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Original Article

Mesenchymal stem cell treatment in hyperoxia-induced lung injury in newborn rats

Selvi Gülaşı,[1] Aytuğ Atıcı,[1] Şakir Necat Yılmaz,[2] Ayşe Polat,[3] Mustafa Yılmaz,[2] Melisa Türkoğlu Laçin,[4] Gülhan Örekici[5] and Yalçın Çelik[1]

[1]*Department of Pediatrics, Faculty of Medicine, Mersin University, Mersin, Turkey,* [2]*Department of Histology and Embryology, Faculty of Medicine, Mersin University, Mersin, Turkey,* [3]*Department of Pathology, Faculty of Medicine, Mersin University, Mersin, Turkey,* [4]*Advanced Technology Education-Research and Application Center, Faculty of Medicine, Mersin University, Mersin, Turkey and* [5]*Department of Biostatistics and Medical Informatics, Faculty of Medicine, Mersin University, Mersin, Turkey*

Abstract

Background: The aim of this study was to evaluate the effectiveness of tracheally delivered mesenchymal stem cells (MSC) on lung pathology in a hyperoxia-induced lung injury (HILI) model in neonatal rats.

Methods: For the HILI model, rat pups were exposed to 85–95% oxygen during the first 10 days of life. Rats were divided into six groups: room-air normoxia (n = 11); room air, sham (n = 11); hyperoxia exposed with normal saline as placebo (n = 9); hyperoxia exposed with culture medium of MSC (n = 10); hyperoxia exposed with medium remaining after harvesting of MSC (n = 8); and hyperoxia exposed with MSC (n = 17). Pathologic changes, number and diameter of alveoli, α-smooth muscle actin (α-SMA) expression and localization of MSC in the lungs were assessed.

Results: Number of alveoli increased and alveolar diameter decreased in the mesenchymal stem cell group so that there were no differences when compared with the normoxia group (P = 0.126 and P = 0.715, respectively). Expression of α-SMA decreased significantly in the mesenchymal stem cell group compared with the placebo group ($P < 0001$). Green fluorescent protein-positive cells were found in lung tissue from all rats given MSC. Some green fluorescent protein-positive MSC also expressed surfactant protein-C.

Conclusion: Mesenchymal stem cells became localized in damaged lung tissue, and recovery approximated the room air control.

Key words bronchopulmonary dysplasia, hyperoxia, lung injury, mesenchymal stem cell, newborn.

Bronchopulmonary dysplasia (BPD) is a major cause of preterm infant mortality and morbidity leading to heavy economic and emotional burdens on the patient, the patient's family and society. Although the incidence of BPD varies due to the use of different treatment protocols in different clinical centers, it has been reported to be approximately 25% in infants under 1500 g.[1,2] Prevention of BPD is not easy and few effective treatments are available against BPD. BPD was previously described as a lung disease in preterm infants with respiratory distress syndrome who received mechanical ventilation and oxygen treatment. This description has been modified for extremely low-birthweight infants who receive surfactant treatment and have mild lung disease at onset.[3,4] The typical finding in BPD is arrested lung development. Pathology shows reduced septation, fewer and larger alveoli and alveolar underdevelopment. These features are described as "new BPD".

With the knowledge that lung development is interrupted by premature birth[5] and that this results in alveolar simplification (larger but fewer alveoli), reduced septation and impaired vascular growth, recent experimental studies have focused on mesenchymal stem cell (MSC) treatment, which can prevent and repair BPD. It has been demonstrated in rats that injected bone marrow-derived MSC differentiate into parenchymal cells in various non-hematopoietic tissues including the lung.[6–8] Recently, the transplantation of various types of stem/progenitor cells has shown potential in the prevention and treatment of neonatal hyperoxic lung injury.[9–11] Use of this method has been investigated in the treatment of congenital metabolic, neurodegenerative and cardiovascular diseases of newborns.[12,13] Consequently, MSC treatment might be a new therapeutic modality for treatment of hyperoxic neonatal lung injury or BPD. This study provides evidence that tracheally delivered MSC improve alveolar rarefaction, mitigate emphysema and reduce α-smooth muscle actin (α-SMA) expression by myofibroblasts in a hyperoxia-induced lung injury (HILI) model in neonatal rats. We also showed that the engrafted MSC had become established in the damaged lung tissue and transformed into an alveolar epithelial cell phenotype.

Methods

This study was performed at Mersin University, Mersin, Turkey. All procedures were approved by the Mersin University Animal Experimentation Ethics Committee.

