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Original Research Article

Rhubarb alleviates hyperoxia-induced lung injury in neonatal rats with bronchopulmonary dysplasia by inhibiting inflammation

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Abstract

Purpose: *To investigate the effect of rhubarb on hyperoxia-induced lung injury in neonatal rats with bronchopulmonary dysplasia (BPD), and the underlying mechanism.*
Methods: *Sixty 4-day-old neonatal rats were assigned to air control, BPD, and rhubarb intervention groups, with 20 rats in each group. Immunoblotting was employed to assay NF-κB expression. Levels of malondialdehyde (MDA) and SOD were determined spectrophotometrically, while ELISA was used to measure serum levels of IL-6, IL-8 and TNF-α.*
Results: *The peripheral blood levels of TNF-α, IL-8 and IL-1β were markedly higher in BPD-exposed rats than in the air control rats, while peripheral blood levels of TNF-α, IL-8 and IL-1β were reduced in rhubarb intervention rats, relative to BPD-exposed rats. The activity of SOD was markedly lower in lung tissue of BPD rats than in lung tissue of air control rats, while MDA level was markedly elevated in BPD rats ($p < 0.05$). There was marked up-regulation of NF-κB p65 expression in BPD-exposed rats, relative to air control rats, but it was markedly lower in rhubarb intervention rats than in hyperoxia model rats ($p < 0.05$).*
Conclusion: *Rhubarb mitigated hyperoxia-induced inflammation, oxidative stress and lung injury in BPD neonatal rat model by inhibiting oxidative stress and reducing the levels of inflammatory factors.*

Keywords: *Bronchopulmonary dysplasia, Neonatal rat, Rhubarb, Inflammation, Oxidative stress*

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INTRODUCTION

Bronchopulmonary dysplasia (BPD) is a chronic lung disease associated with persistent respiratory distress which is commonly found in premature infants. The disease is characterized by a long treatment cycle and a high fatality rate. Survivors often have many long-term complications such as growth retardation, repeated lower respiratory tract infection and hyper-reactive airway disease. These adverse effects seriously affect the quality of life and long-term prognosis in children, thereby imposing heavy economic burdens on families and society [1]. Mechanical ventilation and oxygen therapy are some of the most widely used therapies in the care of premature infants, although proper lung tissue development is impaired, while long-term exposure to hyperoxia easily leads to lung injury in premature infants [2]. Not much is known about the etiology of BPD. However, according to the latest studies, inflammatory and oxidative stress responses are crucial in the development of BPD [3]. Rhubarb is one of the traditional Chinese herbal medicines. Medical research has shown that rhubarb exerts immune-regulatory, apoptotic, antioxidant and anti-inflammatory effects, and it protects the lungs from chronic lung disease, sepsis, inflammation and trauma due to lung injury [4]. However, not much is known about the protective effect of rhubarb on BPD caused by high oxygen concentration. Based on the foregoing, this research investigated the influence of rhubarb on hyperoxic pulmonary injury due to BPD in a neonatal rat model of hyperoxia, as well as the underlying mechanism.

EXPERIMENTAL

Materials and reagents

Seventy-two 4-day-old neonatal Sprague-Dawley (SD) rats were obtained from the Animal Management Center of Medical College of Jiujiang University. The reagents and assay kits used, and their sources (in parenthesis) were superoxide dismutase (SOD) and MDA assay kit (Nanjing Construction Institute of Bioengineering), rhubarb extract (National Institute for Drugs and Biological Products Verification), assay kits for IL-6, IL-8 and TNF-α (BRAHMS, Germany), and NF-κB p65 antibody (Abcam Biotechnology Limited, UK).

