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ORIGINAL RESEARCH

Effect of Hyperoxia on Pulmonary SIgA and Its Components, IgA and SC

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Abstract
Purpose Oxygen therapy (hyperoxia) is essential for the treatment of some neonatal critical care conditions. The lung is the primary target for the changes induced by hyperoxia. Secretory immunoglobulin A (SIgA), IgA and secretory component (SC) reflect the local immunity in the respiratory tract induced by hyperoxia.
Methods The enzyme-linked immunosorbent assay, immunohistochemistry staining, Western blot and Real-time PCR were used to detect the levels of cytokines, IgA and SIgA in bronchoalveolar lavage as well as IgA and SC/pIgR in pulmonary tissue.
Results The levels of IgA and SIgA in BAL fluid were gradually increased following neonatal rat development. Compared with air-inhaling group, in the hyperoxia group IgA, SIgA and other cytokines except IL-1 in BAL fluid were significantly elevated on the 3rd, 5th and 7th days, but on the 10th day TNF-α, SIgA and IgA rapidly decreased. In the hyperoxia group, both the protein expression of SC/pIgR and the mRNA expression of SC/pIgR were remarkably increased on the 3rd, 5th and 7th days, but were significantly decreased on the 10th day, respectively. Conclusion: The large amount of SIgA, IgA and SC in the early period of hyperoxia might protect the lungs of the neonatal rats against acute pulmonary injury, however, in the late period of hyperoxia, the abruptly drop of SIgA and its component might lead to pulmonary immunity abnormality. In hyperoxia, the increased expression of cytokines might contribute to the expression of IgA and SC.

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Keywords Hyperoxia · Pulmonary immunity · Secretory IgA · IgA · Secretory component

Introduction

Secretory immunoglobulin A (SIgA) is the major immunoglobulin of the respiratory tract and other mucosal surfaces [1, 2]. It binds with bacteria and other pathogens to prevent their adherence to mucosal cell surfaces and their subsequent systemic invasion. SIgA is generally considered to be a non-inflammatory antibody, since it only induces "immune exclusion" and results in "immune balance" among mucosal tissues [3–6]. SIgA level increases to protect against infection after injury [7]. SIgA is composed of IgA, secretory component (SC) and J chain. SC is the extracellular component of the polymeric Ig receptor (pIgR) that is responsible for the transcytosis of newly synthesized IgA (polymeric IgA, pIgA). It is essential for defense against bacteria [8], parasites [9] and for immunologically mediated neutralization of cholera toxin [10]. SC also participates in innate protection against mucosal pathogens [11]. So SIgA and SC play an important roles in innate immunity in intestine, respiratory tract and other mucosal sites.

Oxygen therapy (continuously inhaling a high concentration of oxygen, which results in hyperoxia) is essential for the treatment of some neonatal critical care conditions. Since the lungs are the primary targets of hyperoxia, pathophysiological manifestations of pulmonary oxygen toxicity have been extensively studied in neonatal rats [12]. However, few studies have reported changes in pulmonary immunity induced by hyperoxia. Our previous studies had demonstrated that moderate oxygen induced an increase and hyperoxia induced a decrease of intestinal SC in vivo and in vitro [13] and that ileal SIgA was remarkably increased in neonatal rats under conditions of hyperoxia [14]. In the present work, neonatal rats were

under the hyperoxia condition for 1st, 3rd, 5th, 7th, and 10th days, respectively. While the amounts of pulmonary cytokines (IL-1, IL-4, IL-6, TNF-α, TGF-β), pIgR (SC), IgA and SIgA were determined. The aim of the this work was to gain a better understanding of the hyperoxia effects on the immunological function of the lung.

Material and Methods

Animal Model

Animal model was built as described previously [13]. Briefly, within 12 hours of birth, all pups were randomly divided into air-inhaling group (group that was exposed to room air, FiO_2=0.21, N=68) and hyperoxia group (group that was exposed to 90~95 % O2, FiO_2=0.9~0.95, N=203). In the hyperoxia group, oxygen was continuously delivered into sealed environmental chambers to achieve a constant concentration of 90–95 % oxygen, as confirmed by an oxygen monitor (OM-25ME, MAXTEC, American) daily. The air-inhaling group was exposed to similar environmental conditions, except they inhaled room air. Oxygen and room air were filtered through natrolite to keep the CO_2 concentration below 0.5 % (as confirmed using a DapexGas Monitor, American). To equalize the effect of nursing on the pups' development, the nursing mothers were switched every 24 h among different groups and to eliminate maternal effects between groups (from the air-inhaling group to the hyperoxia group and vice versa). Chambers were opened for 1 h daily to switch the nursing mothers. Room temperature (25~27 °C), humidity (70 %~ 80 %), and daily light–dark cycles were automatically controlled. All animal husbandry, animal handling and procedures were reviewed and approved to conform to the animal Ethics Committee of China Medical University guidelines. At each time point, 10 pups were sacrificed to gain Bronchoalveolar Lavage (BAL) fluid and another 10 pups were sacrificed to dissect their pulmonary tissues.

