Potential effect of amniotic fluid-derived stem cells on hyperoxia-induced pulmonary alveolar injury

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Abstract

Background: With the widespread of Coronavirus Disease 2019 pandemic, in spite of the newly emerging vaccines, mutated strains remain a great obstacle to supportive and preventive measures. Coronavirus 19 survivors continue to face great danger of contacting the disease again. As long as no specific treatment has yet to be approved, a great percentage of patients experience real complications, including among others, lung fibrosis. High oxygen inhalation especially for prolonged periods is per se destructive to the lungs. Nevertheless, oxygen remains the first line support for such patients. In the present study we aimed at investigating the role of amniotic fluid-mesenchymal stem cells in preventing versus treating the hyperoxia-induced lung fibrosis in rats.

Methods: The study was conducted on adult albino rats; 5 pregnant female rats were used as amniotic fluid donors, and 64 male rats were randomly divided into two groups: Control group; where 10 rats were kept in normal atmospheric air then sacrificed after 2 months, and hyperoxia-induced lung fibrosis group, where 54 rats were exposed to hyperoxia (100% oxygen for 6 h/day) in air-tight glass chambers for 1 month, then randomly divided into the following 5 subgroups: Hyperoxia group, cell-free media-treated group, stem cells-prophylactic group, stem cells-treated group and untreated group. Isolation, culture and proliferation of stem cells were done till passage 3. Pulmonary function tests, histological examination of lung tissue under light and electron microscopes, biochemical assessment of oxidative stress, IL-6 and Rho-A levels, and statistical analysis of data were performed. F-test (ANOVA) was used for normally distributed quantitative variables, to compare between more than two groups, and Post Hoc test (Tukey) for pairwise comparisons.

Results: Labelled amniotic fluid-mesenchymal stem cells homed to lung tissue. Stem cells administration in the stem cells-prophylactic group succeeded to maintain pulmonary functions near the normal values with no significant difference between their values and those of the control group. Moreover, histological examination of lung tissues showed that stem cells-prophylactic group were completely protected while stem cells-treated group still showed various degrees of tissue injury, namely; thickened interalveolar septa, atelectasis and interstitial pneumonia. Biochemical studies after stem cells injection also showed decreased levels of RhoA and IL-6 in the prophylactic group and to a lesser extent in the treated group, in addition to increased total antioxidant capacity and decreased malondialdehyde in the stem cells-injected groups.
Background
In the worldwide Coronavirus Disease 2019 (COVID-19) pandemic, supportive therapies including respiratory care for affected patients, especially in more severe cases, are employed [1]. Before the emergence of vaccination, hospitalized patients with COVID-19 developed worsening pneumonia and acute respiratory distress syndrome (ARDS) with high rates of mortality. Patients with severe disease often need oxygenation support with high-flow oxygen (O2) and non-invasive positive pressure ventilation, but the safety of these measures is uncertain. High concentrations of O2 may be needed for prolonged periods that might exceed 2 weeks [2]. Prolonged sub-lethal O2 exposures probably end up with lung fibrosis [3]. Patients worldwide who have survived the pandemic continue to battle various symptoms of the illness, known as post-COVID syndrome, which may vary from mild fatigue and body aches to severe forms requiring long term O2 therapy and lung transplantation due to lung fibrosis [4].

O2 also remains the first-line therapy used for many other acute and chronic respiratory diseases for hypoxic patients. However, target goals for normoxemia are not well defined. Therefore, iatrogenic hyperoxia is a very common situation [5]. Supra-physiological levels of O2 are toxic to the tissues due to exacerbated reactive oxygen species (ROS) production and impairing the homeostasis of multiple cellular processes. Cell injury caused by high O2 levels activates inflammatory cascades, mononuclear cellular infiltration, this is in addition to the oedema that amplifies the tissue damage. The lung is the first—but not the only- organ affected by hyperoxia [6]. The alveolar epithelial and alveolar capillary endothelial cells are vulnerable targets for ROS injury caused by it [7]. The acute lung injury following hyperoxia was thoroughly investigated [5], yet the chronic sequelae still need to be discovered.

Amniotic fluid (AF) is an attractive source of mesenchymal stem cells (MSCs) for therapeutic applications. It can be safely collected during or at the end of gestation, with no ethical concerns and low risk of tumorigenicity [8]. AF-MSCs exhibit low immunogenicity and high anti-inflammatory properties. They are able to self-renew, highly proliferative, and have a broad differentiation potential, making them amenable for cell-based therapies [9].

Material and methods
Aim of the study
In this study, we aim to investigate the chronic sequelae of hyperoxia on lung alveolar structure and respiratory functions. Additionally, exploring the possible protective and therapeutic role of AF-MSCs on hyperoxia-induced pulmonary alveolar injury.

Experimental animals
The study was conducted on Sprague–Dawley adult albino rats; 5 pregnant female rats were used as amniotic fluid donors, and Sixty-four male rats weighing 150–200 g, 6–8 weeks of age were used for the study. Rats were purchased from the Medical Physiology department experimental animal facility, Alexandria Faculty of Medicine, where they were housed under a 12–12 h light–dark cycle at 25 °C with food and water provided ad libitum. All experiments were conducted in accordance with the approved guidelines set by the Research Ethics Committee of Alexandria Faculty of Medicine, Egypt (IRB No: 00012098- FWA No: 00018699). Membership through Alexandria University in International Council of Laboratory Animal Science organization ICLAS http://iclasm.org/).