Correspondence: Selvi Gülaşı, MD, Department of Pediatrics, Faculty of Medicine, Mersin University, Mersin 33070, Turkey. Email: glaselvi@yahoo.com

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Table 1 Study groups

Groups	No. rats (n)	Group features
Normoxia	11	Room air
Sham	11	Room air; anesthesia and surgery performed on day 11
Hyperoxia + placebo	9	Hyperoxia and 25 μL normal saline as placebo
Hyperoxia + culture medium	10	Hyperoxia and 25 μL culture medium[†]
Hyperoxia + remaining medium	8	Hyperoxia and 25 μL remaining cell culture liquid after harvesting of MSC[‡]
Hyperoxia + MSC	17	Hyperoxia and 10^5 MSC in 25 μL culture medium

[†]Mesencult® MSC Basal Medium (Stem Cell Technologies, Vancouver, BC, Canada). [‡]MSC were kept for 21 days in Mesencult® MSC Basal Medium. MSC, mesenchymal stem cells.

Study groups

Newborn Wistar albino rats were divided into six groups from birth (Table 1). To determine whether MSC were localized in the lungs, the lungs of nine rats in the hyperoxia + MSC (H-MSC) group were removed 1, 2 and 3 weeks (every week three rats were killed and lungs were removed) after MSC treatment. The lungs of the remaining eight rats in H-MSC group were removed on day 60 as in the other groups.

Hyperoxia-induced lung injury

The present hyperoxia-induced lung injury (HILI) model has been described previously.[14] Newborn rats were exposed to hyperoxia (85–95% oxygen) from birth to the 10th postnatal day in a sealed Plexiglas chamber with continuous oxygen monitoring.[15,16] Dams were switched every 24 h between the hyperoxic and normoxic chambers to prevent damage to their lungs and to provide equal nutrition to each litter. Litter size was adjusted to 11 pups to control the effects of litter size on nutrition and growth. Rat pups were fed with their mother's milk. Rat pups were anesthetized with isoflurane at day 11, median neck incision was performed, trachea was found and either 25 μL normal saline (NS) as placebo, culture medium (CM), remaining cell culture liquid after harvesting of MSC (RM) or MSC was given via the trachea, each time the rat breathed, according to group, with Hamilton injector (Fig. 1).

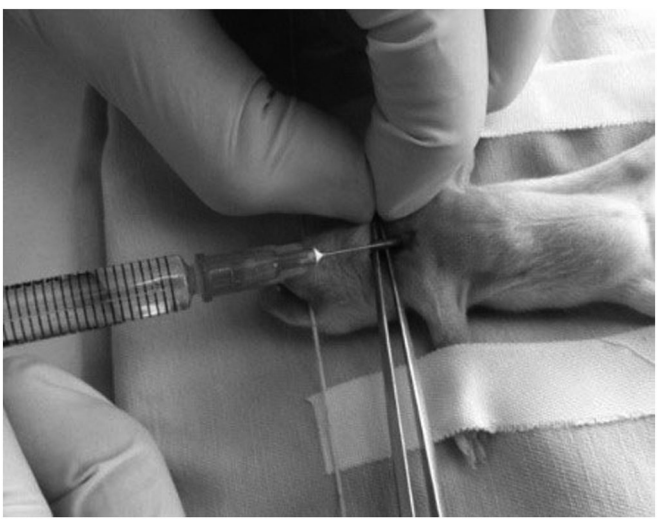

Fig. 1 Median neck incision and tracheal administration route.

MSC

Mesenchymal stem cells were obtained from the bone marrow of healthy adult rats. The cells were marked with CD34 and STRO-1 antibodies (stromal cell surface marker-1, monoclonal mouse antibody, 1:100, Santa Cruz Biotechnology Inc., CA, USA) using an immunofluorescence method. CD34-negative and STRO-1-positive cells were considered to be bone marrow MSC.

Before tracheal administration, the MSC were labeled with green fluorescent protein (GFP) via transfection to enable them to be visualized in tissue. GFP-encoding plasmids that would be used for labeling stem cells were transferred into *Escherichia coli* DH5α strains and amplified. Then, the plasmid was purified. Commercial cationic lipid molecules were used to transfer the plasmids into MSC. According to group, 25 μL NS, CM, RM or MSC was administered tracheally to the rat pups at every inspiration on the 11th day.

MSC presence in lung tissue

On the 60th day of life, rats were killed via anesthetization with ketamine (80 mg/kg) and xylazine (10 mg/kg) and the lungs removed. Rat bodyweight and lung weight were recorded. Lungs were inflated and fixed via the trachea with a 4% formaldehyde solution at a constant pressure of 20 cm H_2O. Middle lobe of the right lung was taken in all groups for standardization. The tissues were separated into two parts. One part was kept in 4% paraformaldehyde solution overnight at +4°C for immunohistochemistry and the other was kept in 10% neutral formalin solution for pathology. Different researchers who were blinded examined the immunohistochemical and pathological changes.

