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Intratracheal transplantation of human umbilical cord -derived mesenchymal stem cells mitigates lung injuries caused by hyperoxia

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ABSTRACT. Bronchopulmonary dysplasia (BPD) is associated with high mortality rate due to lung injury. Recent studies indicate that cell therapy is a promising treatment option for BPD. This study aimed to examine whether lung injuries caused by hyperoxia can be mitigated by intratracheal mesenchymal stem cells (MSCs) transplantation.Newborn Sprague Dawley rats were housed in room air or hyperoxic chambers. Human umbilical cord -derived mesenchymal stem cells (hUC-MSCs) in saline were intratracheally transplanted into the rats through a 30-gauge needle syringe. Lung and brain tissues were harvested for experimental analysis. hUC-MSCs entered the lung tissue but not the brain. Larger congestive alveoli in the lung tissue caused by hyperoxia could be attenuated by intratracheal transplantation of hUC-MSCs. Intratracheal transplantation mitigated hyperoxia-induced inflammatory reactions. hUC-MSC transplantation inhibited apoptosis activation and terminal deoxynucleotidyl transferase dUTP nick end

labeling positive cells in the lungs.Taken together, this study shows that intratracheal transplantation of hUC-MSCs partially mitigates hyperoxia-induced lung injury.

Key words: Bronchopulmonary dysplasia; Umbilical cord -derived mesenchymal stem cells; Lung injuries; brain injuries

INTRODUCTION

Bronchopulmonary dysplasia (BPD) is a chronic lung disease in neonates characterized by arrested alveolar and vascular development. BPD occurs secondary to lung immaturity in premature infants, ventilator-induced lung injury, and hyperoxia, resulting in lifelong impairment of lung function. The pathogenesis of BPD is multifactorial, and its clinical phenotype is variable. Persistent inflammation leads to abnormal lung development, simplification, and fibrosis of the alveolar properties, which play crucial roles in the development of BPD. BPD has several etiologies, and has a high mortality rate owing to the lack of effective treatment (Voynow et al., 2017; Onland et al., 2023). It can also cause chronic respiratory problems. Some interfering factors, such as genetics and preterm birth, play causative roles in the pathogenesis of BPD (Baraldi et al., 2007; Amata et al., 2017). The long-term outcomes of BPD, including lung structural alterations, respiratory tract functional abnormalities, respiratory tract infections, asthma-like symptoms and lung arterial hypertension, must be resolved urgently (Jobe et al., 2016; Metcalfe et al., 2017; Kwinta et al., 2010; Patel et al., 2016). Although numerous studies on BPD have been conducted in recent years, the mechanisms underlying lung injury remain unclear, may be involve in hyperoxia-activated mitochondrial dysfunction, inflammation and apoptosis (Day et al., 2017).

Vitamin A, oxygen supplementation, caffeine, and cell therapy can significantly reduce the risk of developing BPD (Stenson et al., 2016; Hartnoll et al., 2000; Biniwale et al., 2006; Curstedt et al., 2013; Uberos et al., 2014). Cell therapy is the most promising new method for mitigating the lung damage caused by BPD (Weiss et al., 2013; Dobson et al., 2014). Although the role of mesenchymal stem cells (MSCs) remains incompletely defined, extensive evidence suggests that they are an important component in progenitor cell function and repair processes following injury (Chambers et al., 2014). The safety and feasibility of intratracheal transplantation of umbilical cord derived mesenchymal stem cells (hUC-MSCs) in preterm infants has been demonstrated (Willis et al., 2018; Braun et al., 2018). This study, aimed to clarify the effects of intratracheal transplantation of hUC-MSCs on lung injury caused by hyperoxia. The data revealed that intratracheal transplantation of hUC-MSCs mitigated the injuries associated with hyperoxic outcomes. Hence, the intratracheal transplantation of hUC-MSCs may be a promising therapy for BPD.

