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Original Article

Spatial and temporal expression of SP-B and TGF-β1 in hyperoxia-induced neonatal rat lung injury

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Abstract: Objective: Bronchopulmonary dysplasia (BPD) is a severe complication of extreme prematurity that can be caused by hyperoxia inhalation. SP-B and TGF-β have been reported to be implicated in the development of lung. This study aimed to reveal the spatial and temporal expression patterns of these two factors in an animal model of BPD. Methods: Newborn Sprague-Dawley (SD) rats were subjected to hyperoxia conditions to establish an animal model of BPD. The levels of SP-B, TGF-β, MDA and TAOC, as well as the activations of MAPK and PI3K/AKT pathways in lung tissues were monitored during newborn rats prolonged exposure to hyperoxia. Results: We found that hyperoxia exposure significantly induced body weight loss of SD rats. H&E staining for morphometric analyses revealed that hyperoxia arrested alveolar development or loss of alveoli, with fewer and dysmorphic capillaries. mRNA and protein levels of SP-B and TGF-β were high expressed in hyperoxic lung tissues. The concentrations of SP-B and TGF-β in bronchoalveolar lavage fluid were also increased. All these increases begin at the 3th day of hyperoxia exposure. MDA content was increased while TAOC content was decreased in response to hyperoxia. Furthermore, hyperoxia activated p38, and deactivated PI3K and AKT expression. Conclusion: Our research demonstrated that SP-B and TGF-β1 were highly expressed in three levels: mRNA and protein levels in lung tissues, and the release of SP-B and TGF-β1 in bronchoalveolar lavage fluid, beginning at the 3th day of hyperoxia exposure.

Keywords: Bronchopulmonary dysplasia (BPD), hyperoxia, SP-B, TGF-β1, spatial and temporal patterns

Introduction

Oxygen is one of the most widely used therapies in the care of preterm infants as an integral part of respiratory support [1]. However, recent studies have confirmed that inhalation of hyperoxia may cause lung damages [2, 3], which is the main risk factor in the occurrence of bronchopulmonary dysplasia (BPD) [4]. BPD is a severe complication of extreme prematurity that affects 12~32% of infants less than 32 weeks of gestation, with most cases occurring in extremely low birth weight infants [5]. Infants who develop BPD manifest aberrant or arrested pulmonary development and can experience lifelong alterations in cardiopulmonary function [6]. Despite decades of promising research, current clinical management fails to reduce the incidence of BPD [7], which calls for a better understanding of BPD.

The surfactant proteins (SPs), including SP-A, SP-B, SP-C and SP-D, are important components of pulmonary surfactants, which keep normal lung function and prevent lung inflammation [8]. SP-B is a 79-amino acid hydrophobic surfactant protein that its importance for normal lung function has been confirmed by animal studies. In the early stage of hyperoxic lung injury, SP-B maintains surface tension, lowering effects of surfactant in the alveoli or by enhancing pulmonary cell adaptation [9]. Homozygous SP-B -/- deficient mice die of respiratory failure at birth associated with severe pulmonary dysfunction and atelectasis [8].

The transforming growth factor (TGF)-β family consists of three closely related isoforms, *i.e.*, TGF-β1, -β2, and -β3, that exert effects on modulation of cell inflammation, growth inhibition and differentiation, and extracellular matrix

production [10]. Additionally, they are important mediators of the stimulatory and inhibitory cell development pathways that moderate normal early lung patterning [11]. Recent studies have proposed TGF-β1 is implicated in abnormal lung development and fibrosis in newborn mice [10, 12], and its levels are increased in newborn lung injury [13].

Although previous studies have suggested SP-B and TGF-β1 are implicated in the development of lung, the spatial and temporal patterns of these two factor expressions have not been fully revealed in animal model of BPD. Thus, in this study, we used a well-established animal model of BPD [14], to monitor the expression levels of SP-B and TGF-β1 in lung tissues and the concentrations of SP-B and TGF-β1 in bronchoalveolar lavage fluids during newborn rats prolonged exposure to hyperoxia. The findings in this study may helpful for us to better understand the pathogenesis of BPD.