Isolation, culture and proliferative capacity of AF-MSCs
The whole procedure of isolation and characterization was performed at the Center of Excellence for Research in Regenerative Medicine and its Applications (CERM-A), Alexandria Faculty of Medicine.

On day 14 of pregnancy, the female rats were euthanized by an overdose of the inhalation anesthesia. The uterus was dissected, and each gestational sac was opened from the anti-mesenteric side to avoid bleeding from the placental site. Needles of 30-gauge calibration were used to aspirate AF from each sac separately [10]. The AF was then spun at 1400 × g for 5 min to pellet the cells. Pelleted cells were cultured in 60 mm culture dish where 5 ml of low glucose Dulbecco’s Minimum Essential Medium (DMEM, Lonza) supplemented with 10% foetal bovine serum (FBS, Sigma Aldrich) and 1% penicillin/streptomycin (Lonza) were added. Cells were incubated at 37°C and 5% CO2 in a humidified incubator and observed daily under a phase contrast inverted microscope. The media was changed every 2–3 days. Cells were passaged after reaching 80% confluence using 0.25%
(w/v) trypsin/ethylene diamine tetra acetic acid (EDTA) (Lonza), then cultured in T75 cm² flasks [11]. Cell viability was assessed using the trypan blue (Gibco) exclusion test, and cells were counted using a Neubauer hemocytometer [12, 13].

Characterization of AF-MSCs
Immunophenotyping of AF-MSCs using flow cytometer
Cells were characterized using fluorescent-labelled monoclonal antibodies (mAb) for CD45, CD90, and OCT4 markers. Cells at passages 3–5 were trypsinized with 0.25% trypsin–EDTA solution, washed with phosphate-buffered saline (PBS), counted and incubated at room temperature for 30 min in the dark, with monoclonal phycoerythrin (PE)-conjugated antibody for CD45 (Abcam, ab23396, UK), monoclonal fluorescein isothiocyanate (FITC)-conjugated antibody for CD90 (Anti-Thy1.1) (Abcam, ab225, UK), and the monoclonal FITC-conjugated antibody for OCT4 antibody (Abcam, ab181557, UK). Immunofluorescence on the viable cells was analyzed using Becton Dickinson, FACS caliber flow cytometer equipped with Cell Quest software [12, 14].

Colony-forming unit assay
The colony-forming potential of the cultured cells at passage 3 (P3) was tested. In this assay, 500 cells were plated on a 35 mm culture dish in complete media and incubated for 14 days. The cells were then fixed and stained using Crystal Violet (Sigma-Aldrich, USA) at 3% (w/v) in methanol for 5 min at room temperature. The stain was removed, and cells were washed with distilled water. The number of colonies was counted and the colony-forming potential was calculated as follows: Plating efficiency = the number of colonies formed/number of cells plated × 100. All visible colonies were counted; the number of colonies displaying five or more cells was scored under the phase-contrast inverted microscope. A CFU potential of over 40% was considered to be optimal for AF-MSCs culture [15].

Differentiation of AF-MSCs into adipocytes, osteocytes and chondrocytes
Osteogenic differentiation; Cells were plated in an osteogenic differentiation medium containing L-DMEM, 10% FBS, 0.1 μM dexamethasone (Sigma-Aldrich), 200 μM L-ascorbic acid-2-phosphate (Sigma-Aldrich) and 10 mM β-glycerol phosphate (Sigma-Aldrich) for 21 days and induction was confirmed by Alizarin Red S staining.

Adipogenic differentiation; cells were plated in an adipogenic differentiation media containing 1 μM dexamethasone, 58 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 200 μM indomethacin, for 2 weeks then cells were treated with insulin medium (Differentiation medium, containing 10 μg/ml insulin). Medium was changed with fresh insulin medium every 2 days for one week and induction was confirmed by Oil Red O staining.

Chondrogenic differentiation was done using StemX-VivoVR Chondrogenic Base Media (R&D Systems, USA) and StemXVivo Chondrogenic Supplement (R&D Systems, USA). 2.5 × 10⁵ P3 MSCs were re-suspended in chondrogenic-differentiation media then centrifuged at 200 × g for 5 min in a conical falcon tube. The tube’s cap was loosened to allow gas exchange and was incubated upright at 37 °C and 5% CO₂. Every 3 days, the media was changed, and after 28 days, the chondrogenic spheroidal pellet was harvested and stained for assessing the cartilage-specific proteoglycan using Alcian blue 8GX stain [16].

AF-MSCs labelling and tracking
For tracking the injected cells, the cytoplasm/cell membrane of cells prior injection were labelled with a fluorescent probe (chloromethylbenzamido octadecyl indocarbocyanines) (CM-Dil) (molecular probes, Thermo Fisher USA) [17]. Cell Tracker CM-Dil was supplied as 50 μg/vial to be reconstructed in 50 μl dimethyl sulfoxide (DMSO) in a concentration of 1 μg/μl as a stock solution. After the manufacturer protocol; 7 μl of Dil were used to label 1 × 10⁶ cells in 7 ml PBS and incubated in 37 °C for 7 min. After labelling, cells were washed with PBS and re-suspended in fresh medium for IV injection in rats. The rats were then sacrificed 72 h after injection for visualizing the labelled cells in lung tissue under confocal microscopy (Leica microsystems, DMi8, Germany) [18].