MATERIALS AND METHODS

Lentiviral vectors

Lentiviral vectorswere produced by the standard transient transfection of a two-plasmid system into HEK293T cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China). pHBLV-CMVIE-ZsGreen-Puro (Addgene, Beijing, China) was used as the packaging vector. Lentivirus was harvested by collecting the supernatant of a virus-producing cell culture, concentrated using ultracentrifugation, and stored at -80 °C. The lentivirus was used at a multiplicity of infection of 200.

Cells

Human umbilical cord-derived mesenchymal stem cells were obtained from Zhejiang Sidanmu Stem Cell Biotechnology Co. Ltd. (Jinhua, China). hUC-MSCs were cultivated and confirmed for their characteristics using previously established protocols[20]. Briefly, MSCs were isolated from human umbilical cord tissue, and informed consent was obtained from the donors. Cells were grown in a humidified incubator with 5% CO_2 at 37 °C. Cells were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher, Shanghai, China), supplemented with 10% fetal bovine serum (Gibco, USA). Cells were provided with fresh medium every two days. hUC-MSCs were passaged once reached 80–90% confluence to the fourth generation. The hUC-MSCs were characterized by analyzing the expression of CD markers(CD19, CD14, CD34, CD45, CD73, CD90, and CD105). We used lentiviruses to screen cell lines that stably transfected with puromycin, as previously reported methods (Wu et al., 2020).

Rats

This study was approved by the Medical Ethics Committee, School of the Medicine, Jinhua Polytechnic, China. Newborn Sprague Dawley (SD) rats were purchased from Hangzhou Medical College (Hang Zhou, China). Rats were housed in a specific pathogen-free environment at 20-26 °C and 40-70% humidity. To generate the rat model, newborn SD rats were divided into four groups within 6 h after birth: normoxia control (NC, *n*=8), normoxia with intratracheal MSCs transplantation (NM, *n*=8), hyperoxia control (HC, *n*=8),and hyperoxia with intratracheal MSCs transplantation (HM, *n*=8). Rats in the NC and NM group were housed in room air, whereas those in HC and HM group were housed in hyperoxic chambers (95% oxygen). To verify the effect of intratracheal MSCs transplantation, the rats were examined 2 days and 21 days after normoxic and hyperoxic treatments, respectively.

Transplantation

Human UCB-derived MSCs from the 5th passage from a single donor were transplanted at P5. For intratracheal transplantation, the rats were anesthetized with isoflurane and restrained on a board at a fixed angle as described by Chou et al.(Chou et al.,2021). Intratracheal administration was used for transplantation. First, 2.5×10^5 UCB-MSCs in 50 μL normal saline were intratracheally transplanted into the NM and HM groups, while an equal volume of normal saline was intratracheally transplanted into the NC and HC groups. UC-MSCs were administered into the trachea using a 30-gauge needle syringe. After the procedure, the animals were allowed to recover from the anesthesia, and were returned to their dam. No mortality was associated with the transplantation procedure.

Histopathology

The lungs and brain were collected, fixed, and embedded in paraffin overnight at room temperature. Blocks of the optimal cutting temperature compound were sectioned (RM2235, Leica, Germany) and stored in a deep freezer until histopathological analysis. Sections were then cut from the paraffin blocks, and stained with hematoxylin and eosin(HE). Images were captured using a digital camera (DMIL LED Fluo S/N, Leica, Germany).

Enzyme-linked immunosorbent assay

Frozen lung and brain tissues were collected from SD rats and homogenized. The supernatant was collected for enzyme-linked immunosorbent assay (ELISA). An appropriate amount of tissue block was taken, washed in precooled PBS, and transferred to a glass homogenizer placed in ice, after removing the blood. Five to ten milliliters of precooled PBS was added for thorough grinding, the entire process being carried out on ice. The prepared tissue homogenate was centrifuged at 5000 rpm for 5 min, and the supernatant was preserved for the test. Inflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNF-α were measured using ELISA as per the instructions of the manufacturer (MultiSciences, Shanghai, China). The optical density at 450 nm was determined using a microplate reader (iMarK, BioRad, USA). ELISA analyses were replicated in 3 independent experiments.