Immunolabeling was performed on the removed lung tissues using anti-surfactant protein-C (anti-SP-C) primary antibody (cat. no.: sc-13979; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and tetramethylrhodamine (TRITC) conjugated goat anti-rabbit IgG secondary antibody (cat. no.: sc-2091; Santa Cruz Biotechnology). Labeled sections were examined on fluorescence photomicroscopy.

Sections were evaluated and scored for edema, neutrophil and leukocyte infiltration,[17,18] fibrosis,[19] congestion, and bleeding[16,20–23] by blinded pathologist. The scores were defined as follows: normal, 0; minimal, 1; slight, 2; moderate, 3; heavy, 4. The scoring system is shown in Table 2. To asses the formation of alveolar structure and emphysema, five randomly selected fields were examined in all specimens. Mean linear intercept, as a measure of inter-alveolar wall distance, was determined on light microscopy at ×200 magnification. Inter-alveolar wall distance was calculated as follows: the total length of the cross-line

Table 2 Scoring system for evaluation of hyperoxia-induced lung injury

Staging system for fibrosis	
Degree[†]	Histological findings
0	Normal histological findings
1	Increase of vague connective tissue in the interalveolar septa and entire parenchyma observed in <50% of parenchyma. No lung parenchyma damage
2	Increase of vague connective tissue in the interalveolar septa and entire parenchyma observed in >50% of parenchyma. No lung parenchyma damage
3	Increase of prominent connective tissue in the interalveolar septa and entire parenchyma observed in <50% of parenchyma, and accompanied by fibrous bands or isolated fibrous nodules
4	Increase of prominent connective tissue in the interalveolar septa and entire parenchyma observed in >50% of parenchyma, and accompanied by fibrous bands or diffuse fibrous nodules
Staging system of neutrophil infiltration	
Degree	Histological findings
0	Normal histological findings
1	Neutrophil infiltration in sprays in the alveolar septa and alveolar lumen, observed in <25% of lung parenchyma
2	Neutrophil infiltration in sprays in the alveolar septa and alveolar lumen, observed in 25–50% of lung parenchyma
3	Neutrophil infiltration in sprays in the alveolar septa and alveolar lumen, in addition, formation of aggregates in some places, observed in 50–75% of lung parenchyma
4	Diffuse neutrophil infiltration in the alveolar septa and alveolar lumen, observed in >75% of lung parenchyma
Staging system of interstitial and alveolar edema	
Degree	Histological findings
0	Normal histological findings
1	Perivascular expansion in the alveolar septa
2	Patchy edema formation in the alveolar lumen in addition to perivascular expansion in the alveolar septa
3	More widespread edema formation in the alveolar lumen, observed in <50% of the parenchyma in addition to the perivascular expansion in the alveolar septa
4	Edema formation observed in >50% of the parenchyma in addition to perivascular expansion in the alveolar septa
Staging system for congestion and alveolar hemorrhage	
Degree	Histological findings
0	Normal histological findings
1	A few erythrocyte groups in the alveoli lumen and expansion in the alveolar septa, around the vessels
2	Prominent expansion on the vessel lumens in the alveolar septa, erythrocyte clumps in alveolar lumens that do not completely fill the alveoli
3	Erythrocyte leakage outside the vein in the alveolar septa, erythrocyte clumps in alveolar lumens that partially fill the alveoli
4	Hemorrhage points in the alveolar septa, erythrocyte clumps that completely fill the alveoli lumens

[†]0, normal; 1, minimal; 2, slight; 3, moderate; 4, heavy.

(1006 μm) was divided by the number of alveolar walls intersecting the test lines. Mean alveoli, an indicator of alveolar density, was calculated for each sample based on five random fields by counting the number of alveoli in each field and dividing this number by the area of the field. The establishment of MSC into the lungs was evaluated by another blinded researcher.

Myofibroblast α-SMA immunohistochemistry

The α-SMA was measured to assess the degree of lung fibrosis. Monoclonal mouse anti-α-SMA antibody (MS-113P, 1:100 dilution; NeoMarkers, Lab Vision, California, USA) was added to tissue sections. To evaluate fibrosis according to α-SMA expression in a single section, 10 non-overlapping spaces were scored. A 1–4 point semiquantitative scoring scale was used to determine the prevalence of α-SMA expressed cells (*A*) and the intensity of the stain in these cells (*B*).[24] For prevalence (*A*), the scoring criteria were as follows: 1, vague α-SMA-positive cells in the alveolar septa; 2, individual and/or small groups of α-SMA-positive cells in the alveolar septa; 3, groups of α-SMA-positive cells in the alveolar septa consisting only of a few cells; 4, α-SMA-positive cells widespread in the alveolar septa. For intensity (*B*), a separate scoring was system was defined: 1, common weak staining in all areas; 2, areas of regional moderate staining in addition to weak staining; 3, areas of moderate staining in all regions; 4, intensive staining foci in more than half of the areas, as well as moderate

staining. $A \times B$ was then calculated in each case to obtain the total staining score. Weak staining was defined as ≤4, and strong staining as >4.

