Human β-Defensin-2 Improves Hyperoxia-Induced Lung Structural and Functional Injury in Neonatal Rats

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background:
Bronchopulmonary dysplasia (BPD) is a major complication of extreme prematurity, characterized by alveolar simplification and pulmonary malfunction. Hyperoxia-induced lung injury in neonatal rats has been used as a model of BPD, as indicated by lung architectural change and alveolar simplification that resembles clinical feature of BPD. β-defensin-2 (BD2) plays an important role in lung diseases by inhibiting inflammation response. However, little is known about its role in BPD. The aim of this study was to determine the effect of human BD2 (hBD2) gene on hyperoxia-induced animal model of BPD.

Material/Methods:
The neonatal rats were exposed to 90% oxygen (O_2) continuously for 14 days to mimic the BPD-like lung injury. These rats were then randomly assigned to the following four groups: in room air (air), in 90% O_2, in 90% O_2 with null adenovirus vector infection (O_2+Ad), and in 90% O_2 with gene therapy through adenovirus transfected hBD2 (O_2+Ad-hBD2). Morphology of lungs, pulmonary function and expression of inflammatory cytokines on P7, P10, P14, and P21 were documented and compared across the 4 groups.

Results:
The overexpression of hBD2 mediated by the adenovirus vector was successfully constructed. hBD2 gene therapy increased hBD2 mRNA expression, increased radial alveolar count (RAC), lung volume and compliance, decreased mean linear intercept (MLI), tissue damping, and elastance. Furthermore, pro-inflammatory cytokines IL-1β, IL-6, and TNF-α were inhibited and anti-inflammatory cytokines IL-10 was increased in the lungs of rats in O_2+Ad-hBD2 group.

Conclusions:
In hyperoxia-induced rat models of BPD, hBD2 promotes alveolarization and improves pulmonary function. The mechanism may contribute in alleviating inflammation response and inhibiting pro-inflammatory factors including IL-1β, IL-6, and TNF-α.

MeSH Keywords:
Alveolar Process • beta-Defensins • Bronchopulmonary Dysplasia • Hyperoxia • Respiratory Function Tests

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Background

With the improvement in survival of extremely premature infants, the incidence of bronchopulmonary dysplasia (BPD) increases rapidly and BPD has become one of the most common complications of extreme prematurity [1]. BPD is characterized by alveolar simplification, pulmonary developmental arrest, and inflammatory and vascular changes. BPD has long-term poor respiratory, cardiovascular and neurodevelopmental consequences that may persist into adulthood, resulting in increased healthcare cost [2]. The clinical efficiency of treatment for BPD, such as glucocorticoids and diuretics, are limited. In addition, glucocorticoids have many adverse effects, especially impaired neurodevelopmental and grow. Preventing or ameliorating BPD has been a challenge to both clinicians and researchers.

In clinical setting, there is need to give premature supplemental oxygen to prevent and treat respiratory failure in preterm infants. Increased levels of supplemental oxygen in the canalicular and early saccular phases of lung development induce the impaired alveolarization and microvascular development; thus, chronic exposures to high oxygen concentrations in preterm infants increase the risk of BPD. Hyperoxia- induced lung injury in neonatal rats has long been used as a model of BPD, as indicated by lung architectural change and alveolar simplification that resembles clinical feature of BPD [2,3].

Inflammation is involved in the pathological process of BPD when the tissues/organs are exposed to hyperoxia [4]. The combination of pre- and post-natal factors initiates an inflammatory process that is mediated by a variety of molecular mediators such as cytokines in clinical BPD. In BPD models of hyperoxia-exposed neonatal rats, the levels of a variety of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, increased [5–7]. Previous studies have demonstrated that some agents, such as etanercept [8], colchicine [9], and caffeine [10], had favorable effects on alveolarization and/or lung function by alleviating inflammation response in hyperoxia-exposed neonatal rats with BPD.

Recently, β-defensin-2 (BD2) has received much attention due to its ability in inducing and recruiting inflammatory and immune cells, which is mainly produced in mucosa epithelial cells of the bronchus, skin and plays an important role in mucosal defense [11]. Studies have shown that the overexpression of BD2 mediated by the lentiviral vector decreased the level of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, and TNF-α, increased basal expression of anti-inflammatory cytokine such as IL-4, IL-10, and IL-13 and protected lung from infection of *Pseudomonas aeruginosa*, but shRNA targeting BD2 aggregated the damage of lung [12]. hBD2 plays an important role in acute lung injury (ALI), pneumonia, pulmonary cystic

fibrosis, chronic obstructive pulmonary disease (COPD) by antimicrobial, regulating immune responses and cytokines [12–15]. These studies suggest the important role of hBD2 in inflammatory-immune response. Currently, little is known about the effect of hBD2 in neonatal BPD.

In this study, we investigated the effect of hBD2 on lung structure, pulmonary function and inflammatory response in hyperoxia-induced BPD rat model.

Material and Methods

Materials

T cells were obtained from the American Type Culture Collection and grown in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Sprague Dawley rats (180 to 200 g) were supplied by Shanghai Laboratory Animal Center. All rats were maintained in specific pathogen-free static cages with a 12-hour light/dark cycle, constant temperature (25±2°C) and 50% relative humidity, and provided ad libitum access to food and water. All procedures were performed according to protocols approved by the Experimental Animal Ethics Committee of Wenzhou Medical University (wydw2013-0072).