Western blotting

To extract proteins from the lung and brain tissues of SD rats, the lungs and brain were collected, washed in cold PBS and homogenized in RIPA buffer containing the protease inhibitor PMSF for 5 min on ice. The homogenates were centrifuged at 12,000 rpm/min for 20 min at 4 °C. The supernatant was collected and stored at -80 °C. The protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, USA). Protein lysates were separated in a 10% SDS-PAGE gel (Transgen, Beijing, China) using Mini-Protean Tetra System (Bio-Rad) and transferred onto a PVDF membrane. The PVDF membrane was washed, blocked in skimmed milk for 1 h, and incubated with the primary antibody for 2 h at room temperature. The blots were detected with ECL (Thermo Fisher Scientific) and imaged using the ChemiDoc™ Touch Imaging System (BioRad). Antibodies against caspase 3 and GAPDH were obtained from Cell Signaling Technology (Danvers, MA,USA). Western blot analyses were replicated in 3 independent experiments.

TUNEL assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to detect apoptosis using the *in situ* apoptosis detection kit (Chemicon International, Canada). The sections were deparaffinized, rehydrated and washed with PBS. Sections were incubated in 20 μg/mL proteinase-K at room temperature for 15 min. After rinsing within distilled water, the sections were incubated in 3% H_2O_2 for 10 min to block endogenous peroxidase activity. Sections were washed in PBS. TdT reaction enzyme was added for 15-20 min and quenched using a stop/wash buffer. The sections were then washed three times with PBS. An anti-digoxigenin-peroxidase conjugate was added and the sections were incubated in a humidified chamber for 30 min at room temperature. The sections were washed and visualized using DAB. The sections were counterstained with hematoxylin, dehydrated with ethanol and Citrisol V (Thermo Fisher Scientific, Shanghai, China), and mounted.

Statistical analysis

Quantitative data are presented as mean ± SEM. For continuous variables with a normal distribution, comparison among more than 2 groups was done using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. A p-value < 0.05 was considered statistically significant. Data were analyzed using GraphPad Prism 8(GraphPad Software, USA).

RESULTS

hUC-MSCs enter the lung tissue but not the brain

We used SD rats as the typical rat model for this study. hUC-MSCs from a single donor were used for the intratracheal transplantation. To verify the target site of hUC-MSCs, 2.5×10^5 hUC-MSCs in 50 μL of normal saline were intratracheally transplanted into SD rats. On day 2 post-transplantation, the lung and brain tissues of rats were collected, and frozen sections were prepared. Representative photomicrographs of GFP-hUC-MSCs are shown in Figure 1. hUC-MSCs could reach the lung tissue but not the brain. We suspected that UCB-MSCs were unable to cross the blood-brain barrier.

MSCs transplantation mitigates injuries of hyperoxic outcomes

We divided 32 newborn SD rats into four groups: normoxia control (NC),normoxia with intratracheal MSC transplantation (NM),hyperoxia control (HC),and hyperoxia with intratracheal MSCs transplantation (HM). The normoxic group was housed in room air, whereas the hyperoxia group was housed in hyperoxic chambers (95% oxygen). MSCs were intratracheally transplanted into SD rats at P5. Lung and brain tissues were collected at the 2-day and 21-day time points. Then they were subjected to histological study using HE staining. As shown in Figure 2, pathological changes in brain tissue are less obvious, but the hyperoxia groups showed larger and more congestive alveoli in the lung tissue compared with the normoxia groups, and more injuries occurred in the brain tissue on day 2 post-transplantation. Changes caused by hyperoxia were mitigated by intratracheal MSCs transplantation in the HM group. No significant differences were observed between these groups on day 21 post-transplantation.