Establishment of hyperoxia
Rats were housed in airtight glass chambers 66Lx47Wx43H and exposed to chronic intermittent hyperoxia (100% O₂ concentration 6 h/day for 30 days) with O₂ flow rates of 10 L/min and pressure = 1 bar.

Experimental design
Sixty-four male rats were randomly divided into the following groups:

Control group (CG), where 10 rats were kept in normal atmospheric air O₂ then sacrificed after 2 months. Hyperoxia-induced lung fibrosis group, where fifty-four rats were exposed to the chronic intermittent hyperoxia, then randomly divided into the following subgroups:

- Hyperoxia group (HG); 10 rats were sacrificed after 1 month of hyperoxia exposure for the assessment of hyperoxia-induced lung fibrosis model.
• Cell-free media-treated group (CFM-TG); 10 rats received I.V. injection of 1 ml cell-free complete media after induction, then sacrificed after another month.

• Stem cells-prophylactic group (SC-PG); 12 rats received I.V. injection of AF-MSCs (1 × 10^6 cells in 1 ml complete media) [19], 48 h after exposure to hyperoxia then the exposure was continued for the rest of the month. 10 rats were then sacrificed after another month for assessment, while 2 rats were sacrificed 72 h after injection with labelled stem cells for assessing stem cells homing.

• Stem cells-treated group (SC-TG); 12 rats received I.V. injection of AF-MSCs (1 × 10^6 in 1 ml complete media). 10 rats were sacrificed after another month for assessment, while 2 rats were sacrificed 72 h after injection with labelled stem cells for assessing stem cells homing.

• Untreated group (Unt-G); 10 rats left untreated for another month after induction of fibrosis, then sacrificed for assessment.

Pulmonary functions
Tidal volume (VT), minute respiratory volume (MRV) and forced vital capacity (FVC) were assessed using a PowerLab digital data acquisition system at the beginning and the end of the study. The ventilatory parameters were recorded using a pneumotachometer MLT11L (Lab chart 8, AD Instruments, Castle Hill, NSW, Australia) with P1 channel end connected to the outlet of the NP/Whole Body Plethysmography [20].

Lung tissue histological processing
At the end of the experiment, all rats were sacrificed and both lungs were dissected. The left lung was kept frozen at −80 °C for later biochemical study. While the lower right lobe of each right lung was divided into two halves; one half was fixed in 10% neutral-buffered formalin, then processed to obtain (5–6 µm) thin sections [23] for transmission electron microscope (TEM) examination (Jeol 1400 plus Tokyo, Japan).

Biochemical assessment
Assessment of Ras homolog family member A (RhoA) and interleukin-6 (IL-6) expression in lung tissue using western blot [24]
Western blot was used to assess RhoA and IL-6 expression in lung tissue. Lung tissues were homogenized and the tissue lysate was prepared by adding radio-immunoprecipitation cell lysis (RIPA) buffer (Cat. No. AR0105, Bosterbio), Tris (PH 8.0) and protease inhibitor (Cat. No. AR1182, Bosterbio). The lysates were assayed for total protein concentration by Lowry method [25], and stored until analyzed. After protein electrophoresis, protein transfer from sodium dodecyl sulphate polyacrylamide gel onto nitrocellulose membrane was performed by electro blotting. Following transfer, bands were detected using Polyclonal Anti-RhoA Antibody (Cat. No. YPA2321, Biopsies Co), Anti IL-6 Polyclonal Antibody (Cat. No. YPA1248, Biopsies Co.) and AntiBeta Actin (Cat. No. BPA1012, Biopsies Co). Next, the membranes were incubated with the rabbit IgG DAB Chromogenic Reagent Kit (Cat. No. SA2020, Bosterbio). Protein relative band densities ratio were assessed using NIH Fiji program (NIH, Bethesda, MD, USA) and protein expression was normalized against β-actin.

Measurements of biomarkers of oxidative stress
The lung tissues were homogenized in 1–2 ml of 5 mM cold potassium phosphate buffer (pH 7.4), centrifuged at 4000 rpm for 15 min at 4 °C and the supernatant was removed and stored at −80 °C for assay of total antioxidant capacity (TAC) (Cat. No. TA 25 13, Biodiagnostics) and malondialdehyde (MDA) (Cat. No. MD 25 29, Biodiagnostics).

Measurement of TAC
The determination of the antioxidant capacity is performed by the reaction of antioxidants in the sample with a defined amount of exogenously provided hydrogen peroxide (H₂O₂). The antioxidants in the sample eliminate a certain amount of the provided hydrogen peroxide (H₂O₂). The residual H₂O₂ is determined calorimetrically at 510 nm by an enzymatic reaction which evolves the conversion of 3,5, dichloer-2-hydroxybenzenesulphonate to a colored product [26].

