Published in final edited form as:

Biochem Biophys Res Commun. 2018 September 18; 503(4): 2653–2658. doi:10.1016/j.bbrc.2018.08.019.

Intraperitoneal injection of MSC-derived exosomes prevent experimental bronchopulmonary dysplasia

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Abstract

Mesenchymal stromal cell (MSC) derived exosomes mediate tissue protection and regeneration in many injuries and diseases by modulating cell protein production, protecting from apoptosis, inhibiting inflammation, and increasing angiogenesis. In the present study, daily intraperitoneal injection of MSC-derived exosomes protected alveolarization and angiogenesis in a newborn rat model of bronchopulmonary dysplasia (BPD) induced by 14 days of neonatal hyperoxia exposure (85% O₂). Exosome treatment during hyperoxia prevented disruption of alveolar growth, increased small blood vessel number, and inhibited right heart hypertrophy at P14, P21, and P56. *In vitro*, exosomes significantly increased tube-like network formation by HUVEC, in part through a VEGF mediated mechanism. In summary, daily intraperitoneal injection of exosomes increased blood vessel number and size in the lung through pro-angiogenic mechanisms. MSC-derived exosomes therefore have both anti-inflammatory and proangiogenic mechanism to protect the lung from hyperoxia induced lung and heart disease associated with BPD.

Keywords

bronchopulmonary dysplasia; chronic lung disease; exosomes; mesenchymal stromal cells; lung prematurity

1. Introduction

Bronchopulmonary dysplasia (BPD) is a chronic respiratory disease affecting preterm infants born in the late canalicular or early saccular stage of lung development caused, in part, by exposure to high concentration of oxygen (hyperoxia) that results in impaired

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Conflicts of interest

The authors declare no conflicts of interest.

alveolarization and compromised vasculogenesis [1–3]. Patients with BPD often have poor long-term outcomes with increased infections, interstitial fibrotic changes, small airway abnormalities, or emphysema that can persist into adulthood and all treatment options have failed to produce effective clinical outcomes [4, 5].

Preclinical studies with cell-based therapies present a new treatment strategy for acute and chronic lung injury [6–9]. In rodent models of BPD early administration of mesenchymal stromal cells (MSCs) offers protection from lung injury, and their late administration could partially reverse lung injury induced by neonatal hyperoxia exposure [10–12]. MSCs exert their biological effects mainly in a paracrine manner including the release of exosomes [13, 14]. Indeed, MSC-derived exosome treatment of hyperoxia exposed newborn mice blunts the resulting inflammation and stimulates neovascularization and immunosuppression after myocardial injury [12, 15, 16]. VEGF-A is essential for the development of the lung vascular system and alveolarization. VEGF mRNA and protein levels are decreased in premature infants who go on to develop BPD [17, 18]. In animal models of BPD, recombinant human (rh)VEGF partially protects against impaired alveolar growth [19]. Furthermore, postnatal intratracheal adenovirus-mediated VEGF expression in a neonatal rat model of BPD improved survival, promoted lung capillary formation, and improved alveolar development [20]. We hypothesized that intraperitoneal injection of MSC-derived exosomes would attenuate impaired lung growth in a neonatal hyperoxia exposure rat model of BPD by stimulating vascular growth.

2. Materials and Methods:

2.1. Animals:

All animal procedures and protocols were reviewed and approved by the University of Wisconsin Animal Care and Use Review Committee. Time-pregnant Sprague-Dawley rats were obtained from Envigo (Madison, WI) and were allowed to deliver spontaneously. Newborn pups were randomly divided into 4 groups (Table 1). Animals were exposed to either hyperoxia (Hyp; 85% oxygen) or normoxia (Norm; 20.9% oxygen) from immediately after birth to postnatal day 14 (P14). Dams were alternated every 24 hours from hyperoxia to normoxia to minimize oxygen toxicity. Standard rat pellet diet and water were provided ad libitum. Animals were injected intraperitoneally with either 50 μ l exosomes (15 μ g protein in 50 μ l saline equivalent to 3.4×10^9 exosomes) or 50 μ l saline starting one day after birth (P1) and repeated daily until one day after ending hyperoxia exposure (P15) equalizing 14 injections in total. Animals were sacrificed at 3 time points (P14, P21, or P56) (Figure 1A). Tissue analyzed at P14 obtained 12 intraperitoneal exosome injections.

