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Original Article

Effects of vitamin B-6 supplementation on oxidative stress and inflammatory response in neonatal rats receiving hyperoxia therapy

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ARTICLE INFO

Article history:
Received 25 October 2017
Received in revised form
8 January 2018
Accepted 10 January 2018
Available online 2 February 2018

Keywords:
Hyperoxia
Inflammation
Neonate
Oxidative stress
Vitamin B-6

ABSTRACT

Hyperoxia is often used in the treatment of neonates. However, protracted use of hyperoxia leads to significant morbidity. The purpose of this study was to evaluate the effects of vitamin B-6 supplementation on oxidative stress and inflammatory responses in neonatal rats undergoing hyperoxia therapy. The study consisted of 2 parts: a survival study and a vitamin B-6 efficacy study for 16 days. Neonatal rats were randomly divided into either the control group, B-6 group (subcutaneously injected with 90 mg/kg/d of pyridoxal 5′-phosphate [PLP]), O_2 group (treated with 85% oxygen), or O_2 + B-6 group (simultaneously treated with 85% oxygen and 90 mg/kg/d PLP). After the survival study was done, the vitamin B-6 efficacy study was performed with duplicate neonatal rats sacrificed on the 3rd, 6th, 9th, and 16th day. Serum inflammatory cytokines, tissue pathology, and malondialdehyde (MDA) levels were measured. In the survival study, the survival rate of neonatal rats in the control, B-6, O_2, and O_2 + B-6 group on the 16th day were 100%, 100%, 25%, and 62.50%, respectively. The efficacy study showed lung polymorphonuclear granulocyte (PMN) and macrophage infiltration, increased liver hemopoiesis, and higher MDA levels in liver homogenates at days 3 through 16 in the O_2 group. Vitamin B-6 supplementation considerably increased serum inflammatory cytokines in either the 6th or 9th day and decreased liver MDA level before the 6th day. These results indicate that neonatal rats receiving hyperoxia treatment suffered divergent serum inflammatory responses and were in increased liver

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https://doi.org/10.1016/j.jfda.2018.01.004

oxidative stress. Vitamin B-6 supplementation seemed to improve survival rates, change systemic inflammatory response, and decrease liver oxidative stress while neonatal rats were under hyperoxia treatment.

1. Introduction

Hyperoxia is often used in neonatal intensive care units for supportive care. Hyperoxia is defined as an excess of oxygen in tissues and organs (fraction of inspired oxygen > 60%), which can lead to the development of chronic lung disease [1,2], retinopathy, and brain injury in neonates [3,4]. A fetus develops in the uterus, which is a relatively hypoxic environment, and fetal antioxidant capabilities, such as the superoxide dismutase and glutathione antioxidant systems, are immature [5,6]. After birth, some neonates are exposed to hyperoxic conditions, which can increase levels of reactive oxygen species (ROS). This can in turn induce cellular damage through local activation of proinflammatory signaling and the recruitment of inflammatory cells into the vital organs, thereby resulting in uncontrolled tissue injury [7,8].

Accordingly, antioxidative therapy might prevent neonates from hyperoxia-induced complications. Many vitamins with antioxidative actions, such as vitamin A, C, and E, have been evaluated in neonates to prevent hyperoxia-induced injury, but the results have been inconclusive [9–11]. Vitamin B-6 (15–30 mg/kg/d) has been used as an effective agent in the treatment of pyridoxine-dependent seizure in neonates since 1954 [12,13]. In recent decades, vitamin B-6 has been shown to have a crucial role in antioxidant mechanism and inflammatory responses [14–18]. Vitamin B-6 is not only readily available in clinical settings but also a water-soluble vitamin, which might be safer than lipid soluble vitamins (e.g., vitamin A or E) as therapy for neonates.

Vitamin B-6 is a collective term for the metabolically and functionally related pyridoxine, pyridoxamine, and pyridoxal, as well as their phosphorylated forms, pyridoxine 5′-phosphate, pyridoxamine 5′-phosphate and pyridoxal 5′-phosphate (PLP). Pyridoxal 5′-phosphate is the physiologically active coenzyme form of vitamin B-6. Although the exact mechanism has not been fully ascertained, PLP may react with peroxy radicals and thereby scavenge free radicals and inhibit lipid peroxidation through its hydroxyl and amine group on the pyridine ring [14–16,19]. In addition, PLP acts as a coenzyme in the production of cytokines and other polypeptide mediators during inflammatory response [17]. Inadequate vitamin B-6, therefore, might directly decrease its antioxidant capacities or compromise inflammatory responses [20,21].

If neonates are under increased oxidative stress and inflammatory response during hyperoxia therapy [22,23], this could exhaust the use and metabolic turnover of plasma PLP and decrease tissue PLP reserves [24,25]. Therefore, it might be useful to determine whether vitamin B-6 supplementation would have a preventive effect in reducing oxidative stress or inflammatory responses while neonates are receiving hyperoxia therapy. In this study, we imitated clinical conditions by using neonatal rats in a hyperoxic environment. We then evaluated whether vitamin B-6 supplementation had an effect on oxidative stress, inflammatory response, and survival in neonatal rats with hyperoxia therapy.

2. Methods

2.1. Animals and study design

The first part of this study was a survival study and the second part of this study was a vitamin B-6 efficacy study. In the survival study, four pregnant Wistar rats were obtained from BioLASCO Taiwan Co., Ltd. and were raised in the animal center of Changhua Christian Hospital for 1 week before delivery. Sufficient water and normal diet were freely provided to the maternal rats, which were kept in a 12:12-h light–dark cycle. After delivery within 12 h, neonatal rats were randomly divided into four groups: 1) control group, neonatal rats were treated with room air and daily normal saline injections (equivalent volume of PLP); 2) hyperoxia group (O_2 group), neonatal rats were housed in a chamber (air jacket multi-gas incubator, Astec Co., Ltd.) and treated with 85% O_2 and daily normal saline injections; 3) vitamin B-6 group (B-6 group), neonatal rats were subcutaneously injected with PLP (90 mg/kg/d); 4) hyperoxia combined with vitamin B-6 group (O_2 + B-6 group), neonatal rats simultaneously treated with 85% O_2 and daily subcutaneous PLP (90 mg/kg/d) injections. All neonatal rats were fed by maternal rats during the experimental period. Maternal rats were rotated daily between the O_2-exposed rats and room air-exposed rats to avoid O_2 toxicity and to eliminate maternal effects among groups. Body weight and mortality of neonatal rats were monitored daily for 16 days.

