

Consecutive Daily Administration of Intratracheal Surfactant and Human Mesenchymal Stem Cells Attenuates Hyperoxia-induced Lung Injury in Neonatal Rats

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Research

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Abstract

Background: Surfactant therapy is a standard of care for preterm infants with respiratory distress and reduces the incidence of death and bronchopulmonary dysplasia in these patients. Mesenchymal stem cells (MSCs) attenuated hyperoxia-induced lung injury. Surfactant reduced the in vitro viability of human MSCs, and the combination therapy of surfactant and MSCs did not have additive effects on hyperoxia-induced lung injury in neonatal rats. The effects of 2 consecutive days of intratracheal administration of surfactant and MSCs on hyperoxia-induced lung injury were undetermined.

Methods: Neonatal Sprague Dawley rats were reared in either room air (RA) or hyperoxia (85% O₂) from postnatal days 1 to 14. On postnatal day 4, the rats received intratracheal injections of either 20 µL of normal saline (NS) or 20 µL of surfactant. On postnatal day 5, the rats reared in RA received intratracheal NS, and the rats reared in O₂ received intratracheal NS or human MSCs (3 × 10⁴ or 3 × 10⁵ cells). Six study groups were examined: RA + NS + NS, RA + surfactant + NS, O₂ + NS + NS, O₂ + surfactant + NS, O₂ + surfactant + MSCs (3 × 10⁴ cells), and O₂ + surfactant + MSCs (3 × 10⁵ cells). The lungs were excised for analysis on postnatal day 14.

Results: The rats reared in hyperoxia and treated with NS yielded significantly higher mean linear intercepts (MLIs) and cytokine levels and significantly lower vascular endothelial growth factors (VEGFs), platelet-derived growth factor protein expression, and vascular density than did those reared in RA and treated with NS or surfactant. The lowered MLIs and cytokine levels and the increased VEGF expression and vascular density indicated that the surfactant and surfactant + MSCs (3 × 10⁴ cells) treatment attenuated hyperoxia-induced lung injury. The surfactant + MSCs (3 × 10⁵ cells) group exhibited a significantly lower MLI and significantly higher VEGF expression and vascular density than the surfactant + MSCs (3 × 10⁴ cells) group did.

Conclusions: Consecutive daily administration of intratracheal surfactant and MSCs can be an effective regimen for treating hyperoxia-induced lung injury in neonates.

Background

Supraphysiological oxygen is often required to treat newborns with respiratory disorders. However, administering supplemental oxygen to newborn infants with respiratory failure can lead to lung injury. Term-born rat models are appropriate for studying the effects of hyperoxia on preterm infants with respiratory distress because rats are born at the sacular stage, which is approximately equivalent to a human gestational age of 30 weeks [1]. The prolonged exposure of neonatal rats to hyperoxia results in a decrease in alveolarization and vascularization similar to human bronchopulmonary dysplasia (BPD) [2, 3]. The pathogenesis of BPD is multifactorial, and oxygen toxicity plays a crucial role in the process of lung injury leading to BPD [4, 5].

Surfactant therapy is a standard of care for preterm infants with respiratory distress syndrome and can reduce the incidence of death and BPD [6]. Mesenchymal stem cells (MSCs) are multipotent stromal cells that have immunomodulatory, anti-inflammatory, and regenerative properties and have been demonstrated to treat hyperoxia-induced lung injury in newborn animals [7–14]. In this study, we demonstrated that the addition of surfactant reduced the in vitro viability of human MSCs through mitochondrial dysfunction and that a combination therapy of surfactant and MSCs had no additive effects on lung development in neonatal rats exposed to hyperoxia [14]. The effects of 2 consecutive days of intratracheal administration of surfactant and MSCs on hyperoxia-induced lung injury were undetermined. We hypothesized that consecutive daily administration of intratracheal surfactant and MSCs improves lung development and that high doses of MSCs enhance this effect on experimental BPD in neonatal rats. The aim of this study was to investigate the effects of an animal-derived surfactant (Survanta) and consecutive daily administration of human MSCs on hyperoxia-induced lung injury in neonatal rats.

Methods

Surface tension

Survanta (AbbVie Inc., North Chicago, IL, USA) was prepared through lipid extraction from minced bovine lungs and contained approximately 84% phospholipids, 1% hydrophobic surfactant proteins (SP-B and SP-C), and 6% free fatty acids. The solution was supplied at a 25 mg/mL concentration of phospholipids suspended in a 0.9% sodium chloride solution. The surface tension of Survanta (25 mg/mL), Survanta (12.5 mg/mL), and Survanta (25 mg/mL) and the human MSCs (1.5×10^6 cells/mL) was determined at a volume of 2 mL by using a surfactometer (Amherst Electronics, Buffalo, NY, USA). Each sample was measured three times.

Isolation of human mesenchymal stem cells

The MSCs were isolated from human umbilical cords, as previously described [12]. The MSCs were characterized by analyzing the expression of cluster of differentiation (CD) markers (CD44, CD73, CD90, CD105, CD11b, CD19, CD34, and CD45) and the human leukocyte antigen D-related complex through flow cytometry (BD Stemflow hMSC Analysis Kit, BD, Franklin Lakes, NJ, USA). An examination of trilineage differentiation capability (osteocytes, chondrocytes, and adipocytes) and a karyotyping revealed positive results.