Materials and methods

Animals

Thirty clean grade of pregnant Sprague-Dawley (SD) rats were purchased from the Experimental Animal Center of Nantong University (Nantong, China). The rats were maintained under laboratory conditions with free access to standard diet, sterile water and controlled temperature (24°C). All the animal experiments were approved by our local Animal Ethics Committee, and were conducted in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Study design

24-hour-old rat pups were used in all experiments, and were randomly divided into two groups (n = 15 rats/group): 1) Control group, in which newborn rats and mothers were placed in Plexiglas chamber in which the oxygen concentration was maintained at a FiO_2 = 0.21 (normoxia) for 14 days; 2) Hyperoxia group, in which mothers and pups were placed in chamber with FiO_2 = 0.60 (hyperoxia) conditions [15]. Mothers in these two chambers were exchanged once a day to avoid the impacts of hyperoxia on maternal feeding.

Sample collection

At the 3th, 7th and 14th day, 5 newborn rats were randomly selected form each group and were weighted. Rats were anesthetized by intraperitoneal (IP) injection with chloral hydrate (3 mL/kg; Qingdao Yulong Seaweed, Qingdao, China), and were killed by a blow to the head. The left lung, and the middle and posterior lobes of right lung were removed.

Lavages of the left lungs were performed 3 times with 0.3 mL PBS (pH 7.4). The bronchoalveolar lavage fluids were centrifuged at 800×g for 15 min, and the supernatants were stored in -20°C before use.

The middle lobes of right lung were fixed with 4% paraformaldehyde solution for 24 h, and then were embedded in paraffin. The tissues were cut into 4~6 μm sections and used for hematoxylin and eosin (H&E) staining and immunohistochemical staining. Posterior lobes of right lung stored at -80°C were used for qRT-PCR and Western blot analyses.

Histological and immunohistochemical assessment

Lung lobe sections were stained by H&E for general histological examination. Alveolarization was assessed by performing radial alveolar count (RAC), according to the method previously described [16, 17]. Five counts were performed for each sample, and the average of 5 high-power fields was randomly selected.

For immunohistochemistry, lung lobe sections were first blocked with 5% normal goat serum (LianShuo Biological, Shanghai, China) for 1 h at room temperature. Then sections were incubated with primer antibodies against SP-B (cat. no. ab40876) and TGF-β1 (cat. no. ab92486) (Abcam, Cambridge, MA, USA) overnight at 4°C and followed by incubation with the secondary antibody goat anti-rabbit IgG (cat. no. ab-150077) for 4 h at room temperature. The staining was analyzed with the Image Pro Plus 6 (Media Cybernetics, Rockville, MD, USA).

ELISA

SP-B and TGF-β1 concentrations in bronchoalveolar lavage fluids were quantified by using

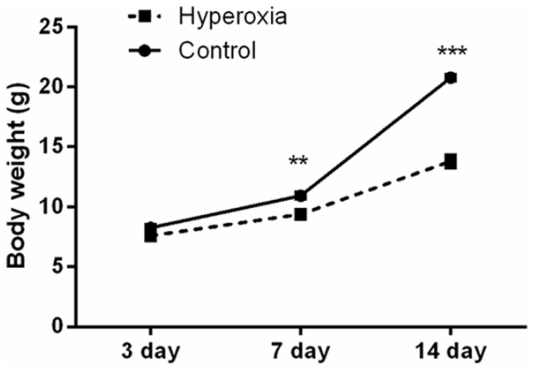

Figure 1. Changes in the body weight of newborn rats after hyperoxia exposure for 3, 7, and 14 days. n = 5 per group. Values are means ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Control group.

their correspondingly commercial ELISA kits (CD Creative Diagnostics, NY, USA) according to the manufacturer's prtocol. Cat. No. DEIA-BJ2273 was used for SP-B detection and cat. No. SEIA1362 was used for TGF-β1.

