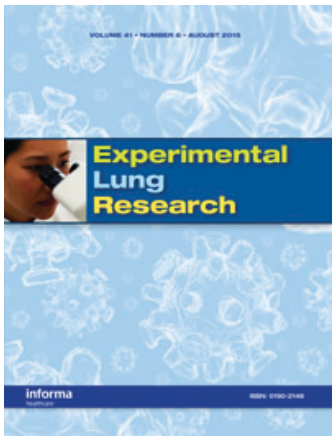

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Attenuation of hyperoxia-induced lung injury in neonatal rats by 1α,25-Dihydroxyvitamin D_3

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ORIGINAL ARTICLE

Attenuation of hyperoxia-induced lung injury in neonatal rats by 1α,25-Dihydroxyvitamin D_3

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ABSTRACT

Background: Mounting evidence suggests that Toll-like receptor (TLRs) plays an important role in oxidative stress and is implicated in the pathogenesis of hyperoxic lung injury. 1α,25-Dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), the hormonally active form of vitamin D, not only plays an essential role in mineral balance, but also possesses immunomodulatory and antioxidant properties. Besides, Vitamin D_3 is involved in the regulation of TLRs signaling. The present study was designed to investigate whether 1,25$(OH)_2D_3$ attenuates hyperoxia-induced lung injury by regulating TLRs signaling in neonatal rats. *Methods*: Pups were divided into four groups: normoxia control group (NC), normoxia plus 1,25$(OH)_2D_3$ treatment group (ND), hyperoxia control group (HC), and hyperoxia plus 1,25$(OH)_2D_3$ treatment group (HD). Lung tissues were collected for histological examination and detection of mRNA and protein expressions. *Results*: Treatment of hyperoxia-exposed animals with 1,25$(OH)_2D_3$ resulted in significantly increased body weight and reduced hyperoxia-induced lung injury. Moreover, 1,25$(OH)_2D_3$ significantly downregulated the expression of TLR4, NF-κB, and the inflammatory cytokines TNF-α, IL-1β, and IL-6. *Conclusions*: 1,25$(OH)_2D_3$ could attenuate hyperoxia-induced lung injury in neonatal rats, possibly by regulating TLR4/NF-κB signaling.

KEYWORDS 1,25-Dihydroxyvitamin D_3, hyperoxia, lung injury, TLR

INTRODUCTION

Despite significant advances in perinatal medicine, including the introduction of surfactant therapy and new ventilation strategies, the neonatal mortality resulting from bronchopulmonary dysplasia (BPD) remains relatively high. BPD is a multifactorial disease and hyperoxia, or oxygen toxicity, is known to play a key role in its pathogenesis. Exposure of the developing lung to high concentrations of oxygen inhibits alveolus formation and decreases lung surface area, leading to alveolar and capillary hypoplasia [1, 2]. Hyperoxia exposure leads to the production of reactive oxygen species (ROS) causing lung injury via oxidation of cellular macromolecules including DNA, proteins, and lipids.

At the cellular level, excessive ROS-induced oxidative stress could activate a number of signaling pathways, including inflammatory signaling pathways such as Toll-like receptor (TLR) signaling. TLRs are the crucial players in the innate immune response to microbial invaders [3]. Mounting evidence suggests that some TLRs can sense oxidative stress and are important contributors to some diseases. For example, recent studies demonstrated that TLR4-deficient mice were protected from hepatic or cardiac injury after ischemia and reperfusion [4, 5]. Additionally, TLR4 deficiency was thought to be protective against various types of lung injury induced by ischemia-reperfusion as well as LPS [6, 7]. What is more, hyperoxia-exposure was found to up-regulate the expressions of TLR2 and TLR4 [8]. Ligand engagement of TLR causes activation of the nuclear factor-kappa B

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(NF-κB) transcriptional complex to induce an inflammatory response. NF-κB heterodimer consists of p50 and p65 subunits, and activation of NF-κB induces the release and degradation of the inhibitory protein I-κB from the dimeric complex, followed by phosphorylation of NF-κB p65 and its translocation to the nucleus [9]. In the nucleus, NF-κB binds to corresponding sites to regulate transcription of many proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 [10, 11].