Animal model and experimental groups

This study was approved by the Animal Care Use Committee of Taipei Medical University (LAC-2019-0396). Time-dated pregnant Sprague Dawley rats were housed in individual cages with ad libitum access to laboratory food and water, kept on a 12:12-h light–dark cycle, and allowed to deliver vaginally at term. Within 12 h of birth, the litters were pooled and randomly redistributed to the newly delivered mothers; the pups were then randomly assigned to room air (RA) or oxygen-enriched atmosphere (85% O₂) groups for

postnatal days 1–14. The nursing mothers were rotated between the 85% O₂ and the RA groups every 24 h to prevent oxygen toxicity in the mothers and to eliminate differing maternal effects between the groups. An oxygen-rich atmosphere was maintained in a transparent 40 × 50 × 60-cm³ plexiglass chamber receiving continuous O₂ at 4 L/min. The oxygen concentration inside the hyperoxic plexiglass chamber was continuously monitored using an oxygen sensor (Coy Laboratory Products, Grass Lake, MI, USA). On postnatal day 4, the rats received intratracheal injections of either 20 µL of normal saline (NS) or 20 µL of surfactant (Survanta, AbbVie Inc.), corresponding to approximately 50 mg/kg of phospholipids (Fig. 1). On postnatal day 5, the rats reared in RA were treated with NS and those reared in O₂ received intratracheal injections of 20 µL of NS, human MSCs (3 × 10⁴ cells), or human MSCs (3 × 10⁵ cells) in 20 µL of NS. Six study groups were examined: RA + NS + NS, RA + surfactant + NS, O₂ + NS + NS, O₂ + surfactant + NS, O₂ + surfactant + MSCs (3 × 10⁴ cells), and O₂ + surfactant + MSCs (3 × 10⁵ cells). The lungs were excised for histological, western blot, and cytokine analyses on postnatal day 14.

Intratracheal administration of surfactant and human MSCs

For intratracheal transplantation, the rats were anesthetized with isoflurane and restrained on a board at a fixed angle as described by Chen et al. [15]. On postnatal day 4, the rats received intratracheal injections of either 20 µL of normal saline (NS) or 20 µL of surfactant. On postnatal day 5, the rats reared in RA received intratracheal NS, and the rats reared in O₂ received intratracheal NS or human MSCs (3 × 10⁴ or 3 × 10⁵ cells).

Lung histology

The lungs were placed in 4% paraformaldehyde, washed with phosphate-buffered saline, and then serially dehydrated in increasing concentrations of ethanol before being embedded in paraffin. To standardize the analyses, lung sections were taken from the right middle lobe. Sections of tissue weighing 5 µm were stained with hematoxylin and eosin, examined using light microscopy, and assessed for lung histology. The mean linear intercept (MLI), an indicator of the mean alveolar diameter, was assessed in 10 nonoverlapping fields [14].

Immunohistochemistry of lung vascular endothelial growth factor and von Willebrand factor

Immunohistochemical staining was performed on the 5-µm paraffin sections through immunoperoxidase visualization. After routine deparaffinization, heat-induced epitope retrieval was performed by immersing the slides in 0.01 M sodium citrate buffer (pH 6.0). To block the endogenous peroxidase activity and the nonspecific binding of antibodies, the sections were preincubated for 1 h at room temperature in 0.1 M phosphate-buffered saline containing 10% normal goat serum and 0.3% H₂O₂. The sections were then incubated for 20 h at 4°C with rabbit polyclonal anti-von Willebrand factor (vWF) antibodies (1:100; Abcam, Cambridge, MA, USA) or rabbit polyclonal anti-vascular endothelial growth factor (VEGF) antibodies (1:50; Santa Cruz Biotechnology, Inc., CA, USA) as primary antibodies. The sections were then treated for 1 h at 37°C with biotinylated goat antimouse or antirabbit IgG (1:200, Jackson

ImmunoResearch Laboratories Inc., West Grove, PA, USA). After the reagents from an avidin–biotin complex kit (Vector Laboratories, Inc., CA, USA) produced a reaction, the reaction products were visualized with a diaminobenzidine substrate kit (Vector Laboratories Inc.) in accordance with the recommendations of the manufacturer. Pulmonary vessel density was determined by counting the number of vessels with positive vWFs stained in an unbiased manner by using a minimum of four random lung fields at $\times 400$ magnification [16].

Western blot analysis of growth factors

The lung tissues were homogenized in ice-cold buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, and a protease inhibitor cocktail (complete minitables; Roche, Mannheim, Germany). The samples were sonicated and then centrifuged at 500 *g* for 20 min at 4°C to remove cellular debris. Proteins (30 μ g) were resolved on 12% SDS-PAGE gels under reducing conditions and electroblotted to a polyvinylidene fluoride membrane (ImmobilonP, Millipore, Bedford, MA, USA). After blocking with 5% nonfat dry milk, the membranes were incubated with antibodies against VEGF (1:1000; Santa Cruz Biotechnology, Inc.), platelet-derived growth factor subunit B (PDGF-B; 1:1000; Santa Cruz Biotechnology, Inc.), or anti- β -actin (1:20,000; Sigma-Aldrich, St. Louis, MO, USA) and subsequently with horseradish peroxidase-conjugated goat antirabbit IgG or antimouse IgG (Pierce Biotechnology, Rockford, IL, USA). Densitometric analysis was performed with AIDA software to measure the intensity of VEGF, PDGF-B, and β -actin bands.

Lung cytokine levels

The lung tissue was homogenized in 1 mL of ice-cold lysis buffer containing 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.01 M deoxycholic acid, and a complete protease cocktail inhibitor. Cell extracts were centrifuged, and the levels of interleukin (IL)-1 β and IL-6 in the supernatants were measured with an enzyme-linked immunosorbent assay kit (Cloud-Clone Corp., Houston, TX, USA).

Statistical analysis

The data are presented as means \pm standard deviations. Statistical analyses were performed using one-way ANOVA with the Bonferroni post hoc test for the multiple-group comparisons. The survival rate was evaluated by using the Kaplan–Meier method, and the log-rank test was used for the intergroup comparisons. Differences were considered statistically significant when $P < 0.05$.

Results

Surface tension

The surface tensions for the 25 mg/mL Survanta, the 12.5 mg/mL Survanta, and the 25mg/mL Survanta with the human MSCs (1.5×10^6 cells/mL) were 24.7 ± 1.7 , 26.7 ± 3.8 , and 34.9 ± 3.5 mN/m, respectively.

The 25 mg/mL Survanta with the human MSCs exhibited a significantly higher surface tension than did the 25 mg/mL Survanta ($P = 0.005$) and the 12.5 mg/mL Survanta ($P = 0.011$).