qRT-PCR

Total RNA from lung tissue samples was extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthetized by using Super M-MLV Reverse Transcriptase Kit (Bioteke Corporation, Beijing, China). qRT-PCR was performed in ABI PRISM 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) by using Platinum SYBR® Green® Super Mix-UDG Kit (Life Technologies, NY, USA). Primers for TGF-β1 and SP-B were synthesized by Generay Biotech Co. Ltd. (Shanghai, China) and theirs sequences were as follows: TGF-β1 (F: 5'-AGC CCG AAG CGG AAC TAC TAT-3' and R: 5'-AGC CCG AAG CGG ACT ACT AT-3'); SP-B (F: 5'-CTG GTC ATC AAC TAC TTC CA-3' and R: 5'-TGT GTG TGA GAG TGA GGG TGT AAG-3'). Data were analyzed according to the classic $2^{-\Delta\Delta Ct}$ method [18], and were normalized to GAPDH expresion in each sample.

Malondialdehyde (MDA) and total antioxidant capacity (TAOC) analysis

The right lung tissue samples were homognized in cold saline at 4°C, and the homogenate was centrifuged at 800×g for 15 min, and the supernatants were collected for detection of MDA and TAOC contents. MDA, a lipid peroxide degradation product, which can be condensed with thiobarbituric acid to form a red product [19]. The content of MDA in lung tissue samples were detected by using the MDA test kit (cat. No. A003-4; Jiancheng Bioengineering Research Institute, Nanjing, China) according to the manufacturer's instructions. TAOC was measured by the method of ferric reducing/antioxidant power assay by using TAOC test kit (cat. No. A015-1; Jiancheng Bioengineering Research Institute). Levels of MDA were expressed as nmol/g protein and TAOC activity was expressed as U/g protein.

Western blot

Total proteins were extracted from lung tissue samples by using lysis buffer (Beyotime, Shanghai, China). Protein samples were mixed with a sample buffer (Beyotime) and boiled for five minutes. Equal amounts of protein samples (30 μg) were dissolved in sodium dodecyl sulfate polyacr-ylamide gel (SDS-PAGE) and transferred to nitrocellulose mebrane (Millipore, Bed-ford, MA, USA). The membranes were blocked with 5% non-fat milk for 1 h at room temperature, and then were incubated with primer antibodies against SP-B (cat. no. ab40876), TGF-β1 (cat. no. ab92486), p-p38 (cat. no. ab47363), p38 (cat. no. ab170099), p-PI3K (cat. no. ab182651), PI3K (cat. no. ab191606), p-AKT (cat. no. ab38449), AKT (cat. no. ab18805) and GAPDH (cat. no. ab9485) (Abcam) overnight at 4°C. Following by incubation with the secondary antibody goat anti-rabbit IgG (cat. no. ab150077) for 1 h at room temperature, the positive signals in membranes were visualized by Super Signal Femto (Pierce, Rockford, IL, USA).

Statistical analysis

All data are provided as means ± SEM from five independent assays in triplicate. Statistical differences between control group and hyperoxia group were analyzed by SPSS version 13.0 program (SPSS Inc., Chicago, IL, USA) using one-way analysis of variance (ANOVA). *P*-value < 0.05 was considered as statistically significant results.

Results

Hyperoxia exposure induces lung injury

Hyperoxia exposure of newborn rats results in the disruption of lung structure, affecting alveo-

