Original Research Article

Expression levels of reactive oxygen species, NF-κBp65 and TGF-β1 and their correlations in bronchopulmonary dysplasia in neonatal rats

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Abstract

Purpose: To study the levels of reactive oxygen species (ROS), nuclear transcription factor-κBp65 (NF-κBp65) and TGF-1, and their correlation in bronchopulmonary dysplasia in neonatal rats.
Methods: Twenty (20) pregnant rats were randomly divided into study and normal groups. Radial alveolar counts were carried out at 2, 8 and 15 days of age. The levels of ROS expression in the lung tissues of the two groups were assayed by ELISA while immunohistochemistry was used to determine the expressions of TGF-β1 and NF-κBp65 in neonatal lung tissues of the two groups. Pearson correlation test was used to analyze correlations amongst ROS, TGF-β1 and NF-κBp65 in the neonatal lung tissues.
Results: At days 8 and 9 after birth, radial alveolar count was significantly lower in study rats than in control rats (p < 0.05). Expression levels of ROS, TGF-β1 and NF-κBp65 in study group were markedly raised at days 2, 4 and 8 after birth, relative to control (p < 0.05).
Conclusion: The levels of ROS, TGF-β1 and NF-κBp65 in bronchopulmonary dysplasia in neonatal rats are significantly and positively correlated, and are higher than those in normal rats. This provides a scientific basis for development of drugs for bronchopulmonary dysplasia.

Keywords: Neonatal rats, Bronchopulmonary dysplasia, Lung tissue, NF-κBp65, TGF-β1, Correlation

INTRODUCTION

Bronchopulmonary dysplasia, a chronic pulmonary disease in premature infants, is characterized by alveolar and pulmonary microvascular dysplasia due to inflammation [1]. A new type of bronchopulmonary dysplasia with complicated pathogenesis refers to stunted or stagnant lung development in premature babies. Research suggest that this new type of bronchopulmonary dysplasia is the result of a combination of multiple factors [2].

Studies have found that ROS, TGF-β1and NF-κBp65 are closely associated with the etiology of bronchopulmonary dysplasia. Reactive oxygen species (ROS) which are involved in the NF-κBp65 pathway, activate S6 kinase, induce phosphorylation of IκB, and dissociate NF-κBp65, thereby causing nuclear translocation

and regulation of the transcriptions of TGF-β1and other factors [3]. It has been suggested that ROS may play crucial roles in lung tissue damage [4]. It is known that TGF-β1 is a multifunctional cytokine that regulates cell growth and differentiation, and its appropriate expression plays an important role in normal lung development [5]. In this study, ROS, NF-κBp65 and TGF-β1 in bronchopulmonary dysplasia in neonatal rats, and their correlations, were determined.

EXPERIMENTAL

Animals

Sixty healthy Sprague-Dawley rats of mean weight 197 ± 23 g were purchased from Hunan Slake Jingda Experimental Animal Co. Ltd. (production license no. SCXK (Xiang) 2016-0002). They were housed in a laboratory with temperature of 23 ± 2 ℃, humidity of 57 ± 3 ℃, and photoperiod of 12-h day/12-h night, and were permitted *ad libitum* access to feed and drinking water during a 1-week period of adaptive feeding.

This research was approved by the Animal Ethical Committee of Department of Pediatrics/Neonatal, Hebei General Hospital, Shijiazhuang, PR China (approval no. 201833983), and was carried out in line with "Principles of Laboratory Animal Care" (NIH 85-23, 1985) [6].

Reagents and equipment

The reagents and instruments used, and their makers/suppliers (in brackets) were: Fluorescence microscope (Guangzhou Mingmei Photoelectric Technology Co. Ltd, model MF43); electronic balance (Shenzhen Jinzhonggang Industrial Co. Ltd, model EX125ZH); Slicer (Zhejiang Jinhua Huasu Technology Co. Ltd); Centrifuge (Guangzhou Jiidi Instrument Co. Ltd, model jidi-4d-ws); Oxygen tank (Beijing Fuyi Electric Co. Ltd); Refrigerator (Qingdao Haier Company, model: bcd-196tmpi); NF-Bp65 polyclonal antibody (Beijing Zhongshan Jinqiao Biotechnology Co. Ltd); rabbit anti-mouse TGF-antibody 1 polyclonal antibody (Beijing Baiao Lebo Technology Co. Ltd), and immunohistochemical staining kit (Shanghai Dingjie Biotechnology Co. Ltd).