Survival rate

All the rats reared in RA and treated with NS or surfactant survived (Fig. 2). In the O₂ + NS + NS group, one, one, three, and one rats died on postnatal days 5, 9, 10, and 11, respectively. In the O₂ + surfactant + NS and O₂ + surfactant + MSCs (3×10^4 cells) groups, one rat each died on postnatal days 6 and 8. In the O₂ + surfactant + MSCs (3×10^5 cells) groups, one rat each died on postnatal days 9 and 10. The rats reared in hyperoxia and treated with NS exhibited a significantly lower survival rate than did those reared in RA and treated with NS or surfactant ($P < 0.05$). Treatment with surfactant and treatment with surfactant and MSCs augmented the hyperoxia-induced decrease in the survival rate, but the differences in survival rate were not statistically significant. The survival rates were comparable among the RA + NS + NS, RA + surfactant + NS, O₂ + surfactant + NS, O₂ + surfactant + MSCs (3×10^4 cells), and O₂ + surfactant + MSCs (3×10^5 cells) groups.

Body and lung weight and lung-to-body-weight ratio

The body and lung weights and the lung-to-body-weight ratios on postnatal day 14 were comparable among the six study groups (Table 1).

Histology results

Fig. 3 presents the lung tissue sections stained with hematoxylin and eosin on postnatal day 14. The rats reared in hyperoxia and treated with NS exhibited large thin-walled air spaces and yielded a significantly higher MLI than did those reared in RA and treated with NS or surfactant (Fig. 3a). Treatment with surfactant and treatment with surfactant and MSCs (3×10^4 cells) significantly diminished the hyperoxia-induced increase in the MLI. The O₂ + surfactant + MSCs (3×10^5 cells) group exhibited a significantly lower MLI than did the O₂ + surfactant + NS and the O₂ + surfactant + MSCs (3×10^4 cells) groups (Fig. 3b). Fig. 4 shows representative lung sections stained for vWF on postnatal day 14. The rats reared in hyperoxia and treated with NS yielded a significantly lower vascular density than did those reared in RA and treated with NS or surfactant. Treatment with surfactant and MSCs (3×10^4 or 3×10^5 cells) significantly augmented the hyperoxia-induced decrease in vascular density. The surfactant + MSCs (3×10^5 cells) treatment more significantly increased vascular density compared with the surfactant + MSCs (3×10^4 cells) treatment.

Immunohistochemistry and western blotting of VEGF

The VEGF immunoreactivities were primarily detected in the endothelial cells (Fig. 5a). The rats reared in hyperoxia and treated with NS exhibited significantly lower VEGF protein expression than did those reared in RA and treated with NS or surfactant (Fig. 5b). Treatment with surfactant and MSCs (3×10^4 or 3×10^5

cells) significantly augmented the hyperoxia-induced decrease in VEGF protein expression compared with treatment with NS.

Western blot analysis of PDGF

Fig. 6 shows the representative western blot of PDGF-A and PDGF-B. The rats reared in hyperoxia and treated with NS exhibited significantly lower PDGF-A and PDGF-B protein expression than did those reared in RA and treated with NS or surfactant. Treatment with surfactant and MSCs (3×10^5 cells) significantly augmented the hyperoxia-induced decrease in the PDGF-A and PDGF-B protein expression compared with treatment with NS.

Lung cytokine levels

The rats reared in hyperoxia and treated with NS yielded significantly higher IL-1 β and IL-6 levels on postnatal day 14 than did those reared in RA and treated with NS or surfactant on postnatal (Fig. 7). The treatment with surfactant and the treatment with surfactant and MSCs significantly diminished the hyperoxia-induced increase in IL-1 β and IL-6 levels.

Discussion

On postnatal day 14, our in vivo neonatal rat model demonstrated that neonatal hyperoxia exposure during the first 2 weeks of life impaired alveolarization and angiogenesis. The intratracheal administration of surfactant on postnatal day 4 and MSCs (3×10^4 or 3×10^5 cells) on postnatal day 5 improved alveolarization and angiogenesis in the neonatal rats exposed to hyperoxia. The surfactant and MSCs (3×10^5 cells) treatment was more successful in improving alveolarization and angiogenesis than the surfactant and MSCs (3×10^4 cells) treatment was. The main findings of this study were that the intratracheal administration of MSCs on the day following administration of surfactant improve lung development and that high doses of MSCs amplify the therapeutic effects on experimental BPD in neonatal rats compared with the low doses of MSCs.

Although term-born rats have structurally immature lungs, they are functionally mature and require no surfactant treatment. In one study, neonatal mice exposed to hyperoxia for 4 days exhibited disruptions to type II cell proliferation, which produced pulmonary surfactant [17]. In another study, hyperoxia during the first 3 days of life induced inflammatory cell infiltration in alveolar spaces and increased the wet-to-dry lung weight ratio in neonatal Sprague Dawley rats [18]. The results of these studies have suggested that hyperoxia reduces surfactant production and induces lung inflammation in newborn animals. Pulmonary surfactant is a mixture of phospholipids, surfactant-associated proteins, and neutral lipids, which modulate pulmonary inflammation and stabilize the alveoli by reducing surface tension [19]. In our study, the administration of surfactant on postnatal day 4 diminished the hyperoxia-induced increase in MLI and lung cytokines in the neonatal rats. The surfactant treatment did not augment the hyperoxia-induced decrease in pulmonary vascular density. These results support the idea that pulmonary

surfactant fulfills an essential role in the lungs for both host defense mechanisms, such as modulating pulmonary inflammation, and for improving alveolarization [14, 20].

Surfactant therapy has become the standard of care for preterm infants with respiratory distress syndrome and can reduce the combined outcomes of death and BPD [6]. Compared with delayed surfactant treatment, early surfactant treatment was more effective in reducing mortality, air leak, BPD, and BPD or death in preterm infants [21]. Although surfactant has a unique spreading property and can reduce surface tension. The addition of a surfactant reduced the in vitro viability of human MSCs, and the combination therapy of surfactant and MSCs did not exhibit any additional benefits to lung development in neonatal rats exposed to hyperoxia [14]. For this reason, we administered intratracheal surfactant and MSCs on 2 consecutive days and found that the intratracheal administration of surfactant on postnatal day 4 and MSCs on postnatal day 5 improved alveolarization and angiogenesis in the neonatal rats exposed to hyperoxia. The time interval between the administration of surfactant and the MSCs for achieving optimal therapeutic effects was not determined. Future studies are required to evaluate the effects of different time intervals on hyperoxia-induced lung injury.

