Human Amniotic Fluid Stem Cells Protect Rat Lungs Exposed to Moderate Hyperoxia

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Summary. Background: Treatment of bronchopulmonary dysplasia (BPD) remains as yet an unmet clinical need and recently stem cells have been proposed as a therapeutic tool in animal models. We investigated the role of amniotic fluid stem cells (AFS) in an adult rat model of hyperoxia lung injury. Methods: Fifty Sprague–Dawley rats were, at birth, randomly exposed to moderate hyperoxia or room air for 14 days and a single dose of human amniotic fluid stem (hAFS) or human Fibroblasts (hF), cells was delivered intratracheally (P21). At P42 animals were euthanized and lung tissue examined using histology, immunohistochemistry, PCR, and ELISA. hAFS cells characterization and homing were studied by immunofluorescence. Results: In rats treated with hAFS and hF cells 16S human rRNA fragment was detected. Despite a low level of pulmonary hAFS cell retention (1.43 ± 0.2% anti-human-mitochondria-positive cells), the lungs of the treated animals revealed higher secondary crest numbers and lower mean linear intercept and alveolar size, than those exposed to hyperoxia, those left untreated or treated with hF cells. Except for those treated with hAFS cells, moderate hyperoxia induced an increase in protein content of IL-6, IL-1β, as well as IFN-γ and TGF-1β in lung tissues. High VEGF expression and arrangement of capillary architecture in hAFS cell group were also detected. Conclusions: Treatment with hAFS cells has a reparative potential through active involvement of cells in alveolarization and angiogenesis. A downstream paracrine action was also taken into account, in order to understand the immunodulatory response.

Key words: hyperoxia; hAFS cells; transplantation; lung repair.

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INTRODUCTION

The pathogenesis of bronchopulmonary dysplasia (BPD) is multifactorial, suggesting that multiple approaches might be considered to help reduce its incidence and severity. Hyperoxia is one of the primary risk factors leading to the onset of BPD, but the oxygen toxicity goes together with genetic risk factors and airways inflammatory cell responses. Clinically, BPD in preterm infants is a challenging condition and disorders may persist beyond childhood leading to lung diseases in adults. Children and adolescents born preterm show impaired lung function which in many cases leads to the development of chronic respiratory illnesses.

In rats, lung damage caused by 0.6 FiO\textsubscript{2} exposure, namely moderate oxygen exposure, in contrast to 0.95 during the first 14 days of life does not completely arrest lung cell proliferation and is associated with parenchymal thickening, as observed in BPD. Isolated developmental arrest in alveolarization may be caused by more subtle changes in signaling pathways without evidence of inflammation or necrosis, which are likely to happen under moderate hyperoxia. Meanwhile, there is increasing evidence of the value of stem cells as a potential treatment for neonatal lung diseases. Presently adult mesenchymal stem cells (MSCs) and umbilical cord blood cells show particular promise for therapy produces limited results. AFS cells isolated from cord blood, adult MSCs from BM are rare, difficult to expand and therefore banking of these cells for therapy produces limited results. AFS cells were demonstrated to resemble MSCs in many respects including surface marker expression and differentiation potential. AFS cells are likely to exhibit immunomodulatory capabilities as MSCs. Direct contact with AFS cells inhibits lymphocyte activation and cell-free supernatants, derived from AFS cells cocultured with total blood monocytes or IL-1β, a cytokine released by monocytes in the inflammatory response, which also inhibited lymphocyte activation.

As a consequence, in this work we have explored the therapeutic effect of human amniotic fluid stem (hAFS) cells in a rat lung injury model, after hyperoxia exposure during the neonatal period. So far AFS cells have been shown to be capable to differentiating into several cell types, such as respiratory epithelium, chondrocytes, adipocytes, neural cells, and endothelial cells. To trigger lung injury and to inhibit alveolar growth, we opted for moderate levels of hyperoxia rather than the higher exposure levels used in previous studies.

Our hypothesis was that hAFS cells might heal hyperoxia-challenged rats in a late alveolarization phase. Amelioration of lung histopathology and hAFS cell retention were evaluated. A downstream paracrine action was also taken into account, noting the immunomodulatory response and angiogenesis.

