) but not by PBS treatment (Fig. 6e).

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BMSCs suppressed lung inflammation of hyperoxic newborn rats

Hyperoxia-exposed animals showed higher levels of TNF-\( \alpha \) and TGF-\( \beta 1 \) compared with the normoxia group, \( p < 0.05 \), and which were alleviated by BMSCs treatment (Fig. 7a,b). Furthermore, there was no significant difference of IL-10 in lung between hyperoxia-exposed animals and normoxia group, \( p > 0.05 \). Hyperoxia-exposed but BMSCs treatment animals presented a significant increase in the level of IL-10 compared with the respective PBS controls on post-injection day 3, \( p < 0.05 \) (Fig. 7c).
Discussion

In the current era, with the advent of surfactant treatment and gentler modes of ventilation, the pathology of BPD has evolved into a new pattern of injury characterized by impaired alveolarization and dysmorphic vasculogenesis (27). BMSCs are a heterogeneous subset of stromal stem cells in bone marrow that can be isolated and cultured in vitro with the ability of rapid amplification and self-renew. In addition to their multilineage differentiating capability, BMSCs produce immunosuppressive cytokines and growth factors that may help in the reparative process (28). BMSCs are capable of releasing a wide repertoire of potent mediators, such as vascular endothelial growth factor, keratinocyte growth factor, hepatocyte growth factor, IL-10 and so on, which may mediate the protective properties of BMSCs in the lung (13, 29–31). Intravenous administration of BMSCs attenuates the severity of lung damage, reduces lung inflammation and fibrosis, and increases survival in adult rats after bleomycin and endotoxin-induced lung injury. Our results provide evidence that intravenous administration of BMSCs may have the therapeutic benefit in a neonatal rat model of BPD.

Intravenous administration resulted in various engraftment rates ranging from 0% to 20% (32–36). The recruitment of stem cells to the lung, the extent of stem cell engraftment, and the effect of injury on these processes remain undetermined. van Haaften et al. (37) and Aslam et al. (38) have reported striking protective effects of BMSCs therapy despite low engraftment rates may be largely mediated through the production of paracrine mediators. In the present study, BMSCs first home to the lung after i.v. infusion. The ratio of BMSCs in lung of hyperoxic rats was higher, but these cells in both groups of cell treatment declined as time went on, and after 14 days, BMSCs were only detected in lung of hyperoxic rats. As lacking of quantitative assays on DNA of GFP from lungs, and furthermore GFP expression in combination with lung markers did not show that the cells are functional lung cells (data were not showed), we cannot propose that donor BMSCs extensively replace injured

95% O₂ showed the histological findings of BPD. BMSCs were administered after the animals had been already exposed to high oxygen for seven days and conferred protection of improving weight gaining, preserving normal alveolar number, and suppressing lung inflammation. Our results provide evidence that intravenous administration of BMSCs may have the therapeutic benefit in a neonatal rat model of BPD.

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lung cells to effectively improve lung architecture. BMSCs may promote lung tissue repair through other protective effects.

To investigate whether BMSCs modulate the inflammatory response to hyperoxia in the developing neonatal lung, we quantified cytokine levels in lung tissue homogenate with ELISA. Inflammation is considered the key mediator of alveolar simplification and lung inflammation. Lung treated with BMSCs has smaller and more numerous alveoli, thinner septa, and less inflammation. Normoxia group on post-natal day 10 (a); hyperoxia group on postnatal day 10 (b); normoxia group plus PBS on post injection day 14 (c); BMSCs-treated hyperoxic group on post-injection day 14 (d); PBS-treated hyperoxic group on post-injection day 14 (e).