In this study, the administration of surfactant and the administration of surfactant with human MSCs to the hyperoxia-exposed rats significantly improved lung development in the surviving animals, although the survival rate did not significantly improve. The differences in the survival rates between rats treated with surfactant and those treated with surfactant and MSCs were not significant on postnatal day 14. The rats reared in hyperoxia and treated with NS exhibited a low survival rate after postnatal day 5. The treatment with surfactant and treatment with surfactant and human MSCs (3×10^5 cells) maintained the survival rate from postnatal days 5 to 9. These results suggest that an additional dose of MSCs is required to maintain the survival rate.

In this study, we determined the levels of VEGF, PDGF-A, and PDGF-B expression and elucidated the mechanisms that mediate the MSCs' effects because their mRNA and protein expression decreased in the lungs of newborn animals exposed to 14 days of hyperoxia [22–24]. VEGF is a potent endothelial cell mitogen that regulates angiogenesis and alveolar development [25]. PDGF is crucial to alveolarization of normally developing lungs [26]. We demonstrated that the rats reared in hyperoxia and treated with NS exhibited significantly lower levels of VEGF, PDGF-A, and PDGF-B protein expression than did those reared in RA and treated with NS or surfactant. Treatment with surfactant and MSCs augmented the hyperoxia-induced decrease in the VEGF, PDGF-A, and PDGF-B protein expression levels. These results suggest that treatment with MSCs enhanced vascular and alveolar development in the neonatal rats through the induction of growth factors.

Conclusions

Consecutive daily administration of intratracheal surfactant and human MSCs likely attenuated hyperoxia-induced defective alveolarization and angiogenesis by increasing VEGF expression. High doses of MSCs enhanced the therapeutic effects more effectively than the low doses of MSCs. Consecutive

daily administration of intratracheal surfactant and MSCs can be an effective regimen for treating hyperoxia-induced lung injury in neonates.

Abbreviations

BPD: bronchopulmonary dysplasia; IL: interleukin; MLI: mean linear intercept; MSCs: mesenchymal stem cells; NS: normal saline; O₂: oxygen-enriched atmosphere; PDGF: platelet-derived growth factor, RA: room air; VEGF: vascular endothelial growth factor; vWF: von Willebrand factor

Declarations

Ethics approval and consent to participate

The study was approved by the Animal Care and Use Committee at Taipei Medical University (LAC-2019-0396).

Consent for publication

All authors consent to publication.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CMC and HCC contributed to the study design, data collection, execution of the study, data analysis and interpretation, and preparation of the manuscript. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

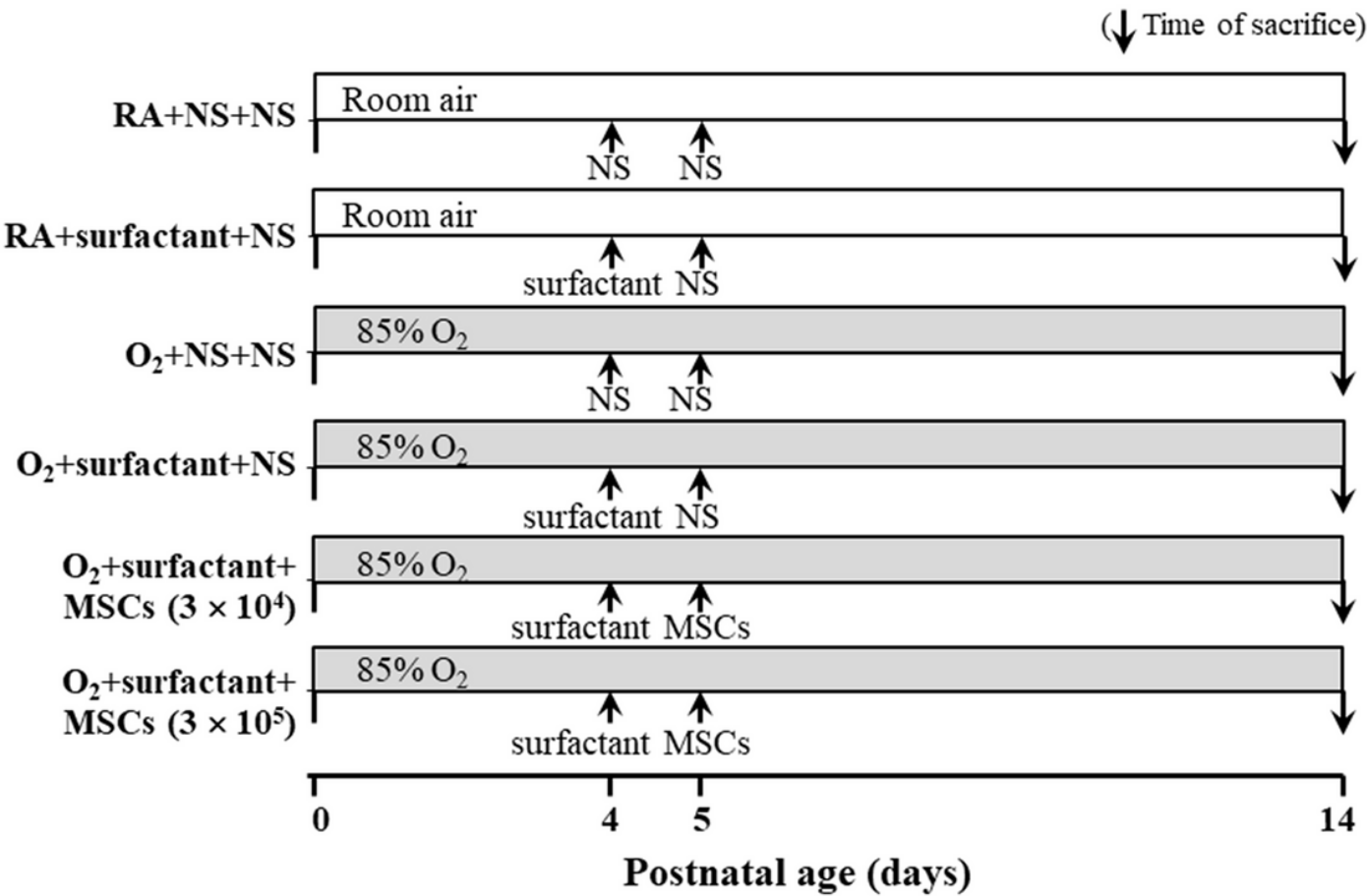


Figure 1

Diagrammatic representation of the experimental design showing the study timeline and the rat pup treatment groups.

