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Expression of transforming growth factor-β1 in neonatal rats with hyperoxia-induced bronchopulmonary dysplasia and its relationship with lung development

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ABSTRACT. The aim of this study was to detect the expression of transforming growth factor-β1 (TGF-β1) in neonatal rats with hyperoxia-induced bronchopulmonary dysplasia (BPD) and to explore its relationship with lung development. Forty-eight rats (2-3 days old) were randomly divided into a hyperoxia group and a control group (N = 24) which were then fed in ≥95% oxygen atmosphere and air, respectively. On the 1st, 3rd and 7th days of hyperoxia exposure, morphological changes of lung tissues were observed under an optical microscope. TGF-β1 mRNA and protein levels in lung tissues were detected by real-time quantitative polymerase chain reaction and western blot, respectively. With increasing time of hyperoxia exposure, the hyperoxia group gradually suffered from pathological changes such as poor development of lung tissues, alveolar simplification, decrease in the number of alveoli, and hindered pulmonary microvascular

development. On the 7th day of hyperoxia exposure, TGF-β1 mRNA and protein levels (relative to β-actin) of the hyperoxia group (0.34 ± 0.19 and 0.21 ± 0.09, respectively) were significantly lower than those of the control group (0.83 ± 0.45 and 0.57 ± 0.45, respectively; $P < 0.05$). TGF-β1 participates in the pathogenesis of BPD as an important regulatory factor during pulmonary vascular development.

Key words: Transforming growth factor-β1; Hyperoxia; Bronchopulmonary dysplasia; Lung development

INTRODUCTION

Bronchopulmonary dysplasia (BPD) is the most common chronic lung disease of premature infants and is caused by mechanical ventilation and oxygen therapy after acute respiratory distress. In some extreme cases it may lead to severe complications. The mortality rate of BPD is over 30% because of chronic lung dysfunction and lung dysplasia (Balasubramaniam et al., 2007; Mailaparambil et al., 2010). Increase in the survival rate of premature infants can be attributed to progress in mechanical ventilation techniques, application of exogenous surfactants and glucocorticoids, and improvements in nutritional support and nursing. However, the incidence rate of BPD continues to rise annually, which seriously affects the survival and quality of life of newborns (Gortner et al., 2013). Currently, BPD is pathologically typified by alveolar and pulmonary vascular dysplasias. Most previous studies have focused on alveolar but not pulmonary vascular development (Xu et al., 2011). As an essential factor that regulates cell proliferation and differentiation, transforming growth factor-β1 (TGF-β1) can promote the proliferation and phenotypic modulation of vascular smooth muscle cells and fibroblasts, and is closely associated with the onset of many types of cardiovascular diseases (Tahira et al., 2002). In animal models, TGF-β1 can induce the gene expression and secretion of vascular endothelial growth factor that, as we know, is a key signaling factor for angiogenesis and vascular remodeling (Wen et al., 2003). In this study, we detected the expression of TGF-β1 in the lung tissues of neonatal rats with hyperoxia-induced BPD and explored its relationship with lung development.

MATERIAL AND METHODS

Experimental animals and modeling

Four clean-grade, healthy pregnant Sprague-Dawley rats (Experimental Animal Center of Guangzhou Medical University, China) naturally gave birth to 55 neonatal rats from which 48 were selected (2-3 days old, either sex) and randomly divided into a hyperoxia group and a control group (N = 24, each).

An oxygen tank was made by our group, on which an air inflow hole, an air outflow hole and an oxygen-measuring hole were set. The air inflow hole was connected to oxygen and the oxygen-measuring hole was connected with a CY-100 digital oxygen measurement device (Jiande Lida Instruments Factory, Zhejiang Province, China). Solid barium hydroxide was put in the oxygen tank to adsorb carbon dioxide, with the temperature maintained at 22°-26°C and the humidity kept at 65-75%. For the hyperoxia group, 24 neonatal rats and 2 female