Activation of NF-κB in hyperoxia has been observed in several lung cell types, including lung epithelial cells, human pulmonary artery endothelial cells, and in rat and murine lungs [12]. Ogawa Y and colleagues reported that the lack of TLR4 signaling suppressed hyperoxia-induced neutrophil accumulation into the lungs as well as NF-κB translocation and decreased inflammatory mediators, including TNF-α and IL-6 [13]. It is likely that TLR/NF-κB signaling is involved in the pathogenesis of hyperoxic lung injury.

1α,25-Dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), the hormonally active form of vitamin D$_3$, not only plays an essential role in mineral balance, but also has immunomodulatory and antioxidant properties. VitaminD$_3$ treatment could decrease the formation of ROS and reduce oxidative injury in some animal models of oxidative stress-related diseases [14–16]. Pretreatment with vitamin D3 or its analogs was found to ameliorate acute lung injury by inhibiting neutrophil recruitment and lowering IL-6 levels in lungs [17, 18]. It was reported that vitamin D$_3$ was involved in the regulation of TLRs and NF-κB signaling. More recently, studies showed that vitamin D$_3$ suppressed the expression of TLR2 and TLR4 protein and mRNA in human monocytes [19]. 1,25(OH)$_2$D$_3$ inhibits NF-κB activation by stabilizing inhibitor I-κBα mRNA and reducing phosphorylation in human airway smooth muscle cells [20]. However, whether 1,25(OH)$_2$D$_3$ treatment has effect on hyperoxia-induced lung injury remains unknown.

In the present study, we explored if that treatment with 1,25(OH)$_2$D$_3$ could attenuate hyperoxia-induced lung injury and examined its association with TLRs/NF-κB signaling regulation in neonatal rats.

MATERIALS AND METHODS

Animals

All procedures and protocols were approved by the Institutional Animal Care and Use Committee, Huazhong University of Science and Technology. Timed-pregnant Sprague–Dawley rats were kept in a 12:12-h dark-light cycle and allowed to have access to food and water ad libitum. The rat pups were delivered naturally at full-term gestation, pooled, randomized, and returned to the nursing dams. The pups were divided into four groups and treated as follows: normoxia control group (NC, $n = 10$); normoxia plus 1,25(OH)$_2$D$_3$ treatment group (ND, $n = 10$); hyperoxia control group (HC, $n = 10$); and hyperoxia plus 1,25(OH)$_2$D$_3$ treatment group (HD, $n = 10$).

The pups in NC and ND were kept in room air, while the pups of HC and HD were housed in a transparent Plexiglas chamber and exposed to hyperoxia ($\geq$95% O$_2$) for 7 days as described previously [21]. The oxygen concentration in the Plexiglas chamber was monitored continuously with an oxygen analyzer. Pups in ND and HD groups were intraperitoneally injected with 1,25(OH)$_2$D$_3$ (0.5 μg/kg/day; Sigma, St. Louis, MO, USA) in 0.9% saline from postnatal day 1 to postnatal day 7 as reported previously [22]. Pups in NC and HC groups received 0.9% saline as placebo in the same manner. Nursing dams were rotated between hyperoxia and room air-exposed litters every 24 hours to prevent oxygen toxicity in the dams. Weight, evidence of developing disease and deaths were checked on daily basis.

Tissue Preparation

Pups were killed on postnatal day 7, and the left lungs were infused with 4% paraformaldehyde via a tracheal catheter at 20 cm H$_2$O of pressure for 5 min and then fixed in 4% paraformaldehyde solution overnight at 4°C as described previously [23]. Fixed lung tissues were paraffin-embedded and cut into 5 μm sections. The right lungs were harvested for total RNA and protein extraction.