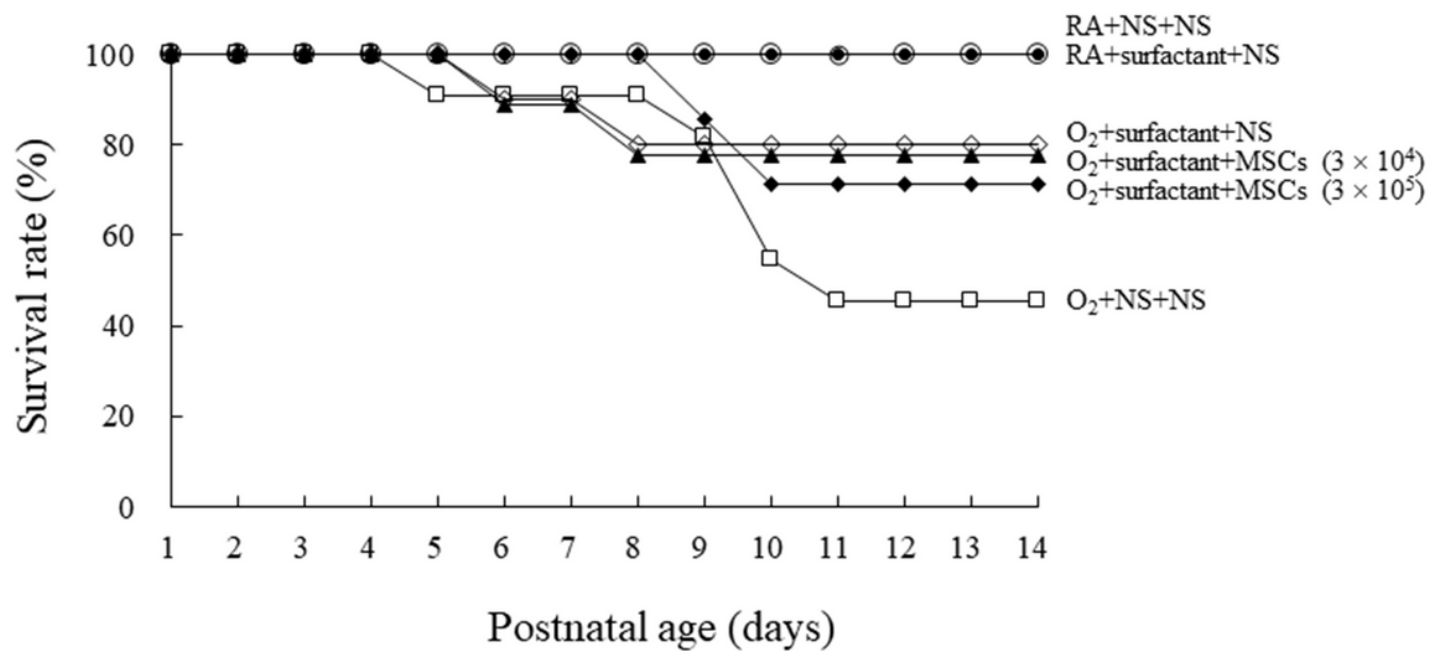


Figure 2

Effects of surfactant and human MSCs on the survival rate on postnatal day 14.