Intratracheal transplantation of MSCs attenuates inflammatory responses in the lung tissue

To further understand the effects of hyperoxia and intratracheal MSCs transplantation, the levels of inflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNF-α were detected in the lung tissue. The data indicated that the levels of these cytokines in the hyperoxic group were higher than those in the normoxic groups on the day 2 (Figure 3A). After the intratracheal MSCs transplantation, the effects of hyperoxia were alleviated in the HM group. In contrast, as shown in Figure 3B, there were few differences between these groups on day 21 post-transplantation. These data showed that intratracheal MSCs transplantation attenuated the inflammatory reaction caused by hyperoxia.

MSCs transplantation inhibits caspase 3 activation caused by hyperoxia

Caspases are proteolytic enzymes that are widely known for their roles in controlling cell death and inflammation. Caspase 3play an important role in apoptosis. To evaluate whether the expression of caspase 3 was altered at the protein level, the lungs and brain of SD rats were collected at various time points post-transplantation and subjected to western blot analysis. As shown in Figure 4A and 4E, the level of caspase 3 in the hyperoxia groups was increased compared with that in the normoxia groups and was attenuated by intratracheal MSCs transplantation at day 2 post-transplantation in the lung. The expression of caspase 3 expression in the lung tissue of SD rats treated for 21 days (Figure 4B and F), the brain of SD rats treated for 2 days (Figure 4C and 4G) and

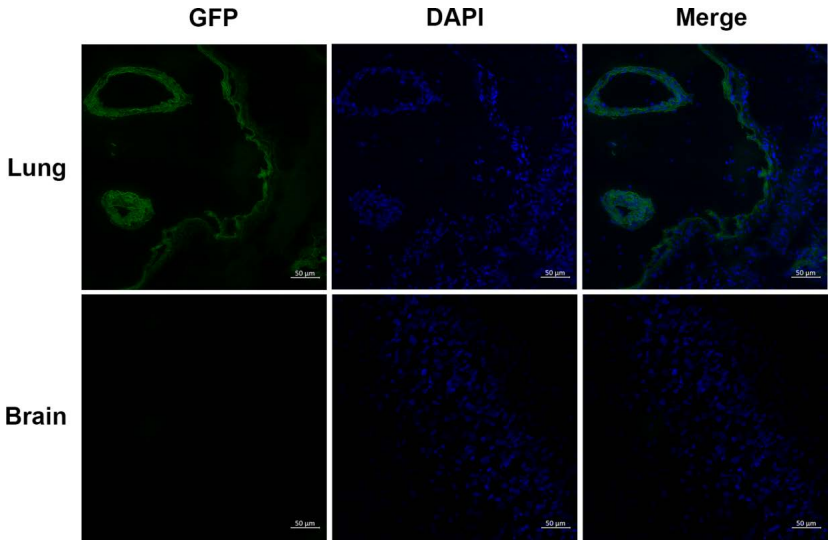

Figure 1. hUC-MSCs enter the lung tissue but not the brain. The lung and brain tissues were taken from SD rats with intratracheal transplantation of GFP-hUC-MSCs. The expression of GFP-hUC-MSCs was viewed in a fluorescence microscope (scale bar, 50μm).