Construction of hBD2 expressing vector

The full-length (236 bp) cDNA of hBD2 was obtained by reverse transcription polymerase chain reaction (RT-PCR) with primers: 5'-AGGTCGACTCTAGAGGATCCCGCCACCATGAGGGTCTTGTATCTC-3' and 5'-TCCTTGTAGTCCATACCTGGCTTTTTGCAGCATTTTG-3'. The PCR product was then ligated to the linear adenoviral vector (BamHI/AgeIdigestion, Shanghai Genechem Co., Ltd., China) with green fluorescent protein (GFP) to generate Ad-hBD2. The ligation mixture was transformed into the competent *Escherichia coli* DH5α strain and the positive clones were selected. The plasmid was extracted and then analyzed by polymerase chain reaction (PCR). The result of sequencing was consistent with anterior hBD2. According to the manufacturer's instructions, the expressing adenoviral vector and adenovirus package plasmid mix were co-transfected into 293T producer cells using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). The transfection efficiency was evaluated by fluorescent microscopy of enhanced GFP expression and viral titer was measured by gradient dilution.

Establishment of hyperoxia animal models

Hyperoxia exposure was performed using procedures as described previously [16]. In brief, the neonatal rats were exposed to air in the room (21% O_2) or to hyperoxia (90% O_2)
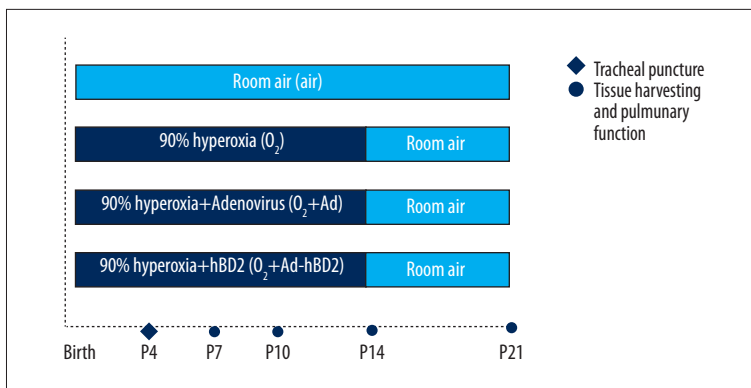
Figure 1. Experimental procedure

in a sealed Plexiglas chamber with continuous O_2 monitoring. Dams were rotated (air to hyperoxia and vice versa) every 24 hours to obviate the effects of hyperoxia to the adult female rats. After hyperoxic exposure for 14 days, the animals were transferred to room air for another 7 days. In this study, the neonatal rats were randomly assigned as follows: in room air (air), in 90% oxygen (O_2), in 90% O_2 with null adenovirus vector infection (O_2+Ad), in 90% O_2 with adenovirus transfected hBD2 (O_2+Ad-hBD2, Figure 1).

Administration of adenovirus intratracheal *in vivo*

The rats in the O_2+Ad-hBD2 group were anesthetized by an intraperitoneal injection of chloral hydrate anesthesia (4%, 0.5 mL/100 g). Subsequently, the rats were incised through the neck exposing their trachea. After that, 1E+7 PFU/mL hBD2 adenovirus solution (25 µL) was delivered into the airways through intratracheal puncture with a short, 30-gauge needle (Becton-Dickinson) on postnatal day 4 (P4). The rats in O_2+Ad group were treated with same dose of null adenovirus.

Measurement of hBD2 mRNA in rat lungs

hBD2 gene expressions in pulmonary on P7, P10, P14, and P21 were analyzed by quantitative real-time (qRT)-PCR). Briefly, total RNA was isolated from the whole lung tissue using TRIzol regent (PuFei, Shanghai, China) and reversely transcribed into cDNA using M-MLV Reverse Transcriptase kit (Promega, USA). The mRNA level of hBD2 was detected by qPCR using hBD2 primer pairs: 5'-TTCCTGATGCCTCTTCCA-3' and 5'-GAGACCACAGGTGCCAAT-3' as well as control β-actin primer pairs: 5'-TGACTTCAACAGCGACACCCA-3' and 5'- CACCCTGTTGCTGTAGCCAAA-3', respectively. The assay was carried out using the 2-step qPCR system (Roche, Basel, Switzerland) according to the manufacturer's instructions. First, the reaction mixture was incubated at 42°C for 60 minutes and then at 70°C for 10 minutes. Second, an aliquot (0.6 µL) of the resulting cDNA was amplified by PCR in a 12 µL reaction. PCR was performed at the conditions of 94°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and

extension at 72°C for 2 minutes. β-actin was used as a reference to normalized hBD-2 levels by the $2^{-\Delta\Delta Ct}$ method.

Assessment of pulmonary function

Invasive pulmonary function test was performed using the flexiVent system (SCIREQ, Montreal, Quebec, Canada). Neonatal rats were anesthetized with an intraperitoneal injection of Pentobarbita (1%, 0.01–0.02 mL/g body, Sigma, America) to suppress spontaneous breath. After tracheostomy was performed, a needle (20-gauge for neonatal rats at P7, 12-gauge for other time points) was inserted into the airway and connected to the flexiVent. The following assays were performed automatically: 1) total lung capacity perturbation (mean displaced volume, V end/wt); 2) snapshot perturbation (dynamic resistance, Rrs); dynamic compliance (Crs) and dynamic elastance (Ers); 3) prime-8 forced oscillation (central airway resistance, Rn), tissue damping (G) and elastance (H); and 4) pressure-volume loops: total lung capacity (A) and static compliance (Cst). The perturbation was performed until 3 acceptable measurements (coefficient of determination > 95%) were recorded in each individual subject, of which an average was calculated.

Lung morphology

After pulmonary function testing, anesthetized neonatal rats were euthanized. The left lungs were fixed in 4% paraformaldehyde solution through the trachea under a constant pressure of 25 cm H_2O for 5 minutes. The trachea was then ligated, and the lungs were immersed in paraformaldehyde at 4°C for 24 hours. The lung sections (4 µm) were stained with hematoxylin and eosin (H&E) to reveal the lung morphometry including radial alveolar count (RAC) and the mean linear intercept (MLI). The RAC measurement was performed according to Cooney and Thurlbeck [17].