rats were placed inside the oxygen tank, where oxygen flow was controlled at 6 L/min and the concentration was maintained ≥95% and continuously monitored. For the control group, 24 neonatal rats and 2 female rats were put inside a standard rat cage and continuously fed in the same room. Drinking water and food were added at a fixed time every day. Additionally, bedding and solid barium hydroxide were refreshed when needed. The growth, development, respiration, and activities of the rats were recorded. To prevent the female rats from oxygen poisoning-induced decreases in feeding and nursing abilities, they were switched on a regular basis. On the 1st, 3rd, and 7th days, 8 neonatal rats were randomly selected from each group and sacrificed after anesthesia through intraperitoneal injection of 10% chloral hydrate (8-10 mg/kg), after which lung tissues were sampled.

Sampling and treatment

The neonatal rats were sacrificed after anesthesia. After the thoracic cavity was scissored open, bilateral lungs were rapidly taken out and rinsed repeatedly with normal saline. Then, tissues of the right lung were put in an Eppendorf tube, frozen with liquid nitrogen, and stored at -80°C before real-time quantitative polymerase chain reaction (PCR) and western blotting. Tissues of the left lung were fixed in 4% paraformaldehyde for 24 h, washed with water, dehydrated with serial concentrations of ethanol, made transparent with dimethylbenzene, paraffin-embedded, and prepared into 5-μm thick sections.

Morphological observation of lung tissues

The sections were stained with hematoxylin and eosin (H&E), deparaffinized, hydrated, stained, sealed, and mounted under a light microscope to observe the morphological changes of lung tissues. Ten sections were selected from each sample and 10 visual fields were randomly selected from each section. Alveolar number, diameter, area, and septal thickness were measured with the Image Pro-Plus (IPP) software (Media Cybernetics, USA).

Detection of TGF-β1 mRNA levels in lung tissues

TGF-β1 mRNA levels were detected by real-time quantitative PCR. Total RNA was extracted from lung tissues with a one-step method using TRIzol reagent and reverse-transcribed into cDNA with a reverse transcription kit (TaKaRa, Japan) for PCR amplification. The amplified products were resolved on 2% agarose gel electrophoresis, stained with ethidium bromide, and observed under a UV lamp. Primers for TGF-β1: forward, 5'-CTTTGTACAACAGCACCCGC-3', reverse, 5'-GTCAAAAGACAGCCACTCAGG-3', resulting in an amplified product of 135 bp. Primers for β-actin (internal reference): forward, 5'-ACGGTCAGGTCATCACTATC-3', reverse, 5'-TGCCACAGGATTCCATACC-3', resulting in an amplified product of 109 bp. The PCR mixture (25 μL) consisted of 12.5 μL Premix Ex Taq (2X), 0.5 μL PCR forward primer (10 μmol), 0.5 μL PCR reverse primer (10 μmol), 1 μL fluorescent probe solution, 0.5 μL ROX reference dye II (50X), 2 μL template (cDNA solution) and 8 μL dH_2O. PCR conditions were: denaturation at 95°C for 5 min; 40 cycles of 95°C for 5 s and 52°C for 20 s; and extension at 72°C for 5 min. Relative mRNA levels of each gene were obtained based on cycle threshold and the relative standard curve. Relative mRNA expression levels are represented by the ratio of the content of the target gene to that of the internal reference gene (β-actin).

Detection of TGF-β1 protein expression in lung tissues

TGF-β1 protein expression levels in lung tissues were detected by western blot. Total protein was extracted from lung tissues, the content of which was measured by plotting a standard curve with the BCA method. Then, the protein was separated by SDS-PAGE and transferred to a PVDF membrane by a semi-dry procedure. Afterwards, the membrane was blocked in 1X TBS containing 5% skimmed milk for 1 h, and incubated with rabbit anti-rat TGF-β1 and β-actin primary antibodies (Abcam, USA) (1:1000 dilution in 5% skimmed milk) at room temperature for 1 h. The next day, the membrane was incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG secondary antibody and HRP-labeled anti-biotin antibody (1:1000 dilution in 5% skimmed milk) at room temperature for 1 h. The target proteins were detected by the chemiluminescence method. The membrane was then exposed to X-ray film, fixed, and scanned by the Image-Pro Plus Version 6.0 software (Media Cybernetics, USA) to quantitatively analyze the gray values of the blots. Relative protein expression level was represented by the ratio of gray value of the target protein to that of the internal reference protein.