Lung Histopathology

For lung morphometric analysis, lung sections from the left lower lobes were stained by standard HE method for histological and morphometrical examinations. Images of each section were captured by employing a Magnafire digital camera through an Olympus CKX41 microscope (Olympus Corporation, Tokyo, Japan) and then saved for late analysis. The morphometric analysis of lung samples was performed by a researcher blind to the experimental conditions. Alveolarization was assessed in terms of radial alveolar count (RAC) by using the method reported by Emery and Mithal [24]. From the center of the respiratory bronchiole, a perpendicular was drawn to the edge of the acinus (as defined by a connective tissue septum or the pleura), and the number of septa intersected by this line was counted. Septal thickness was

measured on images captured at 400 × magnification by employing the Image Pro software in accordance with a previously described method [25].

RNA Isolation and Quantitative Real Time PCR

Total RNA was extracted from frozen lung tissues by using Trizol reagent (Invitrogen). Total RNA (1 μg) was reversely transcribed in a 20 μL reaction by using a first-strand cDNA synthesis kit by following manufacturer's protocol (Invitrogen). The real time-PCR was performed on a Bio-Rad CFX96 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each reaction included diluted first-strand cDNA, specific primers (Invitrogen) (Table 1), THUNDERBIRD SYBR qPCR Mix (Toyobo Co., Ltd., Osaka, Japan), ROX and RNase-free water. The thermal cycle profile was 95°C for 3 minutes, followed by 45 cycles of 95°C for 30s, 58°C for 30 seconds, and 72°C for 30 seconds, with RNase-free water serving as negative control. The sequences of the designed primers were as follows:
TLR2-sense-5'-GAATGCAGATCTCACCGATAAAAGT-3' and
TLR4-sense-5'-CAAGAAGCAACAACTTTGACCTG-3'; and
antisense-5'-CCTGTGAGGTCGTTGAGGTTAG-3';
TNF-?-sense-5'-GGTTCCGTCCCTCTCATACACT-3'; and
antisense-5'-CAGTAGACAGAAGAGCGTGGTG-3';
IL-1?-sense-5'-GCTATGGCAACTGTCCCTGAAC-3'; and
antisense-5'-CACGGGCAAGACATAGGTAGCT-3';
IL-6-sense-5'-GTTGCCTTCTTGGGACTGATGT-3' and
antisense-5'-ATACTGGTCTGTTGTGGGTGGT-3';
GAPDH-sense-5'-GGTGCTGAGTATGTCGTGGAGT-3' and
antisense-5'-CAGTCTTCTGAGTGGCAGTGAT-3'.

Enzyme-Linked Immunosorbent Assay

Sections of the lungs were rapidly prepared after sacrificing and kept at –80°C until assayed. The levels of TNF-α, IL-1β, and IL-6 were determined by enzyme-linked immunosorbent assay (ELISA) according to kit instructions (Jingmei Biotech Co. Ltd., China).

Western Blotting

Total protein was extracted from lung tissues with a RIPA buffer in accordance with manufacturer's protocol (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). The protein concentrations were measured by BCA protein assay (Beyotime, China). Samples of total protein (50–100 μg) were fractionated by SDS-PAGE on 10% Tris-glycine precast gradient gels (Invitrogen Technology, Shanghai, China) and then transferred to nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were incubated overnight at 4° with respective primary antibodies against TLR2, TLR4, and phosphorylated NF-κB p65 (Santa Crus Biotechnology, Santa Cruz, CA, USA), and then incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibodies. The protein bands were detected by using enhanced chemiluminescence kit (ECL, Pierce, Thermo Fisher Scientific Inc, Rockford, IL, USA). The intensities of protein bands were quantificated by BandScan.

Statistical Analysis

Data were expressed as means ± SD, and two-way analysis of variance (ANOVA) was used to assess the differences between treatments (placebo, $1,25(OH)_2D_3$) and conditions (room air and hyperoxia) and the interaction between them. Post-hoc testing was completed using Tukey test, with significance set at $P < .05$.

RESULTS

Effect of $1,25(OH)_2D_3$ on Survival and Body Weight

The survival rate in the hyperoxia control group was 90% (9/10). The survival rate of all other groups was 100%. Although there was no difference found in birth weight among groups, in pups subjected to hyperoxia body weight was significantly decreased as compared to pups under normoxia at postnatal day 7. There was a significant interaction between hyperoxia exposure and $1,25(OH)_2D_3$ treatment on final pup weights at day 7. The body weight of $1,25(OH)_2D_3$-treated pups exposed to hyperoxia was increased by over than 20**%** when compared to hyperoxia control group ($P < .05$) (Figure 1).