Establishment of the rat model of BPD

The 60 neonatal rats were assigned to air control, air rhubarb, hyperoxia (model), and model + rhubarb groups, with 15 rats/group. The air control and air rhubarb control groups were exposed to air, while BDP was established in rats in the high oxygen group and high oxygen + rhubarb group via exposure to 60 % oxygen, with humidity maintained at 50 - 70 %, and the temperature kept at 25 - 26 ºC in an atmosphere of < 0.5 % CO_2. In addition, rats in hyperoxia + rhubarb group and air rhubarb control received rhubarb suspension (600 mg/kg), once every day. Rats in other groups received equivalent doses of physiological saline through gavage, once daily. This research was approved by the Animal Ethical Committee of Medical College of Jiujiang University according to "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1985) [5], the approval number is MCJU2020022.

Sample collection

At age of 21 weeks, each rat was subjected to jugular bleeding for collection of 1 mL of blood. After centrifugation, the supernatant was kept in a -20 ºC refrigerator prior to analysis. After blood collection, the rats were sacrificed under 10 % chloral hydrate anesthesia. The chest cavities were opened, and the right lung tissues were excised and transferred to -80 ºC refrigerator. The left lung samples were fixed with 4 % paraformaldehyde overnight for hematoxylin-eosin (H&E) staining.

Examination of lung histomorphology

Lung tissues were processed histologically, stained with H&E, and sealed, after which lung tissue morphologies of neonatal rats in the 3 groups were examined under a light microscope. Radioactive alveolar count (RAC) was carried out to evaluate the degree of alveolar development, which was averaged in 5 random observation fields for each specimen.

Determination of peripheral blood levels of inflammatory indices

Venous blood (1 mL) was drawn from each rat, and serum was isolated by centrifuging in a serum separator at 3000 rpm for 20 min. Then, serum concentrations of inflammatory factors were determined with ELISA assay kits in strict compliance with the kit instructions.

Determination of levels of oxidative stress indices in lung tissue

A 10 % lung tissue homogenate was prepared at 4 ºC. The homogenate was spun for 20 min at 3000 rpm, and the clear extract was retained for analysis. Protein concentration in supernatant was measured with BCA method, while MDA and SOD levels were determined spectrophotometrically using assay kits in strict compliance with the manufacturer's protocols.

Western blot assay for NF-κB protein expression

Pulmonary tissue protein extraction was done using RIPA lysis buffer. After centrifugation, lysate protein concentration was determined using BCA procedure, after which equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis for 90 min. This was followed by transfer to PVDF membranes which were blocked by incubation with 5 % non-fat milk. Then, following incubation for 12 h at 4 ºC with appropriate 1º immunoglobulins, the films were incubated with HRP-linked 2º immunoglobulin, followed by color development, and analysis of relative expression levels using Bio-Rad image laboratory software.

Statistical analysis

Statistical analysis was done using SPSS 20.0. Measurement data are presented as mean ± SD, and comparison amongst groups was done using ANOVA, while comparison between groups was done with pairwise LSD test or Tamhane test. Values of $p < 0.05$ indicated statistical significance.

RESULTS

Morphological changes in lung tissues in each group

Results from H&E staining showed that after exposure to hyperoxia, pulmonary tissue morphology of rats in BPD-exposed rats was distorted: the alveolar volume was increased, while the number alveoli was decreased, with disappearance of the normal alveolar structure, widening of the alveolar septum, and presence of pulmonary bullae. In the air control group, the lung tissue structure was intact, the volume and number of alveoli were normal, with normal alveolar structure, and normal alveolar spaces. The pathological changes in lung tissue structure in rhubarb intervention group exposed to high oxygen model group were significantly reduced.

Radial alveolar count (RAC) was markedly reduced in BPD-exposed rats, relative to air control rats. However, RAC in rhubarb intervention group was significantly higher than that in hyperoxia model group ($p < 0.05$). These results are presented in Table 1.

Table 1: Comparison of radial alveolar counts among the three groups (n = 20)

Group	Radial alveolar counts
Air control	10.74±2.64
BPD	5.66±1.82
Rhubarb intervention	9.38±2.20
F	27.440
P-value	0.000

Levels of inflammatory indices in peripheral blood of rats in each group

Inflammatory factor levels were significantly up-regulated in peripheral blood of BPD rats, relative to air control rats, but were markedly reduced in peripheral blood of rhubarb intervention group, relative to BPD group ($p < 0.05$; Table 2).