Statistical analysis

All data were analyzed by SPSS13.0 (Chicago, IL, USA). The categorical data are reported as means ± standard deviation. Intergroup comparisons for the data conforming to normal distribution were performed by two independent sample *t*-tests. Comparisons among multiple groups were conducted with one-way analysis of variance. Pairwise comparisons were carried out by using least significant different *t*-test. Intergroup comparisons for the data not conforming to normal distribution were performed by Wilcoxon rank sum test. $P < 0.05$ was considered statistically significant.

RESULTS

General state of animals

The control group rats grew normally, with the body weight and body length increasing by 2-3 g/day and 1.0-1.5 cm/day, respectively. With extended time of hyperoxia exposure, the hyperoxia group gradually became emaciated and sluggish, with the body weight and body length rising slowly. Two rats in the hyperoxia group died on the 5th and 6th days, respectively. The total mortality rate was 8.33%. The dead rats were discarded and lung tissues were not sampled.

Morphological changes of lung tissues

Under a low-power microscope (100X magnification), lung tissues of the control group did not undergo obvious pathological changes. The alveoli were clearly structured and uniformly sized. The alveolar space was small, without liquid or inflammatory exudate, and the alveolar septum was thick. In the hyperoxia group, lung tissues had disordered structures. With increased time of hyperoxia exposure, the alveolar wall became attenuated, and the alveoli decreased and became structurally simplified and non-uniformly sized. Some alveoli

fused and became enlarged. The alveolar area was reduced and the diameter of alveolar space evidently increased. There was exudation of a small number of inflammatory cells inside the alveolar space and a thin alveolar septum. Since interstitial lung fibroblasts and collagen-like substance increased locally, differentiation and development of the lung were blocked (Figure 1). Under a high-power microscope (400X magnification), the control group had integral alveolar walls and there was no evidence of exfoliated cells inside the alveoli. In contrast, alveoli of the hyperoxia group had erythrocytes, macrophage exudate, and exfoliated lung epithelial cells. Additionally, the alveolar septum was thickened, interstitial cells increased, and small blood vessels dilated and became congested (Figure 2).

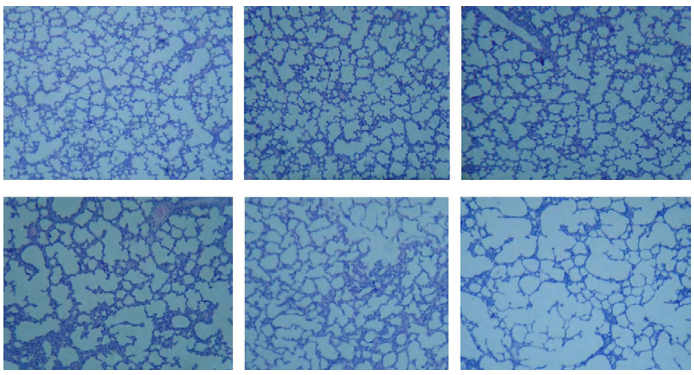

Figure 1. Morphological changes in lung tissues under the low-power microscope (H&E; 100X). Top: control group; bottom: hyperoxia group. From left to right: 1st day, 3rd day, and 7th day.

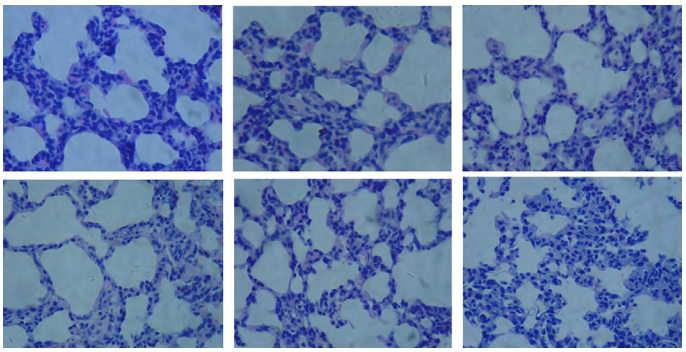

Figure 2. Morphological changes in lung tissues under the high-power microscope (H&E; 400X). Top: control group; bottom: hyperoxia group. From left to right: 1st day, 3rd day, and 7th day.