2.2. MSC isolation

MSCs were harvested from rat bone marrow and maintained in culture at 37°C in a humidified atmosphere containing 5% CO₂ changing medium every 3–4 days. Adherent cells at passage 3 were characterized according to main criteria defined by ISCT, such as differentiation capability to osteoblasts, adipocytes and chondrocytes and expression of rat MSC specific markers (anti-CD45-DL350, anti-CD34-PerCP, CD11b-AF700, CD105-

AF647 (Novus Biologicals, Littleton, CO) and CD90-BV510 (Biolegend, San Diego, CA) by flow cytometry [21, 22] (Supplemental Figure 1A and 1B).

2.3. Exosome isolation and characterization

Passage 3 MSCs at 80% confluent cultures were rinsed with phosphate buffered saline (PBS) and then incubated in serum free α MEM for 24 hours. The culture supernatant was centrifuged for 30 min at 20,000g at 4°C, the pellet was discarded, followed by a centrifugation for 70 min at 100,000g at 4°C. The pellet was resuspended in PBS protein concentration was quantified (Bicinchoninic Acid Protein Assay Kit; Thermo Fisher, WI). Exosome size and number was determined by nanoparticle tracking (Nanosight, Malvern Instruments, Worcestershire, UK) and transmission electron microscopy (TEM; Philips CM120) [23, 24]. Exosomal markers (CD9, CD63, and CD81) were confirmed by western blot analysis.

2.4. Tissue preparation and immunohistochemistry:

The heart was dissected into right ventricular free wall (RV) and left ventricle (LV) plus septum (S) wall and weighed. RV to LV+S weight ratio was calculated as the Fulton index (FI). The right lung was dissected and flash frozen, the left lung was inflated and fixed by instillation of 4% formaldehyde solution at a constant inflation pressure of 25 cm H₂O for one hour and processed into paraffin blocks. Transverse serial sections (5 μ m) were cut, deparaffinized, and either stained with routine hematoxylin & eosin (H&E), or analyzed by immunohistochemistry. Sections were analyzed using anti-von Willebrand Factor (vWF; 1:1000; Dako, Santa Clara, CA) or anti-CD31 (1:500; R&D, Minneapolis, MN) primary antibodies. Antibody detection was carried out using ImmPRESS anti-rabbit Ig HRP (Vector Laboratories, Burlingame, CA). Finally, the signal was visualized with DAB and sections counterstained with 1% methyl green for contrast.

2.5. Assessment of airway and alveolar size and density by MLI

Four tissue sections (5 μ m) per rat were chosen randomly and were stained with hematoxylin and eosin. From each section 10 microscopic random fields of the distal lung were analyzed and level of distal air space dimensions was determined by measuring the mean linear intercept (MLI) [25].

2.6. Tube formation assay

The angiogenic potency of MSC-derived exosomes was assessed by tube formation assay. HUVECs (1.5×10^4 cells/well) were seeded in growth factor reduced high concentrated BD Matrigel matrix-coated (10 μ l/well; Corning, Thermo Fisher, Waltham, MA) μ -slide (ibidi USA, Madison, WI). Cells were incubated with either exosomes (10 μ g/well) alone or in combination with activity neutralizing rat anti-VEGF antibody or Isotype control (R&D systems, Minneapolis, MN) overnight to allow the formation of tube-like structures with serum-free medium as a control. The images of the capillary network were acquired under a light microscope at 4 $\times$ objective lenses and total tube lengths formed were measured using ImageJ (National Institutes of Health, MD, USA). A tube formation assay was performed in 5 replicates and 2 independent experiments.

2.7. Western blot analysis

Isolated exosomes were lysed in Radio-Immunoprecipitation Assay (RIPA) buffer supplemented with proteinase inhibitors, resolved over SDS-PAGE, and then transferred to nitrocellulose membrane (Bio-Rad, CA). Proteins were detected with primary antibodies: anti-CD9, anti-CD63, anti-CD81, or anti-VEGF-A (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), followed by HRP-conjugated secondary antibodies. Chemiluminescent signals from immunoreactive bands were visualized using the ECL system.