MATERIALS AND METHODS

Cell Preparation

Human AFS cells were isolated and cultured from human amniotic fluid, as previously reported. Briefly, following the guidelines established by the Hospital Ethical Committee (protocol number 451P/32887), consenting volunteer donors gave fresh backup samples of routine amniocentesis (2–3 ml). The cells were plated in Petri dish (3.5 cm diameter) and once adherent cells reached approximately 60–70% of confluence, they were detached with Trypsin 0.05–EDTA 0.02 w/v (Biochrom AG, Berlin, Germany) and magnetically selected for human anti-mouse c-kit (Santa Cruz Biotechnology, Inc., Delaware, CA). Cells were characterized by citofluorimetric analysis using the following antibodies: CD73-PE, CD-90-FITC, CD105-FITC, HLA-ABC-FITC, and HLA-DR-PE all purchased from BD and FITC, and HLA-DR-PE all purchased from BD and SSEA-4 (Santa Cruz Biotechnology, Inc.) with secondary antibody 488 conjugated (Invitrogen, Milan, Italy). Cells were differentiated in osteogenic and adipogenic medium and Von kossa and oil-red-o staining were performed to verify the attained differentiations.

Human primary fibroblasts, hF, used as negative control, were anonymously obtained from foreskin circumcision samples and kindly provided by the Laboratory of Gene Therapy, Women’s and Children’s Health Department, University of Padua, Padua, Italy. Cells were selected by adhesion and maintained in Petri dishes (10 cm diameter) with complete DMEM and 15% fetal bovine serum. When required cells were detached with Trypsin 0.05–EDTA 0.02 w/v (Life Technologies, Milan, Italy) and resuspended in PBS in aliquots of 1.5 × 10\textsuperscript{6} cells, ready for the injection.

Hyperoxia Exposure

Female wild-type Sprague–Dawley (SD) rats (Harlan Italy, Udine, Italy) and their offspring were housed and handled in accordance with the recommendations of the Public Health Office release of December 17, 2009, number 220/2009-B under the supervision of the local Ethical Committee. The study was conducted on male and female rat pups kept together with their nursing mother in conventional facilities. Adequate care for
their health and well-being was provided in accordance with Italian Law 116/92. Within the first 12 hr of birth (P0), pups and dams (or foster mothers) were placed in transparent polished acrylic chambers provided with software enabling the continuous monitoring of 

\[ \text{O}_2 \text{ and CO}_2 \] (BioSpherix, OxyCycler model A84XOV, Redfield, NY). The normobaric oxygen concentration was set at FiO\(_2\) = 0.6 until postnatal day (P) 14. The animals were fed ad libitum and exposed to alternating 12-hour light/dark cycles, humidity level was maintained at 50%, and ambient temperature at 24°C.

**Experimental Design**

Fifty newborn rats were randomly distributed between four experimental groups (Fig. 1A):

- Room air, \(n = 10\), pups were raised in normoxia at FiO\(_2\) = 0.21, for 42 days (control group);
- Untreated hyperoxia, \(n = 12\), pups were exposed to FiO\(_2\) = 0.6 from birth for 14 days, then to room air for 28 days;
- Hyperoxia + hAFS cells, \(n = 18\), pups were exposed to hyperoxia for 14 days and room air for 28 days, and intratracheally administered with hAFS cells at P21;
- Hyperoxia + HF, \(n = 10\), pups were exposed to hyperoxia for 14 days, then to room air for 28 days, and intratracheally administered with HF at P21.

At P42 (after 21 days in room air following hAFS or HF cell treatment) animals were euthanized and lung tissue was collected.

**Transplant Procedure**

Following an anterior neck incision under isoflurane anesthesia, the trachea was punctured with a 30G needle and each animal in the hAFS cell group received a single dose of \(1.5 \times 10^6\) cells resuspended in 30 μl of phosphate buffered saline (PBS, pH 7.4); similarly animals transplanted with HF cells were administered \(1.5 \times 10^6\) cells in PBS, pH 7.4. At P42 all animals were deeply anesthetized with a 1:1 combination of zolletil and xylazine and euthanized. All efforts were made to minimize suffering.