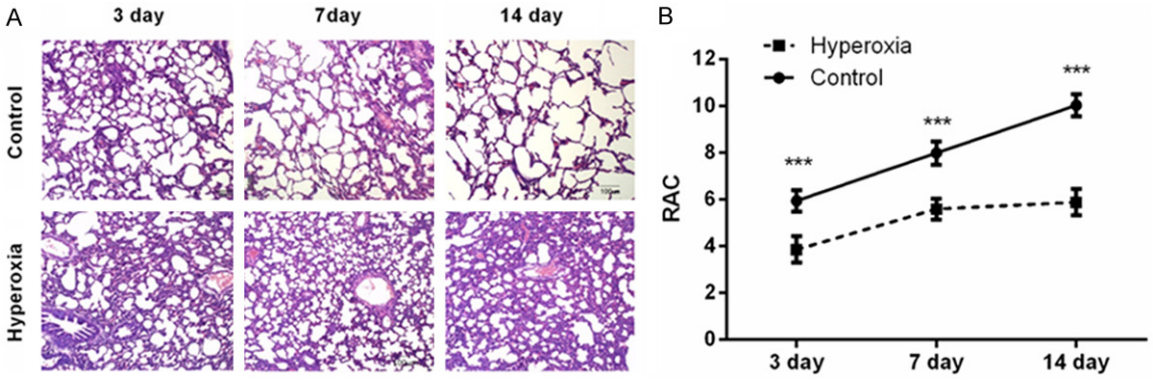

Figure 2. The disruption of lung structure after newborn rats exposed to hyperoxia for 3, 7, and 14 days. A. H&E staining for morphometric analyses of newborn rat lung. B. The radial alveolar counts (RAC) from newborn rat lung. n = 5 per group. Values are means ± SEM. ***P < 0.001 vs. Control group.

larization and vascularization, a condition that strongly resembles "new BPD" in premature infants [2, 3]; this was also confirmed in this study. As results shown in **Figure 1**, following 7 and 14 days of hyperoxia exposure, body weight of newborn rats were much lower than those in control group ($P < 0.01$ and $P < 0.001$). Histopathological changes in lung tissues were monitored, and as shown in **Figure 2A**, lung tissues in control group showed uneven pink at the 3th day, and this pink changed to more uniformed at the 7th and 14th day. The color of tissues in Hyperoxia group was rather deep than those in Control group at the 3th day, and became pale at the 7th and 14th day; in addition, petechial hemorrhages were observed on the surface of the lung at the 7th and 14th day. Further, we found that hyperoxia significantly decreased RAC when compared with control group after 3, 7, and 14 days of exposure ($P < 0.001$; **Figure 2B**).

Hyperoxia up-regulates SP-B and TGF-β1

ELISA was performed to detect the concentrations of SP-B and TGF-β1 in bronchoalveolar lavage fluids. As shown in **Figure 3A** and **3B**, much higher contents of SP-B and TGF-β1 were found in Hyperoxia group when compared with Control group ($P < 0.001$). Data from immuhistchemical assessment were coincident with the results from **Figure 3A** and **3B** showed that, SP-B and TGF-β1 were highly expressed after hyperoxia exposure (**Figure 3C-F**; $P < 0.001$). In addition, the expression levels of these two factors in lung tissues were also detected by qRT-PCR and Western blot analyses. As results shown in **Figure 4A-C**, both the mRNA and protein levels of SP-B and TGF-β1 were up-regulated after hyperoxia exposure ($P < 0.001$).

Hyperoxia increases MDA, decreases TAOC and monitors MAPK and PI3K/AKT pathways

Next, we asked whether hyperoxia could alter the concentrations of MDA and TAOC in lung tissues. As results shown in **Figure 5A** and **5B**, MDA content was much higher while TAOC content was much lower in Hyperoxia group when compared with Control group ($P < 0.01$ or $P < 0.001$). Further, the expression levels of main factors in MAPK and PI3K/AKT pathways were detected. Western blot analycal results showed that (**Figure 5C**), the phosphorylation level of p38 was remarkably up-regulated, and the phosphorylation levels of PI3K and AKT were notably down-regulated following hyperoxia exposure. The total levels of p38, PI3K and AKT were unaffected.

Discussion

Supplemental oxygen is widely used in the treatment of neonatal respiratory failure [1]; however, long-term of high-concentration of oxygen may cause BPD [4]. In the present study, newborn SD rats were exposed to hyperoxia conditions. We found that hyperoxia exposure significantly induced body weight loss of SD rats. Besides, H&E staining for morphometric analyses revealed that hyperoxia arrested alveolar development or loss of alveoli, with fewer and dysmorphic capillaries, which are the characteristics of BPD [20]. All these suggested