2.8. Statistical analysis

Values are expressed as means $\pm$ SD. Intergroup differences at a single time point were assessed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. Data that did not show a significant interaction of time were analyzed with multiple t-test. To compare changes between multiple time points Two-way ANOVA with Tukey's multiple comparisons test was used. Calculations were done using GraphPad Prism (GraphPad Software, San Diego, CA). Values with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Exosomes ameliorate hyperoxia induced lung injury

MSCs used for exosome harvest expressed CD90 (66%), CD105 (46% + 16% double + with CD11b), and no CD45 or CD34, but some expressed CD11b (23%) (Supplemental Figure 1). The characterization of exosomes showed that $1.0\text{--}3.4 \times 10^9$ particles/ 10^6 cells with mean diameter 179nm and the visible spherical structures were $<200\text{nm}$ (Figure 1A&B). Further, exosomes expressed the characteristic markers CD63, CD9, and CD81 (Figure 1C). All pups survived the hyperoxia exposure and the daily injections (see experimental design in Figure 1D). Hyperoxia/Saline (Hyp/Sal) exposure of newborn rats induced a significant simplification of the alveolar structure with alveolar septal wall thickening and strong cellular infiltrates compared to Normoxia/Saline (Norm/Sal) exposed animals at all time-points analyzed (Figure 2A). Norm/Sal exposed animals at P21 showed multiple areas of secondary crest formation leading to subdivision of the airspace at the end of the saccular phase whereas in Hyp/Sal exposed lungs airspaces are enlarged with less airspace subdivision, thickened alveolar walls, and increased cellular infiltrates (Figure 2). Injection of exosomes during hyperoxia (Hyp/Exo) prevented most of these changes (Figure 2A). Norm/Exo rats did not show any changes in lung structure at any day analyzed (Supplemental Figure 1C). MLI confirmed the histological findings. There was a significant interaction between age and group ($p < 0.0001$). MLI in the Hyp/Sal group was significantly higher compared to the Norm/Sal group at all time-points, P14, P21 and P56 (Figure 2B). Exosome injection preserved alveolar growth in hyperoxia exposure rats at P21 and P56 as evidenced by similar MLI values in the Hyp/Exo group compared to the Norm/Sal group. However, it was significantly higher in the in the Hyp/Exo group compared to the Norm/Sal at P14 (Figure 2B). In contrast, exosome depleted MSC tissue culture medium (Hyp/Edm) decreased MLI significantly compared to Hyp/Sal but remained significantly higher than Hyp/Exo at P21 (Figure 2C).

3.2. MSC-derived exosomes enhanced lung blood vessel density and reduce RV hypertrophy

Hyperoxia exposure significantly diminished growth of blood vessels in the P14 lung compared to the Norm/Sal group (Figures 3A&B). The difference was still significant between these groups at P21 but disappeared at P56. Intraperitoneal injection of exosomes increased the number of small vessels in the Hyp/Exo group somewhat compared to the Hyp/Sal group at P14, but remained significantly lower compared to the Norm/Sal group (Figure 3A, 3B, Supplemental Figure 2). At P56 the number of small blood vessels in the lung was not different between the three groups (Norm/Sal; Hyp/Sal; Hyp/Exo) although the vessels remained smaller in diameter compared to the Norm/Sal group at P56 (data not shown).

Corresponding with the reduced blood vessel growth, RV hypertrophy suggesting pulmonary hypertension was prominent at all time-points. FI was elevated in the Hyp/Sal group compared to the Norm/Sal group at P14 (Figure 3C). Exosome injection significantly reduced the FI in the Hyp/Exo group compared to the Hyp/Sal group at P14 and P21 but not significantly at P56. Exosome depleted medium had no protective effect on FI (Figure 2C), or right ventricular hypertrophy (Figure 3D).

3.3. MSC-derived exosomes contain VEGF and induce angiogenesis

MSC-derived exosomes *in vitro* stimulated extensive capillary network formation in HUVECs as compared to controls (Figure 4A). Capillary tube length in exosome treated HUVECs was significantly higher compared to controls (Figure 4B). Exosome induced tube formation was decreased nearly to control level by anti-VEGF antibodies whereas isotype control antibodies showed no effect. Western blot analysis revealed the presence of VEGF protein in the MSC conditioned media but not in the exosome depleted media (Figure 4C).

4. Discussion

At term the rat lung is in the saccular stage of development and is ready for breathing normal air without any negative impact on alveolar or vascular development. Exposure of newborn rats to hyperoxia disrupts both alveolarization and angiogenesis displaying BPD like pathology, including alveolar simplification, reduction in blood vessels density, and pulmonary hypertension [12]. Exosome treatment can be applied by intravenous, intratracheal, subcutaneous, or intraperitoneal injection. A single intravenous injection of exosomes in preclinical models partially protects rodent lungs from hypoxia induced pulmonary hypertension or hyperoxia induced BPD [12, 26]. Intratracheal instillation of exosomes protects lungs from endotoxin induced lung injury [27]. The timing, route, and dosing are key issues that need to be addressed to optimize the treatment as discussed by Willis et al [5].