Establishment of animal model and animal groups

Following 1 week of adaptive feeding, female rats were kept in cages with male rats at a ratio of 2:1, until the female mice became pregnant. A total of 40 pregnant rats were obtained. These were assigned to normal and study groups, using the random number table method. In the observation group, pregnant rats and neonatal rats were put in an oxygen tank for continuous inhalation of high concentration of oxygen, and normal diet and water were provided. Rats in normal group were fed under normal air conditions.

Study indicators

At 2, 4 and 8 days after birth, 6 neonates from each group were weighed and sacrificed. Lung tissues of the neonates in both groups were excised and weighed. Body weight and lung weight of neonatal rats in the two groups were recorded and compared.

The lung tissues were fixed in formalin solution, and paraffin sections were prepared for embedding. Following H&E staining, lung tissue histomorphological changes in the two groups of neonatal rats were examined using light microscopy.

Radiative alveolar counts were monitored at three time points after birth: days 2, 4 and 8.

At each of these time points, small sections of lung tissue were taken, cut into smaller sections, and homogenized. The supernatant fraction was kept in a low-temperature refrigerator, and subsequently used for assay of ROS expressions with ELISA.

Paraffin sections of rat lung tissues were cut into about 4-cm thick slices. The tissue sections were placed flat on glass slides and oven-dried at 65℃, rinsed with phosphate buffer, stained, dehydrated, cleared, paraffinized and blocked. The expressions of NF- Bp65 and TGF- TGF-TGF- 1 were determined using immunohistoche-mistry. Light-yellow, brownish-yellow and tan colors in the cytoplasm were used as indices of the degree of positive cells. Six fields of view were selected at high magnification and used to calculate the mean optical density at each time interval.

The correlations amongst ROS, NF-κBp65 and TGF-β1 expression in lung tissues of newborn rats were analyzed with Pearson correlation test.

Statistical analysis

Measurement data were compared amongst groups using means of single factor and multiple samples, while two-group comparison was

carried out with independent sample *t*-test. Counting data were compared using t-test. The correlations amongst ROS, NF-κBp65 and TGF-β1 expressions were analyzed with Pearson correlation test. All statistical analyses were done with SPSS 21.0 software package. Results of statistical comparisons were considered significant at $p < 0.05$.

RESULTS

Body and lung weights of neonatal rats

Table 1 shows that body and lung weights of neonatal rats were raised in both groups, but the increase was higher in the normal rats. Compared with the normal group, body and lung weights of neonatal rats in the study group were markedly reduced at days 2, 4 and 8 of age ($p < 0.05$).

Table 1: Post-litter body and lung weights of neonatal rats (g, n = 6)

Group		2 days	4 days	8 days
Normal	Body weight	6.92 ± 0.29	8.98 ± 0.43	15.73 ± 1.12
	Lung weight	0.16 ± 0.03	0.21 ± 0.03	0.33 ± 0.03
Study	Body weight	6.42 ± 0.27	8.05 ± 0.58	13.11 ± 0.62
	Lung weight	0.12 ± 0.02	0.16 ± 0.02	0.27 ± 0.02

Values are mean ± SD

Histomorphological changes in lung tissues of neonatal rats

In the normal group, the lung tissue structure of neonatal rats was intact and devoid of hyperemia, edema and inflammatory cell infiltrations. In contrast, in the observation group, there were clear evidence of alveolar septum in the lung tissues of the neonatal rats. Moreover, the alveolar septum was widened, with presence of hyperemia, edema and infiltration of inflammatory cells. These results are depicted in Figure 1.
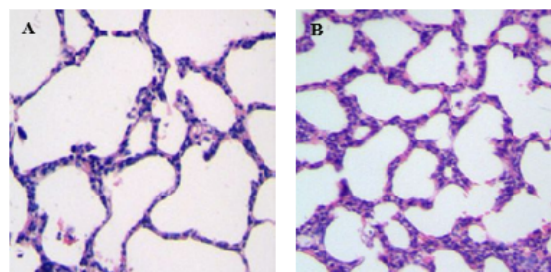

Figure 1: Histomorphological changes in lung tissues of neonatal rats in the two groups. A: lung tissue of neonatal rats in normal group; B: lung tissue of neonatal rats in the observation group

Radiative alveolar counts of neonatal rats

Table 2 shows that there was no significant difference in radial alveolar count between the study group and the normal group at 2 days of age ($p > 0.05$). However, radial alveolar count in study group was markedly lower than that in normal group at 8 days and 15 days after litter ($p < 0.05$).