Statistical analysis

Shapiro–Wilks test was used to confirm that the sample had normal distribution, and ANOVA was used for between-group comparison. For dual comparison, Dunett *t*-test was preferred. In addition, chi-squared test was used to analyze the relationship between groups and scores. Level of significance was set at $P < 0.05$, and SPSS 11.5 version (SPSS Statistics for Windows, Version 11.5. Chicago: SPSS Inc., USA) was used for statistical analysis.

Results

Rat weight in all groups was compared with that in the normoxia (N) group and with each another (Fig. 2). The mean weight of hyperoxia-exposed rats on day 10 was statistically significantly lower than that of N group rats (all $P < 0.001$). There were no differences in mean weight on day 60 ($P = 0.086$).

Lung weight as a sign of edema and lung congestion was investigated via between-group comparison (Fig. 2). Compared with the N group and with each other, there was a statistically significant increase in the mean lung weight of the hyperoxia + placebo (H-P; $P = 0.006$) and hyperoxia + CM (H-CM groups ($P = 0.037$), with no significant difference in the lung weight of the hyperoxia + RM (H-RM) or hyperoxia + MSC (H-MSC) groups. Lung weight/bodyweight was compared between groups. Compared with the normoxia groups, there was a statistically significant increase in mean lung weight/bodyweight of the H-P group ($P = 0006$), with no significant difference in mean lung weight/bodyweight of the H-CM, H-RM or H-MSC groups.

The mean number and diameter of alveoli were compared between groups (Fig. 3). There was no difference between the normoxia and H-MSC groups in mean number of alveoli ($P = 0.126$). The mean number of alveoli in the H-P ($P = 0.003$), H-CM ($P < 0.001$) and H-RM ($P = 0.015$) groups was statistically significantly reduced compared with the N and S groups. There was no difference between the normoxia and H-MSC groups in mean alveolar diameter ($P = 0.715$). There was, however, a statistically significant increase in the mean alveolar diameter of the H-P ($P = 0.012$) and H-CM ($P < 0.001$) groups.

Edema, fibrosis, congestion and leukocyte infiltration were scored on pathology (Fig. 4). The percentage of rats with first-degree (minimal) edema was statistically significantly higher in the H-P (77.8%), H-CM (80%) and H-RM (75%) groups than in the N group (9%); ($P = 0.007$, $P = 0.004$ and $P = 0.013$,