Measurement of MDA
Lipid peroxidation is determined as thiobarbituric acid which reacts with MDA in acidic medium to form thiobarbituric reactive product measured calorimetrically at 534 nm [27].
Statistical analysis of data
Data was fed to the computer and analysed using IBM SPSS software package version 20.0 (Armonk, NY: IBM Corp). Qualitative data were described using number and percent. The Kolmogorov–Smirnov test was used to verify the normality of distribution. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. Significance of the obtained results was judged at the 5% level. F-test (ANOVA) was used for normally distributed quantitative variables, to compare between more than two groups, and Post Hoc test (Tukey) for pairwise comparisons [28].

Results
Characterization of AF-MSCs
Morphological characterization
The cell cultures were monitored daily using phase-contrast inverted light microscope. In primary culture (P0), cells were small and rounded, then became spindle in shape after 72 h of culture, displaying a heterogeneous population and reached 70–80% confluence in approximately 10–12 days. With passaging, cell growth was accelerated and cell morphology changed, exhibiting large, flattened and spindle-shaped cells. Mitotic rounded cells appeared, demonstrating proliferation. At P3 the culture represented a homogenous fibroblast-like cell monolayer (Fig. 1A, B).

Colony forming unit-fibroblast (CFU-F) assays
After 5–7 days of incubation, cells gradually proliferated into small colonies. Two weeks post-seeding, large colonies were seen with crystal violet staining (Fig. 1C). The colony-forming assay showed that each dish with 100 cells gave 93% ± 1.35 of colonies after 14 days.

Immunophenotyping of AF-MSCs by flow cytometry
FACS analysis for AF-MSCs at P3 showed that 95.09% of the cultured cells expressed the mesenchymal multipotent CD90 surface marker. 97.49% of cells expressed the pluripotent surface marker OCT4, while they were almost negative for the CD45 hematopoietic marker shown only in 0.9% of cells (Fig. 1G).

Differentiation of AF-MSCS
AF-MSCs were successfully differentiated into adipocytes, osteocytes and chondrocytes-like cells noted with the characteristic positive staining of fat droplets with Oil red stain (Fig. 1D), Ca\[^{2+}\] deposits with Alizarin stain (Fig. 1E) and collagen-specific proteoglycans with Alcian blue (Fig. 1F), respectively.

Homing of AF-MSCS into lung tissue
Labelled AF-MSCs were tracked under the laser scanning confocal microscope in lung tissue 72 h after injection. Red fluorescent cells were seen in the lung tissue (Fig. 2A–C).

Pulmonary function assessment
The HG showed a significant decrease in MRV, FEV1 and FVC by 50%, 30% and 40% and an increase in RR and FEV1/FVC by 10% & 13% compared to the CG (p < 0.001) demonstrating that the induced model had affected the pulmonary functions. The CFM-TG and Unt-G showed no significant improvement in pulmonary function tests as compared to HG and showed a 46% and 50% decrease in the MRV, 30% in FEV1 and 40% and 36% in FVC compared to the CG respectively. An increase in RR by 11% and FEV1/FVC by 12% in these two groups (CFM-TG and Unt-G) as compared to CG (p < 0.001) further proved that pulmonary functions remained deteriorated with no significant improvement whether spontaneously Unt-G or when treated with CFM. However, stem cells administration in the SC-PG succeeded to maintain MRV, FEV1, FVC, FEV1/FVC as well as RR near the normal values with no significant difference between their values and those of the CG. Moreover, treatment with stem cells after one month of O\(_2\) therapy in the treated group increased the MRV, FEV1 as well as FVC by 50%, 14%, and 33% in comparison to the HG, and decreased RR by 3% yet this improvement did not reach normal levels as there was significant difference between their values and those of the CG (p < 0.001) (Fig. 2).

Histological assessment
Haematoxylin and eosin (H&E) stain
Light micrographs of the CG revealed normal lung architecture, patent alveoli and thin interalveolar septa. The alveoli were lined with flat squamous type I pneumocytes and bulging cuboidal type II (Fig. 3A, B). HG showed atelectasis, thickened interalveolar septa with interstitial pneumonia, congested blood vessels and red blood corpuscles extravasation in the alveolar lumen and the septa. Few type I pneumocytes were seen. On the other hand, many type II pneumocytes with their rounded nuclei were further depicted (Fig. 3C, D). The CFM-TG showed microscopic changes similar to the HG, with collapsed alveoli, thick septa with multiple interstitial cells having vacuolated cytoplasm. Most nuclei lining the alveoli were rounded together with perivascular cellular infiltration (Fig. 3E, F). The SC-PG showed almost normal appearance of the lung.
architecture with thin interalveolar septa and patent alveoli. Flat squamous type I pneumocytes and bulging eosinophilic type II were also seen (Fig. 3G, H). The SC-TG revealed mostly patent alveoli. The interalveolar septa were mildly thickened and some interstitial cells showed vacuolation (Fig. 3I, J). The Unt-G depicted an appearance similar to the HG with atelectasis and thickened interalveolar septa containing vacuolated interstitial cells. The alveoli were mostly lined with deeply-stained nuclei (Fig. 3K, L).
Masson's trichrome stain
Photomicrographs of the CG showed normal collagen distribution in the interalveolar septa, perivascular and peribronchial. (Fig. 4A). On the other hand, HG showed excessive deposition of collagen (Fig. 4B). Similarly, CFM-TG showed an apparent increase in stained areas of collagen deposition within the interalveolar septa (IAS) (Fig. 4C). On the other hand, SC-PG revealed limited and focal distribution of collagen stained areas within the IAS (Fig. 4D). Likewise, SC-TG showed limited stained areas within the IAS and perivascular as compared to CG (Fig. 4E). Contrarily, the Unt-G depicted moderate to severe stained areas within the IAS, indistinguishable from the HG.