Tissue Preparation

The neonatal rats were sacrificed by an intraperitoeal injection of pentobarbital sodium. To standardize analysis, the right lung lower lobe was fixed in situ with 4 % paraformaldehyde in phosphate-buffered saline (pH 7.4) and then was embedded in paraffin. The rest of the right lung was harvested for pIgR mRNA analysis. The left lung was harvested for protein analysis.

Bronchoalveolar Lavage (BAL)

The sacrificed neonatal rats were placed in a supine position. Ice-cold normal saline (NS) was gently instilled by syringe until resistance was felt. After 1–2 min, the NS was withdrawn and collected. Each animal was lavaged five times. Lavage fluid was collected, centrifuged to remove cells and debris. The supernatants were stored at −80 °C.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA kits was employed to measure the concentrations of TNF-α (KeyGEN, Nanjing, China), IL-1, IL-4, IL-6 and TGF-β (RB, American), IgA and SIgA (IBL, Hamburg, Germany), following the manufacturer's instructions. The absorbance of each solution was determined at a wavelength of 450 nm. Assay sensitivities as well as intra- and inter-assay coefficients were determined for all of the biomarkers tested. They are as follows: 8 ng/L, 9 % and 15 % for IL-1 and IL-6; 5 ng/L and 9 % and 15 % for IL-4; 10 ng/L and 9 % and 15 % for TGF-β; 5 ng/ml and 9.5 % and 9.5 % for TNF-α; 30 ng/ml and 9 % and 11 % for IgA; 3.0 ng/ml and 8 % and 10 % for SIgA, respectively.

Immunohistochemistry for the Detection of Pulmonary Tissue IgA and SC

Paraffin-embedded sections of the pulmonary tissues were deparaffinized, rehydrated, and incubated with rabbit anti-rat SC (Bethyl, American) and goat anti-rat IgA (Sigma, American). The slides were rinsed three times with PBS between incubations, and sections were counterstained with hematoxylin. In the negative control, the primary antibody was replaced with PBS. The median absorbance values of IgA and SC were determined using the Image analysis software (Shanghai, China) after scanning.

Protein determination The BCA Protein Assay Kit (Beyotime, shanghai, China) was used to determine protein concentration of pulmonary tissue according to the manufacturer's instructions.

Western Blot for Analysis of pIgR Protein in Pulmonary Tissues

Proteins extracted from pulmonary tissues were separated using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked with Tris-buffer containing 50 ng/L skim milk and probed with rabbit anti-rat SC antibody (Bethyl, American) or β-actin (Sigma, American) followed by a peroxidase-conjugated secondary antibody. They were then incubated with an enhanced chemiluminescent substrate and exposed to X-OMAT film (Perkin Elmer, American). Images were scanned and densitometry was analyzed for pIgR protein levels normalized to

β-actin using the Tanon GIS-2020 software (Tanon, Shanghai, China).

Quantitative Real-time Polymerase Chain Reaction of Pulmonary pIgR mRNA

Total RNA was extracted from pulmonary tissue of neonatal rats using an RNA Mini Kit from Takara (Takara Biotechnology Co., Dalian, China). The quality of extracted RNA was determined by the number and size of the bands obtained using agarose gel electrophoresis. cDNA was synthesized using 100 ng RNA. The levels of individual RNA transcripts were quantified using quantitative real-time polymerase chain reaction (PCR). The primers for SC/pIgR were as follows:

pIgR-F: 5′- CGATGGTGACTCTCGCTGGA -3′;
pIgR-R: 5′- TTGCACGGATAGTGGCAGGA -3′;
GAPDH-F: 5′-AATGGTGAAGGTCGGTGTG-3′;
GAPDH-R: 5′-TGAAGGGGTCGTTGATGG-3′.