Cytokine measurements in lung tissue

After addition of lysis buffer, the frozen lung tissues were homogenized on ice using a homogenizer and the supernatant
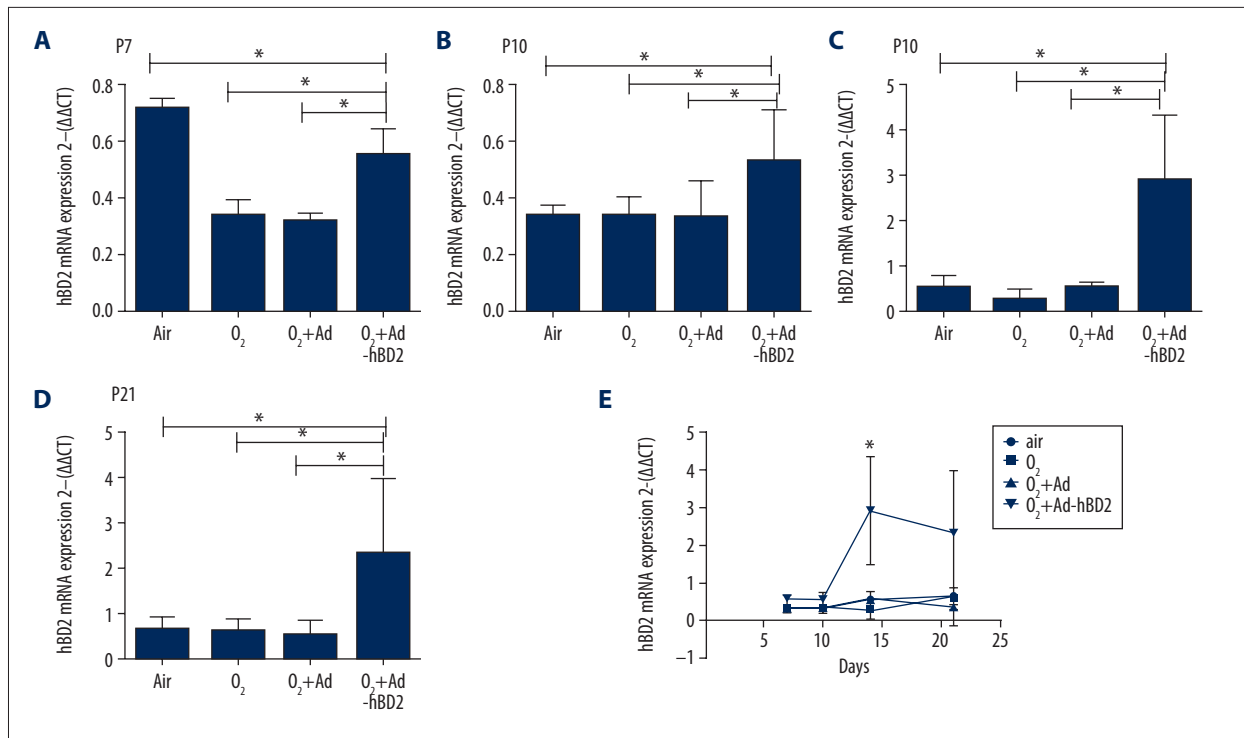
Figure 2. Relative hBD2 mRNA levels in rats lung on different days. The results showed the expressions of hBD-2 mRNA in the air, O_2 and O_2+Ad groups were very low. However, its expression was much higher in the O_2+Ad-hBD2 groups on P7, P10, P14, and P21 (**A–D**) (all $P<0.05$). The line graph showed that the highest mRNA expression of hBD2 was on P14 in the O_2+Ad-hBD2 groups and sustained at high levels subsequently (**E**, * $P<0.05$, n=6).

was collected for the following assays. The levels of IL-1β, IL-6, TNF-α, and IL-10 were measured with enzyme linked immunosorbent assay (ELISA) kit (RayBiotech, Inc., Guangzhou, China). Quantification of relative cytokine expression was normalized with BSA protein assay and expressed as pg/mL of cytokine.

Statistics analysis

Statistical analysis was performed using the SPSS 25.0 software. Values were expressed as the mean ± standard deviation relative to that of vehicle for each condition. All data were analyzed using one-way ANOVA followed by least significant difference tests for between group comparisons. A P value <0.05 was considered statistically significant.

Results

hBD2 expressing vectors

Based on sequencing results, the hBD2 expressing vector was successfully constructed 24 hours after transfection. The fluorescent microscopy assay proved the high transfection efficiency of hBD2 expressing vectors. In addition, the viral titer was also measured, with the concentration of 1E+9 PFU/mL.

hBD2 gene was efficiently delivered into the pulmonary of rats

There was little hBD2 mRNA expression in the air group, O_2 group and the null vector group (O_2+Ad). However, significant increment in hBD2 mRNA expressions were detected in the lung tissue on P7, P10, P14, and P21 (all the $P<0.05$ compared to other groups, respectively) (Figure 2A–2D) in adenovirus transfected hBD2 group (O_2+Ad-hBD2). The line graph showed that the highest mRNA expression of hBD2 was on P14 in the O_2+Ad-hBD2 group and sustained at high levels subsequently, and mRNA expressions of hBD2 in other groups had no significant changes (Figure 2E).

hBD2 improved pulmonary function in hyperoxia-induced BPD rat model

Hyperoxia impaired the lung function *in vivo* and hBD2 significantly reduced the effect of hyperoxia and improved the lung function. As shown in Table 1, Ad-hBD2 decreased H on P7. It further increased Crs, decreased Ers, G, and H on P10. hBD2 effectively increased lung volume on P14 by increasing mean displaced volume, A and Crs, decreasing H, Ers, and G. However, Rrs, Cst, and Rn were not affected by hBD2 therapy. On P21, Ers was decreased when treated with Ad-hBD2 compared to that in the control group.