Morphometric analysis
Morphometric analysis of trichrome-stained sections The Morphometric analysis of trichrome-stained sections was performed by calculating the percentage area of greenish blue-stained collagen. There was a significant increase in the percentage area of collagen deposition in HG and CFM-TG ($p < 0.001$). On the other hand, SC-PG
and SC-TG showed a significant decrease in the area percentage of collagen deposition in comparison to HG, reaching the control levels (p < 0.001). Moreover, Unt-G showed statistically significant increase in area percentage of collagen deposition in comparison to CG, SC-PG and SC-TG, with no significant difference to that seen in HG (Fig. 4G).

**Morphometric analysis of the alveolar surface area**  
To assess the degree of alveolar collapse, the alveolar surface area was measured morphometrically. There was a significant decrease in surface area of alveoli in HG and CFM-TG versus the CG (p < 0.001). On the other hand, SC-PG and SC-TG showed a significant increase in the surface area of the alveoli in comparison to HG. These results were almost the same shown in CG (Fig. 4H).

**Morphometric analysis of interalveolar septal thickness**  
The interalveolar septal thickness was similarly measured to evaluate the degree of interruption of blood-air barrier. SC-PG and SC-TG showed no significant difference in inter-alveolar septal thickness in comparison with CG. Yet, there was a significant decrease of septal thickness in comparison to HG (p < 0.001) (Fig. 4I).

**Electron microscopic results**  
Electron micrographs of CG lungs showed thin inter-alveolar septum (IAS) with few interstitial cells (ISCs)
Type I pneumocytes had flattened euchromatic nuclei (Fig. 5B). Type II pneumocytes showed central euchromatic nucleus, prominent microvillous border, multiple lamellar bodies, and mitochondria (Fig. 5A, C). Intercellular junctions were observed between the two cell types (Fig. 5C). Blood-air barrier (BAB) appeared thin (Fig. 5A, C).

On the other hand, the HG depicted variable degenerative changes in the lung parenchyma. The IAS was obviously thickened with multiple ISCs having heterochromatic nuclei (Fig. 5D–F) and fibroblasts with collagen (Fig. 5D–F) and elastic fibers deposition (Fig. 5E). In addition to obvious interstitial oedema (Fig. 5E, G). Type I pneumocytes appeared having irregular heterochromatic nuclei and electron-dense cytoplasm (Fig. 5E, G). Type II pneumocytes had blunted microvilli, vacuolated cytoplasm, and abnormal, sometimes empty lamellar bodies (Fig. 5D, E, G). Many type II pneumocytes were seen extruded into the alveolar lumina (Fig. 5D). Apoptotic bodies and peripheral nuclear chromatin clumps were often seen (Fig. 5G).
Electron micrographs of CFM-TG revealed a picture similar to that of the HG. The IAS was also thickened with multiple ISCs showing irregular heterochromatic nuclei (Fig. 5H, I) and collagen deposition (Fig. 5H, J, K). Degenerating type I pneumocytes with irregular surface (Fig. 5I), numerous type II pneumocytes having irregular heterochromatic nuclei (Fig. 5I, K). Some had dilated perinuclear cisternae (Fig. 5K). Many of them contained empty small lamellar bodies (Fig. 5I, L) or large vacuoles (Fig. 5I). Extraembryonic RBCs in the alveolar lumina (Fig. 5L) that were mostly collapsed (Fig. 5I, L).

Electron micrograph of SC-PG showed marked preservation of alveolar ultrastructure with patent alveoli, thin BAB and intact intercellular junctions (Fig. 6A). Type I pneumocyte had large euchromatic nuclei and few rough endoplasmic reticulum cisternae (Fig. 6B). Large bulging type II pneumocytes showed their characteristic microvilli and typical lamellar bodies. Intercellular junctions were kept intact.

Electron micrograph of SC-TG showed marked improvement of alveolar ultrastructure. Thin interalveolar septum containing few collagen, and patent alveoli were the prominent features (Fig. 6D). Thin BAB can be seen (Fig. 6D, E). Type I pneumocytes showed flattened euchromatic nuclei. However, some showed large slightly bulging nuclei (Fig. 6E, F respectively). Moreover, dilated rough endoplasmic reticulum cisternae were depicted in few type I pneumocytes (Fig. 5E). Type II pneumocytes showed their distinctive microvillus border, and numerous well-formed lamellar bodies (Fig. 6G, H). However, some type II pneumocytes showed small nuclei with peripheral chromatin clumps (Fig. 6H) and dilated perinuclear cisternae (Fig. 6G). Moderate amounts of collagen could be depicted in the IAS (Fig. 6G).