Table 2: Radial alveolar count on days 2, 8 and 15 after delivery of neonatal rats (n = 6)

Group	Age (days)	Radiative alveolar counts
Normal	2	14.12 ± 0.32
	8	16.44 ± 0.72
	15	19.61 ± 0.66
Study	2	13.94 ± 0.32
	8	12.27 ± 0.38[ab]
	15	11.91 ± 0.52[abc]

[a]$P < 0.05$, vs normal rats; [b]$p < 0.05$, vs normal rats at 8 days of age; [c]$p < 0.05$, vs normal rats at 15 days of age

Expressions of ROS, TGF-β1 and NF-κBp65 in lung tissues of neonatal rats

Relative to normal rats, ROS and expressions of NF-κBp65 and TGF-β1 in study group were significantly increased at days 2, 4 and 8 after birth ($p < 0.05$), as shown in Table 3 and Figure 2.

Table 3: ROS, TGF-β1 and NF-κBp65 levels in lung tissues of two groups of neonatal rats (n = 6)

Group	Age (days)	ROS (IU/mL)	NF-κBp65	TGF-β1
Normal	2	382.55 ± 6.86	0.14 ± 0.03	0.11 ± 0.03
	4	387.17 ± 6.99	0.16 ± 0.03	0.12 ± 0.04
	8	388.04 ± 8.51	0.17 ± 0.03	0.13 ± 0.04
Study	2	636.38 ± 6.43[a]	0.25 ± 0.02 [a]	0.19 ± 0.03 [a]
	4	640.95 ± 8.67[ab]	0.27 ± 0.04 [ab]	0.20 ± 0.04 [ab]
	8	646.15 ± 9.69[abc]	0.28 ± 0.03 [abc]	0.22 ± 0.05 [abc]

[a] $P < 0.05$, vs normal rats; [b]$p < 0.05$, vs normal rats at 4 days after birth; [c]$p < 0.05$, vs normal rats 8 days after birth
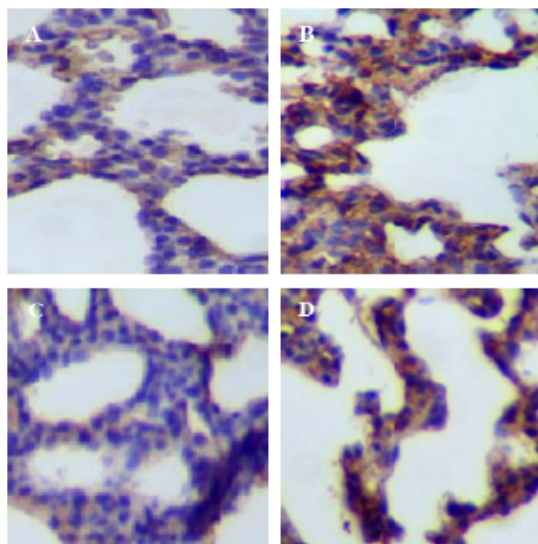
Figure 2: Expressions of ROS, TGF-β1 and NF-κBp65 in the lung tissues of the two groups of neonatal rats. A: NF-κBp65 expression in neonatal rat lung tissue in normal group; B: expression of NF-κBp65 in lung tissue of neonatal rats in observation group; Figure C: expression of TGF-β1 in neonatal rat lung tissue in normal group; D: expression of TGF-β1 in neonatal lung tissue of rats in the study group

Correlations amongst ROS, TGF-β1 and NF-κBp65 in neonatal rat lung tissues

As shown in Table 4, there were positive correlations amongst the expression levels of ROS, NF-κBp65 and TGF-β1 in the lung tissues of the neonatal rats ($p < 0.05$).