On the 1st, 3rd, and 7th days, the hyperoxia group had significantly decreased alveoli, increased alveolar diameter, decreased alveolar area, and attenuated alveolar septum ($t = 2.94\text{-}51.69$; $P < 0.05$). With prolonged time of hyperoxia exposure, the hyperoxia group had significantly decreased and enlarged alveoli, reduced alveolar area, and thickened alveolar septum ($F = 62.83\text{-}852.06$; $P < 0.05$). The control group had similar lung morphologies from the 1st to the 7th day ($F = 0.14\text{-}1.67$; $P > 0.05$) (Table 1).

Table 1. Number, diameter, and area of alveoli and thickness of alveolar septum (N = 8, means ± SD).

Item	1st day	3rd day	7th day	F value	P value
Number of alveoli					
Control group	136.21 ± 5.67	135.65 ± 5.56	139.35 ± 7.79	0.33	0.721
Hyperoxia group	124.42 ± 4.12	98.37 ± 6.12	82.12 ± 4.11	154.55	0.000
t value	4.25	12.78	18.11		
P value	0.001	0.000	0.000		
Diameter of alveoli					
Control group	56.13 ± 3.65	56.79 ± 3.92	57.03 ± 3.51	0.14	0.877
Hyperoxia group	70.27 ± 4.05	81.17 ± 3.91	93.11 ± 3.77	62.83	0.000
t value	7.32	12.56	19.35		
P value	0.000	0.000	0.000		
Area of alveoli					
Control group	19.14 ± 0.54	19.37 ± 0.55	19.26 ± 0.98	0.18	0.839
Hyperoxia group	11.65 ± 0.59	7.14 ± 0.38	2.53 ± 0.34	852.06	0.000
t value	26.57	51.69	46.00		
P value	0.000	0.000	0.000		
Thickness of alveolar septum					
Control group	10.41 ± 0.91	9.43 ± 1.47	9.89 ± 0.68	1.67	0.215
Hyperoxia group	9.21 ± 0.67	5.59 ± 0.71	4.65 ± 1.01	70.80	0.000
t value	2.94	6.72			
P value	0.011	0.000	0.000		

Z = 2.86, 2.36; P < 0.05.

TGF-β1 mRNA and protein levels in lung tissues

With increasing time of hyperoxia exposure, TGF-β1 mRNA and protein levels were decreased. From the 1st to the 7th day, the control group had similar levels of TGF-β1 mRNA and protein (H = 0.08, 0.86; P > 0.05), but the hyperoxia group had significantly different levels (H = 2.10, 4.85; P < 0.05). On the 7th day, the hyperoxia group had significantly lower TGF-β1 mRNA and protein levels than those of the control group (Z = 2.86, 2.36; P < 0.05) (Table 2 and Figure 3).

Table 2. TGF-β1 mRNA and protein levels (N = 8, means ± SD).

Item	1st day	3rd day	7th day	H value	P value
TGF-β1 mRNA expression levels					
Control group	1.00 ± 0.57	0.93 ± 0.78	0.83 ± 0.45	0.08	0.932
Hyperoxia group	1.01 ± 0.99	0.85 ± 0.41	0.34 ± 0.19	2.10	0.035
Z value	0.02	0.17	2.86		
P	0.977	0.871	0.014		
TGF-β1 protein levels					
Control group	1.01 ± 0.87	0.99 ± 0.54	0.57 ± 0.45	0.86	0.441
Hyperoxia group	0.96 ± 0.56	0.75 ± 0.45	0.21 ± 0.09	4.85	0.021
Z value	0.74	0.77	2.36		
P	0.475	0.445	0.047		