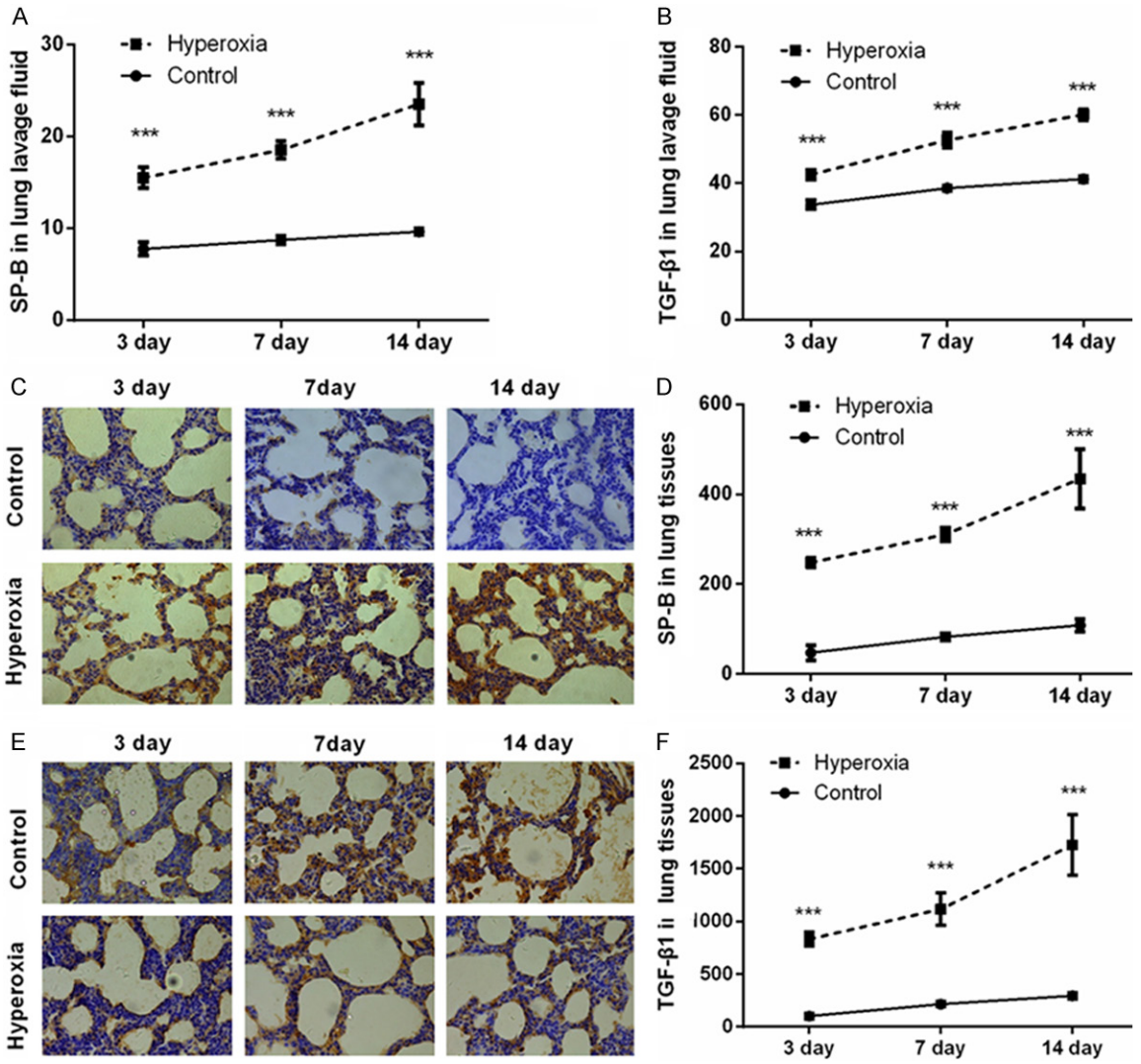

Figure 3. Expression levels of SP-B and TGF-β1 in bronchoalveolar lavage fluids and lung tissues after newborn rats exposed to hyperoxia for 3, 7, and 14 days. A, B. ELISA assay for detection the concentrations of SP-B and TGF-β1 in bronchoalveolar lavage fluids. C-F. Immunohistochemistry for detection the expression levels of SP-B and TGF-β1 in lung tissues. One representative slide per group is shown. n = 5 per group. Values are means ± SEM. $^{***}P < 0.001$ vs. Control group.

that an animal model of BPD was established in newborn SD rats by using hyperoxia conditions.