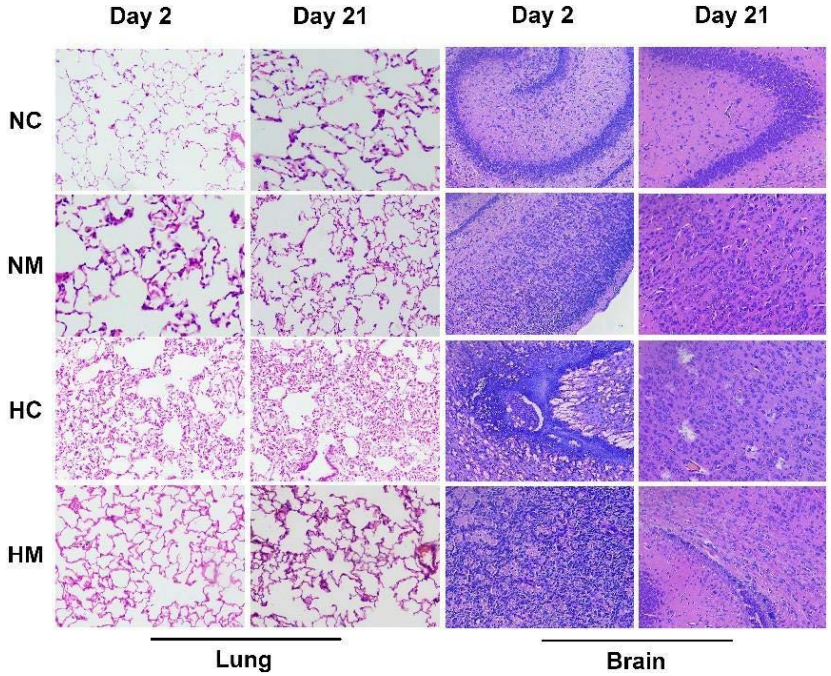

Figure 2. MSCs transplantation mitigates injuries of hyperoxia outcomes. SD rats were treated by hyperoxia and MSC transplantation for 2 days and 21 days. The lung and brain were collected and subjected to histological study using HE staining. NC, normoxia control; NM, normoxia with intratracheal transplantation of GFP-hUC-MSCs; HC, hyperoxic control; HM, hyperoxia with intratracheal transplantation of GFP-hUC-MSCs.

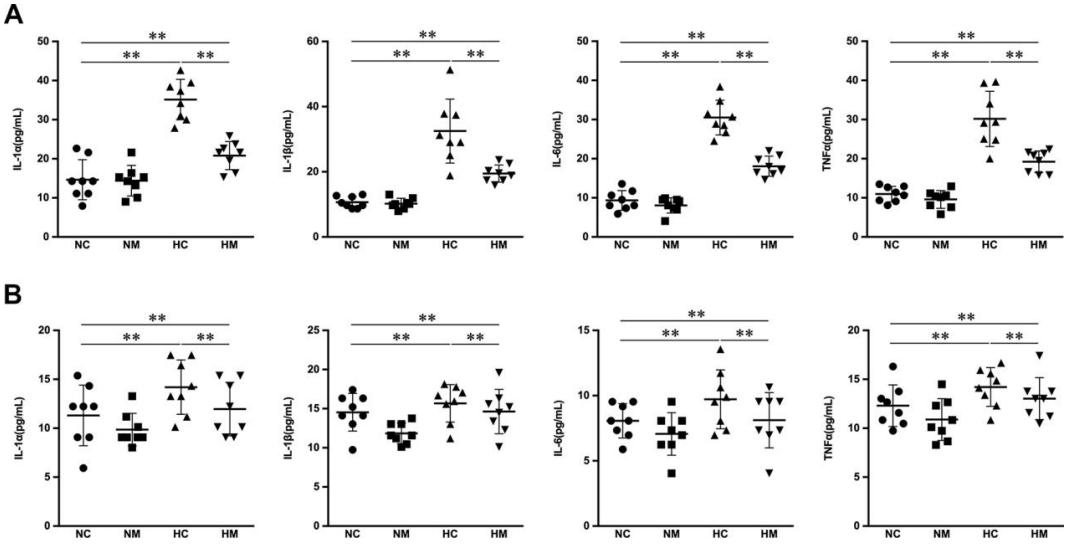

Figure 3. Intratracheal transplantation of MSCs attenuates inflammatory responses in the lung tissue. The SD rats were treated by hyperoxia and MSCs transplantation for (A) 2 days and (B) 21days. Levels of inflammatory cytokines IL-1α, IL-1β, IL-6 and TNF-α in the lung tissue were measured using ELISA. Experiments were repeated three times. ***P*<0.01.