The second part of this study was a vitamin B-6 efficacy study. In the first run of the efficacy study, we repeated the survival study design for 16 days, and neonatal rats of each group were sacrificed on the 16th day. Phenobarbital was injected intraperitoneally before the rats were sacrificed. In the second run of the efficacy study, another 6 pregnant Wistar rats were obtained and raised in the animal center of Changhua Christian Hospital for 1 week before delivery. Within 12 h after delivery, neonatal rats were randomly divided into 4 groups, as in the survival study. Four fertile maternal rats were selected and rotated daily between the oxygen and room air-exposed neonatal rats. On the 3rd, 6th, and 9th day, neonatal rats of each group were sacrificed. Neonatal rats with poor activity in each group were sacrificed in priority in order to keep the experimental numbers in each

group even. Blood and tissue samples were collected after anesthesia. Blood samples were drawn from the heart, transported on ice, and separated into plasma and red blood cells within 30 min through low-speed centrifugation (3000 rpm, 15 min, 4 °C). Tissues were immediately homogenized in phosphate-buffered saline. The homogenized solution was then centrifuged (12,000 rpm, 4 °C, 10 min). The supernatant was then carefully removed for analysis. All samples were stored frozen (−20 °C) until analysis.

All animal experiments were conducted in accordance with the rules of the Institutional Animal Care and Use Committee (IACUC) of Changhua Christian Hospital, Changhua, Taiwan (IACUC Approval No. CCH-AE-103-010).

2.2. Administration and dosage selection of PLP

Pyridoxal 5′-phosphate was purchased from Shinlin Sinseng Pharmaceutical Co., Ltd. (Taoyuan, Taiwan). The concentration of PLP solution was 10 mg/mL. A human equivalent dose calculation method was based on body surface area for dose selection. Because 15 mg/kg/day of PLP injection has been determined to be safe in the treatment of pyridoxine-dependent seizure in human, we then estimated that 90 mg/kg/day was appropriate for neonatal rats [13,26]. Additionally, because research has indicated that the lethal dose of pyridoxine aspartate given via the subcutaneous route for rats is approximately 3000 mg/kg, the choice of dosage in the present study was thought to be reasonable [27,28].

2.3. Measurement of oxidative stress and inflammatory response

Oxidative stress was estimated as levels of malondialdehyde (MDA). Lung and liver MDA was measured in terms of thiobarbituric acid reactive substances based on the method described by Lapenna et al. [29].

Plasma inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), interleukin-6 (IL-6), interleukin 17α (IL-17α), macrophage inflammatory protein 3α (MIP-3α), monocyte chemoattractant protein-1 (MCP-1), and vascular endothelial growth factor (VEGF) were detected using the Bio-Plex Immunoassay Multiplex System (Suu-Flower CO., Ltd. [Taichung, Taiwan]). This system uses different detectable bead sets as substrate capturing analytes in solution and employs fluorescent methods for detection. In addition to plasma cytokine levels, we used western blot technique to quantity IL-6 protein levels in lung and liver homogenates to detect tissue inflammatory cytokine levels.

2.4. Pathology of lung and liver tissues

After the right main bronchus was ligated, right middle lung tissue was removed from each neonatal rat for the pathologic examination. The remaining tissue was left for the preparation of lung homogenate. The liver tissue was cut into two blocks for the pathological examination and tissue homogenates. Pathological samples were put into 4% paraformaldehyde (Sigma Chemical Co., Saint Louis, MO, USA) in room temperature for 2 weeks. They were then embedded in paraffin, cut, and stained through the hematoxylin and eosin method. We observed pathological changes of the liver and lung by using a Nikon 80i research microscope. The number of lung polymorphonuclear granulocyte (PMN), macrophage, and liver hemopoietic foci were assessed in five nonoverlapping fields at a × 400 magnification for each animal.

2.5. Statistical analyses

Data were analyzed using the SAS statistical software (version 9.3; The SAS Institute Inc., Cary, NC, USA). Differences of weight, cytokine, and oxidative stress levels among groups were determined through one-way analysis of variance on ranks, and Tukey test was used for the *post hoc* analysis. The Kaplan–Meier method was used to evaluate the survival of neonatal rats, and the log-rank test was used to compare the survival differences among groups.

Multiple linear regression analysis was used to assess the effect of different treatments on oxidative stress indicator or cytokine levels. The treatment was set as the independent variable and oxidative stress indicator or cytokine levels were set as the dependent variable after adjusting for the study period. All data were presented as means ± standard error. Statistical significance was set as $p < 0.05$.

3. Results

3.1. Effect of vitamin B-6 on survival rate in neonatal rats exposed to hyperoxia

In the survival study, 47 neonatal rats (27 males and 20 females) were born from 4 maternal rats. In our earlier experiments, the survival rate was 20% in neonatal rats exposed to hyperoxia for 14 days. For the poor survival rate and to further confirm the effect of B-6 on the survival, we doubled the experimental numbers in the O_2 and O_2 + B-6 group in this part of study. Neonatal rats were randomly divided into either the control group (n = 7, 3 males, 4 females), B-6 group (n = 8, 4 males, 4 females), O_2 group (n = 16, 10 males, 6 females), and O_2 + B-6 group (n = 16, 10 males, 6 females). Mean weight changes of neonatal rats in different treatment groups from birth to day 16 are shown in Table 1. There was no significant weight difference among groups on day 0. Although mean weight of neonatal rats gradually increased during the study period, mean weight gain of neonatal rats in the O_2 and O_2 + B-6 groups slowed after day 2 compared with control and B-6 groups. Plasma PLP was only measured in 3 neonatal rats in each group due to the blood volume was limited. Plasma PLP concentration was 462.09 ± 72.99, 909.57 ± 521.23, 1003.27 ± 645.60, and 9957.06 ± 15,864.57 in control, B-6, O_2, and O_2 + B-6 groups, respectively (plasma PLP concentration significantly increased in vitamin B-6 supplemented groups, $p < 0.01$).

Fig. 1 illustrates the survival curve of the four groups. No neonatal rats died during the study period in the control and B-6 groups. However, neonatal rats started to die at day 7 in the O_2 group, and only 4 neonatal rats in this group survived at the end of study period (day 16). By contrast, while 1 neonatal rat died at the day 8, 11 neonatal rats survived at the end of the study in the O_2 + B-6 group. Log rank test showed O_2 group had statistically low survival rate compare to control ($p = 0.01$), B-6

Table 1 – Weight changes of neonatal rats in different treatment groups during the survival study.