**Histology and Immunofluorescence**

**Lung Histology, Morphometric Analysis and Immunohistochemistry**

Following terminal anesthesia a catheter was placed into the trachea, and the lungs were inflated and maintained at 30 cmH\(_2\)O pressure with 4% paraformaldehyde in PBS for at least 45 min. A ligature was tightened around the trachea to maintain pressure after removal of the tracheal cannula. Lungs were immersed in paraformaldehyde solution overnight, and a 0.5-cm thick section of the left lower lobe was embedded in paraffin. Lung sections 4 μm thick were stained with hematoxylin and eosin. For each subject, we examined six sections and three fields per section. Photomicrographs were obtained on a field of 864 × 648 pixels at 20× magnification, with a Leica DM 4000B microscope (Leica, Solms, Germany) integrated with a camera (Leica DFC 280). Lung histopathology analyses were performed by two independent researchers blinded to the treatment strategy, using ImageJ, a public domain Java image-processing program (http://rsb.info.nih.gov/ij), as previously reported.27 In particular in each section,
from specific plug-in which leads to the evaluation of the skeletonized air spaces into each high-power field (hpf) the averaged alveolar size was evaluated by considering the alveolar minimum and maximum diameter and excluding the areas of large airways or vessels from analysis. The intra-alveolar distance was measured as the mean linear intercept by standard method, utilizing the same plug-in, by dividing the total length of lines drawn across the lung section (grid), by the number of intercepts encountered. A cell counter was applied for assessing the secondary crests number/hpf. Lung histopathology analyses were performed by two independent researchers blinded to the treatment strategy, using ImageJ.

Anti-CD31 (1:50; DAKO Italia s.p.a., Milan, Italy) immunohistochemistry was performed. After incubation with anti-mouse serum overnight (Envision, DAKO), 3,3'-diaminobenzidine (DAB, Sigma–Aldrich, Milan, Italy) containing H₂O₂ was used and counterstained with haematoxylin. Sections incubated without the primary antibody showed no immunoreactivity, confirming the specificity of the immunostaining. Analysis of the microvessel density (MVD) was blindly performed in 10 hpf at 20× of one representative slide. Vascular structures were considered those with positive reaction and a visible lumen or well-defined linear vessel shape. They were mainly small capillaries, with narrow lumen and very thin walls, located in the alveolar walls. The mean MVDs, expressed as number of positive structures for hpf, were calculated for each case and for the entire samples.

**Immunofluorescence Staining and hAFS Cells Lung Homing**

Anti-human mitochondria antibody Ab-2 (anti-hMIT) clone MTC02 (1:100; Thermo Fisher Scientific, Fremont, CA), rabbit anti-human C-protein surfactant antibody (hSFTPC) (1:200; ProteinTech Group, Chicago, IL), rabbit anti-rat fibronectin polyclonal antibody (1:200; Abcam, Cambridge, UK) were detected by immunofluorescence in 4 µm sections, incubated for 45 min at 25°C, then rinsed in PBS.

Alexa Fluor 488-labeled goat anti-mouse isotype-specific antibodies diluted 1:3000 (Molecular Probes, Eugene, OR) and Alexa Fluor 594-labeled goat anti-rabbit IgG antibodies diluted 1:2000 (Molecular Probes) were used as secondary antibodies. The percentage of human anti-mitochondria-positive cells was determined among lung cells (number of DAPI stained nuclei) per field. Ten random fields were chosen for each lung (on six different sections). Immunofluorescence was evaluated under the Leica DM 4000B microscope, integrated with LAS (Leica Application Suite) software using 3 dapi/green/orange filters. Specific stains for hSFTPC and anti-hMIT were taken using a laser scanner confocal microscope (Model TCS-SL; Leica, Wetzlar, Germany).

**Duplex PCR**

We applied a method based on a duplex PCR product amplified from mitochondrial DNA corresponding to the common human and rat cytochrome b, cyt b, (359 bp) and a 16S rRNA (157 bp) fragment, human-specific. DNA was extracted from the lung tissue using the Chelex Method or CST FORENSIC DNA PURIF KIT (Invitrogen) following the manufacturer’s instructions. The sequences of primers used in a single PCR reaction are: cyt b forward 5'-CCA ACC ATC TCA GCA TGA TGA AA-3'; cyt b reverse 5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3'; 16S rRNA forward 5'-CAA TTG GAC CAA CAA TCT ACC-3'; 16S rRNA reverse 5'-GTG AGG GTA ATG ACT TGT-3'. After an initial denaturation step of 1 min at 94°C, samples were amplified in a Perkin Elmer 9700 thermocycler, for 40 cycles of 5 sec at 94°C, 30 sec at 50°C, and 40 sec at 72°C, followed by a final elongation step of 3 min at 72°C. The PCR reaction mix was as follows: 0.4 mM each of cyt b primers, 0.6 mM each of 16S rRNA primers, 1.25 U Taq polymerase (Applied Biosystems, Foster City, CA), 0.2 mM each dNTPs, 2.5 ml 10× Taq Buffer (Applied Biosystems), in a final volume of 25 ml.