The primers and fluorescent probes for pIgR and internal reference (GAPDH) were purchased from Takara. The PCR conditions were as follows: a preliminary cycle at 95 °C for 10 s, followed by 45 cycles at 95 °C for 5 s and at 60 °C for 20 s, followed by 1 min at 60 °C and 5 s at 95 °C. We also confirmed that the efficiency of amplification for each target gene (GAPDH) was 100 % in the exponential phase of PCR. The mRNA levels were normalized to GADPH mRNA according to the following formula: relative levels of pIgR mRNA=$(2^{-(CTpIgR\text{-}CT\ GAPDH)})\times 100$ %. Pulmonary mRNA levels of the hyperoxia groups were compared with those of air-inhaling group.

Statistical Analysis

For each experiment, at least 10 mice at different time point were tested. Data on group differences were reported as the means±SD. Significant differences between treatment groups were determined using the *t*-test.

Results

The Pathological Changes of Lung Tissues

In the air-inhaling group (Fig. 1a), the lung mucosal epithelium was simple columnar or cubical epithelium, and mucosal epithelium was integrity, morphology was regulation. In the hyperoxia group (Fig. 1b–f), the lung alveolar dilated and breaked, and mucosal epithelium was integrity, and its morphology was irregulation in the late period of hyperoxia (red arrow in Fig. 1). The histological findings suggested a decreased elasticity.

Analysis of Cytokines in BAL Fluid

Compared with those of the air-inhaling group, the expression level of IL-1 remained relatvely invariant on the first 5 days, and a mild increase was observed on the 7th and 10th days. But this increase was not significantly different (Fig. 2a). The expression level of IL-4 was significantly elevated (through day 1 to 10) and peaked on the 10th day (Fig. 2b). The expression level of IL-6 were remarkably elevated on 5th and 7th days, but not on the 1st, 3rd, and 10th days (Fig. 2c). TNF-α levels increased on the 3rd, 5th, and 7th days (Fig. 2e), while TGF-β stayed relatively constant at the first 3 days, and increased significantly on the 5th, 7th, and 10th days (Fig. 2d).

The Content of IgA and SIgA in BAL Fluid

In the air-inhaling group, IgA (Fig. 3a) and SIgA (Fig. 3b) were increased gradually in a day-dependent manner. In the hyperoxia group, IgA and SIgA were significantly increased compared with those of the air-inhaling group on the 3rd, 5th, and 7th days. On the 10th day, the IgA level quickly decreased to a similar level with that of the air-inhaling group, while the SIgA level droped notably lower than those of the other groups in hyperoxia.

Pulmonary IgA and SC Staining

Immunochemistry staining of IgA in the cytoplasm of airway epithelial cells was pale in the air-inhaling group (Fig. 4a–e). In the hyperoxia group, IgA staining was pale on the 1st (Fig. 4f) and 10th (Fig. 4j) days; was yellow on the 3rd day (Fig. 4g); was brown on the 5th day (Fig. 4h); and was dark brown on the 7th day (Fig. 4i). As shown in Fig. 5a–e, in the air-inhaling group, the staining of SC was yellow in the cytoplasma and on the membranes of the airway epithelial cells. SC staining of the airway epithelial cells from the hyperoxia group were dark brown on the 3rd, 5th and 7th days and light brown on the 10th day (Fig. 5f–j). The median absorbance value showed that the level of pulmonary IgA in the hyperoxia group was significantly increased compared with those of the air-inhaling group on the 5th, and 7th days ($P<0.01$). But on the 10th day, the level of IgA in the hyperoxia group was close to that in the air-inhaling group. The mean absorbance values of SC in the hyperoxia group was significantly increased compared with those in the air-inhaling group on the 3rd, 5th, and 7th days ($P<0.01$). But on the 10th day, it was remarkably decreased compared with those of 3rd, 5th, and 7th days in the hyperoxia group ($P<0.01$; Fig. 6a and b).