Table 4: Correlations amongst the expression levels of ROS, NF-κBp65 and TGF-β1 in neonatal rat lung tissues

Index	ROS	NF-κBp65	TGF-β1
ROS	-	0.936	0.878
NF-κBp65	0.936	-	0.816
TGF-β1	0.878	0.816	-

DISCUSSION

Bronchopulmonary dysplasia which was first named by Northway, is a frequently-occurring chronic pulmonary disease in premature infants [7]. Currently, it is believed that it may be closely related to premature delivery, positive pressure ventilation, high concentration of *yang* and pulmonary infection [8]. Oxygen is important in the treatment of neonates, but due to immature lungs and low antioxidant enzyme activities in neonates, prolonged and high concentration of oxygen therapy often results in lung injury, leading to alveolar retardation and bronchoalveolar dysplasia [9]. The purpose of this study was to investigate the expression levels of, and correlations amongst ROS, NF-κBp65 and TGF-1 in bronchopulmonary dysplasia in neonatal rats, using a rat model of bronchopulmonary dysplasia.

It has been reported that the younger the gestational age, the lower the birth weight, and the higher the incidence of bronchopulmonary dysplasia, which may be related to neonatal pulmonary dysplasia [10]. Due to the low oxygen environment before the birth of the fetus, the antioxidant capacities of tissues and cells of premature infants are low. Thus, under high oxygen atmosphere, the imbalance in the oxidant-antioxidant ratio, and the susceptibility to bronchopulmonary dysplasia are significantly accentuated. In this study, the body weights and lung weights of neonatal rats were increased in both groups, but the weight gain was higher in the normal group.

Compared with the normal group, body weights and lung weights of rats in the study group were significantly reduced at days 2, 4 and 8 after birth. The lung tissues of newborn rats had normal architecture, and were without hyperemia, edema and inflammatory cell infiltration. However, in the study group, alveolar septum in the lung tissues of rats was widened, and hyperemia, edema and inflammatory cell infiltration were observed. There was no significant difference in radial alveolar count between the study and normal groups at 2 days of age. However, radial alveolar count in the study rats was markedly lower than that in the normal group at 8 and 15 days of age. These results indicate that high oxygen tension causes bronchopulmonary dysplasia in neonatal rats.

Reactive oxygen species (ROS) comprise strong oxidants and oxygen-containing free radicals, and peroxides that easily form free radicals [11]. Under normal circumstances, excess ROS are neutralized *in vivo* by antioxidants such as glutathione peroxidase, superoxide dismutase and other antioxidant enzymes. It has been reported that lung injury may be associated with ROS [12,13]. It is known that NF-κBp65, which plays an important role in immune response, inflammatory response and apoptosis, is a transcription factor widely distributed in a variety of cells [14]. It usually binds to the inhibitor I B to form an inactive cytoplasmic heterodimer. When cells undergo stimulation, I B is phosphorylated and NF-κBp65 is dissociated and rapidly transferred to the nucleus where it mediates expressions of target genes [15]. The transforming growth factor-β1 (TGF-β1) is a member of the TGF-β1 family. A variety of cells

in the lungs, including alveolar macrophages and epithelial cells secrete TGF-β1 [16].

In this study, ROS, TGF-β1 and NF-κBp65 levels were significantly increased in the study group at days 2, 4 and 8 after birth, relative to normal rats, and the expression levels of ROS, NF-κBp65 and TGF-β1 in study group were significantly increased at the three time points. These results suggest that the pathogenesis of bronchopulmonary dysplasia in neonatal rats is significantly correlated with levels of ROS, NF-κBp65 and TGF-β1.

CONCLUSION

This study has established positive correlations amongst TGF-β1, ROS and NF-κBp65 levels in bronchopulmonary dysplasia in neonatal rats. Moreover, these expressions are significantly higher in bronchopulmonary dysplasia neonatal rats than in normal rats. Thus, these findings provide a scientific basis for the development of new drugs for bronchopulmonary dysplasia.

DECLARATIONS

Acknowledgement

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Yaying Cheng conceived and designed the study, Xin Wang, Meng Sun, Chan Wang, Youning Zheng, Yaying Cheng collected and analyzed the data, while Xin Wang wrote the manuscript.

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