**RT-PCR**

For angiogenesis analysis the total RNA in the sample was extracted using RNA Trizol according to the manufacturer’s protocol (Invitrogen) and the concentration was measured (Nanodrop, Wilmington, DE) at 260 nm. One microgram of total RNA from each lung sample was reverse transcribed into cDNA and real-time PCR-amplified with VEGF and GAPDH primers by the SYBR Green I method (Applied Biosystems). Reactions (12.5 µl 2× SYBR Master Mix, 300 nM primers and template in a total volume of 25 µl) underwent denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min. Plasmids containing VEGF and GAPDH fragments were used as templates for standard curves. VEGF mRNA levels for each sample were normalized using GAPDH as an internal control. The sequence of primers used was as follows: VEGF (for- 5'-ATGACGAGGGCCTTGAGTGTG-3'; rev- 5'-CCTATGTGCTGGCCTTGGTGAG-3') and GAPDH (for- 5'-ACACCCACTCCTCACCCTT-3'; rev- 5'-TCCACCACCTCCTGTTGCTGTA-3').

**Cytokines ELISA Immunoassay**

At the experimental endpoint, lungs were harvested and homogenized in 300 µl of phosphate buffer pH 7.4
and centrifuged at 15,000 g for 10 min at 4°C with protease inhibitor, and were finally centrifuged to remove debris and supernatant before storage at −80°C. Total protein content was measured in the supernatant by Bradford’s method.29 (Bio-Rad Laboratories, Hercules, CA). The supernatant was then diluted to a final volume of 500 μL with 0.9% NaCl saline containing 4% BSA (Sigma–Aldrich, St Louis, MO).

Concentration of rat IL-6, IL-1β, IFN-γ, and TGF-β1 in lung homogenates was measured using ELISA assay kits according to the manufacturer’s instructions (Bender Medsystems, Vienna, Austria). Assay standard concentration ranges were 31–2,000 pg/mL.

Data Analysis and Statistics

Data are given as means ± SE. Box plot was used to show the median, the interquartile ranges and minimum/maximum values on average of the body weights. Scatter-plot graphics were made for morphometric data distribution. The ANOVA test and the Neuman–Keuls multiple comparison test were used for assessment of differences among groups. A P-value <0.05 was considered statistically significant (GraphPad Prism 5.04 Software San Diego, CA).

RESULTS

All the 50 newborn rats randomly distributed between the four experimental groups (Fig. 1A) survived both during oxygen exposure and after cell administration procedures. They all gained weight throughout the P42 after birth with no differences among the groups (Fig. 1B).

hAFS Cells Colonize Damaged Lungs

Human AFS cells used in this study were characterized by the stable presence of stromal cell markers as CD90 (Thy-1), CD105 (endoglin, TGFbeta receptor), and CD73, highly expressed (90%) along the entire period of culture. More than 30% of the AFS cells at the time of injection were also Stem cell Embryonic Antigen-4 (SSEA-4) positive and they also expressed HLA-ABC while were negative for HLA-DR confirming their low immunogenicity profile (Fig. 2A). hAFS cells used in the study showed, after appropriate culture conditions, to be able to undergo adipogenic and osteogenic differentiations (Fig. 2A).