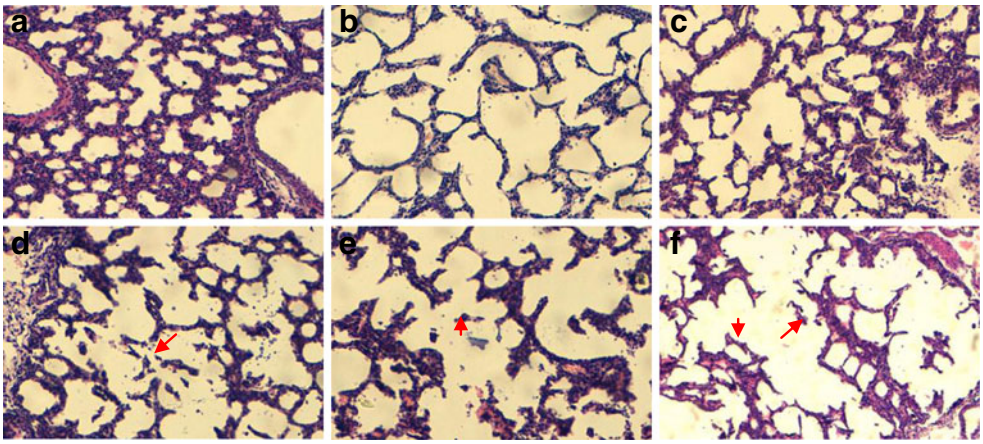

Fig. 1 The pathological changes of pulmonary tissues. (**a**) air group; (**b**) 1st day in hyperoxia; (**c**) 3rd day in hyperoxia; (**d**) 5th day in hyperoxia; (**e**) 7th day in hyperoxia; (**f**) 10th day in hyperoxia

pIgR Protein of Pulmonary Tissue Detected by Western Blot

Three subunits of pIgR protein (85KD, 92KD, 107KD) were detected (Fig. 7a). In the air-inhaling group, the 85KD pIgR protein was increased in a day-dependent manner, while the 92 KD pIgR protein remained at a lower level until the 7th day when a sudden increase appeared. Densitometry analysis (pIgR densitometry/β-actin densitometry) showed that both the expression of the 85KD (Fig. 7b) and 92KD (Fig. 7c) pIgR proteins in the hyperoxia group were significantly increased on the 1st, 3rd, 5th and 7th days. But it was significantly decreased on the 10th day compared with those of the 3rd, 5th and 7th days in the hyperoxia group ($P<0.01$).

pIgR mRNA of Pulmonary Tissues in Hyperoxia Neonatal Rats

The mRNA expression of pulmonary pIgR in the hyperoxia group on the 3rd, 5th and 7th days were 8.67±2.85, 8.46±2.36, and 18.60±3.34, respectively. In the hyperoxia group, the mRNA expression of pulmonary pIgR was significantly