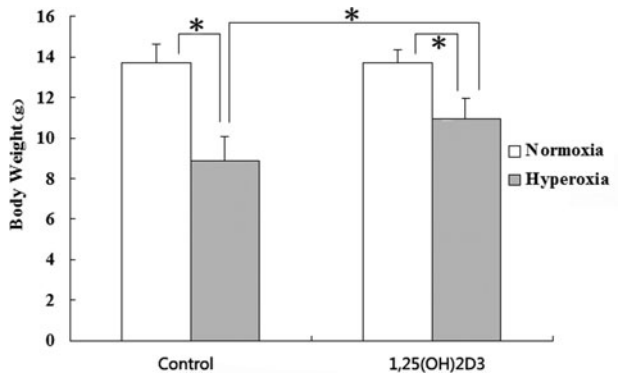

FIGURE 1. Effect of treatment with 1,25(OH)$_2$D$_3$ on body weight in newborn rats exposed to hyperoxia for 7 days. By two-way ANOVA there was a significant interaction effect observed between treatments (placebo, 1,25(OH)$_2$D$_3$) and conditions (room air and hyperoxia). Post hoc analyses indicated that hyperoxia-exposed animals were smaller, even upon treatment with 1,25(OH)$_2$D$_3$, as compared to animals maintained under normoxia ($^*P < .05$). Compared with hyperoxia control animals body weight was increased by 20% in 1,25(OH)$_2$D$_3$-treated animals under hyperoxic conditions ($^*P < .05$).

Lung Morphometry

Figure 2 shows that, compared to animals in the hyperoxia-exposed control group, treatment of hyperoxia-exposed animals with 1,25(OH)$_2$D$_3$ resulted in a conspicuous improvement in lung morphology. Specifically, exposure to hyperoxia significantly increased septal thickness and decreased number of RAC compared with air-exposed animals ($P < .05$). On the other hand, after treatment with 1,25(OH)$_2$D$_3$, the septal thickness was reduced and RAC increased significantly in comparison with hyperoxia-exposed, placebo-treated animals ($P < .05$).

Down-Regulation of TLR4 but not TLR2 Expression by 1,25(OH)$_2$D$_3$

Real time-PCR was performed to determine the effects of hyperoxia and 1,25(OH)$_2$D$_3$ treatment on TLR4 and TLR2 mRNA expressions. At the same time, the protein levels of TLR4 and TLR2 were examined by Western blot analysis. As shown in Figure 3, exposure of pups to hyperoxia markedly increased the expression of lung TLR4 protein and mRNA, independent of treatment of 1,25(OH)$_2$D$_3$ or placebo ($P < .05$). 1,25(OH)$_2$D$_3$ treatment, to some extent, reduced the hyperoxia-induced TLR4 expression ($P < .05$). 1,25(OH)$_2$D$_3$ treatment did not exert any effect on TLR2 expression, at both protein and mRNA levels (data not shown).

Expression Level of Activated NF-κB Protein in Lung Tissues

Western blotting showed that exposure to hyperoxia for 7 days elevated protein expression of NF-κBp65 in lungs as compared with exposure to normoxia ($P < .05$). Nonetheless, 1,25(OH)$_2$D$_3$ treatment significantly decreased the protein level of phosphorylated NF-κBp65 under hyperoxia ($P < .05$) (Figure 4).

Effects of 1,25(OH)$_2$D$_3$ on Hyperoxia-Induced Proinflammatory Cytokine Expression in the Lung

Real time-PCR revealed that levels of TNF-α, IL-1β, and IL-6 mRNA in the lungs were significantly increased 7 days after hyperoxic exposure ($P < .05$) (Figure 5 A, C, and E). Comparably, the protein expression levels of these proinflammatory cytokines were up-regulated by hyperoxia exposure, and the results were further confirmed by ELISA (Figure 5 B, D, and F) ($P < .05$).