Table 1. Invasive lung function results.

flexiVent parameter	Air	O2	O$_2$+Ad	O$_2$+Ad-hBD2
P7				
Mean displaced volume				
Vend/wt, mL/kg	27.33±7.09	27.25±5.60	26.50±2.35	27.75±2.36
Snapshot				
Rrs, cm H$_2$O/mL	2.22±0.58	3.16±0.40*	3.70±0.82**	3.14±0.89*
Ers, cm H$_2$O/mL	49.44±12.56	87.56±8.44**##	86.24±15.63**##	56.15±10.09
Crs, mL/cm H$_2$O	0.099±0.049	0.011±0.003**	0.011±0.004**	0.015±0.004**
Prime-8				
Rn, cm H$_2$O/mL	0.944±0.416	1.504±0.328*	1.662±0.322**	1.488±0.234*
G, cm H$_2$O/mL	8.91±1.30	13.15±3.17*#	14.34±4.84*#	9.35±1.35
H, cm H$_2$O/mL	25.07±7.05	44.18±14.03*#	47.37±13.47*#	28.14±10.09
Pressure volume loop				
A, mL	0.53±0.17	0.43±0.08	0.34±0.07	0.47±0.24
Cst, mL/cm H$_2$O	0.019±0.004	0.014±0.005	0.015±0.008	0.015±0.008
P10				
Mean displaced volume				
Vend/wt, mL/kg	28.33±3.08	25.79±6.60	23.67±3.27	27.83±5.95
Snapshot				
Rrs, cm H$_2$O/mL	1.45±0.32	1.89±0.081**	1.99±0.31**	1.68±0.21
Ers, cm H$_2$O/mL	53.75±6.23	69.33±12.70*#	77.06±14.53**##	54.27±6.83
Crs, mL/cm H$_2$O	0.018±0.002	0.012±0.003**##	0.013±0.003**#	0.017±0.004
Prime-8				
Rn, cm H$_2$O/mL	0.531±0.144	0.714±0.090	0.736±0.321	0.665±0.202
G, cm H$_2$O/mL	9.04±0.82	11.48±2.24*#	11.39±1.35*#	9.25±0.93
H, cm H$_2$O/mL	30.97±5.96	49.08±15.53*#	47.64±8.43*#	31.79±5.82
Pressure volume loop				
A, mL	0.64±0.38	0.31±0.03*	0.35±0.09*	0.39±0.05*
Cst, mL/cm H2O	0.053±0.018	0.022±0.002*	0.019±0.003*	0.022±0.003*
P14				
Mean displaced volume				
Vend/wt, mL/kg	27.50±3.60	20.58±4.13*#	18.28±2.68**#	23.20±5.30
Snapshot				
Rrs, cm H$_2$O/mL	1.09±0.57	1.46±0.70*#	1.68±0.86*#	1.17±0.49
Ers, cm H$_2$O/mL	48.12±25.78	79.51±26.10*#	96.25±24.00**##	50.83±16.35
Crs, mL/cm H$_2$O	0.024±0.013	0.014±0.005*#	0.012±0.004*#	0.022±0.006

Table 1 continued. Invasive lung function results.

flexiVent parameter	Air	O2	O$_2$+Ad	O$_2$+Ad-hBD2
Prime-8				
Rn, cm H$_2$O/mL	0.079±0.068	0.539±0.428*	0.859±0.458**	0.317±0.221
G, cm H$_2$O/mL	9.20±2.71	11.37±2.37*#	11.66±2.10*#	9.00±1.48
H, cm H$_2$O/mL	30.63±13.42	48.41±16.01*#	48.56±15.24*#	24.77±6.65
Pressure volume loop				
A, mL	0.45±0.16	0.36±0.08*#	0.33±0.07*#	0.44±0.06
Cst, mL/cm H$_2$O	0.066±0.041	0.026±0.013*	0.015±0.003*	0.042±0.037
P21				
Mean displaced volume				
Vend/wt, mL/kg	33.66±13.59	19.59±6.43*#	20.31±4.51*#	31.92±4.69
Snapshot				
Rrs, cm H$_2$O/mL	1.24±0.21	5.83±3.72*#	5.44±2.45*#	1.04±0.38
Ers, cm H$_2$O/mL	19.60±4.44	32.07±10.67***#	28.84±5.38*#	20.06±4.08
Crs, mL/cm H$_2$O	0.186±0.063	0.035±0.013*#	0.038±0.008*#	0.167±0.041
Prime-8				
Rn, cm H$_2$O/mL	0.332±0.102	0.676±0.145*	0.713±0.078*	0.495±0.255
G, cm H$_2$O/mL	3.24±0.50	6.07±2.40*#	7.28±1.99*#	4.03±0.85
H, cm H$_2$O/mL	14.42±2.64	27.02±6.91*#	25.19±5.02*#	16.12±2.06
Pressure volume loop				
A, mL	0.96±0.022	0.61±0.16*#	0.56±0.25*#	0.92±0.32
Cst, mL/cm H$_2$O	0.083±0.016	0.038±0.020*	0.041±0.017*	0.052±0.017*

Date presented as mean ±SEM. * $P<0.05$, ** $P<0.01$, versus air group. # $P<0.05$, ## $P<0.01$, versus O$_2$+Ad-hBD2, n=6. Rrs – dynamic resistance; Crs – dynamic compliance; Ers – dynamic elastance; Rn – central airway resistance; G – tissue damping; H – elastance; A – total lung capacity; Cst – static compliance.