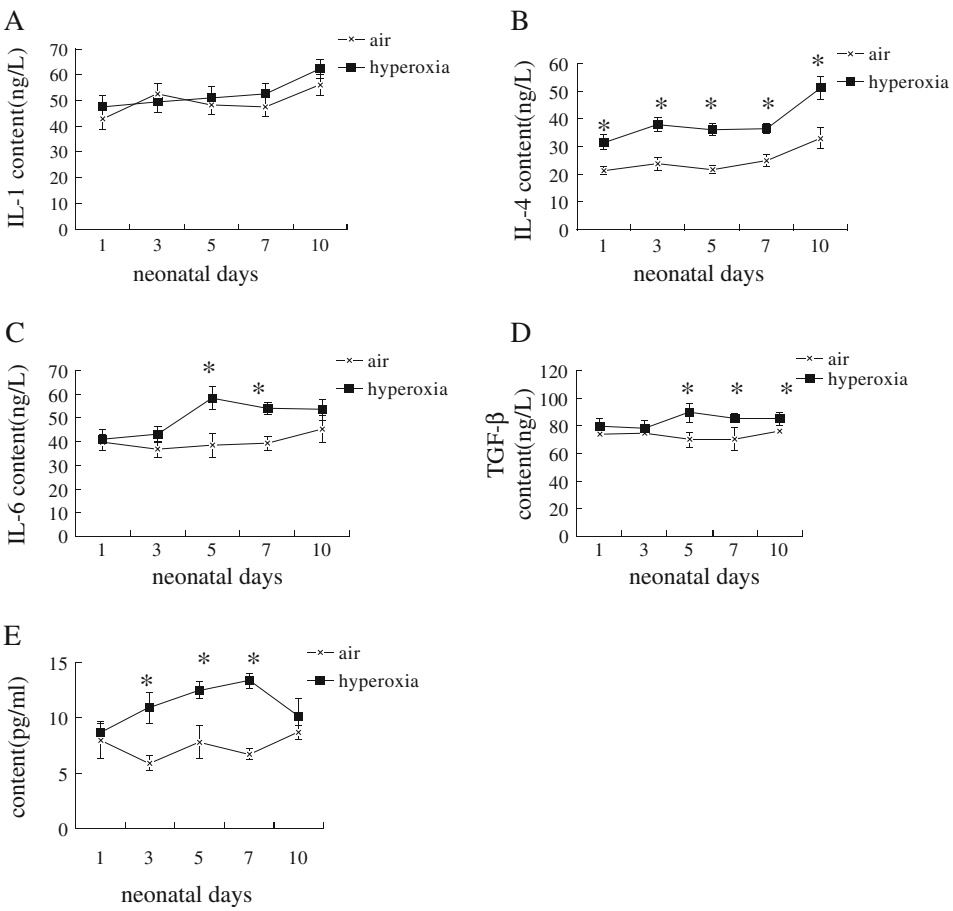

Fig. 2 The levels of cytokines in BAL. The levels of cytokines in BAL were compared and analyzed on various neonatal days. (**a**) IL-1; (**b**) IL-4; (**c**) IL-6; (**d**) TGF-β; (**e**) TNF-α; *$P<$ 0.01 compared with the air-inhaling group, IL-4, IL-6 and TGF-β of hyperoxia group were significantly elevated; TNF-α of hyperoxia group was significantly increased on the 3rd, 5th, and 7th days ($n=10$ in each time point)

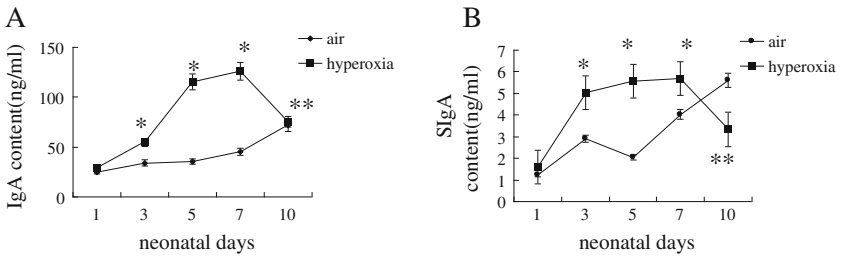

Fig. 3 The levels of IgA and SIgA in BAL. (**a**) IgA; (**b**) SIgA. The content of IgA and SIgA in BAL were compared and analyzed on each neonatal day. *$P<0.01$ compared with the air-inhaling group, IgA and SIgA of hyperoxia group were significantly increased on the 3rd, 5th, and 7th days; **$P<0.01$ IgA and SIgA were significantly decreased on the 10th compared with those of the 3rd, 5th, and 7th days in hyperoxia group($n=10$ in each time point)

increased on the 3rd, 5th and 7th days compared with that of the air-inhaling group (3rd day 3.14±0.82; 5th day 4.59 ±2.32; 7th day 4.27 ±0.46; $P<0.01$). However, on the 10th day, the mRNA expression of pulmonary pIgR (4.29±3.22) was notably lower than those of the other groups in hyperoxia and that of the 10th day in the air-inhaling group (6.82±7.84) ($P<0.01$; Fig. 8).

Discussion

Hyperoxia is an indispensable therapeutic measure in intensive clinical neonatal disease. But it is also a major factor of lung injury. Early exposure to hyperoxia results in progressive lung disease in premature infants [12]. This lung disease is associated with an initiation phase of injury, followed by an inflammatory phase and a destructive phase and ends at a final fibrotic stage [15]. In general, 6 to 48 h of hyperoxia is sufficient to cause marked pulmonary inflammation in infant rats [16]. Hyperoxia induces lung injury characterized by the development of pulmonary edema and inflammation [17]. The acute lung injury caused by hyperoxia is characterized by severe endothelial damage, alveolar epithelial injury, and increased pulmonary permeability [18]. We guessed that the mechanisms that caused the pathological change may be associated with changes of pulmonary local immune function.

IgA is synthesized and assembled to form pIgA by pulmonary subepithelial or glandular interstitial plasma cells. SIgA is formed by the association of pIgA and pIgR (SC), which is produced by the ciliated cells of bronchial epithelialium and the serous cells of submucosal glands. This association with pIgR (SC) has been shown to protect SIgA from proteolytic degradation. In this study, IgA and SIgA in BAL fluid were gradually increased following neonatal rat development in the air-inhaling group. In the hyperoxia group, IgA and SIgA in BAL fluid were significantly increased on the 3rd, 5th, and 7th days, however, were quickly decreased on the 10th day. The immunohistochemical results were similar with those of BAL fluid. This proved that the increased IgA in BAL was not local transcytosis of IgA but was IgA produced by pulmonary subepithelial or glandular interstitial plasma cells. Since one molecule of the pIgR is consumed for every molecule of SIgA released into luminal secretions, the increased synthesis of pIgR may contribute to the higher secretion of IgA