Electron micrograph of Unt-G revealed thickened interalveolar septa with many interstitial cells having irregular small nuclei with peripheral chromatin clumps (Fig. 6I, J), and dilated perinuclear cisternae (Fig. 6I). Interstitial oedema was obvious (Fig. 6I, J). Alveolar and interstitial macrophages were also depicted with their numerous lysosomes and pseudopodia (Fig. 6I). Type I pneumocytes with irregular heterochromatic nuclei and type II pneumocytes with fused lamellar bodies were also seen. (Fig. 6I). Some type II pneumocytes appeared with electron-dense cytoplasm, folded irregular heterochromatic nuclei and few almost empty lamellar bodies (Fig. 6K, L). Some showed dilated perinuclear cisternae (Fig. 6I, K), many cytoplasmic vacuolations (Fig. 6I) and still others were extruded into the alveolar lamina. (Fig. 6K).

**Biochemical assessment**

*Western blot results for RhoA and IL-6 expression in lung tissue*

RhoA was significantly increased in HG and CFM-TG compared to the CG while it was significantly decreased in the SC-PG and SC-TG compared to HG and CFM-TG ($p < 0.001$). Its value in Unt-G was significantly increased compared to all studied groups. IL-6 was significantly increased in HG, CMF-TG and Unt-G compared to the CG, while it was significantly decreased in the SC-PG and SC-TG compared to HG and CFM-TG ($p < 0.001$) (Fig. 7A–C). Full length blots are included in the Additional file 1 Fig. S1.
**Oxidative state assessment**

The study showed that MDA was significantly increased in HG and CMF-TG compared to the CG, while it was significantly decreased in the SC-PG compared to HG and CMF-TG ($p<0.001$). On the other side, TAC was significantly decreased in HG and CFM-TG groups than in the CG, while it was significantly increased in SC-PG, SC-TG and Unt-G compared to HG and CFM-TG ($p<0.001$). Moreover, it showed no significant difference in CG compared to SC-PG, thus reaching normal levels (Fig. 7D, E).
Discussion
Although O2 therapy is the first line of treatment for many hypoxaemia-related diseases, its elaborate and uncontrolled administration can do more harm than good to the patient’s condition. In the present study, we implemented our experimental design to induce lung fibrosis upon the already proved model of hyperoxic lung injury. The effects of hyperoxia are assumed to be time and dose dependent [29]. So, unlike most earlier studies, which used continuous short-term hyperoxia [30, 31], this study used intermittent prolonged hyperoxia for the induction of lung fibrosis. The study tracked the structural, physiological and biochemical long-term sequelae of this model of induced lung fibrosis. In this experiment, hyperoxia was coupled with a surge in the oxidative stress, is another cause of increased IAS thickness. This in turn leads to loss of pulmonary barrier functions represented by a decrease in MRV, FEV1 and FVC and an increase in RR and FEV1/FVC compared to the CG, SC-PG and SC-TG. Mach et al. demonstrated that the first structure appeared to be affected by hyperoxia is the vascular endothelial cell [21], and probably this was the cause of capillary congestion and massive RBCs extravasation into the alveoli as well as in the IAS that were commonly encountered in our histological sections in both HG and CFM-TG. Recruitment of immune cells to the lung following oxidative stress, is another cause of increased IAS thickness. This in turn leads to loss of pulmonary barrier function.

With the induction of this oxidative burst, mainly by activated inflammatory cells, the secretion of proinflammatory chemokines and cytokines by resident macrophages and epithelial cells is followed [33]. So in the present study, the hyperoxia-induced lung injury was accompanied by pulmonary inflammatory responses as evidenced by increased IL-6. Strong correlation may exist between IL-6 expression and pulmonary injury/inflammation as it poses a strong proinflammatory effect [34]. These findings were further confirmed with the histological picture of the lung in these groups. Where the light micrographs of CFM-TG were indifferent from the HG; both showed alveolar and vascular congestion, diffuse alveolar septa with alveolar damage and collapse, intense cellular infiltration denoting interstitial pneumonia. Thickened interalveolar septa with deposition of interstitial collagen fibers were the worst of all changes. So with the large amounts of ROS generated from mitochondrial electron transport or NADPH oxidase-catalyzed reactions during hyperoxia [3], damaged mitochondrial membranes become more permeable for pro-apoptotic components which then pass to the cytoplasm and contribute to the excess ROS. Subsequently, interstitial pulmonary oedema and impaired gas exchange by means of alveolar collapse and disintegration of the alveolar-capillary barrier occur [35]. This was obviously reflected on the rats’ pulmonary functions and thus their mortality rates which was higher in these groups (8%). By the end of the study, surviving animals appeared sick, fatigued and irritable with significant deterioration of pulmonary functions.
function, producing more inflammation and pulmonary oedema that are characteristics of hyperoxic lung injury [31]. Also, According to Kallet et al. [36], after prolonged exposure to a toxic O2 tension, the alveolar cells become hyperplastic and hypertrophied, causing a great increase in the thickness of the alveolar walls. These changes persist even after removal to normal O2 tensions as demonstrated in the Unt-G.

During lung injury, type II pneumocytes can proliferate and differentiate into type I cells to compensate for
damaged cells [37]. When the delicate homeostatic balance is disturbed by oxidative stress, damage of nucleic acids especially in the proliferating type II pneumocytes, might occur. Moreover, damage of proteins, and lipids leading to changes in membrane permeability and fluidity, progressing to lytic damage and cell death [35]. Furthermore, several proteomic investigations have demonstrated significant changes in cellular migration, differentiation, and proliferation proteins in type II pneumocytes following hyperoxic injury [38].