Weight (g)	Control (n = 7)	B-6 (n = 8)	O_2 (n = 16)	O_2 + B-6 (n = 16)
Day 0	6.86 ± 0.38	7.00 ± 0.76	7.06 ± 0.57	7.00 ± 0.52
Day 1	7.86 ± 0.69	7.75 ± 0.46	7.44 ± 0.51	7.31 ± 0.70
Day 2	9.43 ± 0.54^{a}	9.00 ± 0.54^{a,b}	8.25 ± 0.68^{b}	8.25 ± 0.78^{b}
Day 3	10.57 ± 0.54^{a}	10.88 ± 0.64^{a}	9.31 ± 0.7^{b}	9.25 ± 0.93^{b}
Day 4	13.43 ± 0.79^{a}	12.50 ± 0.93^{b}	10.31 ± 0.79^{c}	9.88 ± 0.96^{c}
Day 5	15.57 ± 0.54^{a}	14.29 ± 0.95^{b}	10.94 ± 1.00^{c}	10.63 ± 1.09^{c}
Day 6	17.43 ± 0.79^{a}	17.00 ± 1.07^{a}	12.19 ± 1.22^{b}	12.13 ± 1.31^{b}
Day 7	19.57 ± 0.98^{a}	19.00 ± 1.31^{a}	13.07 ± 1.03^{b} (n = 15)	12.38 ± 1.31^{b}
Day 8	22.57 ± 0.98^{a}	21.38 ± 1.51^{a}	14.36 ± 1.08^{b} (n = 14)	14.21 ± 1.63^{b} (n = 14)
Day 9	25.29 ± 0.95^{a}	23.13 ± 1.55^{b}	15.31 ± 1.44^{c} (n = 13)	15.00 ± 1.84^{c} (n = 14)
Day 10	27.14 ± 1.35^{a}	25.50 ± 1.60^{a}	16.83 ± 2.25^{b} (n = 12)	15.86 ± 2.07^{b} (n = 14)
Day 11	30.43 ± 1.13^{a}	28.25 ± 1.67^{a}	18.40 ± 2.12^{b} (n = 10)	17.00 ± 3.64^{b} (n = 14)
Day 12	32.57 ± 1.27^{a}	30.25 ± 1.49^{a}	22.83 ± 2.04^{a,b} (n = 6)	18.36 ± 2.85^{b} (n = 14)
Day 13	35.00 ± 1.00^{a}	32.50 ± 1.85^{b}	25.40 ± 2.07^{c} (n = 5)	19.93 ± 3.00^{d} (n = 14)
Day 14	37.29 ± 1.50^{a}	34.75 ± 1.75^{a}	28.60 ± 1.95^{a,b} (n = 5)	21.39 ± 3.07^{b} (n = 13)
Day 15	39.43 ± 1.40^{a}	36.38 ± 1.51^{b}	30.40 ± 1.95^{c} (n = 5)	22.42 ± 2.97^{d} (n = 12)
Day 16	41.29 ± 1.25^{a}	38.75 ± 1.75^{a}	35.00 ± 2.83^{b} (n = 4)	23.91 ± 3.08^{c} (n = 11)

^{a,b,c,d} Values with different superscript letters are significantly different among different treatment groups at same day; $p < 0.05$.

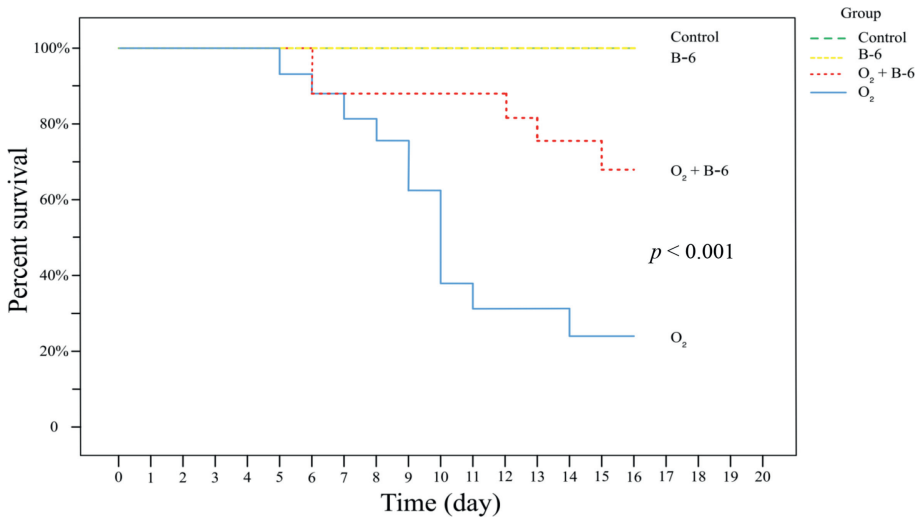

Fig. 1 – Kaplan–Meier curve of survival of four experimental groups for efficacy study.

($p < 0.01$), and O_2 + B-6 ($p = 0.04$) groups, while survival rate in O_2 + B-6 group had no difference when compared with either control or B-6 groups.

We duplicated the design of the survival study in the first run of the efficacy study. A total 52 neonatal rats (29 males and 23 females) were born from 4 maternal rats. Neonatal rats were randomly divided into four groups: the control group (n = 10, 5 males, 5 females), B-6 group (n = 10, 6 males, 4 females), O_2 group (n = 16, 9 males, 7 females), and O_2 + B-6 group (n = 16, 9 males, 7 females). Four female neonatal rats died in the O_2 group during this part of the study.

During the 2nd run of the efficacy study, 77 neonatal rates were born from 6 maternal rats. A total 60 neonatal rats were selected and randomly assigned to the control group (n = 15); B-6 group (n = 15); O_2 group (n = 15); and O_2 + B-6 group (n = 15), with 7 males and 8 females in each group. Four of 6 maternal rats were selected and rotated daily between the O_2 and room air-exposed neonatal rats. On the 3rd and 6th day, 2 male and 3 female neonatal rats were sacrificed in each group. On the 9th day, 3 male and 2 female neonatal rats were sacrificed in each group. No neonatal rats died during the 2nd run of the efficacy study. Table 2 lists body weight and adjusted tissue weight of the four groups in the efficacy study. Similar to what was observed in the survival study, the increase of body weight was much slower in both O_2 and O_2 + B-6 groups compared with control and B-6 groups. The O_2 + B-6 group had the lowest body weight of the four experimental groups on day 16. Likewise, the lung-to-body weight ratio decreased on 6th day but conversely increased on days 9 and 16. The PLP treatment had no effect on liver weight but tended to decrease lung-to-body weight ratio on day 16.

3.2. *Vitamin B-6 supplement had influence on serum inflammatory cytokine*

The levels of inflammatory markers are shown in Table 3. In addition to IL-17α, different cytokine levels were substantially lower at day 16 than at day 3 in each group. Among the

Table 2 – Weight changes and adjusted tissue weight of neonatal rats in different treatment groups during the efficacy study period.