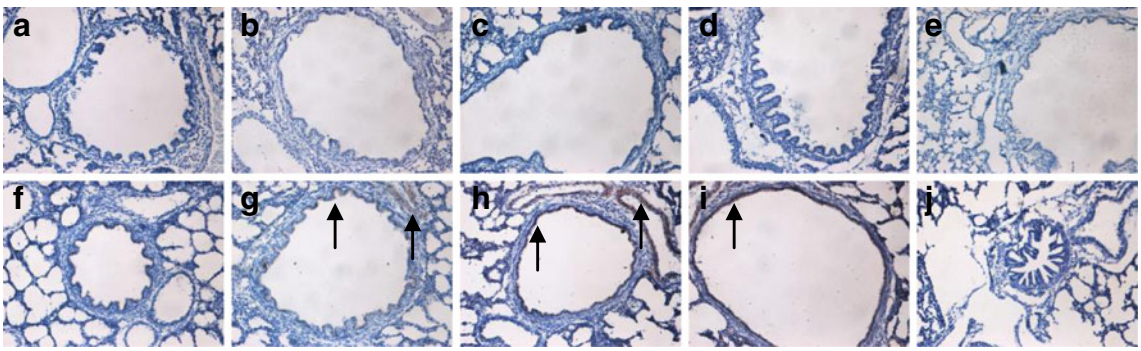

Fig. 4 IgA staining of pulmonary tissues. **a**–**e**, Pulmonary IgA staining was pale in the air-inhaling group ($n=10$ in each time point). The staining results indicated that in the air-inhaling group, pulmonary IgA in the neonatal rats was much less. **f**–**j**, Pulmonary IgA staining in the hyperoxia group ($n=10$ in each time point). IgA staining was pale on the 1st (**f**) and 10th (**j**) days; was yellow on the 3rd day (**g**); was brown on the 5th day (**h**); and was dark brown on the 7th day (**i**) (see *arrows*). Magnification bars=50 μm($n=10$ in each time point)

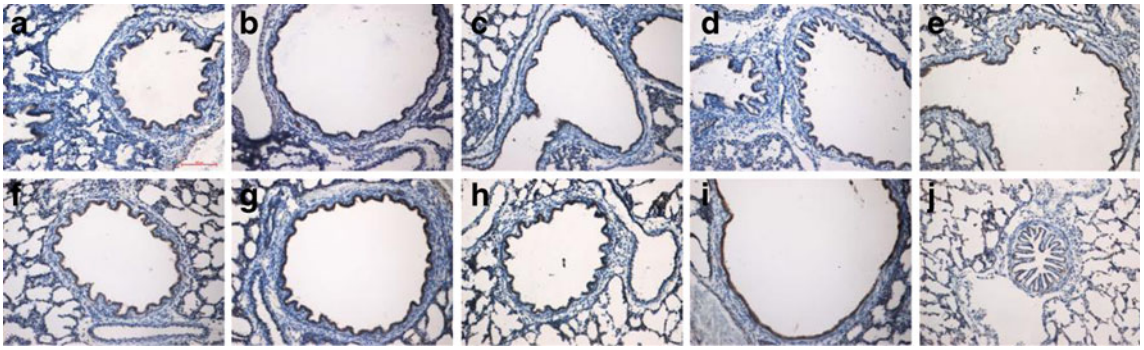

Fig. 5 SC staining of pulmonary tissues. **a**–**e**, Pulmonary SC staining was brown in the air-inhaling group(n=10 in each time point). The staining results indicated that pulmonary SC in neonatal rats was secreted when they were born. **f**–**j**, Pulmonary SC staining was dark brown in the hyperoxia group(n=10 in each time point). Magnification bars=50 μm (n=10 in each time point)

[19]. It is reported that the initiation phase of hyperoxia represented a sensitively balanced activation of damaging and protective systems in lung tissue [20]. SIgA can control mucosal inflammatory responses and therefore protect host tissues from damage [21]. Thus we speculated that in the early period of neonatal rats exposed to hyperoxia, large amounts of IgA and SIgA were produced to defend against acute pulmonary injury. *Polosukhin* et al. reported that SIgA levels in BAL from patients with chronic obstructive pulmonary disease were reduced [22]. He deemed that bronchial epithelial remodeling led to abnormalities in SIgA trafficking to the airway that impaired local host defenses [22]. Another report also stated that SIgA in bronchial secretions was decreased in cystic fibrosis [23]. So we considered the decrease of SIgA at the 10th day of hyperoxia as a pulmonary epithelial impairment.