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**Fig. 7** Effect of stem cell treatment on biochemical markers. **A** Western blot analyses of IL-6 and RhoA in lung tissue in all groups. β-actin was used for normalization. Images of the bands where obtained from the same gel with no cropping in between the bands. Full length blots are included in the Additional file 1 Fig. S1. **B, C** Representative graphs for relative expression of IL-6 and RhoA respectively. **D, E** Representative graphs for quantification of MDA and TAC in lung tissue of all experimental groups. Data is represented in mean. n = 3 in all groups. a: significant compared to CG. b: significant compared to HG. c: significant compared to CFM-G. d: significant compared to SC-PG. e: significant compared to SC-TG. pairwise comparison bet. Each 2 groups using Post Hoc Test (Tukey). Significance at p ≤ 0.05. Error bars represent S.E.M. Stem cell treatment significantly reduced IL-6, RhoA and increased TAC, while stem cell prophylaxis significantly reduced MDA. Abbreviations: CG; control group, HG; Hyperoxia group, CFM-G; Cell-free media-treated group, SC-PG; Stem cells-prophylactic group, SC-TG; Stem cells-treated group, Unt-G Untreated group, IL-6; interleukin 6, RhoA; Ras homolog family member A, MDA; malondialdehyde, TAC; total antioxidant capacity.
Although resident fibroblasts and circulating progenitors can certainly contribute to the hyperplasia of fibroblasts and the subsequent pathology, an additional source for pulmonary fibroblast formation might be through the differentiation of type II pneumocytes through the process of epithelial-to-mesenchymal transition (EMT) [30]. EMT is a biologically important process involved in tissue remodeling during organogenesis, but the reversion of terminally differentiated epithelium to its mesenchymal origin has been implicated in organ fibrosis [30].

The electron micrographs of HG and CFM-TG revealed variable degenerative changes, confirming the LM results, where type I pneumocytes were infrequently encountered. This can be attributed to the direct toxic effect of hyperoxia on these cells [39]. HG as well as the CFM-TG showed marked hypertrophy of type II pneumocytes with their rounded nuclei, on the expense of the flat type I. This is in line with other authors’ findings who proved the morphological change of type II to type I pneumocytes as a mechanism to compensate for their loss; probably through an intermediate cell stage that may express markers from both cell types [40].

The presence of vacuoles of different sizes were a prominent feature observed in both HG and CFM-TG under the EM in the cytoplasm of interstitial cells but also in the pneumocytes. They were also noticed in the SC-TG and the Unt-G. Presence of vacuoles is generally thought to represent degenerative changes [41]. For example, misfolding of endoplasmic reticulum (ER) proteins by the oxidative environment, leads to ER stress with distended and pinched off vesicles and tubules. Moreover, accumulation of triglycerides within the damaged mitochondria or within the cytoplasm, decreased lipid transport and their accumulation in the cytoplasm, all these changes would manifest as vacuolization [41].

Finally, our EM results in HG, CFM-TG and Unt-G showed prominent lung fibrosis, that probably was an ultimate consequence of pulmonary O₂ toxicity. Similarly, Hemnes et al. showed in their study that excessive production and deposition of extracellular matrix proteins, e.g., collagen-I, was the most important feature of pulmonary fibrosis in hyperoxia-induced lung injury [39].

In harmony with the fibrosis that was encountered in the EM results, the small GTPase RhoA, that is essential in the regulation of various cellular functions including formation of F-actin stress fibers and focal adhesion complexes and transcription of genes containing serum response element [42] was significantly overexpressed in HG and CFM-TG [30]. This is consistent with the study showing that ROS-dependent RhoA activation is responsible for the increase in collagen-I synthesis in hyperoxic lung fibroblasts of mouse lungs [43].

MSCs derived from amniotic tissue have characteristics of both adult and embryonic stem cell, they express the embryonic cell marker OCT4, thus they possess a higher regenerative power than adult sources. In addition to their immunomodulatory, anti-inflammatory and antioxidant functions, AF MSCs lack the ethical concerns for their use, unlike embryonic stem cells [44].

Devine and colleagues attempted to study the different targets of systemic MSCs injection. In their study, after intravenous (IV) injection of MSCs into rats’ tails, most of the cells were trapped in lung and only a minor fraction of the cells (less than 3%) engrafted in other tissues [45]. Moreover, Chang et al. demonstrated in two of their studies on bronchopulmonary dysplasia, that the IV MSCs administration route is as effective as the intra-tracheal one [46, 47]. This helped our team to confidently rely on the IV route of injection in the present study. Indeed, AF-MSCs were shown in the lung tissue 72 h after their injection further confirming our IV injection choice.

In our study, the administration of stem cells prior to induction of fibrosis in the SC-PG helped in maintaining the pulmonary function tests within normal values. Moreover, their administration after the hyperoxia-induced fibrosis helped to ameliorate the pulmonary functions’ deterioration. This was supported by the significantly decreased MDA and increased TAC levels denoting the maintenance in the redox oxidation balance, together with a reduction in IL-6 level in the lungs denoting an inhibition of pro-inflammatory cytokine production, as well as a reduction in the level of RhoA and these were consistent with relevant studies [3, 48]. Moreover, the histological results went in line with the previous results suggesting the prevention of hyperoxia-induced fibrosis in the lung with stem cells preconditioning as well as slight improvement of the already formed fibrosis in SC-TG.