	Control	B-6	O_2	O_2 + B-6
Body weight of mechanistic study (g)				
Day 3	10.60 ± 0.24^{a}	10.60 ± 0.25^{a}	9.40 ± 0.25^{b}	9.20 ± 0.37^{b}
Day 6	15.20 ± 0.37^{a}	14.00 ± 0.45^{a}	$14.20 \pm 0.37^{a,b}$	13.60 ± 0.24^{b}
Day 9	23.80 ± 0.80^{a}	23.80 ± 0.80^{a}	19.40 ± 0.93^{b}	18.40 ± 0.51^{b}
Day 16	35.73 ± 0.72^{a}	37.21 ± 0.84^{a}	25.64 ± 0.75^{b}	19.18 ± 0.53^{c}
Adjust spleen weight (g/g body weight)*1000				
Day 3	3.71 ± 0.42	3.55 ± 0.38	3.50 ± 0.27	3.91 ± 0.17
Day 6	5.55 ± 0.41^{a}	7.99 ± 0.23^{b}	$6.64 \pm 0.65^{a,b}$	$7.13 \pm 0.38^{a,b}$
Day 9	6.95 ± 0.11^{a}	$7.10 \pm 0.56^{a,b}$	$7.93 \pm 0.38^{a,b}$	8.52 ± 0.27^{b}
Day16	$3.78 \pm 0.17^{a,b}$	4.14 ± 0.12^{a}	$3.91 \pm 0.10^{a,b}$	3.43 ± 0.21^{b}
Adjust lung weight (g/g body weight)*100				
Day 3	1.92 ± 0.07	1.87 ± 0.10	1.85 ± 0.05	1.85 ± 0.04
Day 6	1.83 ± 0.04^{a}	1.76 ± 0.12^{a}	1.36 ± 0.04^{b}	$1.51 \pm 0.14^{a,b}$
Day 9	1.67 ± 0.06	1.69 ± 0.05	1.67 ± 0.18	1.39 ± 0.10
Day16	1.32 ± 0.16^{ab}	1.08 ± 0.03^{b}	2.05 ± 0.16^{c}	$1.73 \pm 0.06^{c,a}$
Adjust liver weight (g/g body weight)*100				
Day 3	3.37 ± 0.06	3.72 ± 0.06	3.38 ± 0.02	3.08 ± 0.12
Day 6	3.10 ± 0.10	3.12 ± 0.15	3.34 ± 0.11	3.05 ± 0.22
Day 9	$3.28 \pm 0.09^{a,b}$	3.40 ± 0.06^{a}	$3.91 \pm 0.08^{b,c}$	4.05 ± 0.26^{c}
Day16	$3.02 \pm 0.06^{a,b}$	3.10 ± 0.09^{a}	$2.97 \pm 0.05^{a,b}$	2.85 ± 0.05^{b}

Values are presented as mean ± standard error. ^{a,b,c} Values with different superscript letters are significantly different among different treatment groups at same day; $p < 0.05$; N = 5 in each group on day 3, 6 and 9; N = 10 in control and B6 group on day 16; N = 12 in O_2 group on day 16; N = 16 in O_2 + B-6 group on day 16.

different treatment groups, neonatal rats under hyperoxia exposure (O_2 group) had divergent performance on IL-1β, IL-6, IL-17α, TNF-α, macrophage colony-stimulating factor (M-CSF), MIP-3α, and VEGF levels. As an example, hyperoxia elevated MIP-3α on day 9 but inversely decreased it on day 16. Also, IL-6 was elevated in the O_2 and O_2 + B-6 groups on day 9 but was undetectable in all groups on day 16. Additionally, TNF-α and M-CSF in the O_2 group also had lower values than in the control group on day 16. After PLP supplementation, IL-1β increased from day 3 to day 9 and MCP-1 increased on the 6th day. In comparison with the O_2 group, supplemental PLP in hyperoxia helped maintain serum inflammatory cytokine levels close to those of the control group on day 16.

3.3. *Vitamin B-6 decreased hepatic oxidative stress under hyperoxia*

Table 4 displays oxidative stress indicator and IL-6 levels in liver and lung tissues of different treatment groups. Exposure to a hyperoxic environment increased oxidative stress (i.e., MDA level) in liver tissue during days 3–16 day and on day 3 in lung tissue compared with the control groups. However, liver and lung MDA levels tended to decrease with time within the group even under high oxygen conditions in the hyperoxia group. In contrast to oxidative stress status in the liver and lung, hyperoxia temporally lowered IL-6 in liver homogenate on day 9 but had no effect on lung IL-6 in our work. Injection of PLP protected the liver from oxidative stress on the 3rd day to the 6th day.

3.4. *Pathologic change after hyperoxia exposure and vitamin B-6 supplement*

The pathological changes of the liver and lung in different treatment groups are shown in Table 5 and Figs. 2 and 3. The size of alveolar sac, level of PMNs and macrophage infiltration in lung tissue was considerably larger and increased in both O_2 and O_2 + B-6 groups compared with control groups. PMNs peaked on the 3rd day and macrophages peaked on the 9th day. The levels of liver hemopoiesis were significantly higher at the 3rd day than at the 6th, 9th, and 16th days in each group. Hyperoxia exposure was found to prolong the duration of liver hemopoiesis up to 16 days. Supplemental PLP did not change PMNs and macrophage infiltration in the lung, but it increased the duration of liver hemopoiesis up to 6 days.

3.5. *Influence of vitamin B-6 supplement on early oxidative stress and cytokine levels*

Multiple linear regression was done to assess the effect of different treatments on oxidative stress indicator and cytokine levels after adjusting for the study period before 9 days (Table 6). Using the control group set as the reference group, hyperoxia (O_2 group and O_2 + B-6 group) had substantial effects on liver MDA, serum TNF-α, lung PMN, and macrophage levels. Vitamin B-6 supplement was correlated with increased serum IL-1β. Hyperoxia combined with vitamin B-6 treatments (O_2 + B-6 group) increased serum IL-17α and VEGF values.

4. Discussion

Hyperoxia-induced complications and molecular changes have been widely discussed [1–4]. However, to the best of our knowledge, this is the first study to evaluate whether vitamin B-6 supplementation had beneficial effects on hyperoxia-induced oxidative stress and inflammatory response in neonatal rats. Consistent with previous studies,

Table 3 – Serum cytokine measurements of neonatal rats in different treatment groups.