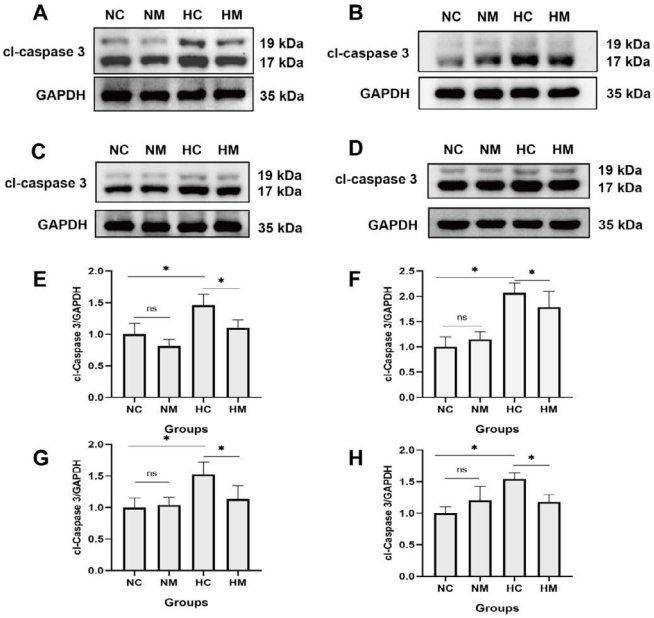

Figure 4. MSCs transplantation inhibits caspase 3 activation caused by hyperoxia. The lung and brain of SD rats were collected at various time points of post-treatment and subjected to western blot analysis. (A,E) The caspase 3 expression in the lungs of SD rats treated for 2 days and the relative level was quantified; (B,F) The caspase 3 expression in the lungs of SD rats treated for 21 days and the relative level was quantified; (C,G) The caspase 3 expression in the brain of SD rats treated for 2 days and the relative level was quantified; (D,H) The caspase 3 expression in the brain of SD rats treated for 21 days and the relative level was quantified. **P*<0.05.

brain of SD rats treated for 21 days (Fig. 4D and H) also showed the same trend. Taken together, these results suggested that MSCs transplantation inhibited hyperocia-induced apoptosis activation.

The hyperoxia-induced increase in TUNEL-positive cells in the lung and brain was significantly mitigated by MSCs transplantation

One marker of apoptosis is the generation of free 3'-hydroxyl termini on DNA via cleavage of chromatin into single and multiple oligonuleosome-length fragments. The TUNEL assay can label the exposed termini of DNA, thereby exhibiting nuclei containing fragmented DNA. To confirm that intratracheal MSCs transplantation can attenuate apoptosis caused by hyperoxia, lung and brain tissues were collected and subjected to a TUNEL assay. The number of TUNEL-positive cells in the lungs and brain of the hyperoxia groups was higher than that in the normoxia groups and intratracheal MSCs transplantation could mitigate the effect caused by hyperoxia at various time points post-transplantation (Figure 5), indicating that hyperoxia-induced apoptosis in the lungs and brain was significantly mitigated by MSCs transplantation.