We report that multiple intraperitoneal injections of rat MSC-derived exosomes restore alveolar and vascular growth and prevent right ventricular hypertrophy induced by neonatal hyperoxia as evidenced by normal MLI, increased small vessel density, and normal Fulton's Index. We also report that rat MSC-derived exosomes enhance vascular tube formation in

vitro and that this is mediated, in part, through a VEGF dependent mechanism. These findings suggest that MSC-derived exosomes can protect the neonatal lung from hyperoxia induced arrest of alveolar and vascular development. These findings also suggest that repeated dosing of MSC-derived exosomes may be more efficacious than a single IV or intratracheal administration. This is the first study to report the results of multiple intraperitoneal dosing in a model of BPD. In contrast to the use of human MSC-derived exosomes, we used syngeneic rat MSCs to procure exosomes. This allowed performing repeated injections without inducing rejection of allogeneic products and maintained the presence of exosomes over a longer period. Intravenous and intratracheal injection carries exosomes to the lung directly. In contrast, intraperitoneal injection requires the exosomes to reach the lung by entering the blood stream or lymphatic system first. Several tracking studies with exosomes have shown that after intravenous injection of labeled exosomes they accumulate in the lung but largely in liver and spleen [28, 29]. Intravenous exosome treatment has a very short half-life, within 30 minutes most exosomes will disappear from the serum [30]. To maintain a more constant level of exosomes over a longer period of time, we chose to administer MSC-derived exosomes daily intraperitoneally for 14 days during hyperoxia exposure.

Macrophages are an important target of exosomes in the mouse model of BPD. A single bolus of exosomes suppressed both pro-inflammatory M1- and anti-inflammatory M2-like macrophages in a dose dependent manner [12]. However, exosomes contain multiple proteins, RNA species and lipids that have all been implicated in their mechanism of action. We found that exosomes induced tube formation in endothelial cells *in vitro* through a VEGF dependent mechanism. This data supports a proangiogenic effect of exosomes and provides a possible mechanism for the increase in the number of small blood vessels seen in this model of BPD. This supports the hypothesis that MSC-derived exosomes can preserve lung vascular and alveolar growth during exposure to neonatal hyperoxia in this experimental model of BPD.

There are several potential limitations of our study. First, animals were born full-term rather than premature in this model of BPD. However, the alveolar phase of lung development in the newborn rodent occurs during the first 3 weeks of postnatal life validating this model to study the effect of hyperoxia on lung development [31]. Second, we utilized ultracentrifugation of conditioned media from MSCs to enrich exosomes rather than isolation of just the exosomal fraction. We can't exclude the presence of other small microvesicles in our preparation. Third, our model utilized a slightly lower level of neonatal hyperoxia exposure (85%) as compared to other investigators (>95%). We chose this level of hyperoxia to more closely mimic the lung structural changes that are seen in human infants with BPD in the post-surfactant era that has focused clinically on limiting oxygen exposure and toxicity. We see a milder impairment of lung structure with lower MLI elevation and reduced fibrosis and interstitial injury similar to the "new BPD". We also did not fully explore the effects of exosomes on neonatal hyperoxia induced lung inflammation and epithelial cell dysfunction. A more thorough investigation in each of these areas is needed in future studies to elucidate fully the complex mechanisms at work with exosomal therapy. Finally, we delivered the MSC-derived exosomes intraperitoneally and not directly to the developing lung. This leaves open the possibility that the protective effect of the MSC-

derived exosomes may be through modulation of the systemic effects of neonatal hyperoxia exposure rather than lung specific mechanisms.

Our study demonstrates that MSC-derived exosomes administered daily during the first 14 days of life protected the developing lung from hyperoxia-induced impairment in alveolar and vascular growth, and the development of right ventricular hypertrophy. The mechanism of this protective effect is in part through VEGF that is carried within the exosomes that results in improved vascular growth. The increased understanding of the MSC-secretome will lead to new strategies for treatment of neonatal lung diseases like BPD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding

This work was supported by The National Heart, Lung, and Blood Institute (NHLBI) grant #: R01 HL115061-01 (Eldridge), the University of Wisconsin Carbone Cancer Center Support Grant P30 CA014520, and the Special BD LSR Fortessa Grant #: 1S100OD018202-01.

Abbreviations:

BPD	Bronchopulmonary Dysplasia
HUVEC	Human umbilical vein endothelial cell
VEGF	Vascular endothelial growth factor
MSC	mesenchymal stem cell
Hyp	hyperoxia
Norm	normoxia
EXO	exosome
RV	right ventricle
LV	left ventricle
S	septum wall

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Figure 1. Exosome Characterization.