pIgR is synthesized as a 90–100 KD precursor protein that matures to 100–120 KD [1]. Human pIgR is expressed as two different molecular weight forms, 92 and 107KD [24]. The 107KD pIgR is a fully glycosylated Golgi form of the protein, but the 92KD pIgR is immature form. SC, which is the extracellular component of the pIgR, is a 80-85KD protein [25, 26]. It has been reported that mouse pIgR is a 120KD protein and SC is a 94KD protein [23]. In the hyperoxia group the 85KD and 92KD SC protein were dramatically increased on the 3rd, 5th and 7th days and were significantly attenuated on the 10th day. This could demonstrate that pIgR was involed in transcytosis of IgA and lysis SC induced by hyperoxia. 107KD pIgR was gradually increased in a day-dependent manner in air-inhaling group, but in the hyperoxia group its expression was undetectable with Western blot. This could reflect the fact that there was less matured pIgR and a large amount of pIgR involving in the synthesis of SIgA in hyperoxia group. Furthermore, the mRNA expression of pIgR were up-regulated in a day-dependent manner in the early period but were decreased in the late period of hyperoxia. This suggests that hyperoxia may result in the destruction and/or damage of respiratory epithelium. In early stage of damage, a large amount of pIgR was secreted to form SIgA to protect the lung from acute injury. However, in late stage of damage, the respiratory epithelium was destroyed so that the secretion of pIgR was decreased.

The key regulators of IgA and pIgR expression are cytokines. In the present study IL-4 was significantly

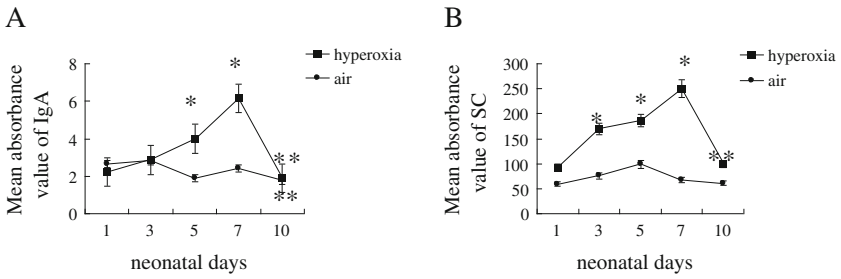

Fig. 6 The median absorbance values of SC and IgA. Compared with the air-inhaling group, the levels of IgA (on the 5th, and 7th days) and SC(on the 3rd, 5th, and 7th days) in the hyperoxia group were significantly increased (P<0.01). However, on the 10th day, in the hyperoxia group the level of IgA was close to that of the air-inhaling group and the level of SC was remarkbly decreased compared with those of 3rd, 5th, and 7th days (P<0.01); Data were reported as the means±SD (t-test) (n=10 in each time point)