	Control	B-6	O_2	O_2 + B-6
Interleukin-1β (pg/mL)				
Day 3	194.91 ± 116.83^{b}	486.35 ± 102.87$^{a;**}$	121.14 ± 45.76$^{b;**,*}$	118.22 ± 14.73$^{b;**}$
Day 6	254.03 ± 152.28^{b,c}	861.11 ± 24.05$^{a;*}$	42.75 ± 7.10$^{c;**,*}$	826.69 ± 40.32$^{a;*}$
Day 9	108.40 ± 95.22^{b}	715.32 ± 57.51$^{a;*}$	232.76 ± 105.01$^{b;*}$	709.61 ± 49.70$^{a;\dagger}$
Day 16	26.98 ± 5.73^{a,b}	11.16 ± 2.39$^{ab;\dagger}$	9.92 ± 2.05$^{b;**}$	30.11 ± 7.19$^{a;**}$
Interleukin-6 (pg/mL)				
Day 3	14.62 ± 3.88	13.75 ± 2.25	18.10 ± 1.65	16.46 ± 1.65
Day 6	13.94 ± 3.17	13.75 ± 1.62	15.59 ± 3.70	15.53 ± 3.70
Day 9	–	–	147.74 ± 112.32	82.14 ± 60.41
Day 16	–	–	–	–
Interleukin-17α (pg/mL)				
Day 3	9.21 ± 1.56*	8.82 ± 1.77*	11.35 ± 2.02*	13.32 ± 0.90*
Day 6	4.13 ± 1.44**	4.72 ± 1.48*	5.19 ± 1.11*	7.39 ± 0.86*
Day 9	1.37 ± 0.83$^{b;**}$	0.84 ± 0.62$^{b;**}$	2.27 ± 0.73$^{b;*}$	5.08 ± 0.51$^{a;**}$
Day 16	45.22 ± 6.91†	41.19 ± 7.55**	24.92 ± 2.12**	42.97 ± 6.53**
TNF-α (pg/mL)				
Day 3	218.90 ± 42.56*	293.56 ± 54.69*	358.51 ± 104.20*	326.32 ± 29.54*
Day 6	100.34 ± 56.06$^{b;*,**}$	264.64 ± 68.71$^{a,b;*,\dagger\dagger}$	336.50 ± 77.78$^{a,b;*}$	398.00 ± 53.93$^{a;*}$
Day 9	39.84 ± 34.29$^{b;**}$	29.63 ± 17.64$^{b;**,\dagger\dagger}$	57.50 ± 29.01$^{b;**}$	192.85 ± 21.63$^{a;**}$
Day 16	17.49 ± 6.13$^{a;**}$	7.10 ± 0.99$^{ab;**,\dagger\dagger}$	4.22 ± 0.60$^{b;\dagger}$	7.71 ± 1.50$^{ab;\dagger}$
MCP-1 (pg/mL)				
Day 3	3199.08 ± 834.02*	6823.08 ± 1714.71*	2688.65 ± 273.20*	3500.54 ± 483.15*
Day 6	2450.62 ± 685.01$^{b;*}$	7879.55 ± 1096.23$^{a;*}$	2517.58 ± 530.00$^{b;*}$	4888.56 ± 1071.72$^{b;*,**}$
Day 9	6340.74 ± 935.01**	7178.72 ± 1748.07*	5079.97 ± 904.29**	6027.39 ± 414.49**
Day 16	1150.17 ± 307.71*	829.99 ± 140.67**	711.25 ± 88.04†	1257.00 ± 315.70†
M-CSF (pg/mL)				
Day 3	260.76 ± 24.63	256.12 ± 34.96*	257.51 ± 26.14*	251.21 ± 15.51*
Day 6	241.48 ± 50.62	245.35 ± 24.88*	238.61 ± 22.40*	221.77 ± 31.27*
Day 9	262.01 ± 11.84	208.01 ± 12.59*	203.52 ± 13.46*	223.68 ± 14.11*
Day 16	131.33 ± 34.32^{a}	63.75 ± 6.49$^{ab;**}$	24.64 ± 2.20$^{b;**}$	68.81 ± 19.26$^{ab;**}$
MIP-3α (pg/mL)				
Day 3	44.72 ± 9.84*	43.68 ± 6.08	51.91 ± 8.80	51.33 ± 8.04**,*
Day 6	8.67 ± 2.46*	29.44 ± 7.54	45.30 ± 13.24	35.31 ± 4.67*
Day 9	32.78 ± 0.98$^{b;*}$	25.14 ± 3.54^{b}	82.18 ± 18.50^{a}	81.58 ± 13.28$^{a;**}$
Day 16	76.68 ± 10.13$^{a;**}$	26.54 ± 4.38^{b}	22.36 ± 5.47^{b}	26.54 ± 4.38$^{b;*}$
VEGF (pg/mL)				
Day 3	106.76 ± 11.65*	103.92 ± 18.52*	74.58 ± 2.29*	116.75 ± 5.39*
Day 6	82.43 ± 6.92*,**	96.39 ± 12.53*,**	120.47 ± 24.19**	124.32 ± 25.40*
Day 9	68.38 ± 5.46$^{b;*,**}$	58.12 ± 5.44$^{b;**}$	67.53 ± 9.39$^{b;*}$	109.93 ± 11.90$^{a;*}$
Day 16	44.24 ± 14.43**	12.27 ± 3.17†	11.81 ± 2.78†	41.55 ± 13.25**

Values are presented as mean ± standard error. TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor. *,**,†,†† Values with different superscript symbols are significantly different within the group; $p < 0.05$. ^{a,b,c,d} Values with different superscript letter are significantly different among different treatment groups at same day; $p < 0.05$. – Value were undetectable by Bio-Plex immunoassay multiplex system. N = 5 in each group on day 3, 6, 9; N = 10 in control and B6 group on day 16; N = 12 in O_2 group on day 16; N = 16 in O_2 + B-6 group on day 16.

we did observe that hyperoxia exposure reduced body weight gain, induced pathological changes to the lung and liver, and increased the mortality rate of neonatal rats. We further found that hyperoxia exposure induced organ oxidative stress and had variable effects on serum inflammatory cytokine levels (first increased and then decreased). Daily injection of 90 mg/kg PLP could protect against increased oxidative stress, balance decreased serum inflammatory responses, and improve survival outcomes in neonatal rats receiving hyperoxia therapy.

In the clinical setting, newborn babies received hyperoxia treatment because of a variety of diseases. High oxygen exposure itself may not directly lead to mortality in newborns but may cause complications. However, in the animal study, the mortality rate of neonatal rats during 16 days of hyperoxia treatment was 75% in our survival study. A previous study examined oxygen-induced injury and found mortality rates of roughly 40% after 4 weeks in neonatal rats and 57% after 2 weeks in neonatal mice [30,31]. The injection of PLP seemed to have a protective effect and reduced the mortality rate (43.75%) during the 16 days of hyperoxia treatment. Although the reasons for this protective effect are not fully understood, vitamin B-6 might play a key role in antioxidant and inflammatory mechanisms.

The animal model used in the present study was based on the Northway protocol, which is a typical research model for neonatal chronic lung disease [8]. Compatible with previous findings, lung PMNs of neonatal rats increased earlier than macrophages, and the lung progressed to fibrotic change with a decreased number of alveoli and enlarged terminal airways after hyperoxia exposure [31]. Similar to the findings of Marconi et al., our pathology results also showed the time

Table 4 – Oxidative stress indicator and interleukin-6 level of neonatal rats in tissues in different treatment groups.