Treatment with 1,25(OH)$_2$D$_3$ suppressed hyperoxia-induced increases in lung TNF-α, IL-1β, and IL-6 mRNA levels by 39%, 46%, and 35%, respectively (Figure 5 A, C, and E). Moreover, administration of 1,25(OH)$_2$D$_3$ also reduced TNF-α, IL-1β, and IL-6 protein levels in lung tissues by approximately 20%, 7 days after exposure to hyperoxia as compared with treatment with placebo (Figure 5 B, D, and F).

DISCUSSION

High-concentration oxygen has been commonly used for the treatment of respiratory insufficiency. However, protracted exposure to high-dose oxygen is known to induce lung tissue damage [26, 27], especially in premature infants who are susceptible to BPD. To explore the role of vitamin D$_3$ in hyperoxia-induced lung injury, in this study, we successfully established a classical model of BPD. Newborn rat pups were exposed to hyperoxia for 7 days and the animals developed lung injuries, as verified by decreased alveolarization, septal thickening, reduced branching complexity, and inflammatory cellular influx that mimicked the findings in human infants with BPD.

Vitamin D$_3$ is functionally important in modulating the innate and adaptive immune responses. Vitamin D$_3$, as an immunomodulatory steroid hormone, works directly on dendritic cells, monocytes, macrophages, B cells, and T cells [28, 29]. Recent studies have shown that administration of vitamin D$_3$

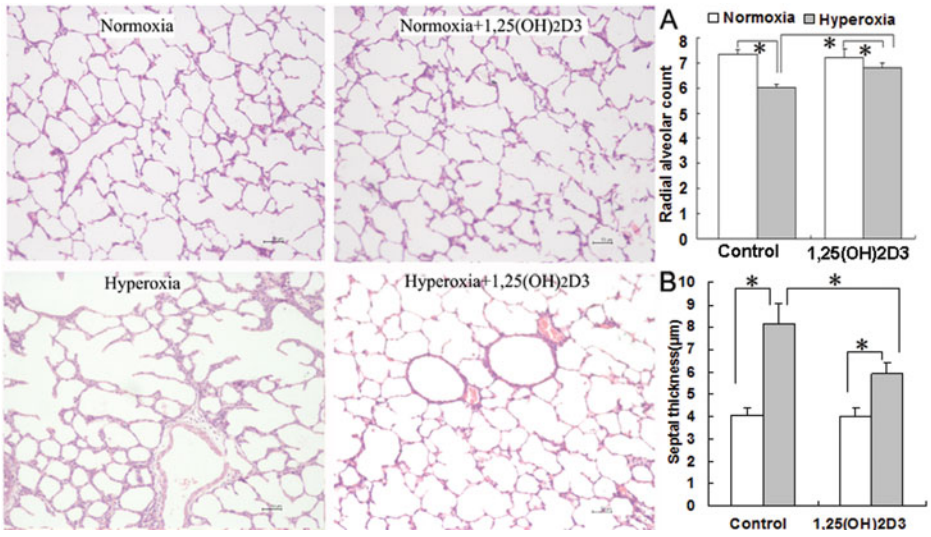

FIGURE 2. Representative light micrographs showing effects of hyperoxia and $1,25(OH)_2D_3$ treatment on lung histopathology of newborn rats (H&E, magnification, ×10), scale bar, 50 μm. Alveolarization was assessed by radial alveolar counts (A). Septal thickness was measured on images captured at 400 × magnification (B). Statistical analysis was performed using two-way ANOVA to test the effect of treatments (placebo, $1,25(OH)_2D_3$) and conditions (room air and hyperoxia). A significant interaction effect was observed between the two factors. Multiple comparison of these data showed hyperoxic exposure decreased RAC and increased septal thickness and $1,25(OH)_2D_3$ treatment increased RAC and decreased septal thickness when compared to hyperoxia control group under hyperoxic condition ($P < .05$) ($^*P < .05$).