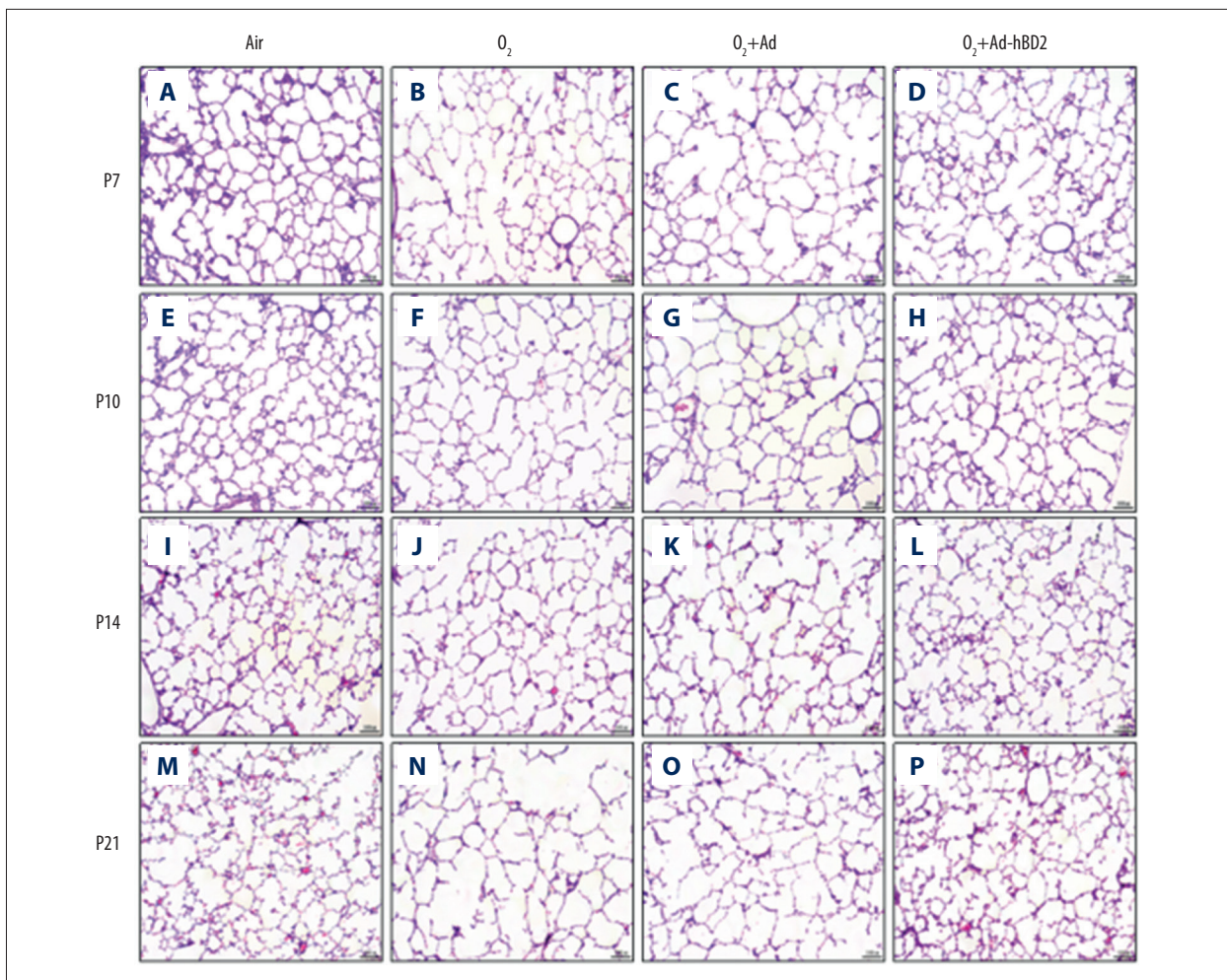## hBD2 promoted the alveolarization in hyperoxia-induced BPD rat model

The exposure of newborn rats to hyperoxia (90%) from birth to P14 impaired alveolar growth by increasing air spaces and reducing alveolar. However, Ad-hBD2 treated animals maintained normal alveolarization (Figure 3A–3P). On P7, the RAC value in the air group was higher than the other three groups ($P<0.05$). On P10, P14, and P21, the RAC values in the air group were higher than those in the O$_2$ and O$_2$+Ad groups (all $P<0.05$), the RAC values in the O$_2$+Ad-hBD2 group increased significantly and were higher than those in the O$_2$ and O$_2$+Ad groups (all $P<0.05$), but were similar to those in the air group (Figure 4A–4D). The results also showed that the RAC values increased as times went by (Figure 4I). In addition, the MLI value

on P10, P14, and P21 in air and O$_2$+Ad-hBD2 groups significantly decreased compared to those on P7, but there were no substantial changes in the O$_2$ and in O$_2$+Ad groups (Figure 4E–4H, 4J). The RAC and MLI assays showed that Ad-hBD2 therapy significantly improves the lung architecture compared to the O$_2$ exposed and O$_2$+Ad exposed groups.

hBD2 attenuated inflammation response in hyperoxia-induced BPD rat model

The trend of pro-inflammatory cytokines expressions did not change significantly in the air group. However, the expressions of TNF-α, IL-1β, and IL-6 in newborn rats were increased after exposure to hyperoxia on P7, P10, P14, and P21 compared to the air group, and Ad-hBD2 therapy significantly decreased

Figure 3. hBD2 promoted the alveolarization in hyperoxia-induced BPD rats model. The exposure of newborn rats to hyperoxia (90%) from birth to P14 impaired alveolar growth by increasing air spaces and reducing alveolar. However, Ad-hBD2 treated animals restored normal alveolarization (**A–P**). All images were magnified 100×, bar=100 μm.

the levels of these inflammatory cytokines. Moreover, IL-10 also increased in the four groups except air group on P7, P10, P14, and P21. In addition, the IL-10 levels seem much higher in Ad-hBD2 therapy group than in O_2 and O_2+Ad groups (P<0.05) (Figure 5). The IL-10 levels were increased on P10 and P14 in O_2+Ad-hBD2 compared to P7, followed by a gradual decreased on P21 (Figure 6).