	Control	B-6	O_2	O_2 + B-6
Liver MDA (μM/mg protein)				
Day 3	$0.53 \pm 0.09^{b;*,**}$	$0.46 \pm 0.09^{b;*,**}$	$1.69 \pm 0.61^{a;*}$	$0.85 \pm 0.24^{b;**,*}$
Day 6	$0.33 \pm 0.03^{b;*,**}$	$0.64 \pm 0.11^{a;*}$	$1.32 \pm 0.66^{a;*,**}$	$0.94 \pm 0.13^{a;*}$
Day 9	$0.29 \pm 0.05^{c;*}$	$0.53 \pm 0.13^{b;*}$	$0.61 \pm 0.13^{b;*,**}$	$1.60 \pm 0.30^{a;\dagger}$
Day 16	$0.16 \pm 0.04^{a;*}$	$0.16 \pm 0.03^{a;**}$	$0.33 \pm 0.04^{b;**}$	$0.33 \pm 0.04^{b;**}$
Lung MDA (μM/mg protein)				
Day 3	$0.27 \pm 0.02^{b;*}$	$0.24 \pm 0.07^{b;*,**}$	$0.78 \pm 0.17^{a;*}$	$0.69 \pm 0.15^{a;*}$
Day 6	$0.25 \pm 0.04^{b;*}$	$0.40 \pm 0.05^{a;**,\dagger}$	$0.29 \pm 0.03^{a,b;**,\dagger}$	$0.33 \pm 0.02^{a,b;**}$
Day 9	$0.47 \pm 0.08^{a;**}$	$0.50 \pm 0.05^{a;\dagger,\dagger\dagger}$	$0.40 \pm 0.05^{a;**}$	$0.18 \pm 0.04^{b;**,\dagger}$
Day 16	$0.14 \pm 0.02^{*}$	$0.15 \pm 0.02^{*}$	$0.11 \pm 0.02^{\dagger}$	$0.12 \pm 0.01^{\dagger}$
Liver interleukin-6 (pg/mL)				
Day 3	$0.41 \pm 0.17^{*}$	$0.49 \pm 0.15^{*,**}$	$0.35 \pm 0.07^{*}$	$0.33 \pm 0.05^{*}$
Day 6	$0.54 \pm 0.10^{*,**}$	$0.78 \pm 0.05^{*,**}$	$0.65 \pm 0.07^{**}$	$0.75 \pm 0.08^{**}$
Day 9	$0.50 \pm 0.05^{a;*,**}$	$0.39 \pm 0.03^{a,b;*}$	$0.33 \pm 0.03^{b;*}$	$0.45 \pm 0.03^{a,b;*,**}$
Day 16	$0.76 \pm 0.08^{**}$	$0.78 \pm 0.11^{**}$	$0.59 \pm 0.06^{*,**}$	$0.73 \pm 0.11^{**}$
Lung interleukin-6 (pg/mL)				
Day 3	$0.55 \pm 0.04^{*,**}$	$0.46 \pm 0.04^{*}$	0.57 ± 0.03	0.58 ± 0.04
Day 6	$0.43 \pm 0.05^{*}$	$0.36 \pm 0.03^{*}$	0.52 ± 0.10	0.55 ± 0.17
Day 9	$0.82 \pm 0.06^{**}$	$0.59 \pm 0.09^{*,**}$	0.80 ± 0.18	0.57 ± 0.06
Day 16	$0.80 \pm 0.07^{**}$	$0.84 \pm 0.09^{**}$	0.71 ± 0.05	0.70 ± 0.04

N = 5 in each group on day 3, 6, 9; N = 10 in control and B6 group on day 16; N = 12 in O_2 group on day 16; N = 16 in O_2 + B-6 group on day 16. Values are presented as mean ± standard error. MDA, malondialdehyde. *,**,†,†† Values with different superscript symbols are significantly different within the group; $p < 0.05$. ^{a,b,c,d} Values with different superscript letter are significantly different among different treatment groups at same day; $p < 0.05$.

Table 5 – Pathological changes of neonatal rats in liver and lung in different treatment groups.

	Control	B-6	O_2	O_2 + B-6
Liver hemopoiesis				
Day 3	$92.13 \pm 1.36^{*}$	$96.20 \pm 2.17^{*}$	$86.60 \pm 7.28^{*}$	$94.40 \pm 7.77^{*}$
Day 6	$14.93 \pm 1.70^{a;**}$	$27.86 \pm 1.53^{b;**}$	$27.87 \pm 2.80^{b;**}$	$30.20 \pm 2.61^{b;**}$
Day 9	$13.33 \pm 1.71^{a;**}$	$13.13 \pm 1.55^{a;\dagger}$	$35.46 \pm 3.47^{b;**}$	$34.84 \pm 2.21^{b;**}$
Day 16	$0.69 \pm 0.06^{a;\dagger}$	$0.65 \pm 0.05^{a;\dagger\dagger}$	$2.26 \pm 0.35^{b;\dagger}$	$1.16 \pm 0.13^{a;\dagger}$
Lung PMN				
Day 3	$3.60 \pm 0.36^{a;*}$	$4.04 \pm 0.40^{a;*}$	$8.84 \pm 0.61^{b;*}$	$10.12 \pm 0.94^{b;*}$
Day 6	$1.92 \pm 0.16^{a;**}$	$2.44 \pm 0.24^{a;**}$	$4.92 \pm 0.25^{a;**}$	$4.80 \pm 0.28^{b;**}$
Day 9	$2.80 \pm 0.11^{a;*,**}$	$2.84 \pm 0.07^{a;**}$	$5.52 \pm 0.50^{b;**}$	$5.45 \pm 0.79^{b;**}$
Day 16	$1.56 \pm 0.40^{a;**}$	$2.37 \pm 0.16^{a;**}$	$5.07 \pm 0.90^{b;**}$	$5.20 \pm 0.21^{b;**}$
Lung macrophage				
Day 3	2.68 ± 0.32^{ab}	2.44 ± 0.32^{a}	$4.04 \pm 0.37^{b,c;*}$	$4.20 \pm 0.39^{c;*}$
Day 6	2.00 ± 0.29^{a}	2.28 ± 0.15^{a}	$4.76 \pm 0.43^{b;*}$	$4.52 \pm 0.50^{b;*}$
Day 9	1.96 ± 0.19^{a}	2.76 ± 0.23^{a}	$6.72 \pm 0.49^{b;**}$	$6.20 \pm 0.26^{b;**}$
Day 16	1.28 ± 0.34^{a}	1.83 ± 0.15^{a}	$6.80 \pm 0.23^{a;**}$	$5.97 \pm 0.27^{b;**}$

N = 5 in each group on day 3, 6, 9; N = 10 in control and B6 group on day 16; N = 12 in O_2 group on day 16; N = 16 in O_2 + B-6 group on day 16. *,**,†,††Values with different superscript letter are significantly different among day 3, 6, 9 and 16 within the group; $p < 0.05$. ^{a,b,c} Values with different superscript symbols are significantly different among different treatment groups at same day; $p < 0.05$. Values are presented as mean ± standard error. PMN, polymorphonuclear leukocyte.

course of liver hemopoiesis under hyperoxia therapy [32]. Vitamin B-6 supplementation did not change the gross lung pathology but did elevate liver hemopoiesis on the 6th day. Because vitamin B-6 is known to be one of the basic substances required for hemopoiesis [33], the mechanism behind PLP and hyperoxia-induced hemopoiesis may be quite different. As such, several mechanisms, including evaluating hemompoietic stem cell activation, must be studied.