ameliorates several diseases associated with oxidative stress [14–16]. In the present study, we demonstrated that $1,25(OH)_2D_3$ reduced hyperoxia-induced lung injury, which was demonstrated by increased RAC and decreased septal thickness. Additionally, we investigated the role of TLR/NF-κB signaling pathway in $1,25(OH)_2D_3$ protection against hyperoxic lung injury. The results showed hyperoxia exposure induced the expression of TLR4 but not TLR2. Meanwhile, we measured the expression of activated NF-κB and found that it was significantly increased in lungs of hyperoxia group as compared with room air group. Treatment with vitamin D3 inhibited TLR4 expression and NF-κB activation in newborn rat under hy-

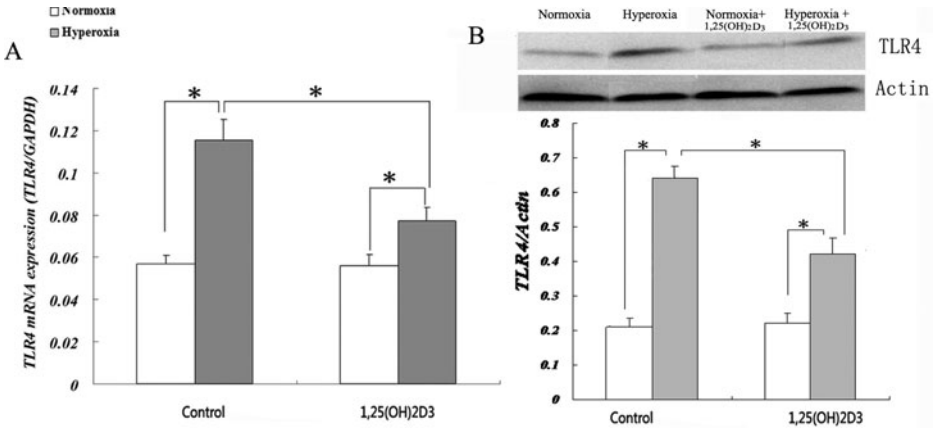

FIGURE 3. Downregulation of the hyperoxia-induced expression of TLR4 by $1,25(OH)_2D_3$. After exposure to either room air or hyperoxia for 7 days, animals were sacrificed. Lung tissue TLR4 mRNA (A) and protein (B) content were measured by both real-time RT-PCR and Western blotting ($^*P < .05$). Statistical analysis was performed using two-way ANOVA. There was a significant interaction effect observed between treatments (placebo, $1,25(OH)_2D_3$) and conditions (room air and hyperoxia). $1,25(OH)_2D_3$ treatment partially suppressed the hyperoxia-induced expression of TLR4 ($P < .05$).

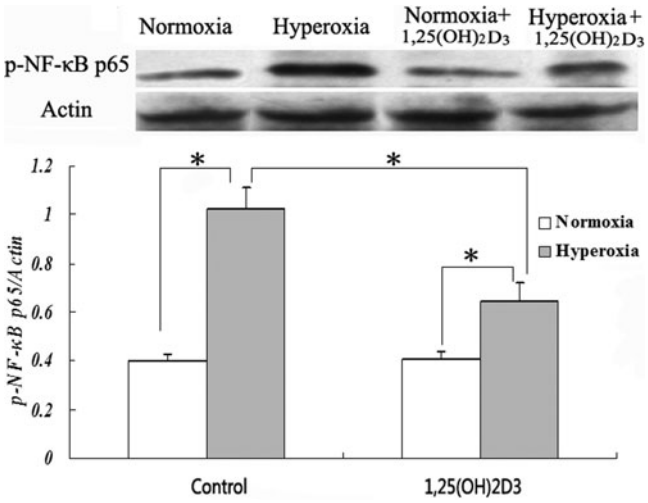

FIGURE 4. Expression of activated NF-κB protein in lung tissues. By two-way ANOVA, there was a significant interaction effect observed between treatments (placebo, 1,25(OH)$_2$D$_3$) and conditions (room air and hyperoxia). Tukey test was used to test the significant differences between the individual groups. Hyperoxia exposure for 7 days resulted in a significant increase in the expression of phosphorylated NF-κBp65 (p-NF-κBp65) protein, and concomitant 1,25(OH)$_2$D$_3$ administration partially prevented these change ($P < .05$). ($^*P < .05$).

peroxic conditions. Moreover, hyperoxia-induced upregulation of TNF-α, IL-1β, and IL-6, which are downstream cytokines of NF-κB, was significantly attenuated in 1,25(OH)$_2$D$_3$-treated rats. These findings suggested that TLR4/NF-κB signaling might play an important role in 1,25(OH)$_2$D$_3$ protecting against hyperoxic lung injury.