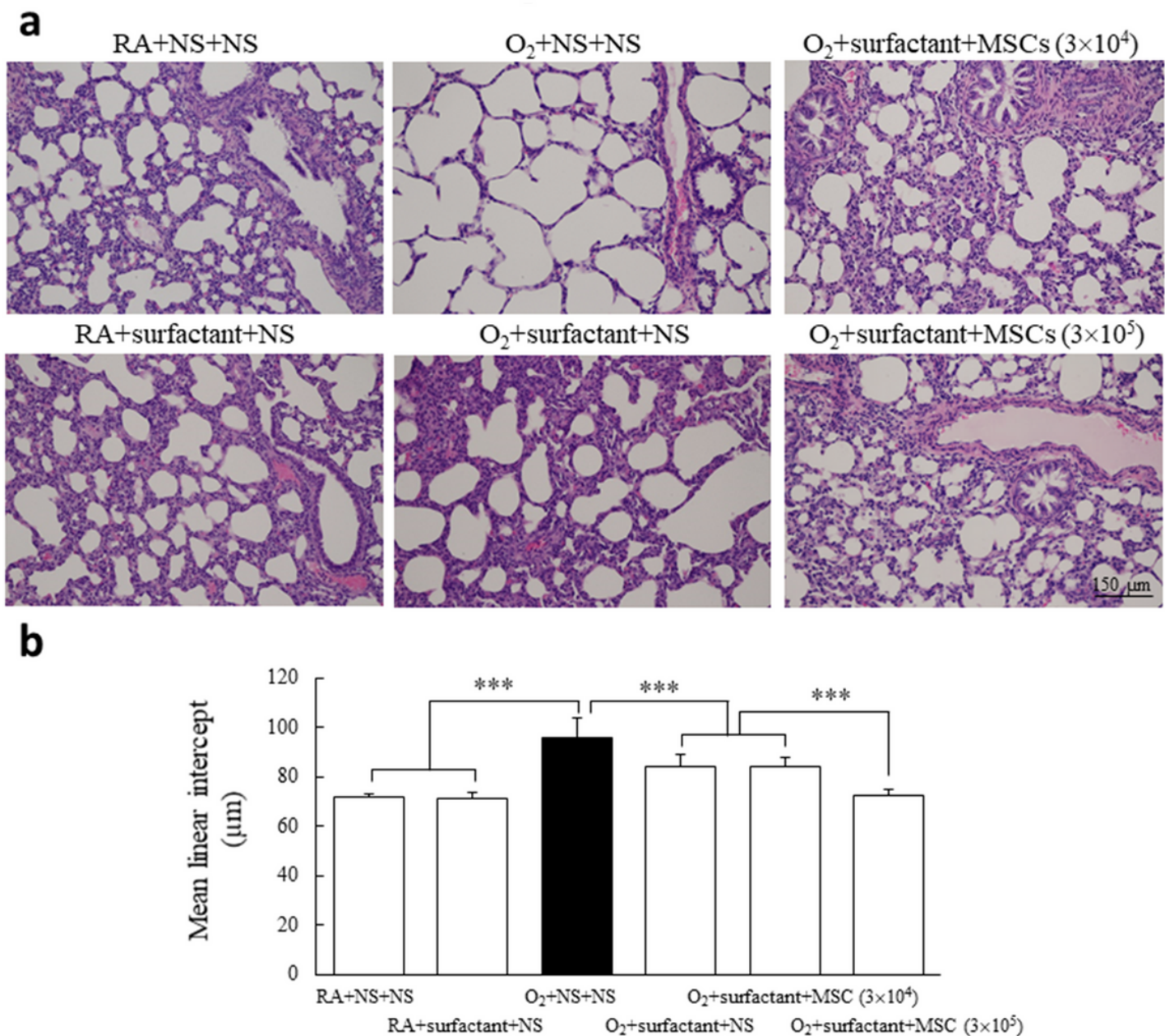


Figure 3

(a) Representative histology and (b) MLI in 14-day-old rats exposed to either postnatal RA or hyperoxia and treated with either NS or surfactant on postnatal day 4 and either NS or MSCs (3×10^4 or 3×10^5 cells) on postnatal day 5. The rats reared in hyperoxia and treated with NS yielded a significantly higher MLI than did those reared in RA and treated with NS or surfactant. Treatment with surfactant and treatment with surfactant and MSCs (3×10^4 cells) significantly diminished the hyperoxia-induced increase in the MLI. The O₂ + surfactant + MSCs (3×10^5 cells) group exhibited a significantly lower MLI than the O₂ + surfactant + NS and the O₂ + surfactant + MSCs (3×10^4 cells) groups. *** $P < 0.001$.

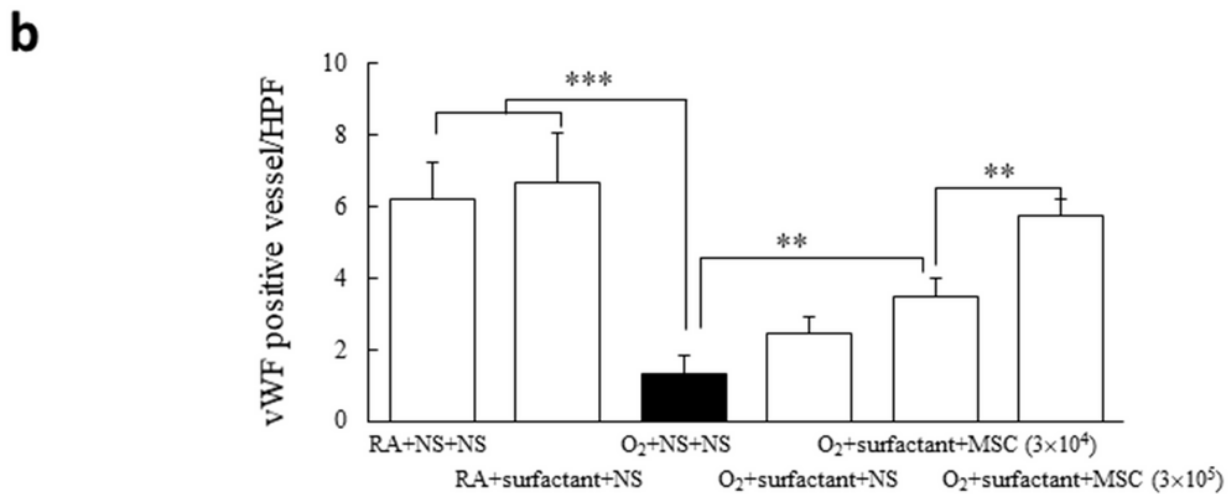
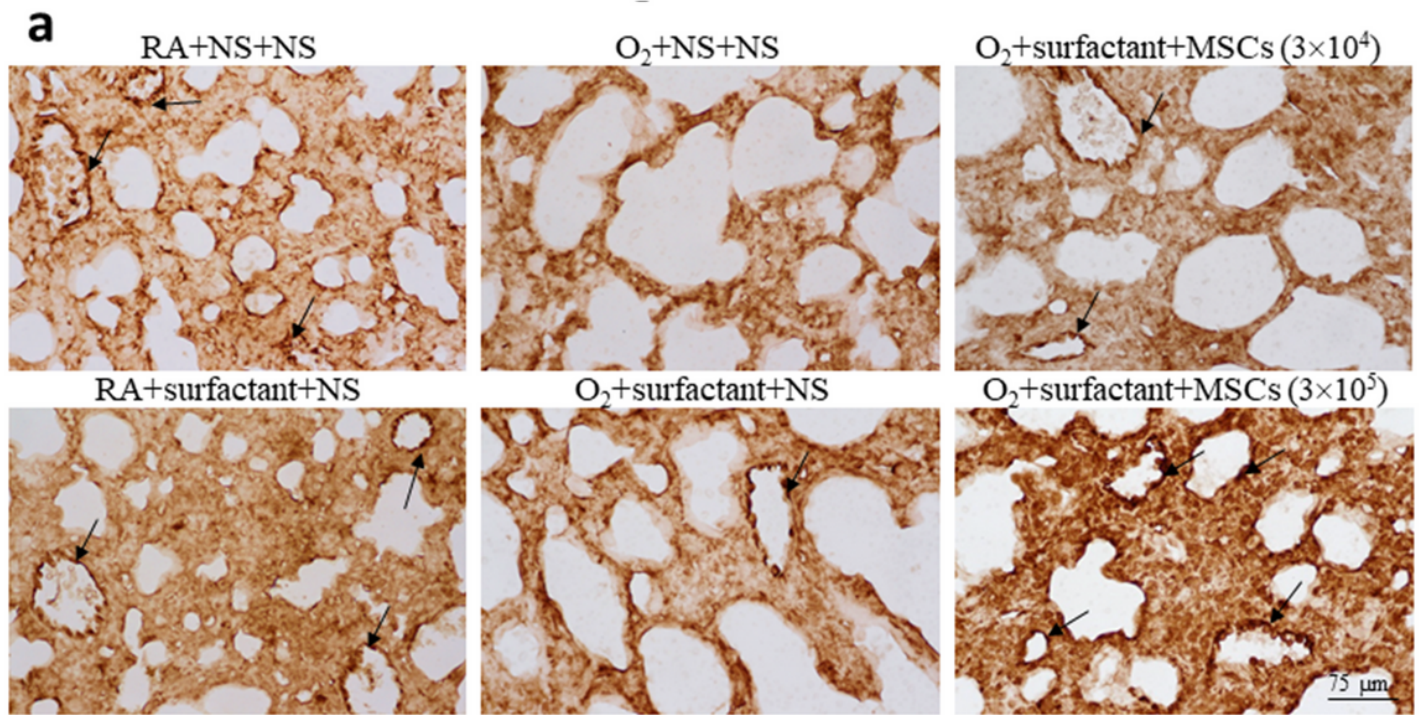