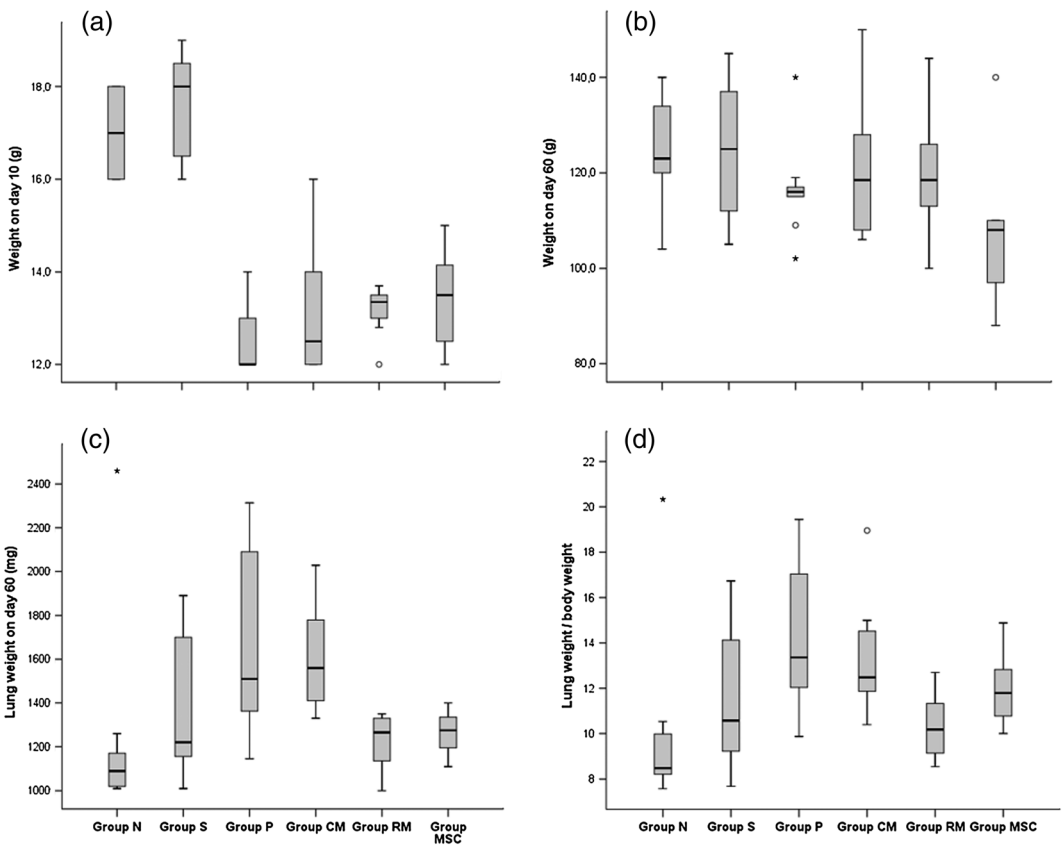

Fig. 2 (a) Mean weight of hyperoxia-exposed rats on day 10 was statistically significantly lower than that of room-air-breathing rats. (b) There were no differences in mean weight on day 60. (c) Mean lung weight in groups H-P and H-CM were significantly increased compared with the two normoxia groups (N and S; $P < 0.05$), with no significant difference in lung weight in groups H-RM or H-MSC. (d) Mean lung weight/bodyweight in the H-P group was significant increased compared with groups N and S ($P < 0.05$). H-CM, hyperoxia + culture medium; H-MSC, hyperoxia + mesenchymal stem cells; H-P, hyperoxia + placebo; H-RM, hyperoxia + remaining culture medium; N, normoxia; S, normoxia sham.

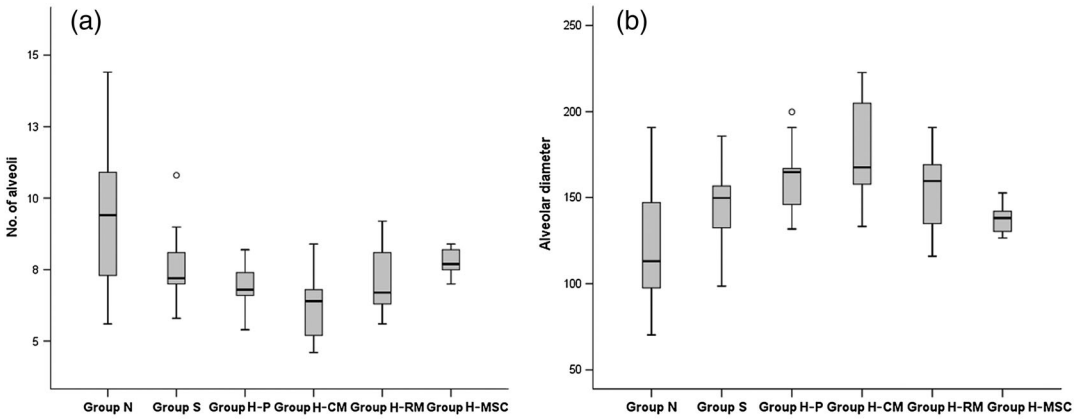

Fig. 3 Mean number and diameter of alveoli were compared with the N group. (a) There was no difference between the N and H-MSC groups in mean number of alveoli. The mean number of alveoli in the H-P, H-CM and H-RM groups was statistically significantly reduced compared with the N group ($P < 0.05$). (b) There was no difference between the N and H-MSC, H-RM groups in mean alveolar diameter, but there was a statistically significant increase in mean alveolar diameter in the H-P and H-CM groups ($P < 0.05$). H-CM, hyperoxia + culture medium; H-MSC, hyperoxia + mesenchymal stem cells; H-P, hyperoxia + placebo; H-RM, hyperoxia + remaining culture medium; N, normoxia; S, normoxia sham.