Immunofluorescence revealed retention of human cells positive for anti-hMIT in all lung sections of animals treated with hAFS cells at P42. Remarkably, cells co-expressing hMIT and hSFTPC were found only in the lungs of animals treated with hAFS cells. At the confocality they appeared in close relationship with secondary crests inside alveoli (Fig. 2B). The percentage

Fig. 2. A: hAFS cells pluripotence marker expression and examples of flow cytometry profile of the injected stem cells; hAFS cells used in the study showed, after appropriate culture conditions, to be able to undergo adipogenic and osteogenic differentiations. B: (a) Retained hAFS cells appeared in rat lung sections stained with anti-human mitochondria (green). Scale bar = 15.9 μm, magnification 40×. B: (b) Lung sections co-labeled (yellow) with anti-human surfactant C-protein (red), and anti-human mitochondria (green), is shown in two representative confocal images identifying the localization of hAFS cells in treated animals. Scale bar = 15.9 μm, magnification 60× (top); scale bar = 23.8 μm, magnification 60× (bottom). B: (c) Polyacrylamide-gel electrophoresis of a duplex PCR product amplified from mitochondrial DNA. The hyperoxia group not treated with human cells, lacks 16S rRNA.

Pediatric Pulmonology
of hAFS cells retained in the lungs accounted for an average of 1.43 ± 0.2% (range: 1.07–3.21%) of the lung alveolar cells (Fig. 2B, b). In hAFS cells and hF-treated rats the presence of two bands (157 and 359 bp) indicates the presence of human cells, whereas a single band (359 bp) in hyperoxia rats indicates a non-human origin of the samples (Fig. 2B, c).

**hAFS Cells Ameliorate Lung Histopathology**

hAFS cells treated animals highlighted a histological pattern with a marked improvement of homogenous alveoli, with the consequent recovery of alveolar growth, in comparison with the untreated hyperoxia group. There was no evidence of inflammatory cells, but the septa were thicker in the untreated and specially in hF-treated hyperoxia groups, with respect to the room air and hAFS cell groups. In contrast hF-treated animals showed no improved alveolar growth and persistent areas of fibrosis (Fig. 3A).

In the morphometric analyses, on average, the alveolar size (μm²) was lower in the room air group (1,049 ± 60.89), when compared to the other groups (1,398 ± 101.8 in hyperoxia, and 2,199 ± 237.6 in hF groups); hAFS cells (1,264 ± 160.4), showed intermediate values between room air and hyperoxia, though lower than hF, P < 0.0001; the value of mean linear intercept (μm) was lower in the room air group (71.64 ± 1.74), when compared to hyperoxia only as well as hF groups (79.69 ± 1.84 and 94.25 ± 2.46, respectively), hAFS-cells treated rats showed intermediate values between room air and hyperoxia (78.97 ± 1.51), though lower than hF group, P < 0.0001.

Similarly, the secondary crests count (n/hpf) was higher in room air and hAFS-cell-treated groups (40.36 ± 2.56 and 31.2 ± 2.36, respectively), rather than hyperoxia and hF-treated groups (27.75 ± 1.84 and 5.6 ± 2.01, respectively), P < 0.0001 (Fig. 3B).

**hAFS Cells Sustain Rearrangement of Capillary Architecture and Exert an Immunomodulatory Response**

Anti-CD31 immunohistochemistry showed a more homogeneous distribution of the capillary network in the normoxia-exposed and hAFS cells treated animals with respect to the other hyperoxic groups. In particular, in normoxia there were numerous small capillaries, with narrow lumen and very thin walls, located in the alveolar walls. In untreated hyperoxia and hF-treated groups the alveolar walls showed some thickenings, where clear capillary structures were more rare and difficult to identify. In the hAFS group, the capillaries of the alveolar walls were more clearly appreciable (Fig. 4B). Morphometric analysis showed a decreased MVD in the untreated hyperoxia (83.72 ± 7.57/hpf) and hF-treated group (67.10 ± 4.55/hpf) in comparison to the room air group (146.7 ± 6.95/hpf) and hAFS group (104.1 ± 6.44/hpf) (P < 0.0001) (Fig. 4A).

Interestingly, VEGF gene expression in the lungs of room air rats (121.5 ± 31.43) and exposed to hyperoxia, but treated with hAFS cells (90.77 ± 18.44) was higher than in untreated hyperoxia (16.77 ± 4.24) and hF-treated (31.49 ± 4.18) rats (P < 0.0004; Fig. 4A). The concentration of tissue IL-6, IL-1β, IF-γ, and TGF-1 decreased at a value close to the room air group level in the hAFS cell group, when compared to the untreated hyperoxia and hF groups, P-values are from 0.01 to 0.038 (Table 1).