SP-B is a major component of SPs that plays important functions in lung throughout life. Previous studies have pointed out that SP-B is abnormally expressed in lung diseases. Chang *et al.*, mentioned that the serum levels of SP-B in respiratory distress syndrome (RDS) were lower than in premature infants without RDS [21], which increasing the risk of RDS in preterm infants. The expression of SP-B is also reported to be reduced in LPS-induced lung injury [22]. Of contrast, in hyperoxia-induced neonatal rat lung injury, SP-B expression was increased during exposure to hyperoxia [23]. Another study reported the similar result that SP-B expression increased sharply in newborn rat lung at 7 and 14 days of hyperoxic exposure [14]. In the current study, the expression of SP-B in response to hyperoxia has been detected in three levels: 1) mRNA, and 2) protein levels in lung tissues, and 3) the concentration in bronchoalveolar lavage fluid. Results showed that reduction of mRNA level of SP-B may decrease the transcription/translation of SP-B protein, and consequently decrease SP-B

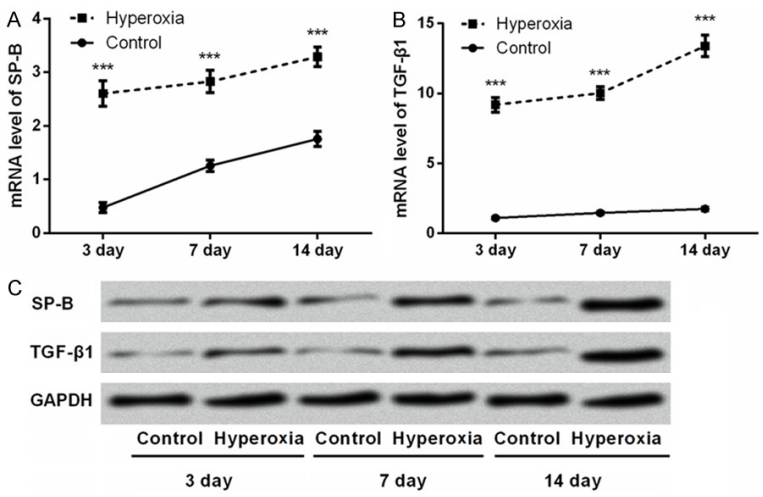

Figure 4. Expression levels of SP-B and TGF-β1 in lung tissues after newborn rats exposed to hyperoxia for 3, 7, and 14 days. A, B. qRT-PCR analysis for detection the mRNA level expressions of SP-B and TGF-β1. n = 5 per group. Values are means ± SEM. $^{***}P < 0.001$ vs. Control group. C. Western blotting for detection the protein level expressions of SP-B and TGF-β1. n = 5 per group. One representative slide per group is shown.