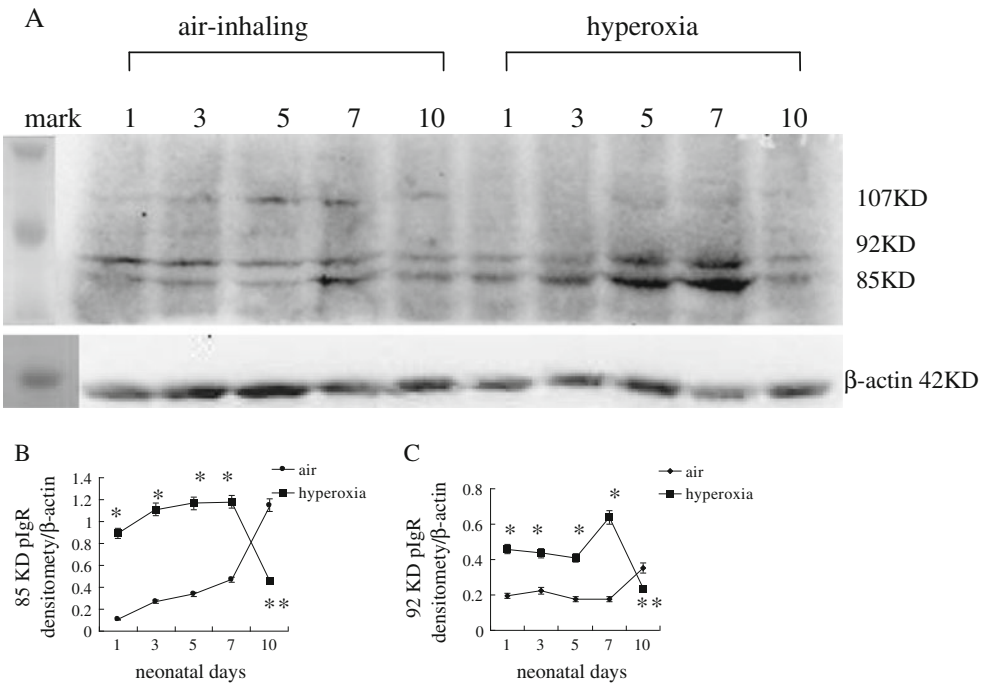

Fig. 7 Effect of hyperoxia on pIgR expression of pulmonary tissues. **a**: specific bands for pIgR. **b**: densitometric analysis of 85KD (n=10 in each time point). **c**: densitometric analysis of 92KD (n=10 in each time point). Both the relative expression rates of the 85KD and 92KD pIgR proteins in the hyperoxia group were significantly increased on the 1st, 3rd, 5th and 7th days(*P< 0.01) but significantly decreased on the 10th day compared with those of the air-inhaling group (**P<0.01).β-actin was served as an internal control. Densitometric measurements were used for analysis of pIgR protein levels normalized to β-actin

elevated in a day-dependent manner, though previous studies had demonstrated that IL-4 was not altered in hyperoxic lung injury in neonatal mice models [27]. *Waheed* et al. reportd that hyperoxia increased TGF in a neonatal rabbit hyperoxia-fibrosis model [28]. Our results showed that TGF-β stayed relatively constant at the first 3 days, but increased significantly in the late period of hyperoxia. So we speculated that TGF-β might be associated with pulmonary tissue impairment in late period of neonatal rats exposed to hyperoxia. Pro-inflammatory cytokines-TNF-α and IL-6 were increased in the hyperoxia group. This was in agreement with previous report [29]. Some authors thought that hyperoxia inducing pulmonary inflammation was mediated by TNF-α [19]. Therefore we concluded that the increased TNF-α would mediate the inflammatory response and lead to impairment of the lung [27]. Furthermore, IL-6 is a central regulator in the inflammatory. It is both a pro-inflammatory and an anti-inflammatory molecule. Its anti-inflammatory effect showed that it confered significant protection from hyperoxic acute lung injury. This protective response appeared to result from the ability of the IL-6 to inhibit hyperoxia-induced cell death [30, 31]. These rapid cytokine response in the setting of hyperoxic injury might play an important role in the adaptation of neonatal lungs to toxicity from prolonged oxygen exposure [27]. Although IL-1 was a multifunctional cytokine involved in immune responses, inflammation and hematopoiesis, in this study there was no significant change in the hyperoxia group.

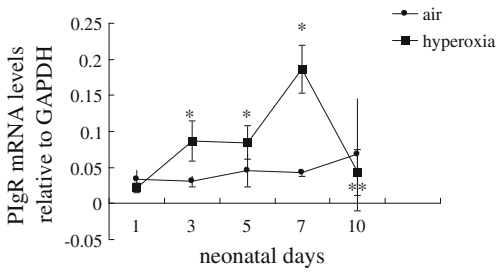

Fig. 8 Relative content of the pulmonary pIgR mRNA. Relative content of pulmonary pIgR mRNA on the 3rd, 5th and 7th days in the hyperoxia group were significantly increased compared with those of the air-inhaling group (*P<0.01). On the 10th day, the relative content of pulmonary pIgR mRNA was notably decreased compared with those of the 3rd, 5th and 7th days in the hyperoxia group(**P< 0.01) (Data were reported as the means ± SD, P<0.01) (n=10 in each time point)

The increased airway IgA after injury is thought to be a protective response and has been associated with increases in airway TNF-α and IL-6 [32]. IL-6 is an inducer of plasma cell development and also influences IL-4 production. IL-4 is known to upregulate IgA production and transport. TGF-β has been shown to be a crucial cytokine for IgA switching [1]. Thus, during the early period, the increase of TGF-β, TNF-α, IL-6 and IL-4 in the hyperoxia group might be factors in the pulmonary IgA increase. The expression of pIgR appear to be upregulated by different cytokines, such as TNF-α [25, 26], IL-4 [33, 34], IL-1 [35] and TGF-β [36]. As mentioned before, cytokines (IL-4, IL-6, TNF-α and TGF-β) were

significantly increased in the hyperoxia group. So we proposed that the increased expression of pIgR was probably related to the effect on epithelia of the higher production of these cytokines in the hyperoxia group.

Conclusions

Taken together, in the early period of hyperoxia, large amounts of IgA, SC and SIgA in the lungs of neonatal rats were produced to defend against acute pulmonary injury, but in the late period of hyperoxia, the decrease of IgA, SC and SIgA might lead to attenuation of pulmonary immunity. In hyperoxia condition, the elevated expression of cytokines might contribute to the expression of IgA and SC.

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