Discussion

In this study, we demonstrate that hBD2 attenuated hyperoxia-induced lung injury in neonatal rats by promoting alveoli maturation, and improving pulmonary function. The mechanism may contribute in alleviating inflammation response and inhibiting pro-inflammatory factors such as IL-1β, IL-6, and TNF-α.

Alveolarization is the mature process of alveoli, which is the principal gas exchange unit. Chronic exposure to high oxygen concentrations in canalicular and early saccular phases of preterm infants can impair alveolarization and microvascular development, resulting in BPD, but low saturation targets may increase death [1]. Neonatal rats exposed to hyperoxia had induced lung architectural changes that resembled clinical features of BPD, which is alveolar simplification [3]. We found that newborn rats exposed to hyperoxia from birth to P14 showed impaired alveolar growth, manifested by enlarging air spaces, and reducing alveolar (as indicated by the increase of MLI and the decrease of RAC), but hBD2 treated animals restored normal alveolarization, and could be detected on P10 and continuously on P21.

Studies in humans suggest that the impairment of pulmonary function in a BPD child will continue into adolescence and even into adulthood [18,19]. In the studies of lung functional
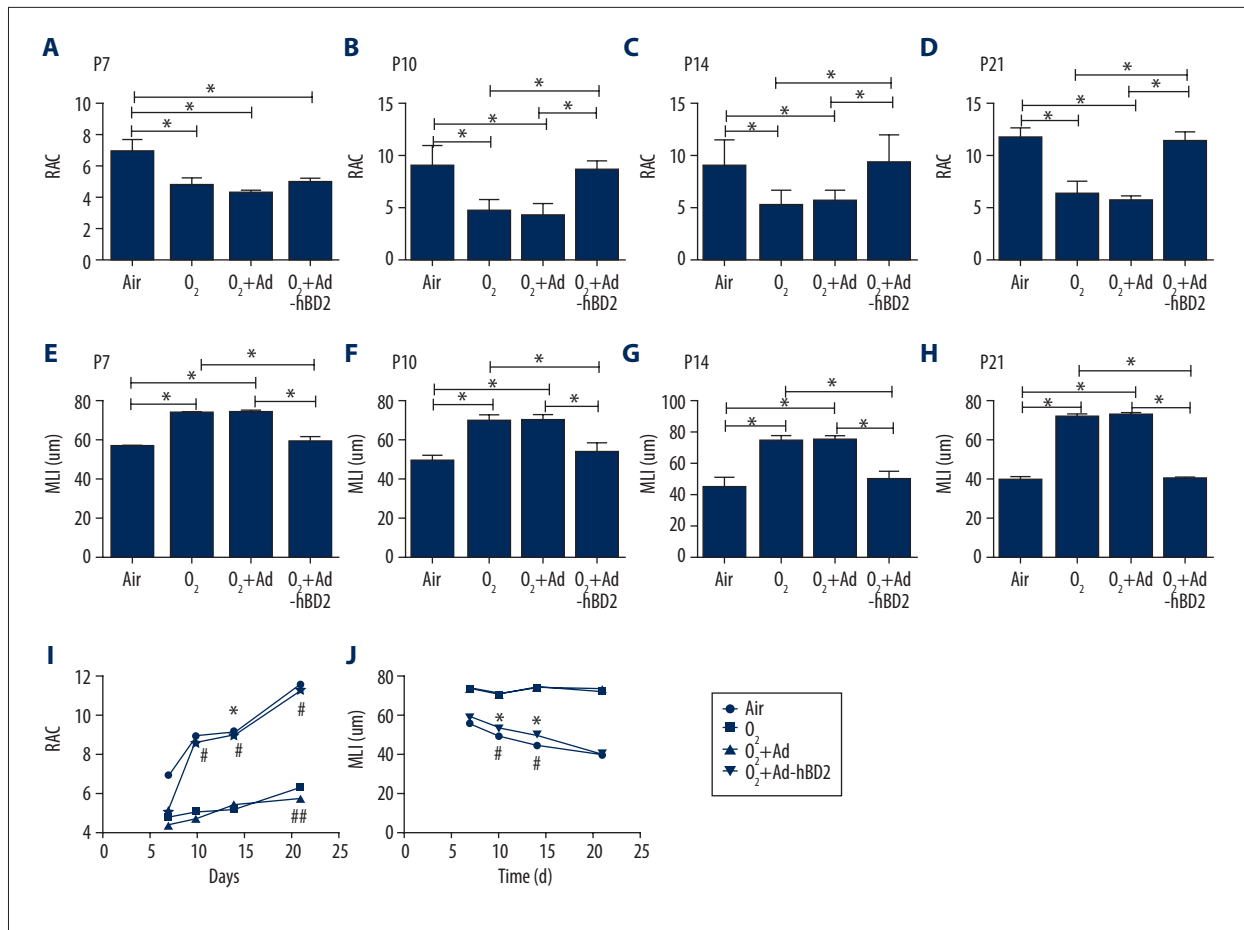
Figure 4. The RAC and MLI assays showed that Ad-hBD2 therapy significantly improves the lung architecture compared to O_2 exposed and O_2+Ad exposed groups. Compared to P7, the RAC value was significantly increased on P14 and P21 in the air group. Moreover, the RAC value was also increased on P10, P14, and P21 in the O_2+Ad-hBD2 group, significantly increased on P21 in the O_2 and O_2+Ad groups (**I**). The MLI on P10, P14, and P21 in the air and O_2+Ad-hBD2 groups were significantly decreased compared to that on P7, but there were not obvious changes in the O_2 and in O_2+Ad groups. * P<0.05 between groups in Figure **A–H**, * P<0.05 compared to that on P7 in air group and **P < 0.05 compared to that on P7 in O_2 group in Figure **I**, **J**. * P<0.05 compared to that on P7 in O_2+Ad-hBD2 group and # P<0.05 compared to that on P7 in air group, n=6.