Figure 4

(a) Representative histology and (b) vascular density in 14-day-old rats exposed to either postnatal RA or hyperoxia and treated with either NS or surfactant on postnatal day 4 and either NS or MSCs (3×10^4 or 3×10^5 cells) on postnatal day 5. The rats reared in hyperoxia and treated with NS yielded a significantly lower vascular density than those reared in RA and treated with NS or surfactant. Treatment with surfactant and MSCs (3×10^4 or 3×10^5 cells) significantly augmented the hyperoxia-induced decrease in the vascular density. Treatment with surfactant and MSCs (3×10^5 cells) more significantly increased vascular density than did the treatment of surfactant and MSCs (3×10^4 cells). ** $P < 0.01$ and *** $P < 0.001$.

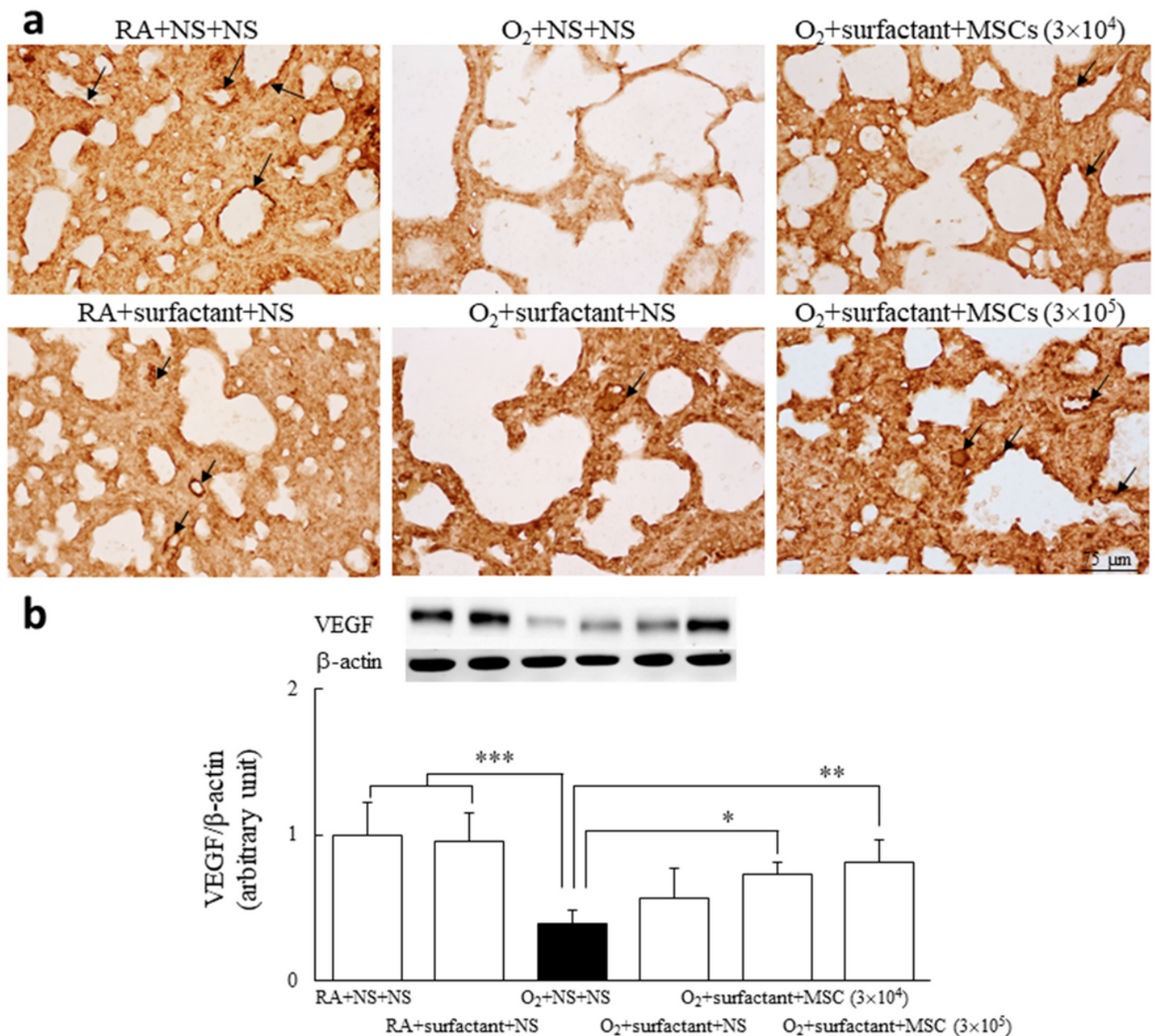


Figure 5

(a) Representative immunohistochemistry of VEGF and (b) representative western blots and quantitative data determined with densitometry for VEGF protein expression in 14-day-old rats exposed to postnatal RA or hyperoxia and treated with NS or surfactant on postnatal day 4 and NS or MSCs (3×10^4 or 3×10^5 cells) on postnatal day 5. The rats reared in hyperoxia and treated with NS exhibited significantly lower levels of VEGF protein expression than did those reared in RA and treated with NS or surfactant. Treatment with surfactant and MSCs (3×10^4 or 3×10^5 cells) more significantly augmented the hyperoxia-induced decrease in the VEGF protein expression levels than the treatment with NS. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