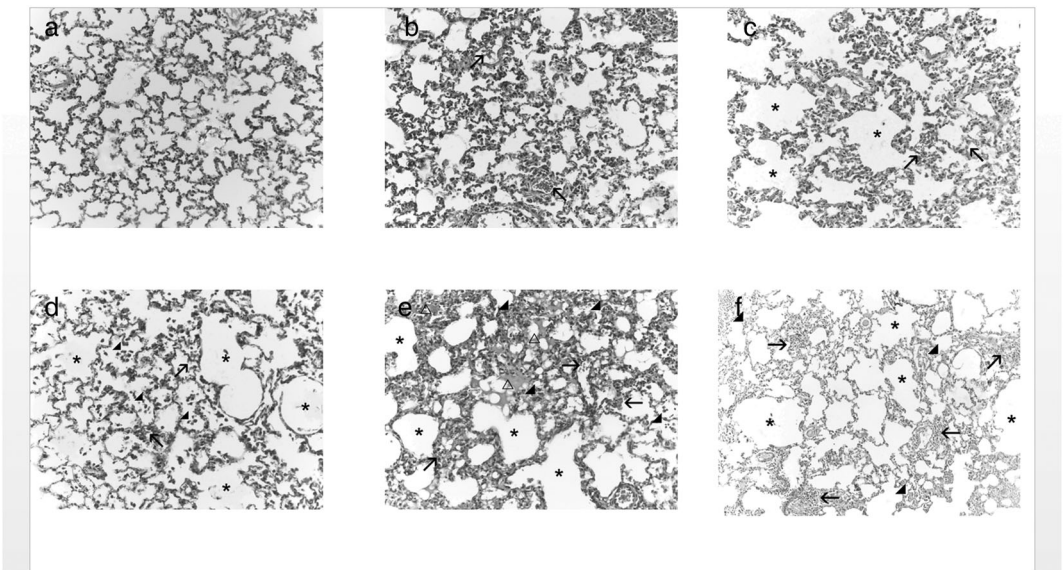

Fig. 4 (a) Normoxia group, normal lung findings; (b) normoxia + sham group, mild congestion with regular lung structure (arrows); (c) hyperoxia + placebo group, lung structure deformation, emphysematous extensions in alveoli (asterisks) and congestion (arrows); (d) hyperoxia + culture medium group, lung structure deformation, emphysematous extensions in alveoli (asterisks), congestion (arrows) and marking for congestion (arrowheads); (e) hyperoxia + remaining culture medium group, lung structure deformation, emphysematous extensions in alveoli (asterisks), congestion (arrows) and inflammatory cell infiltration (arrowheads); (f) hyperoxia + mesenchymal stem cells group, lung structure deformation, sporadic emphysematous extensions in alveoli (asterisks), congestion (arrowheads) and inflammatory cell infiltration (arrows).

respectively). The percentage of rats with first-degree edema in the H-MSC group (50%) was increased compared with the N group, but the difference was insignificant. There were no differences between the groups in terms of second-degree edema, congestion or leukocyte infiltration ($P > 0.05$). No fibrosis was found in any of the groups on HE staining.

The prevalence of α-SMA-expressing cells and the staining concentration in these cells were scored to assess fibrosis (Fig. 5). In the H-P group, both the prevalence of α-SMA-expressing cells and the staining concentration were greater compared with the N group and this difference was statistically significant ($P = 0.002$). The prevalence and staining intensity in the H-RM and H-MSC groups were significantly reduced compared with the H-P group (both $P = 0.004$). Also, the increase in α-SMA expression in the H-CM group was statistically significant compared with the H-RM and H-MSC groups ($P = 0.002$).

Because the time between administration of MSC and removal of the lungs was lengthy and a reduction in GFP-labeled cells was expected, the lungs of three rats in the MSC group were removed at 1 week intervals to be sure that MSC had become established in the lung tissue. This group is created just to show the MSC settlement in the lung and these nine rats (three rats were killed 1 week

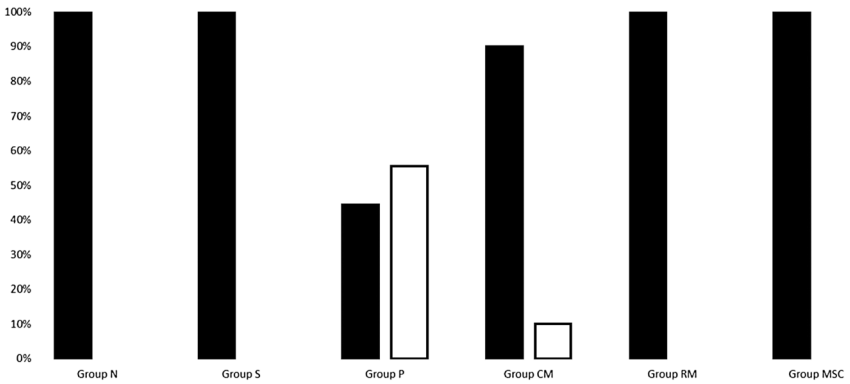

Fig. 5 Prevalence and intensity of α-smooth muscle actin (α-SMA) stain. In the H-P group, both the prevalence of α-SMA expressed cells and the staining intensity were greater compared with the N group ($P < 0.05$). The prevalence and staining intensity in the H-RM and H-MSC groups were significantly reduced compared with the H-P group (both $P < 0.05$). (■) Weak, ≤4; (□) strong, >4. H-CM, hyperoxia + culture medium; H-MSC, hyperoxia + mesenchymal stem cells; H-P, hyperoxia + placebo; H-RM, hyperoxia + remaining culture medium; N, normoxia; S, normoxia sham.

intervals during the three weeks) were not alive until day 60. These rats were killed and they lungs were removed after MSC treatment. GFP-positive cells were seen in the lung tissue of all rats that received labeled cells. On qualitative evaluation the largest number of GFP-positive cells was in rats from which the lungs were removed at the end of the first week; the number of positive cells then decreased with time (Fig. 6). There were very few GFP-positive cells in MSC rats on removal of the lungs at day 60. Type II pneumocytes expressing SP-C were seen in all sections. Double labeling with GFP and SP-C showed that some of the GFP-positive cells also stained for SP-C. We could not determine whether GFP-positive cells that did not stain for SP-C were transformed into other types of lung cell.