**DISCUSSION**

The role of stem cell therapy in animal models of BPD has been studied mainly during the neonatal period. Unfortunately limited data are available on the medium and long term follow-up in a context of moderate hyperoxia and stem cell administration. Therefore, our model has been developed to focus on the chronic lung disease rather than the acute injury. Cell administration was performed at P21, after 2 weeks of moderate hyperoxia and monitored up to P42. This design mimics the late phase of the BPD in a more advanced phase of the disease, which beginning in infancy extends to childhood and adulthood. For the in vivo procedure the optimal number of stem cells proposed and the proper time for instillation were set up as previously investigated at the beginning of the weaning phase (P21; data not shown). Like in postnatal surfactant instillation in neonates, hAFS cells were injected intratracheally, this being shown to be the most efficient way for cell delivery and recruitment.

Rats exposed to moderate hyperoxia for 14 days, showed a variable response to injury in several lung regions. At P42 the lung histopathology was rather well characterized by an arrested alveolarization and dysplastic vascular architecture, similar to the patterns reported in human BPD. Impaired alveolar growth and heterogeneous emphysematous areas were also confirmed in our histopathology panels.

In this study we have described the effects of stem cell administration on lung mean linear intercept, alveolar size, number of secondary crests, pulmonary content of VEGF, and the cytokines profile. In terms of remodeling, hAFS cells triggered an interesting pulmonary microvascular development that is essential for an efficient gas exchange. This was also supported by the alveolar number and size more similar to the lungs of the room air group, with a marked improvement of homogenous alveoli and a consequent recovery of alveolar growth. We detected hAFS cells around the terminal bronchiolar epithelium, in the interstitial spaces and in
the alveolar walls, indicating a selective lung injury tropism. In our study, alveolar type II cells might be speculated to be differentiated, in the co-stained surfactant C-protein and human mitochondria cells. The anti-surfactant antibody stained the inner alveolar and bronchiolar terminal walls; cubic or cylindrically-shaped cells co-stained with anti-human mitochondria and anti-surfactant antibodies, showed a thick cytoplasm edge towards the alveolar space. As we found no evidence of multinucleate hAFS cells, we support the hypothesis of a cell epithelial differentiation rather than fusion. Differently sections from hF-treated rats did not show enough fluorescence signal to be detect, therefore we were unable to assess their percentage of retention,

Fig. 3. A: Representative lungs from the studied groups: Room air, hyperoxia, hAFS and hF cell treated groups. H&E, scale bar = 100 μm, magnification 20×. Lung histopathology analysis at P42. Representative H&E-stained lung sections show that untreated and hF-treated hyperoxia groups were associated with arrested alveolarization, inducing changes in lung morphology with patchy areas of parenchymal thickening and enlarged air spaces (black arrows). Lung sections of hAFS cell treated rats contained smaller, more numerous alveoli (black arrows) and were comparable with those of the room air group. B: Scatterplots of average alveolar size, mean linear intercept, and secondary crests. At P42 the mean alveolar size (μm²) was significantly smaller in the room air and hAFS cell groups, when compared to the hF-treated hyperoxia group; P < 0.0001; *P < 0.05 room air versus hyperoxia and hF-treated groups, 3P < 0.001 hyperoxia versus hF group. The mean linear intercept (μm) was lower in the room air group than in the untreated and hF-treated hyperoxia groups, though the latter was higher than that of the hAFS cell group; P < 0.0001; *P < 0.05 room air versus the three other groups, 3P < 0.001 hyperoxia versus the hF-treated group. The secondary crests (n/hpf) were higher in the room air group than in the untreated and hF-treated hyperoxia groups, hAFS cell-treated showed an increase in secondary crests regarding the hF-treated group, P < 0.0001; *P < 0.001 room air versus the hyperoxia and hF-treated groups, 3P < 0.001 hyperoxia versus the hF-treated group.
which was assumed to be very low. The molecular analysis performed with PCR on mitochondrial DNA in lung parenchyma of hAFS and hF-treated rats, corresponding to the common human and rat cyt b, and a 16S rRNA fragment, suggest that the human cells are likely to move from the site of injection, from the trachea to the bronchi and hence to the interstitium and alveoli. Although both human cell lines were detectable

![Fig. 4](image)