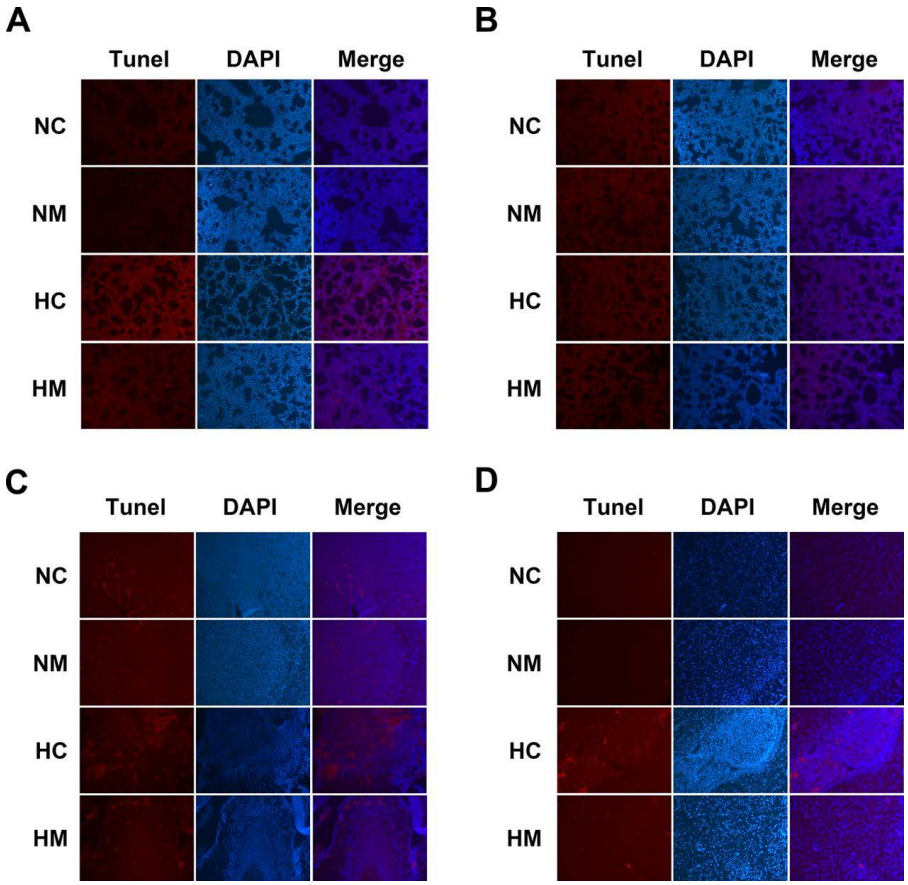

Figure 5. The hyperoxia-induced increase in TUNEL-positive cells in the lung and brain was significantly mitigated by MSCs transplantation. Cells were stained with TUNEL (red) and 4′,6-diamidino-2-phenylindole (DAPI; blue). The samples were (A)lung tissue of 2 days-treated SD rats, (B)lung tissue of 21 days-treated SD rats, (C)brain tissue of 2 days-treated SD rats and (D)brain tissue of 21 days-treated SD rats.

DISCUSSION

BPD is the result of a complex process in which several prenatal and postnatal factors interfere with lower respiratory tract development, leading to severe and lifelong disease (Voynow et al., 2017; Onland et al., 2023). Although advances in perinatal care continue to improve the survival rates of premature infants, they have not consistently resulted in decreased BPD (Onland et al., 2009; Parikh et al., 2007). In addition to chronic lung disease, BPD causes complications that affect growth, cardiovascular health, and neurodevelopment (Majnemer et al., 2000; Schmidt et al., 2003; Natarajan et al., 2012; Lewis et al., 2002). Infants with BPD have higher metabolic demands, a high risk of cardiovascular sequelae including pulmonary arterial hypertension, and an increased risk of poor neurodevelopmental outcomes (Vollsaeter et al., 2015; Caskey et al., 2016). Although numerous studies on BPD have been conducted in recent years, the mechanism underlying lung injury remains unclear.