The biochemical reduction of IL-6 levels suggested that the therapeutic effects of MSCs might be partially mediated through the inhibition of pro-inflammatory cytokine production. Relating our results with the COVID-19 lung destruction, that appears to be associated with a cytokine storm with an increased IL-6 level. This anti-inflammatory effect of MSCs can be a tool to oppose the IL-6 togeth with other pro-inflammatory mediators and thus attenuate the vigorous inflammatory response that might end up with fibrosis and pulmonary failure. While the mild improvement seen in the Unt-G, can be explained by the natural antioxidant capacity inside the cell.

The prophylactic as well as the therapeutic powers of AF-MSCs can be attributed mainly to the paracrine functions of MSCs; through releasing extracellular vesicles or...
exosomes; transferring miRNAs to nearby endothelial and epithelial cells, thereby promoting angiogenesis and alveolar repair [49]. According to Antounians et al., compared to bone marrow MSCs, AF-MSCs exosomes were enriched for miRNAs that are critical for lung development, such as the miR17 ~ 92 cluster and its paralogues (miRs-93, -106, -250, and -363). Moreover, their miRNAs that have previously been reported as dysregulated in hypoplastic lungs, such as miR-33 and miR-200. Many of these miRNAs are conserved across species [50]. That finding made AF the source of choice for MSCs in the current study.

Another possible mechanism of action is through MSCs trans-differentiation through their capability of homing to damaged tissues [51]. One study demonstrated that MSCs engrafted and differentiated to type II alveolar cells in the lung, and their behavior was influenced by the injurious environment [51]. Furthermore, after recruitment to site of injury, AF-MSCs interact with multiple immune cells, such as T and B lymphocytes, natural killer cells and dendritic cells, thus their powerful immunomodulatory properties [52, 53]. Additionally, they possess major anti-inflammatory (through secreting IL-4, IL-6, IL-10, IFN-γ), anti-apoptotic and anti-fibrotic properties [9, 54–56].

**Conclusion**

Prolonged intermittent hyperoxia induces lung fibrosis, which is thought to be secondary to lung injury with excessive ROS production. In this study using an animal model of hyperoxia-induced lung fibrosis, using AF-MSCs as prophylaxis prior to the occurrence of fibrosis, preserved histological, physiological as well as biochemical parameters.

Furthermore, treatment with AF-MSCs markedly improved the histological structure of the lung alveoli, detected by both light and electron microscopes. Additionally, it improved pulmonary function tests and reduced ROS production. Moreover, it decreased inflammatory cells infiltration of lung parenchyma with significant decrease in IL-6, together with decreased fibrotic marker RhoA, subsequently reducing collagen fibrils observed with hyperoxia as evident by reduced the area percentage of collagen deposition.

**Limitations of the current study**

Despite the satisfactory results obtained in the current study, long term follow-up of rats was not applied to monitor whether this improvement will be life-long and will affect the rats’ longevity, and also to monitor any possible latent side effects. Moreover, it would be of utmost benefit to explore whether multiple stem cell injections would have added up to these satisfactory results, or merely a single injection is all it takes as done in the present study. These issues deserve more attention in the future study designs.

**Abbreviations**

AF-MSCs: Amniotic fluid mesenchymal stem cells; IL6: Interleukin-6; RhoA: Ras homolog family member A; LM: Light microscope; EM: Electron microscope; CG: Control group; HG: Hyperoxia group; CFM-TG: Cell-free media-treated group; SC-PG: Stem cell- prophylactic group; SC-TG: Stem cell- treated group; Unt-G: Untreated group; DIC: Differential interference contrast; TV: Tidal volume; MRV: Minute respiratory volume; RR: Respiratory rate; FVC: Forced vital capacity; FEV1: Forced expiratory volume 1.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s13287-022-02821-3.

**Additional file 1**. Supplementary information file (S1) for figure 7: Full length original western blot analysis for IL-6 and RhoA in all studied experimental groups. Beta actin was used for normalization.

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**Author contributions**

AS was the owner of the idea. She was responsible for the study conception and design, performed the histological examination of lung tissues under light and electron microscopes, shared in the interpretation and commenting on histological results, in the conduction and optimization of the hyperoxic exposure and supervised the different steps throughout the research. RAM performed the amniotic stem cells isolation, culture, characterization, imaging and injection into the rats. She also shared in the interpretation of the physiological and biochemical studies and gave conceptual advice. GAM shared in the study design, was the major contributor in writing the manuscript and performed the analytical part of the study. SE was responsible for the experimental model; she performed the hyperoxia exposure, the monitoring of the experimental rats along the study and shared in injecting the rats with stem cells. RS performed the biochemical study including oxidative biomarkers and western blot. NHE carried out the physiological study on the rats including pulmonary function tests and interpreted their results. All authors wrote sections of the manuscript, each in his specialty. All authors read and approved the final manuscript.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Declarations**

**Ethics approval and consent to participate**

The animal study was performed after receiving approval of the Research Ethics Committee for care and use of laboratory animals, approval No. 0201162, IRB code 00012098, FWA code 00018699; membership of Alexandria University in the International Council of Laboratory Animal Science organization (ICLAShttps:// www.hhs.gov/ohrp/assurances/index.html).

**Consent for publication**

Not applicable.
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