assessment in animal model of BPD, Nagatomo et al. [10] reported that preterm rabbits exposed to hyperoxia induced lung functional damage by decreasing lung volume and Crs, increasing Rrs, Ers, G, and H. Hansmann et al. [20] found that lung function was remarkably impaired in hyperoxia-induced mouse BPD with abnormal increase in airway resistance and decrease in dynamic lung compliance at 6 weeks of age. In our study, hyperoxia induced increase in airway resistance, tissue damping, and elastance were detected in Rrs, Rn, Ers, G, and H early on P7 (continuously on P21), a decrease lung volume on P10 (continuously on P21) and mean displaced volume on P14 and P21, a decrease dynamic compliance on P7 (continuously on P21) and static compliance on P10 (continuously on P21). Therefore, compared to lung volume and Cst, the hyperoxia-induced BPD model had more influence on Rn, G, H, and Crs. The hBD2 gene could reverse the impairment of G, H, Crs,

and Ers early on P7 (continuously on P21), could reverse the impairment of Crs began on P10 to P21, could reverse the impairment of mean displaced volume, Rrs and A began on P14 to P21, but Rn could not be restored. The invasive lung function testing provides comprehensive information, allowing a differentiation between airway and parenchymal changes and to exclude the effect of the chest wall when using the constant phase model.

Inflammation is an important risk factor in the pathogenesis of BPD through favoring pro-inflammatory in the imbalance between pro-inflammatory and anti-inflammatory mechanisms [21]. In the present experiment, hBD2 gene promoted alveoli maturation, improved pulmonary function, decreased pro-inflammatory factors IL-1β, IL-6, and TNF-α and increased anti-inflammatory factors IL-10. The results suggest that hBD2
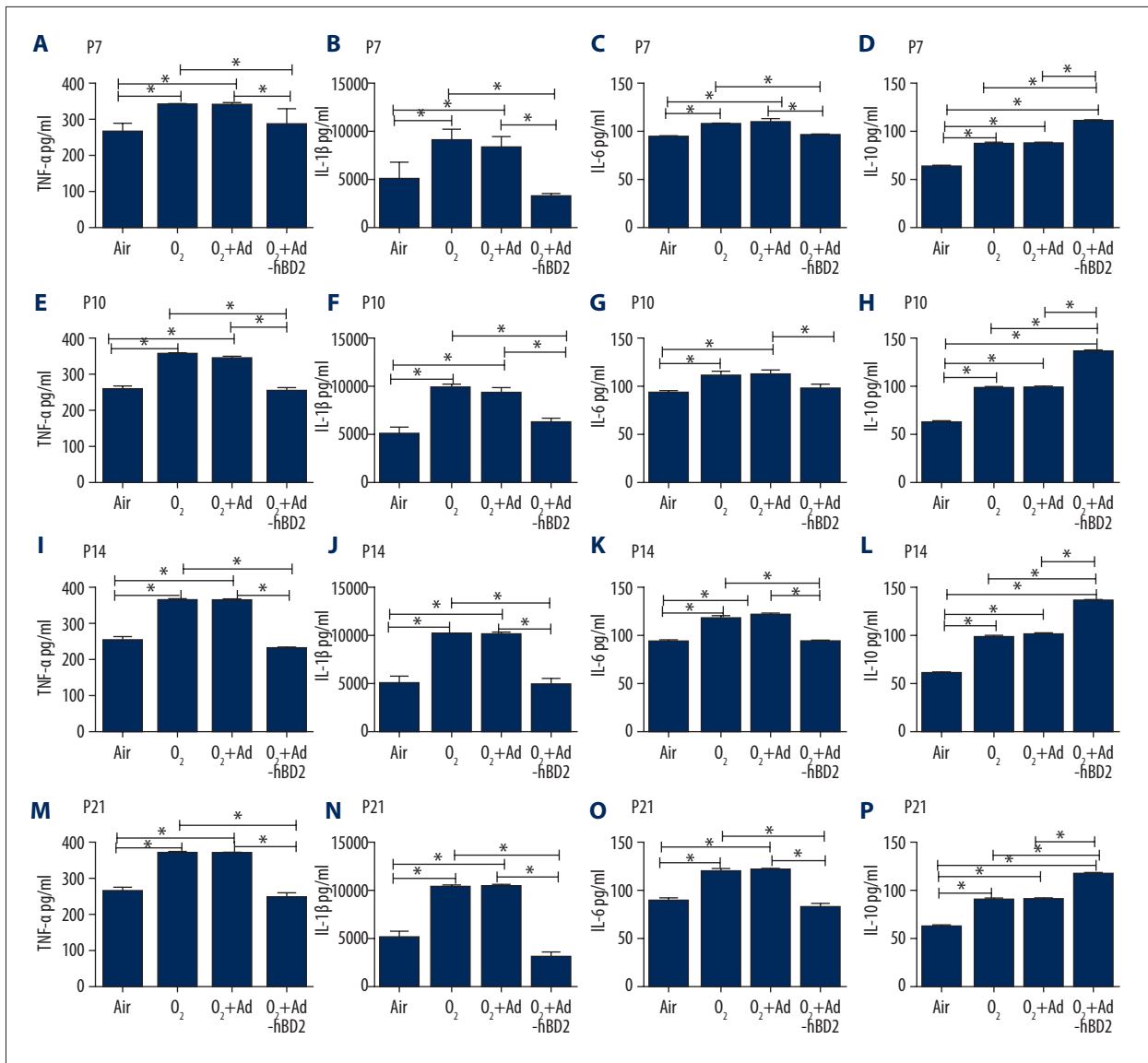
Figure 5. (**A–P**) hBD2 attenuated inflammation response in hyperoxia-induced BPD rats model. The expressions of TNF-α, IL-1β, and IL-6 in newborn rats were dramatically increased after exposure to hyperoxia on P7, P10, P14, and P21 compared to those in the air group. However, Ad-hBD2 therapy significantly decreased the levels of these inflammatory cytokines. Meanwhile, IL-10 also increased in the four groups except the air group on P7, P10, P14, and P21, and the IL-10 levels seem to be much higher in Ad-hBD2 therapy group than in O$_2$ and in O$_2$+Ad groups, * P<0.05, n=6.

may protect from BPD by regulating inflammatory mechanisms in rats.