Discussion

Bronchopulmonary dysplasia develops due to impaired alveolarization in preterm infants and extends into childhood with severe breathing problems. There are no effective treatments; thus, preventive strategies are being investigated. Recent studies of MSC have given rise to the hope of new ways to prevent this disease.

In the present study, we evaluated the effectiveness of tracheally delivered MSC derived from adult bone marrow on lung histopathology, and the localization of MSC in damaged lung tissue in an experimental neonatal rat model of HILI. We also investigated whether the MSC transformed into a type II alveolar epithelial cell phenotype.

Because it is known that hyperoxia in newborn rats interrupts lung development and expansion of the distal air spaces in a manner similar to BPD, we used a HILI model.[25,15] The use of tracheally delivered MSC has been found to improve cell localization compared with intraperitoneal administration; thus, MSC were delivered tracheally in the present study.[6,26]

The MSC were grown in fresh cell culture medium containing growth factors. Because growth factors in the medium can cause structural and functional changes in the lungs, fresh CM with no stem cells was administered to rats with HILI to minimize these effects (H-CM group). Because certain growth factors released from stem cells and other endogenous substances are involved in MSC growth, the RM (after the growth and harvesting of MSC) was administered to rats with HILI to minimize the effects of these factors (H-RM group).[27] RM did not contain MSC, and, in order to evaluate the effects caused by MSC itself, another group, the H-MSC group, was used. In this manner, we investigated whether the present observations were due to the MSC or to paracrine effects. It would also be possible, if it were found that MSC transformed into tumor cells, to consider the RM as a harmless alternative treatment option.

We found that the number of alveoli increased only in the H-MSC group among the hyperoxia-exposed groups. The number of alveoli in the H-MSC group was similar to that in the N and sham (S) groups. The increase in number of alveoli achieved with MSC was not achieved with RM or CM. Alveolar diameter was reduced in the H-MSC and H-RM groups, and there were no

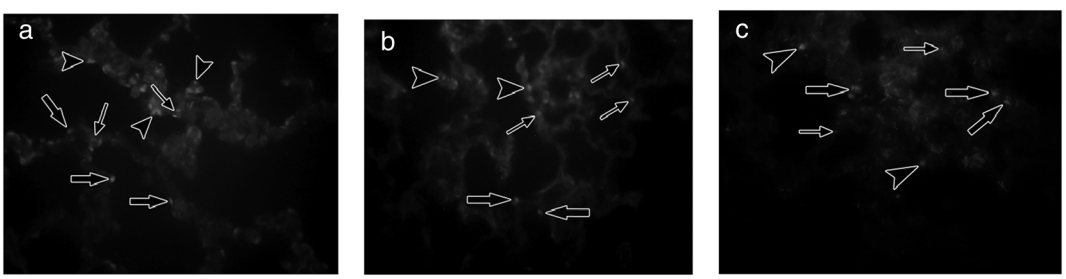

Fig. 6 Staining for green fluorescent protein (GFP) and surfactant protein-C (SP-C) in excised rat lung (a) 1 week, (b) 2 weeks and (c) 3 weeks after mesenchymal stem cell delivery. Arrowheads, GFP and SP-C positive; thin arrows, SP-C positive; thick arrows, GFP positive.

differences compared with the N and S groups. Both MSC and RM had positive effects on emphysema. Hyperoxia reduced septation and number of alveoli, whereas MSC increased the number of alveoli and reduced emphysema; that is, structural integrity was achieved.[10,27] RM only reduced alveolar diameter and did not increase the number of alveoli. MSC prevented arrested alveolar growth in HILI, but other therapeutic benefits such as reducing alveolar diameter are through paracrine activity. Therefore, the number of alveoli increased only in the H-MSC group. Alveolar diameter was reduced in the H-RM group, but, despite the possible effects of some substances released into medium during the proliferation of stem cells, this substance did not increase the number of alveoli. Although recent studies have emphasized that paracrine effect is more important for improvement of the lung structure than MSC themselves, according to the present results the presence of MSC themselves is necessary for alveolar growth.[28] In a study by Sutsko *et al.*, neonatal rats treated with intratracheal MSC were evaluated at 16, 30 and 100 postnatal days, and it was found that although the acute improvement in HILI following MSC therapy may be mainly paracrine mediated, optimum long-term recovery requires the presence of the MSC themselves.[29] This is an important finding given that it provides further insight into the potential of MSC therapy to provide sustained improvement in severe BPD, and opens the door for further evaluation of the paracrine factors released from MSC that may mediate the acute improvement in HILI. In the present study we evaluated lung histopathology at 60 postnatal days, and found that the number of alveoli increased only in the H-MSC group.