Hyperoxia exposure might induce hyperpermeability of pulmonary microvasculature and plasma extravasations leading to pulmonary edema [34]. This might explain why we observed that hyperoxia affected lung development and the relatively small of lung-to-body weight ratios early in hyperoxia therapy but increased lung-to-body weight ratios on the 16th day. In the study conducted by Dionysis et al., mice exposed to 100% oxygen for 14 days also had smaller but heavier lungs than did those lungs exposed to normal room air. Studies have also shown that PLP had an effect on diuresis [35]. Because there were no pathologic differences between the O_2 and O_2 + B-6 groups, the relatively low lung-to-body weight ratio in the O_2 + B-6 group on day 16 might be due to this diuretic effect. Diuresis may alleviate lung edema caused

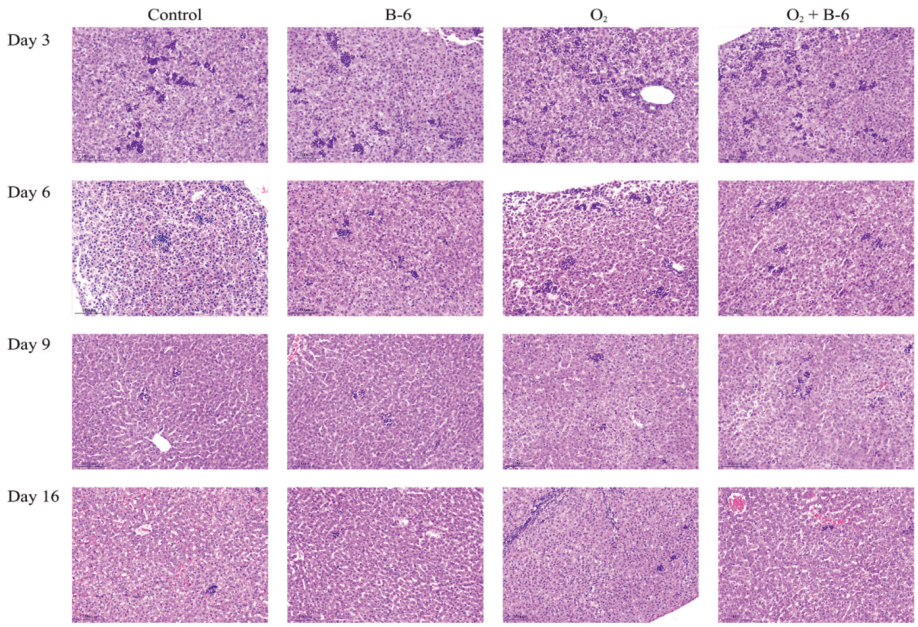

Fig. 2 – Liver pathology among different treatment groups at 3, 6, 9 and 16 days.

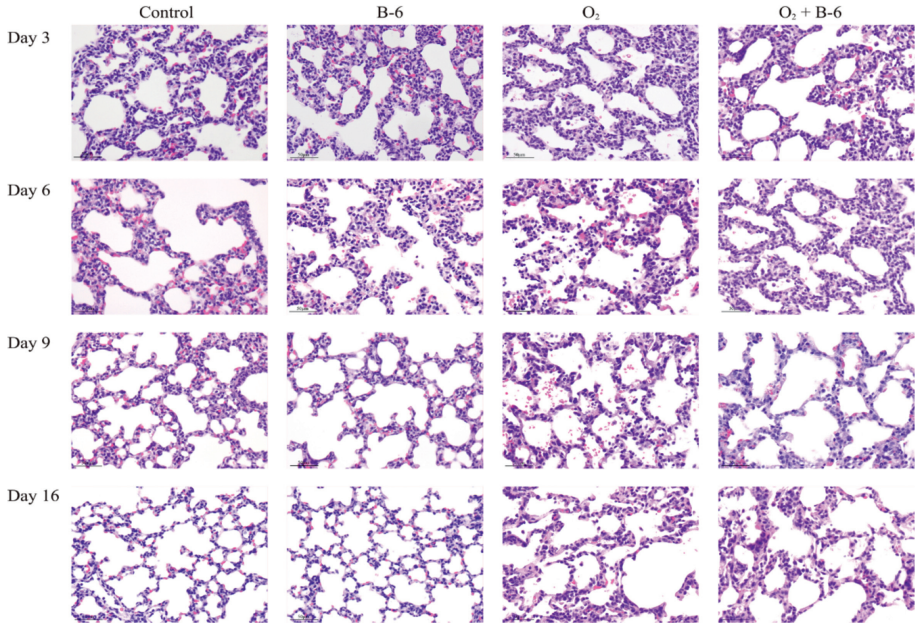

Fig. 3 – Lung pathology among different treatment groups at 3, 6, 9 and 16 days.

by hyperoxia, thereby improving lung compliance and respiratory function. Other evidence of diuresis was that body weight tended to be lower in groups with PLP injection in both the survival and efficacy study.

Oxygen administration over an extended period of time is toxic to lungs, which is known as the Lorrain Smith effect. Hyperoxia can harm the linings of the airway and alveoli and produces a large influx of ROS, which can cause lipid peroxidation, as well as protein and DNA damage. In our study, we observed a transient increase of lung MDA in both O_2 and O_2 + B-6 groups on the 3rd day, which normalized thereafter. Similar to our findings, Nagato et al. evaluated the time course of oxidative damage in mouse lung homogenates, where they found that lung MDA levels were elevated soon after hyperoxia exposure but normalized after 48 h. Management of ROS overproduction requires a good balance between pro-oxidant and antioxidant systems. Vitamin B-6 has been demonstrated to have strong antioxidant effects [14–16,36]. In the liver, the injection of PLP protected the liver from oxidative stress on the 3rd day to the 6th day. However, the antioxidant effect of vitamin B-6 did not persist to the 9th and 16th day in this study. Because vitamin B-6 was metabolized in the liver, whether the adverse effect on oxidative stress was due to overdose of PLP might be a possibility. The optimal dose and

Table 6 – The effects of different treatments on oxidative stress and cytokine levels in first part of mechanistic study.