Mounting evidence suggests that hyperoxia induces lung TLR4 activation and signaling [30, 31]. Consistent with those results, a twofold increase of TLR4 expression in HC pup lungs was detected as compared with that in NC pup lungs at postnatal day 7 in this study. It was confirmed that TLR4-dependent NF-κB activation significantly contributed to hyperoxic lung injury, and the lack of TLR4 signaling suppressed hyperoxia-mediated upregulation of proinflammatory cytokines and neutrophil accumulation in the lung [13]. The C3 mice with a polymorphism in the coding region of TLR4, which disrupts TLR4 function and response to toxicity, shows significantly lower lung edema fluid protein [32]. These findings suggested that inhibiting TLR4 expression might alleviate lung injury. In this study, we observed that vitamin D3 treatment downregulated hyperoxia-induced TLR4 expression, which suggested 1,25(OH)$_2$D$_3$ protected against hyperoxic lung injury partly by suppressing TLR4 signaling. It was reported the level of TLR2 expression was upregulated in various experimental models of oxidative stress such as hemorrhagic shock (HS) and ischemia/reperfusion (I/R) [33]. However, different from TLR4, neither hyperoxia nor 1,25(OH)$_2$D$_3$ treatment exerted any effects on TLR2 expression in this study. Yuehua Li et al. reported that the inflammation is mediated by TLR4 in the early phase and by TLR2 in the late phase in HS-induced acute lung injury [34]. Similarly, TLR2 and 4 might be activated in different stages of hyperoxia-induced lung injury and play a different role in the injury, which needs further study.

All TLR signaling pathways culminate in activation of the transcription factor NF-κB, which regulates the expression of numerous genes involved in a wide array of processes, such as inflammation, immune responses, cell growth and development [35]. Liu YY et al. showed that hyperoxic injury was attenuated when NF-κB activity was inhibited by SN-50 [36]. In an in vitro study, Wright CJ et al. found that nitric oxide decreased cellular toxicity induced by hyperoxia through inhibiting NF-κB activation [37]. These findings suggested that inhibiting NF-κB activation would alleviate hyperoxic lung injury. In this study, we observed significant upregulation of activated NF-κB in newborn rat lung at 7 days of hyperoxia exposure, which was mitigated in the 1,25(OH)$_2$D$_3$ treatment rats under hyperoxic conditions. Several early reports suggested TLR4-mediated signaling pathways mainly stimulate the ac-