Table 2: Levels of inflammatory indices in peripheral blood of rats in each group (pg/L, n = 20)

Group	TNF-α	IL-8	IL-1β
Air control	3.14±0.70	3.36±0.42	9.34±1.76
BPD	5.56±1.08[a]	5.44±0.88[a]	30.28±4.84[a]
Rhubarb intervention	4.32±0.94[b]	4.62±0.74[b]	16.36±2.13[b]
F	34.592	43.952	219.423
P-value	0.000	0.000	0.000

[a]$P < 0.05$, vs air control; [b]$p < 0.05$, vs BPD rats

Amounts of indices of oxidative stress in pulmonary tissues of rats in each group

Superoxide dismutase (SOD) activity was markedly decreased in BPD rats, relative to rats in air control, but MDA level was markedly higher in BDP rats. There was markedly higher SOD activity in lung tissue of rhubarb intervention rats than in BPD rats, but MDA level was higher in air control rats (Table 3).

Table 3: Pulmonary levels of SOD and MDA indices in rats in each group (pg/L, n = 20)

Group	SOD (U/mg)	MDA (μmol/g)
Air control	22.38±1.32	1.57±0.13
BPD	17.52±1.19[a]	1.81±0.23[a]
Rhubarb intervention	21.14±1.13[b]	1.64±0.15[b]
F	86.264	9.903
P-value	0.000	0.000

[a]$P < 0.05$, vs air control; [b]$p < 0.05$, vs with BPD rats

Expression levels of NF-κB P65 in lung tissues of rats

In BPD-exposed rats, the expression level of NF-κB P65 was markedly raised, relative to that in air control rats, but it was markedly down-regulated in rhubarb intervention rats, relative to hyper-oxygen model rats (Table 4).

Table 4: Expression levels of NF-κB P65 in lung tissues of the three groups (n = 20)

Group	NF-κB p65
Air control	0.40±0.12
BPD	0.78±0.25
Rhubarb intervention	0.55±0.17
F	20.775
P-value	0.000

DISCUSSION

Studies have shown that BPD, a chronic pulmonary disease in pre-term babies, adversely influences the thriving and long-term prognosis in children [6]. Appreciable success has been achieved in survival of premature infants due to rapid advances in perinatal medicine such as birth after implementation of protective ventilation strategies, use of exogenous lung surfactants, and use of antenatal corticosteroids, leading to reduction in the incidence of BPD [7]. Clinical studies have shown that the sensitivity of the respiratory tract of surviving premature BPD infants to external stimuli is significantly increased, and they are prone to chronic obstructive pulmonary disease at middle and old ages [8]. Bronchopulmonary dysplasia (BPD) in patients manifests as changes in lung tissue pathophysiology, low vascular density, large alveoli and decreased spacing. These changes lead to symptoms of respiratory failure in BPD children. Thus, there is need for high mechanical ventilation and oxygen therapy so as to alleviate the symptoms of respiratory failure. However, long-term exposure to high oxygen levels increases lung damage in preterm infants, thereby aggravating symptoms of respiratory failure [9,10]. Therefore, it is of clinical significance to seek effective treatment for children with BPD so as to enhance the short- and long-term outcomes of the disease.

Rhubarb is a traditional Chinese medicine. Studies have demonstrated that rhubarb exerts extensive pharmacological effects such as anti-tumor, anti-inflammatory, antioxidant and anti-fibrotic properties [11,12]. Thus, it is widely used in the treatment of a variety of chronic lung diseases due to its potential to improve lung tissue microcirculation, mitigate pulmonary fibrosis, and inhibit airway remodeling [11,12].