	Control	B-6	O_2	O_2 + B-6
Liver MDA (μM/mg protein)	Reference	0.16 ± 0.25	0.82 ± 0.25†	0.75 ± 0.25†
Lung MDA (μM/mg protein)	Reference	0.08 ± 0.09	0.16 ± 0.08	0.07 ± 0.08
Serum interleukin-1β (pg/mL)	Reference	501.81 ± 86.05†	−53.57 ± 86.05	365.72 ± 86.05†
Serum interleukin-6 (pg/mL)	Reference	5.31 ± 39.36	33.98 ± 36.26	10.51 ± 37.03
Serum interleukin-17α (pg/mL)	Reference	−0.07 ± 1.10	1.47 ± 1.05	3.80 ± 1.05†
Serum TNF-α (pg/mL)	Reference	96.64 ± 49.76	142.25 ± 48.21†	190.72 ± 47.67†
Serum VEGF (pg/mL)	Reference	0.29 ± 11.79	1.67 ± 11.79	31.14 ± 11.79*
Liver interleukin-6 (pg/mL)	Reference	0.07 ± 0.08	−0.04 ± 0.08	0.03 ± 0.08
Lung interleukin-6 (pg/mL)	Reference	−0.13 ± 0.08	0.03 ± 0.08	−0.03 ± 0.08
Liver haemopoiesis	Reference	5.60 ± 6.93	9.84 ± 6.93	11.85 ± 7.06
Lung PMN	Reference	0.33 ± 0.56	3.65 ± 0.56†	4.02 ± 0.57†
Lung macrophage	Reference	0.28 ± 0.35	2.96 ± 0.35†	2.71 ± 0.35†

Values are presented as mean ± standard error (N = 5). Multiple linear regression analysis with different treatment as independent variable (control group = 0, vitamin B-6 supplement group = 1, hyperoxia = 2, hyperoxia plus vitamin B-6 supplementation = 3) and oxidative stress indicator or cytokine levels as the dependent variable after adjusting study period. β, regression coefficient. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{\dagger}p < 0.001$.

therapeutic duration of vitamin B-6 in neonatal rats receiving hyperoxia treatment must be further evaluated.

Previous evidence has shown that high oxygen exposure upregulated proinflammatory cytokines, such as IL-1β, TNF-α, and IL-6, in bronchoalveolar lavage fluid (BALF) [37]. Rather than the examination of BALF, we checked serum cytokine levels and found hyperoxia exposure also elevated serum MIP-3α transiently on the 9th day and tended to increase TNF-α on the 6th day. However, these two cytokine levels in the O_2 group substantially dropped on the 16th day to levels that were even lower than those of the control and B-6 groups. In addition to these two cytokines, all measured serum cytokines in the O_2 group on day 16 had the lowest levels of the four experimental groups. Consistent with our findings, previous studies proposed that hyperoxia exposure initially increases the status of systemic inflammatory responses before causing them to fall later [38–40]. Inflammation is a type of host defense. To maintain a certain degree of serum inflammatory cytokine levels while facing stress (i.e., a hyperoxic environment) might be required to increase the survival rate. Vitamin B-6 is involved in the synthesis of nucleic acid, mRNA and protein, it is also associated with differentiation and maturation of monocyte-derived macrophages and T lymphocytes [41]. Since T lymphocytes and macrophages are major sources of many cytokines, vitamin B-6 supplementation under hyperoxia environment might help neonatal rats to maintain a slightly increased cytokine levels in the present study. Plasma PLP deficiency was reported to be an indicator of lower immune response in critically ill patients [42], and vitamin B-6 supplementation could improve immunity in critically ill patients [43]. Although we did observe that the injection of PLP resulted in a transient increase in systemic inflammatory responses, the elevated serum inflammatory cytokine levels in B-6 and O_2 + B-6 groups did not deteriorate local lung pathological presentation in comparison with the control and O_2 groups. Vitamin B-6 supplementation might have somewhat protection on tissue functions through an increase in systemic inflammatory response.

Greater immune responses are not always detrimental. From our data, neonatal rats in control groups were born with elevated TNF-α levels, which might be due to birth stress. The cytokine overexpression in response to hyperoxia has been reported to have a protective effect by attenuating apoptotic signals. Apoptosis had been reported to be one of the mechanisms of hyperoxia-induced injury, as the number of cells undergoing apoptosis increases the longer they are exposed to oxygen and Bcl-2 proteins are known to block the apoptosis pathway [44,45]. Studies indicated that the overexpression of IL-6 induced Bcl-2 expression, disrupted interactions between proapoptotic and antiapoptotic factors, attenuated H_2O_2-induced mitochondrial damage, and prolonged the survival of mice under hyperoxia environment [46–49]. White et al. [50] also proved that parenteral injection of recombinant TNF and IL-1 prolonged the survival of rats in continuous hyperoxia with greater ratios of reduced to oxidized glutathione in lungs. The mechanism of vitamin B-6 supplementation on survival is through antiapoptosis or the glutathione antioxidant system needs further clarified.

Numerous treatments that downregulate inflammatory responses in a nonspecific manner have been investigated in hyperoxia-induced neonatal chronic lung disease, including nonsteroidal anti-inflammatory drugs (NSAIDs) and corticosteroids [7,9]. However, because NSAIDs (e.g., indomethacin or ibuprofen) might increase the risk of gastrointestinal bleeding and renal failure, they are thus not considered as a routine therapy in the clinical settings. Although corticosteroids have been shown to reduce neutrophils in mouse BALF and have been used in short courses to decrease oxygen demand and hasten extubation in neonatal intensive care units, the major concern for the use of corticosteriods is their systemic immunosuppressive effects. If hyperoxia also interfered systemic immunity in a down-regulated direction at the same time [51], the risk of infection or sepsis would be increased. By contrast, PLP supplementation in our study increased the survival rate and regulated systemic inflammation without deterioration or pathological outcomes. The combination of PLP and corticosteroid uses while managing hyperoxia-induced complications could be a possible choice in future clinical practice.

The strength of this study not only provided more pathophysiological information of hyperoxia-induced complications but also offered a new choice in the clinical practice for

considering the use of vitamin B-6 supplementation in neonates under hyperoxia treatment. However, our study has some limitations. The first limitation is the choice of neonatal numbers per treatment group in the second part of the study was made arbitrarily. We attempted to have an even gender distribution, but no previous report on gender difference in the development of bronchopulmonary dysplasia is available. The second limitation was that plasma PLP level was only measured in 3 neonatal rats rather than all neonatal rats in each group of the survival study due to a small amount of blood can be drawn from each neonatal rat. However, plasma PLP concentration ($n = 3$) significantly increased in vitamin B-6 supplemented groups.

In conclusion, neonatal rats receiving hyperoxia treatment suffered divergent inflammatory responses and experienced increased oxidative stress. Vitamin B-6 supplementation had beneficial effects on the inflammatory responses and antioxidative stress. Additional research is needed to determine the mechanism underlying the effect of vitamin B-6 on hyperoxia-induced complications. Further study is needed to determine the optimal timing and dose of vitamin B-6 for neonates who are exposure to hyperoxic conditions.

Funding

The Changhua Christian Hospital provided funding for the experiment. The project number was 105-CCH-IRP-034.

Declaration of conflicting interests

The authors have no conflicts of interest to declare with respect to the research, authorship, and/or publication of this article.

Acknowledgments

The authors thank the center in the Changhua Christian Hospital for their support in this study. We also gratefully acknowledge the cooperation of Chien-Chang Ho and Po-Fu Lee (Department of Physical Education, Fu Jen Catholic University, Taiwan) for their statistics support of this study.

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