Additional oxygen supply is often required for the survival of premature infants in the intensive care unit, which simultaneously increases the risk of adverse lung outcomes (Chess et al., 2006; Mizuno et al., 2007; Endesfelder et al., 2019). Early oxygen exposure is one of the most important factors in BPD development (Wang et al., 2019). Hyperoxia leads to excessive generation of ROS and causes oxidative stress (Pan et al., 2018). Antioxidant therapies are an ideal method for improving this process, with the pathological consequences of oxidative stress (McPherson et al., 2019; Welty et al., 2001). Prevention of prematurity, systematic use of nonaggressive ventilator measures, avoidance of supraphysiologic oxygen exposure and administration of surfactant, caffeine and vitamin A can significantly reduce the risk of BPD development (Biniwale et al., 2006; Curstedt et al., 2013; Uberos et al., 2014). Cell therapy is the most promising new measure to address the lung damage caused by BPD (Dobson et al., 2014). MSCs were described over 40 years ago as bone marrow fibroblast-like cells supportive of the hematopoietic compartment. However, the mechanisms by which MSCs exert their beneficial effects remain poorly understood. MSCs are thought to possess potent immunomodulatory and secretory properties and act in a paracrine manner to modulate endogenous repair (Behnke et al., 2020; Emukah et al., 2019).

In this study, we examined whether hyperoxia-induced lung injury be mitigated by intratracheal hUC-MSCs transplantation. The results showed that intratracheal transplantation of hUC-MSCs attenuated lung injuries caused by hyperoxia, such as larger-sized and congestive alveoli in the lung tissue, elevated levels of inflammatory factors, apoptosis activation and increased TUNEL-positive cells. However, the HE staining results on 21 days post-transplantation showed no obvious differences between the groups. Oxidative stress is one of the most predominant causes of BPD. Hyperoxia activates a cascade of hazardous events, including mitochondrial dysfunction, uncontrolled inflammation, reduced autophagy, increased apoptosis, and the induction of fibrosis (Yang et al., 2021). Recent studies have confirmed that MSCs maybe attenuate BPD injury in early than in late administration by inhibiting inflammation response through down-regulation of the p38MAPK signaling pathway (Zhang et al., 2018); and may possess anti-apoptotic/pro-proliferative capacity in the *in vivo* and *in vitro* models of airway cell injury partly through paracrine secretion of stem cell factor (Li et al., 2017). Hence, we hypothesized that the intratracheal transplantation of hUC-MSCs could be a short-term treatment option. Taken together, this study shows that intratracheal transplantation of hUC-MSCs partially inhibit inflammation and apoptosis to mitigated hyperoxia-induced lung injury. Thus, intratracheal transplantation of hUC-MSCs may be amenable to an ideal therapy for BPD. Evidence from phase II clinical trial demonstrated the safety and feasibility of MSC transplantation for BPD in preterm infants (Ahn et al., 2021). However, many

fundamental questions regarding MSC-based therapies remain unanswered. The source, route and timing of MSCs are critical problems, as is the mechanism by which MSCs target BPD. Clinical trials are needed to determine which MSCs are necessary for improving BPD and which may be dispensable and potentially have undesirable consequences.

Our study found that endotracheal transplantation of hUC-MSCs partially attenuated hyperoxia-induced lung injury. Thus, endotracheal transplantation of hUC-MSCs may be an ideal treatment option for patients with BPD. The source, route, dose and timing of action of hUC-MSCs are key issues and further clinical trials are required to avoid possible adverse effects.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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PATIENT CONSENT FOR PUBLICATION

The patient approved the publication on an open-access basis, understood that it could be accessed freely worldwide, and signed the patient consent form on March 17, 2020.

STATEMENTS & DECLARATIONS

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COMPETING INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception and design. The material preparation, data collection and analyses were performed by Ying Qian, Jingchan Yao, Yiping Lin, Hui Zhang and Lan Wang. Jingchan Yao and Mingxing Ding conceived and designed this study. The first draft of the manuscript was written by Ying Qian and all authors commented on previous versions of the manuscript. All the authors have read and approved the final version of the manuscript.

ETHICS APPROVAL

This study was approved by the Medical Ethics Committee, School of Medicine, Jinhua Polytechnic, China.

AVAILABILITY OF DATA AND MATERIAL

The authors confirm that the data supporting the findings of this study are available within the article [and/or its supplementary materials].

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