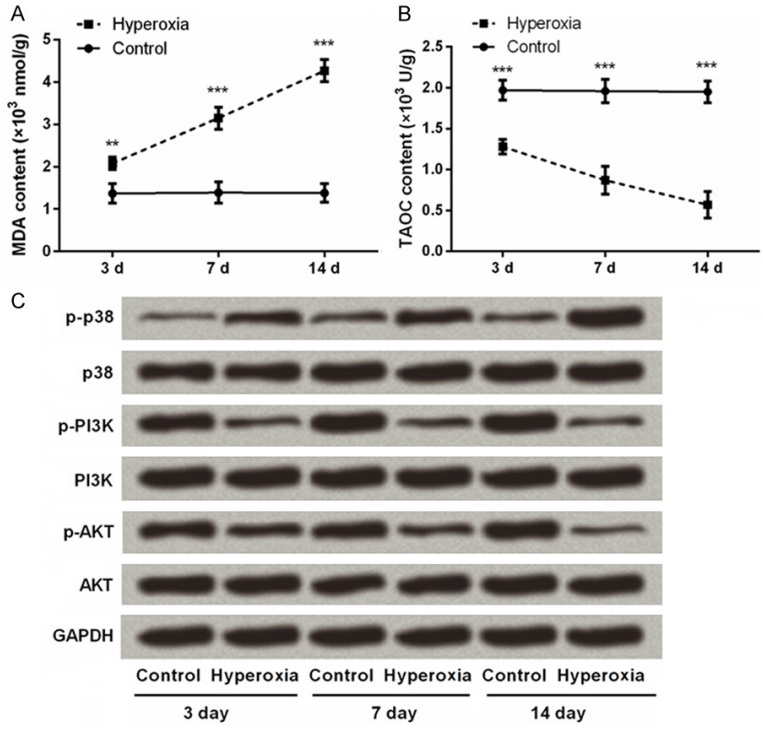

Figure 5. Changes in MDA and TAOC contents and MAPK and PI3K/AKT pathways in lung tissues after newborn rats exposed to hyperoxia for 3, 7, and 14 days. A, B. The concentrations of MDA and TAOC in lung tissues were monitored by using commercial kits. n = 5 per group. Values are means ± SEM. $^{***}P < 0.001$ vs. Control group. C. Western blotting for detection the core protein expressions in MAPK and PI3K/AKT pathways. n = 5 per group. One representative slide per group is shown.

release. It seems that SP-B expression reflect lung injury, that lung injury induced by pulmonary hypoplasia or infection reduced SP-B expression, while lung injury induced by hyperoxia increased SP-B expression, providing an opportunity for use SP-B as a biomarker candidate for distinguish the cause of BPD.

TGF-β1 is a secretory cytokine that binds its receptor TGFβR2, which initiates the TGF-β signaling involved in the regulation of branching and septation phases of lung development [24, 25]. Previous studies have reported the excessive TGF-β1 levels in BPD. For instance, TGF-β1 was increased in bronchoalveolar lavage fluid obtained from infants with chronic lung disease of prematurity [26]. A high level of TGF-β1 immunoreactivity has been observed in the walls of the peripheral lung where alveolarization is diminished by lung injury [13]. These previous studies evidenced that the increase level of TGF-β1 is associated with newborn pulmonary injury. Our data were consistent with these previous studies, suggesting that TGF-β1 was highly expressed after hyperoxia exposure. But, this study provided a more detailed data regarding the abundance of TGF-β1 in different spatial and temporal. That is we revealed that TGF-β1 was highly expressed in three levels: 1) mRNA and 2) protein levels in lung tissues, and 3) the release of TGF-β1 in bronchoalveolar lavage fluid, beginning at the 3th day of hyperoxia exposure.

It is generally believed that MDA value reflects oxidative level, while TAOC value reflects anti-oxidative level. These two indicators are widely used in the estimation of oxidative conditions [27, 28]. Herein, the level of MDA was found to be significantly increased in response to hyperoxia, while TAOC level was significantly reduced after hyperoxia exposure, suggesting oxidative stress was induced in lung tissues. Moreover, hyperxia activated the MAPK pathway and deactivated PI3K/AKT pathway in lung tissues. MAPK and PI3K/AKT are two cell signaling pathways implicated in the survival of pulmonary epithelial cells after hyperoxia exposure [29]. Our data showed that the increase of MDA, the decrease of TAOC, the activation of p38, as well as the deactivation of PI3K and AKT in hyperoxic lung were in sync with the increases of SP-B and TGF-β1 levels. Thus, we boldly propose a hypothesis: increased levels of SP-B and TGF-β1 were stimulated by oxidative stress, and SP-B and TGF-β1 function to lung possibly via MAPK and PI3K/AKT signaling pathways. A large number of investigations are required to verify this hypothesis.

In conclusion, our research demonstrated that SP-B and TGF-β1 were high expressed in three levels: 1) mRNA and 2) protein levels in lung tissues, and 3) the release of SP-B and TGF-β1 in bronchoalveolar lavage fluid, beginning at the 3th day of hyperoxia exposure. SP-B and TGF-β1 might be two potential biomarkers for BPD diagnose.

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Disclosure of conflict of interest

None.

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