**Fig. 4. A:** Left: VEGF gene expression rose in hyperoxic animals due to the hAFS cell transplantation, reaching the room air level, \( P = 0.0004; \) \(* P = 0.004\) room air versus hyperoxia and hF-treated groups, \( ^{\ddagger} P = 0.04\) hyperoxia versus hAFS cells-treated groups. Right: representative data columns of the microvessel density from the studied groups confirmed that the hAFS cell-treated group rose in CD31 positive cells to an intermediate value when compared to the room air and untreated or hF-treated hyperoxia groups, \( P < 0.0001; \) \(* P < 0.001\) room air versus the three other groups, \( ^{\ddagger} P < 0.01\) hyperoxia versus hAFS cell-treated groups. **B:** Immunohistochemistry staining for CD31 demonstrates an higher number of capillaries in the alveolar walls of the room air and hAFS cells treated groups in comparison to the hyperoxia untreated and hF-treated groups. Note the presence in the latter two hyperoxic groups of thickenings of the alveolar walls with very few capillary structures (Scale bar = 100 \( \mu \)m; magnification 20\( \times \)).

**TABLE 1—IL-6, IL-1\( \beta \), IF-\( \gamma \), and TGF-1\( \beta \) Concentrations Were Estimated in the Studied Groups (n = 10 Biopsies in Room Air Group, n = 8 in Untreated, and hF-Treated Hyperoxia, n = 7 in hAFS Cell-Treated Group)**

<table>
<thead>
<tr>
<th>Cytokines (pg/mg of lung tissue)</th>
<th>Room air</th>
<th>Hyperoxia</th>
<th>hAFS</th>
<th>hF</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>261.9 ± 35.75( ^{\ddagger} )</td>
<td>465.3 ± 53.62( ^{\ddagger} )</td>
<td>296.1 ± 62.62</td>
<td>481.3 ± 56.92</td>
</tr>
<tr>
<td>IL-1( \beta )</td>
<td>464.7 ± 58.77( ^{\ddagger} )</td>
<td>803.1 ± 62.52( ^{\ddagger} )</td>
<td>548.4 ± 57.32</td>
<td>819.2 ± 164.0</td>
</tr>
<tr>
<td>IF-( \gamma )</td>
<td>210.3 ± 20.60( ^{\ddagger} )</td>
<td>313.5 ± 31.02</td>
<td>240.4 ± 35.76</td>
<td>362.7 ± 70.42</td>
</tr>
<tr>
<td>TGF-1( \beta )</td>
<td>420.5 ± 59.74( ^{\ddagger} )</td>
<td>644.8 ± 57.72( ^{\ddagger} )</td>
<td>455.3 ± 59.54</td>
<td>673.9 ± 65.65</td>
</tr>
</tbody>
</table>

hAFS cell-administered animals showed intermediate values between untreated or hF-treated hyperoxia groups and room air group.

\( ^{\ddagger} P\)-values from <0.01 to 0.038 room air versus hF-treated groups.

\( ^{\ddagger} \) The same \( P\)-values hyperoxia versus room air groups.
with PCR, a weaker intensity of the amplified band was found in hF-treated samples than hAFS cells. It seems encouraging that the percentage we found of the detected hAFS cells was consistent with that observed in previous works. In fact, the recruitment rate of exogenous stem cells into the lungs remains controversial\cite{52} and is estimated to be from 0.01\% to 0.1\%,\cite{33} though not more than 5\%\cite{12} with the intratracheal route which seems to enhance the retention rate to 5–10\%, when compared to the intravenous route.\cite{12,34} Unfortunately fluorescence techniques, although considered the gold standard, may be a limit and are likely to explain many of the conflicting results.\cite{35} The lack of cell division and proliferation tests could be a limitation of our study and hence this field shall have to be elucidated in further work.

Since a growing number of studies\cite{7,36} have focused on the alveolar vasculature and on factors that may regulate vascular development in BPD, we determined the lung gene expression of VEGF and the content of CD31. In the mature lung, alveolar capillaries are abundant and lie close to the alveolar epithelium, creating a thin air–blood barrier. The mechanisms behind the repair processes may involve a paracrine effect that normalizes VEGF content and improves the alveolar capillary bed architecture. The lower lung VEGF gene expression found after oxygen exposure in untreated animals, confirms that VEGF signaling is disrupted in our model. Since normal VEGF gene expression plays a crucial role in the proper metabolism of the endothelial and alveolar barrier being a pro-angiogenic and endothelial survival factor,\cite{30} it is worth noting that in hAFS cell-treated group the profile we found may reflect a “healthier” state of the lung, more similar to that of the room air controls. Nevertheless some caution needs to be exerted with respect to this interpretation of VEGF content because there is no evidence presented that increased VEGF is coming from the retained hAFS or alveolar type II cells. Finally we are aware that a further limitation of our work is that only a single time point was studied and expression could have been quite different at earlier time points.