Fig. 6. Effect of BMSCs treatment on lung of hyperoxic newborn rats. Representative hematoxylin and eosin–stained lung sections from normoxia, PBS- or BMSC-treated animals and from animals exposed to hyperoxia for seven days and treated with PBS or BMSCs, as indicated (original magnification ×100). Oxygen-exposed lungs display the characteristic features of alveolar simplification and lung inflammation. Lung treated with BMSCs has smaller and more numerous alveoli, thinner septa, and less inflammation. Normoxia group on post-natal day 10 (a); hyperoxia group on postnatal day 10 (b); normoxia group plus PBS on post injection day 14 (c); BMSCs-treated hyperoxic group on post-injection day 14 (d); PBS-treated hyperoxic group on post-injection day 14 (e).

MSCs attenuate lung inflammation

BPD. IL-10 is a cytokine secreted predominantly by monocytes that down regulates the expression of Th1 cytokines, MHC class II antigens, and costimulatory molecules on macrophages. IL-10 has also been reported to inhibit the rolling, adhesion, and transepithelial migration of neutrophils (41). Hyperoxia may induce much cytokine expression such as TNF-α, IL-1β, IL-6, and TGF-β1 and so on. The interaction and induction of these cytokine form a complicated network. IL-10 has been well-described protective effects in lung inflammation (42, 43). In our studies, the concentrations of TNF-α, TGF-β1 increased significantly in lung of hyperoxic group compared with normoxic group, which demonstrated that oxygen exposure resulted in significant inflammation in the lungs of animals, while there was no significant difference of IL-10 in lung between hyperoxia-exposed animals and normoxia group. TNF-α and TGF-β1 showed a trend toward a reduction in the lung of the MSC-treated rats. Furthermore, hyperoxia-ex-
posed but BMSCs treatment animals presented a moderate increase in the level of IL-10 compared with the respective PBS controls, which is consistent with recent reports demonstrating that MSC does increase production of anti-inflammatory cytokines in lung injury (12, 44). Our data show a dramatic suppression of lung inflammation in hyperoxic animals treated with BMSCs and maintaining steady-state levels of cytokines. In addition to increasing IL-10 level, Ortiz et al. (30) suggested that BMSC-secreted interleukin 1 receptor antagonist (IL1ra) represented a key candidate for the observed beneficial effects of BMSCs treatment in bleomycin-induced lung inflammation and fibrosis, and the antiapoptotic effect of BMSCs on neutrophils was shown to depend on IL-6 secretion.

Conclusion

Our results provide evidence for the therapeutic potential of BMSCs in improving weight gaining, preventing arrested alveolar growth, and suppressing lung inflammation in experimental BPD and further support the notion that BMSCs exert their therapeutic benefit partially through downregulation of proinflammatory responses to oxygen. This is the short-term effect of BMSCs against O2-mediated lung injury for designated periods within 14-day timeframe after transplantation. More robust studies are required to dissect the exact mechanism of BMSCs anti-inflammatory action and long-term effect.

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Author contributions

Hongshan Zhang: contributed in an important manner to the study design and performance, data collection and analysis, and writing of the manuscript. Jianpei Fang: conceived of the study, and participated in its design and coordination and helped to draft the manuscript. Haobin Su: participated in the design of the study and performed the statistical analysis. Min Yang: carried out the immunostains. Wenyu Lai: participated in BMSCs harvest and cell culture. Yougang Mai and Yanyun Wu: participated in animal experiments.

Fig. 7. BMSCs suppress lung inflammation of hyperoxic newborn rats. Oxygen exposure resulted in significant increase in level of TNFα and TGF-β1 in lung compared with the normoxic group, which was significant decreased with BMSCs treatment (a, b). There was no significant difference of IL-10 in lung between hyperoxia-exposed animals and normoxia group. hyperoxia-exposed but BMSCs treatment animals presented a moderate increase in level of IL-10 compared with the respective PBS controls on post injection day 3, and then the level was equal to that observed in unjured, normoxic animals on post injection day 14 (c). Data are expressed as mean ± s.d. (n = 8 animals per group). *p < 0.05 each group vs. PBS-treated normoxic group; #p < 0.05 BMSCs-treated hyperoxic group vs. PBS-treated hyperoxic group.
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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1 Material and methods.

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