It has been reported that an increase in lung weight is related to edema and congestion,[15] and there was a statistically significant increase in lung weight in the H-P and H-CM groups in the present study. The H-RM and H-MSC groups had lung weight similar to that of the N and S groups, and RM and MSC treatment had a positive effect on lung weight that was not seen with CM.

A known histopathologic finding in BPD is the first appearance of fibrosis around new blood vessels and loose extracellular matrix granulation tissue at injury repair sites. It is widely accepted that fibroblasts infiltrate the damaged area in fibrosis formation and that these fibroblasts transform into myofibroblasts. Myofibroblasts express α-SMA, which is an important marker used in assessing fibrosis.[30] A study of the anti-fibrotic effects of MSC reported that fibrosis can be assessed by evaluating transforming growth factor β1 (TGF-β1), α-SMA and collagen increases.[31] TGF-β1 is a key fibrogenic cytokine, and activates fibroblasts differentiation into myofibroblasts. The activated myofibroblasts play an important role in the process of lung fibrosis, hence the importance of α-SMA as a key fibroblast myogenic marker. The anti-fibrotic effects of intratracheal MSC in this study were demonstrated by the significant attenuation of hyperoxia-induced increase in α-SMA. Although HE staining showed no fibrosis in the current study, the presence of myofibroblasts was confirmed on immunohistochemistry. This suggests that there was a tendency to develop fibrosis after hyperoxia, but the fibrosis was insufficient to be detected on HE staining. Using α-SMA expression by myofibroblasts to assess fibrosis, we found that α-SMA expression was significantly increased in the H-P group compared with the N and S groups and significantly reduced in the H-CM, H-RM and H-MSC groups compared with the H-P group. Also, the increase in α-SMA expression in the H-CM group was significant when compared with the H-RM and H-MSC groups. This effect might be due to the anti-inflammatory effects of MSC, but the mechanism of action is unclear.

Mesenchymal stem cells were labeled by transfection to enable visualization before tracheal infusion. In a study in which cytomegalovirus was transferred with a GFP-encoding plasmid, GFP expression was found to be 25% 2 weeks after transfection and 10% 3 weeks after transfection.[32] To allow for this reduction over time, the lungs of three MSC group rats were removed at 1 week intervals following 10 days of hyperoxia to be sure that MSC had become established in the lung tissue. GFP-positive cells were seen in the lung tissue of all rats that received labeled cells. Labeling for SP-C showed type II pneumocytes expressing this protein in all sections. Double labeling showed that some GFP-positive cells also expressed SP-C. This suggests that some of the delivered MSC became localized in the damaged tissue, assumed the properties of the cells of that tissue (type II alveolar epithelial cells) and started synthesizing surfactants. But, this findings may also be due to co-localization of MSC and SP-C. The co-localization of GFP-positive MSC with SP-C, a type II pneumocyte-specific marker, was observed in injured lung tissue, suggesting that MSC can differentiate into lung parenchymal cells. This co-localization, however, was observed only in a few cells, and was not sufficient to produce the observed effects. Therefore, it still remain to be elucidated as to whether the engrafted MSC exert their therapeutic effects by inducing direct tissue repair and regeneration of damaged cells. Further studies are required to clarify this.

Finally, it is important to mention the limitations of this study. In the present study rat lungs were removed at 60 postnatal days, and during the recovery period in which the pups were in normoxia, they may have been in a healing process or the lung morphology may have changed due to hypoxic–hyperoxic processes. Although the lungs of three rats had been removed at 1 week intervals following 10 days of hyperoxia to confirm the establishment of MSC in the lungs, the number of GFP-positive cells measured on the 60th postnatal day was very low, and therefore a definitive conclusion is unable to be made. The present study has shown that GFP-positive cells expressed SP-C with double labeling, but the close co-localization of GFP-positive cells with alveolar epithelial cells means that a definitive conclusion regarding cell differentiation is not possible.

In conclusion, tracheally delivered MSC derived from adult rat bone marrow reduced lung weight, increased the number of alveoli, mitigated emphysema and reduced α-SMA expression by myofibroblasts. It also appeared that labeled MSC localized to the lung, came to resemble the cells of the lung tissue. Despite MSC treatment, persistence of some abnormalities may have been due to loss of the supportive extracellular matrix that provides the lung scaffold, and may preclude restoration of normal alveolar structure.

Further studies are needed to evaluate the long-term results of stem cell treatment and whether it has a positive effect on loss of supportive tissues in the lung, and to investigate the optimal

delivery time for stem cells, route of delivery, type of stem cell to be used and the results of combination therapy with growth factors.

Acknowledgment

The authors declare no conflicts of interest.

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