Exosomes concentration and size were analyzed by the Nanosight particle tracking (**A**) and size and shape were analyzed by Electron Transmission Microscopy (**B**). **C**) Western Blot analysis for exosomal markers: CD9, CD63 and CD81 in the isolated exosomes. **D**) Schematic representation of exosome-treatment. Exosomes (Exo); mean linear intercept (MLI); Fulton index (FI).

Figure 2. MSC-derived Exosomes alleviates hyperoxia induced lung destruction.

A) Lungs were collected, processed and sectioned as described in Materials and Methods. Representative micrographs of H&E stained lung sections are shown. Normoxia exposed saline treated (Norm/Sal); hyperoxia exposed saline treated (Hyp/Sal); hyperoxia exposed exosome treated (Hyp/Exo). Solid bar represents 100 μ m. **B&C)** Morphometric analysis of free distance in air spaces in the lung, mean linear intercept (MLI) was performed as described in Materials and Methods. Exosome injections prevented loss of alveolarization at days P14, P21, and P56 comparing three treatment groups: Normoxia exposed saline treated (Norm/Sal; n=4), hyperoxia exposed saline treated (Hyp/Sal; n=4; at P21 n=3) and hyperoxia exposed exosomes-treated (Hyp/Exo; n=4). Two-way ANOVA was used to analyze the treatment and time interaction (**B**). Effect of exosome depleted medium (Edm) at day P21 comparing Hyp/Sal, Hyp/Exo, and Hyp/Edm. One-way ANOVA was used to analyze the effect of Edm (**C**). * indicates a significant difference between the indicated data sets ($p < 0.05$).

Figure 3. A&B) MSC-derived Exosomes improve pulmonary vasculature in hyperoxia-injured lung.

Lung sections were analyzed for small blood vessel number and density by immunohistochemical analysis as described in materials and methods. The presence of small blood vessels in the lung parenchyma was visualized by DAB staining. Antibodies for Von Willebrand Factor (vWF) at day P14 and CD31 at days P21 and P56 were used. solid bar represents 20 μ m. Also see supplemental figure 2 (A). The number of blood vessels of 20 – 100 μ m diameter per field, from at least 12 microscopic fields/group, were counted for each time point. Normoxia exposed saline treated (Norm/Sal), hyperoxia exposed saline treated (Hyp/Sal), and hyperoxia exposed exosomes-treated (Hyp/Exo) (B). *indicates a significant difference between the indicated data sets ($p < 0.05$). Multiple T-test was used to analyze the data. C&D) MSC-derived Exosomes prevented hyperoxia induced right ventricle hypertrophy (RV). Foulton index (FI) was calculated as described in Materials and Methods. Normoxia exposed saline treated (Norm/Sal); hyperoxia exposed saline treated (Hyp/Sal); and hyperoxia exposed exosomes-treated (Hyp/Exo. Two-way ANOVA was used to analyze the treatment and time interaction (C). Effect of exosomes depleted medium (Edm) on RV hypertrophy at day P21 comparing Norm/Sal, Hyp/Sal, Hyp/Exo, and Hyp/Edm. One-way ANOVA was used to analyze the effect of Edm (D). *indicates a significant difference between the indicated data sets ($p < 0.05$).

Figure 4. HUVEC tube formation induced by MSC-derived exosomes.

Tube formation assay was performed as described in Materials and Methods. **A)** Tube-like structures induced significantly higher in exosomes treated cells compared to controls. **B)** The degree of angiogenic induction was quantified for the numerical value of the product of the relative capillary length per microscopic field. Average tube length was determined from 5 different fields and shown as arbitrary units (A.U.). Exosomes (Exo), exosomes + isotype antibody (Exo+isotype) and exosomes + anti-VEGF antibody (Exo+anti-VEGF) * indicates a significant difference between the indicated data sets ($p < 0.05$). T-test was used to compare the data. **C)** Equal amounts of proteins from exosomes and exosome-depleted medium (Supernatant) were analyzed by Western blot analysis for VEGF content. CD63 used as a control.

Table 1:

Neonatal animal groups and treatments

	Experiment 1		Experiment 2		Experiment 3		Total	
Time	Norm	Hyp	Norm	Hyp	Norm	Hyp	Norm	Hyp
P14	4	4	3	3	-	-	7	7
P21	4	3	4	4	3	3	11	10
P56	4	4	4	4	-	-	8	8

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