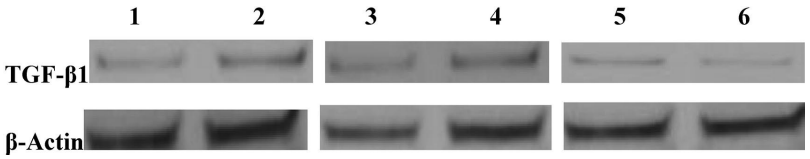

Figure 3. TGF-β1 protein expression levels detected by western blot. *Lane 1* = 1st day of control group; *lane 2* = 1st day of hyperoxia group; *lane 3* = 3rd day of control group; *lane 4* = 3rd day of hyperoxia group; *lane 5* = 7th day of control group; *lane 6* = 7th day of hyperoxia group. β-actin is shown as the internal control.

DISCUSSION

With ever-developing medical techniques, the diagnosis and treatment of BPD has changed dramatically in both pathological and clinical processes. Resulting in mild lung pathological changes and uniform injury areas, BPD primarily manifests as alveolar and vascular dysplasias, such as alveolar simplification, enlargement of the distal air-containing cavity, decrease in capillary bed, and formation of abnormally shaped capillaries (Stenger et al., 2010). We herein established a neonatal rat model of hyperoxia-induced BPD, which accorded with the onset and progression of BPD in premature infants regarding pathological processes and changes. In the vesicular period, lungs of neonatal rats (2-3 days old) can mimic those of premature infants of about 28 weeks gestational age, which are easily inhibited and injured by hyperoxia due to active lung modeling and remodeling. At present, hyperoxia-induced lung injury of animals is usually caused by 95% oxygen atmosphere, short-term and long-term uses of which lead to acute lung injury and BPD, respectively (Coalson, 2003). In this study, with increasing time of hyperoxia exposure, lung tissues gradually underwent blocked alveolarization, including attenuation of the alveolar wall, alveolar simplification, non-uniform alveolar size, and a sharp decrease in alveoli and capillaries, resembling the pathological changes that occur during BPD.

Angiogenesis is indispensable for alveolarization during lung development. Accompanied by alveolar differentiation, the pulmonary vascular network expands and forms an effective air-blood barrier. During premature lung development, premature infants commonly receive mechanical ventilation and oxygen inhalation therapy, which may block alveolar and pulmonary vascular development, decrease the numbers of alveoli and capillaries, and reduce the air/blood exchange area (Guo et al., 2011). Accordingly, early pulmonary vascular dysplasia predominantly controls the pathogenesis of BPD.

As a cell growth factor with many biological effects, TGF-β1 belongs to the superfamily that regulates cell growth and differentiation, and plays a crucial role in tissue formation, development, and repair after injury. Of all the intrapulmonary cells (e.g., alveolar macrophages, fibroblasts, epithelial cells, and endothelial cells) that can secrete TGF-β1, alveolar macrophages play the main role (Li et al., 2006; Wrzesinski et al., 2007). TGF-β1 can stimulate the proliferation and phenotypic modulation of vascular smooth muscle cells, promote vascularization, and increase the synthesis of extracellular matrix and protease inhibitors and expression of other growth factors (Maki et al., 2000). In this study, TGF-β1 mRNA and protein levels decreased in both control and hyperoxia groups from the 1st to the 7th day, but the latter group exhibited a more obvious decrease, without significantly different outcomes on the 7th day. Therefore, TGF-β1 mRNA and protein levels decreased markedly upon hyperoxia-induced BPD, suggesting that TGF-β1 is involved in the regulation of lung development. Such a decrease may result in alveolar degeneration and pulmonary vascular dysplasia (Hegeman et al., 2010; Mohamed et al., 2011; Shen et al., 2013).

In summary, TGF-β1 is an indispensable regulatory factor during pulmonary vascular development, but the underlying mechanism remains elusive and needs in-depth study. Elevating TGF-β1 gene expression in the lung tissues of premature infants by using TGF-β1 or genetic engineering strategies may be conducive to BPD treatment in clinical practice.

Conflicts of interest

The authors declare no conflict of interest.

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