It is acknowledged\cite{37,38} that cell therapy contributes with stem cells as producers of growth factors or modifiers of tissues with generalized effect, a fact today known behind the “stemness concept”. We confirmed that c-kit selected hAFC cells differentiate, when conditioned with adipogenic and osteogenic media, as previously reported\cite{25} and express the characteristic surface markers. It is worth noting that thanks to the low expression of HLA-DR these cells may trigger a lower immune-response,\cite{39} which was also confirmed by the absence of inflammatory areas in the recipients. For this reason we then decided to avoid the immunosuppressant drugs, to preclude interference in stem cell activity.\cite{40,41} In addition the low immune-response and the reported absence of tumorigenesis justify in our experimental design the preclusion of a specific control group in healthy animals administrated with hAFS cells.\cite{23,25,42}

We have also looked at the role of apoptosis in lung remodeling since epithelial cell apoptosis may be a key factor involved in alveolar epithelial regeneration and repair.\cite{8} In our study, we found that the apoptotic index changed significantly in hyperoxia exposed rats versus room air group (data not shown). This is quite an interesting point, as already demonstrated,\cite{43,44} because epithelial cell death is followed by remodeling processes, which consist of epithelial and fibroblast activation, cytokine production, activation of coagulation pathway, neoangiogenesis, and re-epithelialization. We therefore investigated four cytokines normally involved in the immune and inflammatory responses\cite{45} and in the experimental lung injury condition caused by moderate hyperoxia. Interestingly, while IL-6, IL-1β, IFN-γ, and TGF-β1, are significantly increased in hyperoxia and hF-treated rats at P42, the administration of hAFS cells seems to decrease them. Angelini et al.\cite{45} has proven the ability of stem cell therapy to counterbalance the negative effect of the pro-inflammatory response 7 days after the experimental lung injury. Nevertheless a decrease in all the cytokines we studied may still not necessarily indicate proper long term anti-inflammatory effects, instead it may indicate a “less injured” state of the lung, which was achieved by the AFS cell treatment. Here we cannot demonstrate if this late effect is secondary to a citoprotective action or a paracrine secretion mechanism. In light of this we consider that the lack of the assessment of anti-inflammatory cytokines’ wide panel, that is, IL-10, IL-17A,\cite{46} or conditioned media,\cite{21} etc. may represent presently an important limitation of our work and therefore deserves further investigation.

Considering the in vivo models of lung injury,\cite{9,17} the paracrine immunomodulation of stem cells and the protective action in the parenchyma and vascular lung injury result crucial for pulmonary disease. Chronic pulmonary disease occurrence, mainly corresponding to the moderate/severe forms of new BPD, was associated with increased pro-inflammatory and pro-fibrotic/angiogenic cytokines, while mild forms of new BPD were characterized only by the increase of pro-fibrotic/angiogenic cytokines, suggesting a different balance of two pathogenic mechanisms in different phases of the disease.\cite{47} Taken together both cytokine pattern and cell retention are encouraging results, although the low percentage of human cell uptake among alveolar cells detected and the specific mechanism of the anti-inflammatory effect needs to be clarified. The authors consider that the effects they found in the lung, are
predominantly related to a paracrine action of the hAFS cells: further investigation will be needed to ascertain the potential interaction between administration of hAFS and their conditioned media on lung. Taken together these encouraging results candidiate the present work to represent a vital first step in demonstrating the impact that this unique cell population may have on cellular based therapies for lung diseases.

In summary, postnatal treatment with hAFS cells in a moderate hyperoxia exposure, significantly reduces lung pathology disease in young rats by improving alveolarization. Injection of hAFS cells might have a reparative potential, improving angiogenesis with consequent organization of capillary networks. Intratracheal delivery of hAFS cells succeeded to achieve some immunomodulatory effects even for a relatively long time. Taken together, this evidence is encouraging from the perspective of new approaches for the treatment of late BPD disease in humans.

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REFERENCES