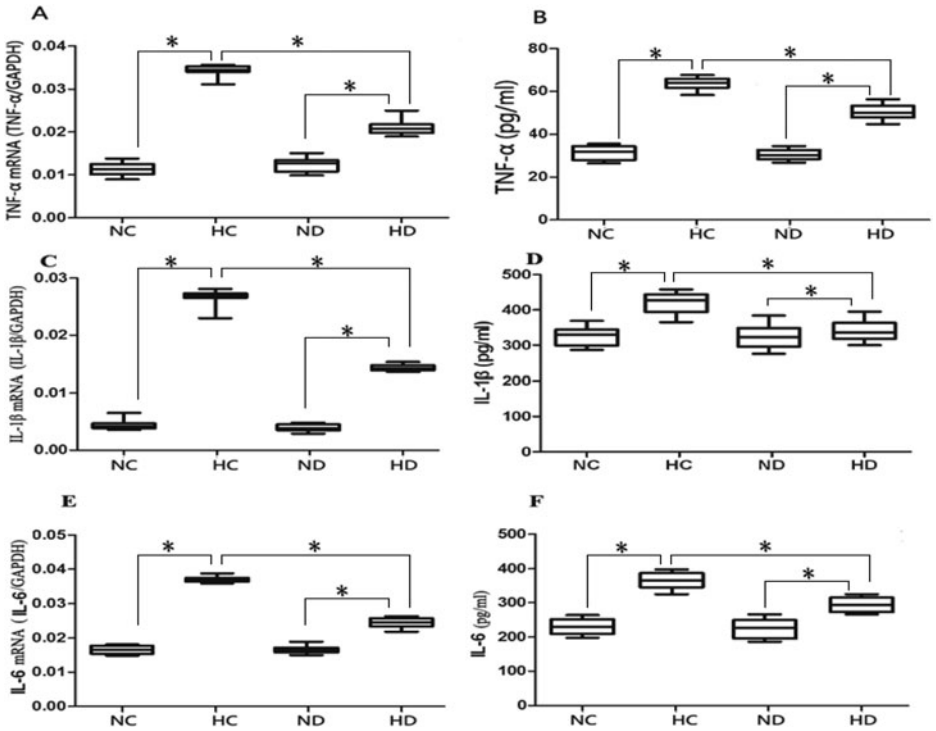

FIGURE 5. Effects of $1,25(OH)_2D_3$ on hyperoxia-induced proinflammatory cytokine expression in the lung. Statistical analysis was performed using two-way ANOVA to test the effect of treatments (placebo, $1,25(OH)_2D_3$) and conditions (room air and hyperoxia). A significant interaction effect was observed between the two factors. Tukey test was used to test the significant differences between the individual groups. Hyperoxia-exposed lungs showed increased TNF-α, IL-1β, and IL-6 expression, and this elevation was attenuated by treatment with $1,25(OH)_2D_3$ ($P < .05$). A, C, E: real time-PCR analysis of TNF-α (A), IL-1β (C), and IL-6 (E) mRNA in lungs with or without $1,25(OH)_2D_3$ treatment under normoxic or hyperoxic condition. B, D, F: ELISA of TNF-α (B), IL-1β (D), and IL-6 (F) protein levels in lungs with or without $1,25(OH)_2D_3$ treatment. NC: normoxia control group; HC: hyperoxia control group; ND: normoxia plus $1,25(OH)_2D_3$ treatment group; HD: hyperoxia plus $1,25(OH)_2D_3$ treatment group ($^*P < .05$).

tivation of NF-κB, which might be responsible for the inflammatory changes during hyperoxia [6, 38]. Since the expression level of activated NF-κB was in parallel with TLR4 expression, we thought that TLR4 signaling could be responsible for NF-κB activation during hyperoxia, and $1,25(OH)_2D_3$ could protect against hyperoxic lung injury partly by suppressing TLR4/NF-κB signaling. It also was possible that the two signaling pathways were suppressed by $1,25(OH)_2D_3$ independently, which need further research.

We observed hyperoxia markedly up-regulated the level of TNF-α, IL-1β, and IL-6. TNF-α plays a critical role in initiating inflammation and enhancing the production of inflammatory mediators and is implicated in the pathogenesis of hyperoxia-induced lung injury [39]. IL-1β and IL-6 also contribute to the pathogenesis of BPD [40]. Meanwhile, TNF-α, IL-1β, and IL-6 were increased in broncho-alveolar lavage fluid harvested from infants who developed chronic lung disease [41]. Many studies demonstrated that TLR/NF-κB signaling is involved in the control of expression of various proinflammatory cytokines expression, including TNF-α, IL-1β, and IL-6 [13,42,43]. In this study, $1,25(OH)_2D_3$ administration decreased the expression of activated NF-κB and its downstream cytokines, TNF-α, IL-1β, and IL-6, in lungs.

In conclusion, in this study, we demonstrated that $1,25(OH)_2D_3$, to some degree, can prevents hyperoxia-induced lung injury. TLR4 plays an important role in sensing oxidative stress and mediating inflammation response. It is possible that $1,25(OH)_2D_3$ decreases the production of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) by suppressing the activation of TLR4/NF-κB signaling, thereby ameliorating hyperoxia-induced lung injury. However, the detailed mechanism by which $1,25(OH)_2D_3$ downmodulates TLR4/NF-κB signaling warrants further investigation.

***Declaration of interest*:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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