As an important regulator of cellular proliferation, differentiation, inflammation and angiogenesis, the nuclear factor kappa B (NF-κB) family of transcription factors regulates many biologic processes that are essential for alveolarization [22], and regulates the transcription of pro-inflammatory and anti-inflammatory cytokines such as IL-6, TNF-α, IL-1β, and IL-10 [23]. Importantly, NF-κB is actively involves in the regulation of hBD2 or rBD2 expression [24]. In this study, hBD2 gene promoted

alveolarization and attenuated inflammation with decrease of IL-1β, IL-6, and TNF-α, which may be regulated by NF-κB. Therefore, NF-κB may involve in the regulation of hBD2 in protection of BPD including the lung cell proliferation, apoptosis and inflammation. A more comprehensive understanding of the potential mechanisms is needed.
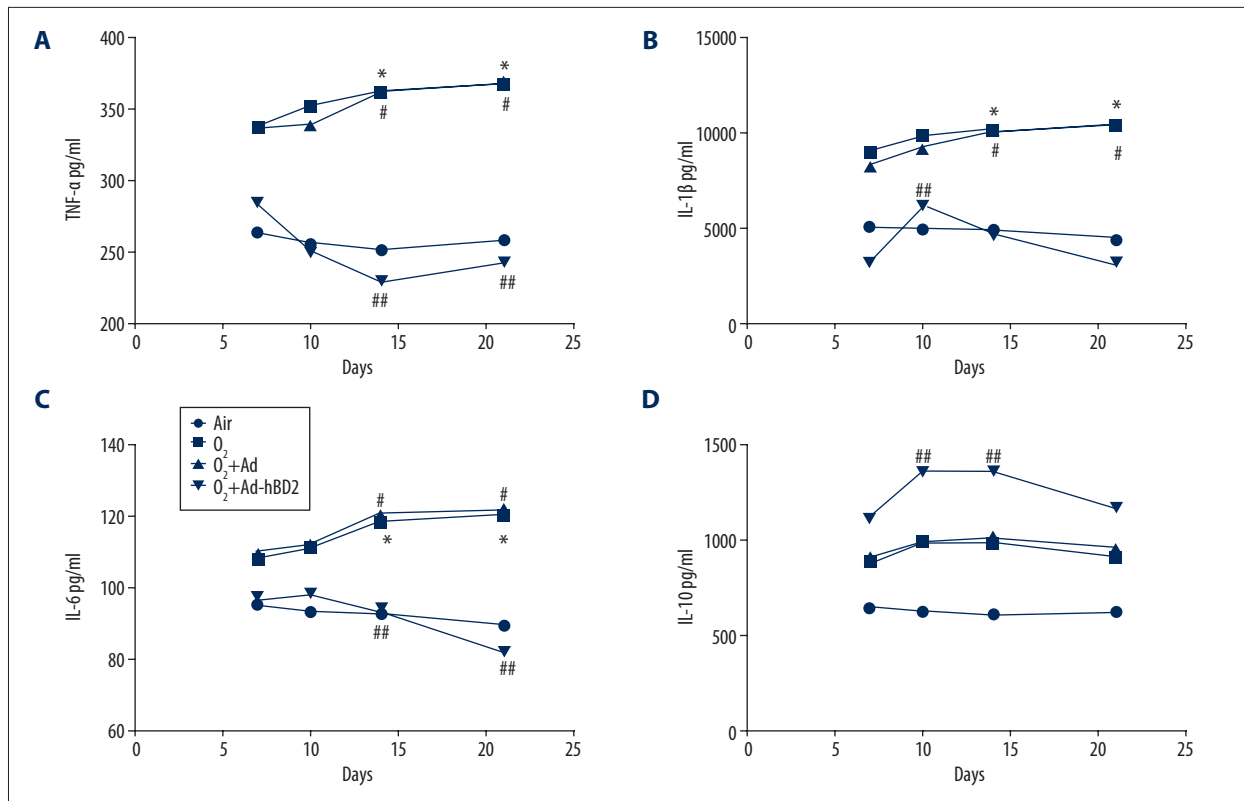
Figure 6. (**A–D**) The therapy of inflammatory cytokine expressions showed that there were no significant changes in air group, but pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 continued increased in the rats exposed to hyperoxia. These increases could be effectively inhibited by Ad-hBD2 therapy, especially on P14 and P21. The IL-10 levels increased on P10 and P14 on O$_2$+Ad-hBD2 group. (compared to P7, * $P<0.05$ for the O$_2$ group, # $P<0.05$ for the O$_2$+Ad group, ## $P<0.05$ for the O$_2$+Ad-hBD2 group), n=6.

Conclusions

hBD2 gene therapy improves alveolarization, pulmonary function and attenuates inflammation in the lung of hyperoxia-induced rats. Hyperoxia-induced lung injury has been a focus of BPD research, which emphasizes the potential of hBD2 in treating BPD in preterm infants. Further studies are needed to understand the potential mechanism involved in NF-κB.

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