In this study, a BPD neonatal rat model was established by exposing the rats to 60 % oxygen. Results of lung histopathology showed that the exposure to high oxygen distorted the lung tissue structure, with increased alveolar volume, reduced alveolar number, absence of normal alveolar structure, widening of alveolar septum, and lung bullae. These alterations are consistent with the characteristic pathological changes in BPD, indicating that the BPD rat model was successfully established. Moreover, it was found that the lung histopathological injury in the BPD rats were significantly decreased after rhubarb intervention, with commensurate increase in RAC. This result suggests that rhubarb attenuated the high oxygen-linked pulmonary histopathological lesion in BPD rats.

The pathogenesis of BPD has not been fully understood yet. However, studies have found that imbalance between oxidant and antioxidant systems is implicated in the occurrence and development of BPD [13]. Superoxide dismutase (SOD) is an important antioxidant enzyme in organisms. It protects the body from oxidative stress by neutralizing excessive free radicals and peroxides. The level of SOD is a reflection of the antioxidant capacity of the body and its capacity to remove oxygen free radicals [14]. Malondialdehyde (MDA is a product of free radical-mediated lipid peroxidation. It causes the cross-linking and polymerization of proteins, nucleic acids and other macromolecules. The level of MDA is an important indicator of the degree of tissue oxidative stress: changes in MDA level indirectly reflect the level of oxygen free radicals and the degree of tissue damage, i.e., the degree of oxidative damage in tissues [15]. In this research, MDA level was markedly raised in BPD rats, relative to rats in air control, while SOD level was markedly reduced in BPD rats. These results suggest that hyperoxia caused BPD lung injury by inducing oxidative stress response. The results of this study also showed that SOD level was markedly raised in lung tissues of rhubarb intervention rats, relative to that in BPD rats, while MDA level was markedly reduced in BPD rats. Thus, rhubarb treatment after lung injury in BPD due to hyperoxia reduced the BPD-induced oxidative stress. Studies have found that emohuol enhanced the activities of glutathione peroxidase and SOD through anti-lipid peroxidation, and it removed oxygen free radicals, thereby reducing the pathological damage caused by these prooxidants in lung tissues [16].

Studies have shown that inflammatory factors are linked to the etiology of BPD. At the critical period of lung development, pulmonary inflammatory response caused by oxygen therapy, mechanical ventilation and infection lead to distortion of lung tissue structure and reduction of alveolar volume and number, leading to reduction of area of gas exchange [17]. It has been reported that the activation of NF-κB signaling pathway is closely related to inflammation [18]. The NF-κB protein produced by NF-κB signaling pathway induces secretion of TNF-α, IL-8, IL1β and other inflammatory cytokines in macrophages, leading to lung inflammation and aggravation of lung tissue injury [19]. In this study, the pulmonary levels of NF-κB protein inflammatory cytokine in peripheral blood of rats in BPD group were significantly higher than those in air control group. These results suggest that hyperoxia caused lung injury in BPD-exposed rats by inducing lung inflammation. Moreover, pulmonary level of NF-κB protein, as well as amounts of TNF-α, IL-8 and IL1β in peripheral blood of rhubarb intervention rats were markedly up-regulated, relative to BPD rats. These data indicate that rhubarb treatment reduced inflammation in BPD rats after hyperoxia-induced lung injury. It has been reported that emoin inhibited the synthesis of peroxisome proliferator-activated receptor γ and toll-like receptor-2-mediated downstream TNF-α, IL-8 and IL1β in NF-κB pathway, and reduced inflammatory cell infiltration, thereby reducing inflammatory responses for several days [20,21].

CONCLUSION

Hyperoxia induced inflammation and oxidative stress response in BPD model rats. However, rhubarb alleviated the high oxygen-mediated pulmonary lesion in neonatal BPD rats by inhibiting oxidative stress response and decreasing levels of proinflammatory cytokines. Therefore, rhubarb extract may be used in treating neonatal hyperoxia-induced lung injury.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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