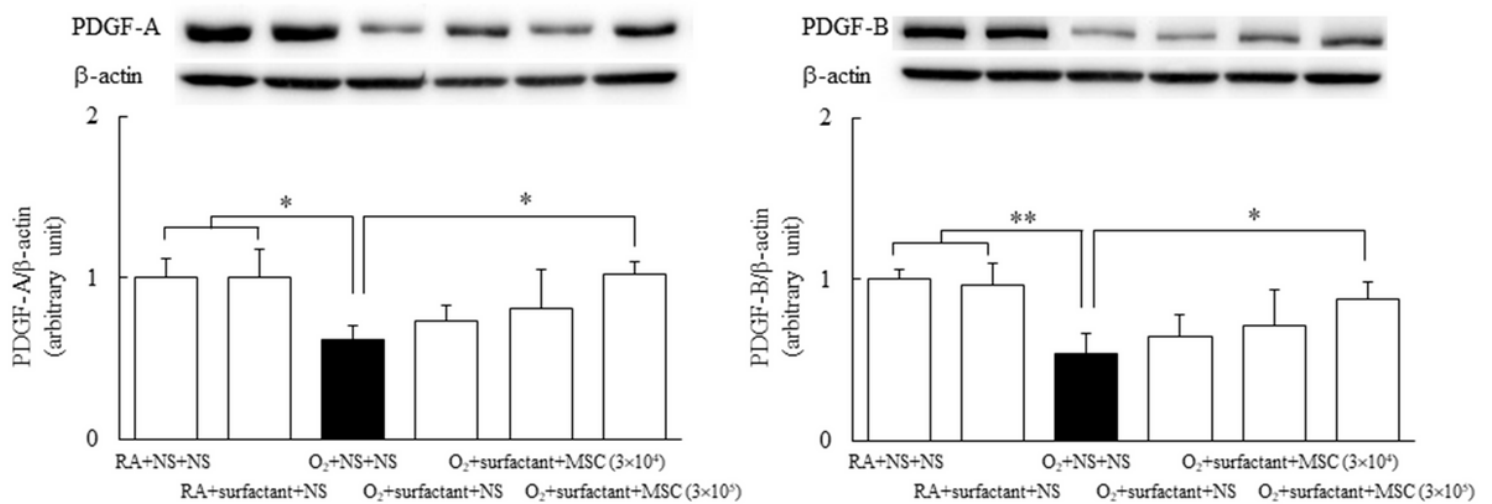


Figure 6

Representative western blots and quantitative data determined with densitometry for PDGF-A and PDGF-B protein expression in 14-day-old rats exposed to postnatal RA or hyperoxia and treated with NS or surfactant on postnatal day 4 and NS or MSCs (3×10^4 or 3×10^5 cells) on postnatal day 5. The rats reared in hyperoxia and treated with NS exhibited significantly lower PDGF-A and PDGF-B protein expression levels than those reared in RA and treated with NS or surfactant. Treatment with surfactant and MSCs (3×10^5 cells) more significantly augmented the hyperoxia-induced decrease in the PDGF-A and PDGF-B protein expression levels than did the treatment with NS. *P < 0.05 and **P < 0.01.

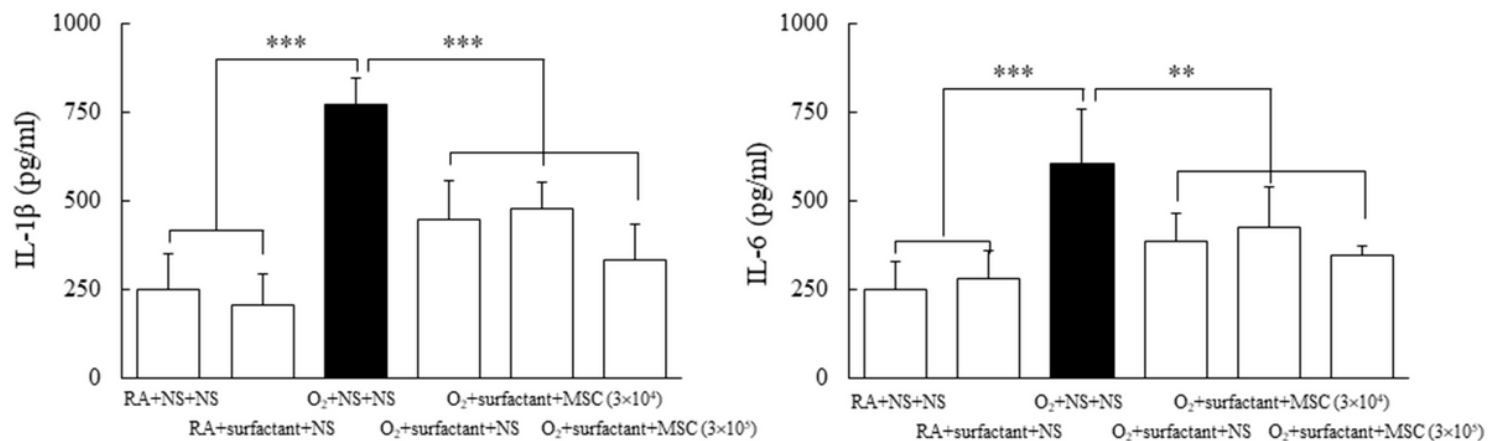


Figure 7

Lung cytokine levels The rats reared in hyperoxia and treated with NS yielded significantly higher IL-1β and IL-6 levels on postnatal day 14 than did those reared in RA and treated with NS or surfactant on postnatal (Fig. 7). The treatment with surfactant and the treatment with surfactant and MSCs significantly diminished the hyperoxia-induced increase in IL-1β and IL-6 levels.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Table1.docx](#)