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Impact of vitamin D on hyperoxic acute lung injury in neonatal mice

Thu T. Tran[1*], Jonathan Davies[2], Richard A. Johnston[3], Harry Karmouty-Quintana[4], Huiling Li[2], Caroline E. Crocker[1], Amir M. Khan[1] and Joseph L. Alcorn[1]

Abstract

Background Prolonged exposure to hyperoxia can lead to hyperoxic acute lung injury (HALI) in preterm neonates. Vitamin D (VitD) stimulates lung maturation and acts as an anti-inflammatory agent. Our objective was to determine if VitD provides a dose-dependent protective effect against HALI by reducing inflammatory cytokine expression and improving alveolarization and lung function in neonatal mice.

Methods C57BL/6 mouse neonates were randomized and placed in room air or hyperoxic (85% O_2) conditions for 6 days. Control, low (5 ng/neonate) and high (25 ng/neonate) doses of VitD were administered daily beginning at day 2 via oral gavage. Lung tissue was analyzed for edema, changes in pulmonary structure and function, and inflammatory cytokine expression.

Results Neonatal mice treated with VitD in hyperoxic conditions had improved weight gain, reduced pulmonary edema and increased alveolar surface area compared to untreated pups in hyperoxia. No significant changes in cytokine expression were observed between untreated and VitD neonates. While changes in surfactant protein mRNA expression were impacted by hyperoxia and VitD administration, no significant changes in alveolar type II cell percentages were observed. At 3 weeks, mice in hyperoxia treated with VitD had greater lung compliance, diminished airway reactivity and improved weight gain.

Conclusions High dose VitD significantly limited harmful effects of HALI. These results suggest that supplementation of VitD to neonatal mice during hyperoxia exposure provides both short-term and long-term protective benefits against HALI.

Keywords Hyperoxia, Acute lung injury, HALI, Vitamin D, Preterm neonate, Inflammatory cytokines, Airway responsiveness

*Correspondence:
Thu T. Tran
Thu.Tran@uth.tmc.edu
[1] Division of Neonatology, Department of Pediatrics, McGovern Medical School, University of Texas Health Science Center at Houston, Houston, TX 77030, USA
[2] Division of Neonatology, Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030, USA
[3] Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, WV 26505, USA
[4] Department of Biochemistry and Molecular Biology, McGovern Medical School, University of Texas Health Science Center at Houston, 6431 Fannin St, MSB 3.230B, Houston, TX 77030, USA

Background

Oxygen is critical medical therapy for many preterm neonates, but this intervention becomes toxic during prolonged administration in high concentrations, known as hyperoxia. Oxygen therapy at high concentrations greater than 60% for a little as 12 h has long raised concerns of unfavorable or even detrimental outcomes such as mortality, pulmonary inflammation, increased reactive oxygen species and long term pulmonary damage [1–3]. There has been extensive research to understand the mechanisms underlying the negative consequences of prolonged exposure to hyperoxia on the lungs,

collectively referred to as hyperoxic acute lung injury (HALI). Development and accumulation of reactive oxygen species (ROS) due to hyperoxia can damage cellular proteins, lipids and DNA, resulting in pulmonary inflammation and tissue injury [4]. Continued exposure reduces expression of surfactant proteins, which leads to pulmonary atelectasis and progression of respiratory distress syndrome (RDS) [5]. Upregulation of the cytokine cascade may cause chronic inflammation and lead to impairment of lung development and airway hyperresponsiveness resulting in increased morbidity and mortality [6]. Hyperoxia during the neonatal period continues to have significant harmful effects into adulthood possibly from dysregulation of the innate immune system and its failure to resolve inflammation [7, 8].

Vitamin D (VitD) stimulates maturation of the lung epithelium and increases the number of type II alveolar cells responsible for surfactant synthesis [9–11]. VitD decreases alveolar cell apoptosis via its paracrine effects on alveolar epithelial differentiation and reduction of ROS through increased expression of peroxidredoxin, nuclear factor erythroid 2–related factor 2 (Nrf2) activation and inhibition of lung lipofibroblast apoptosis [12–15]. VitD also has anti-inflammatory effects and can downregulate toll-like receptor 4 (TLR4) expression, a key receptor central to inflammatory processes [14]. The fetus receives a large portion of its required level of VitD via the placenta during the third trimester, placing preterm neonates at high risk for VitD deficiency with exclusively breastfed infants requiring VitD supplementation [16–19]. Currently, there are different recommendations for VitD supplementation in preterm infants. The American Academy of Pediatrics (AAP) recommends preterm neonates receive 200 – 400 IU/day of VitD while the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) recommends 800 – 1000 IU/day[20, 21]. We hypothesized that VitD supplementation in the neonatal murine HALI model would ameliorate inflammatory cytokine expression and protect lung in a dose-dependent manner.

Methods

Mice

All research utilizing vertebrate animal subjects conforms to the principles enunciated in the Assurance of Compliance document negotiated between the Health Science Center and the Department of Health and Human Services and to regulations promulgated by the U.S. Department of Agriculture. All protocols were certified by the Animal Welfare Committee (AWC) of McGovern Medical School at the University of Texas Health Science Center at Houston (UTHealth), which is the Institutional Animal Care and Use Committee (IACUC) for UTHealth (AWC-20–0067, Houston, TX). Generation of C57BL/6 wildtype (WT) adult and neonatal mice have been described previously [22] and are supplied from our colony housed at the Center for Laboratory Animal Medicine and Care (CLAMC) facility, which is responsible for the health and well-being of laboratory animals.

Murine HALI model

Mouse pups from different time-mated litters were evenly randomized into 6 groups starting at birth (considered day 0): normoxia control, hyperoxia control, normoxia-low dose, hyperoxia-low dose, normoxia-high dose and hyperoxia-high dose. Hyperoxia-exposed animals were placed in sealed chambers (A-Chamber, BioSpherix, Lacona, NY) with oxygen level kept at 85% (ProOx110, BioSpherix, Lacona, NY) from day 0 to day 6 of life [23–26]. Any pups demonstrating excessive stress were removed and euthanized. Nursing dams were switched between hyperoxia and normoxia cages daily to protect the health of dams. Dams were monitored for appropriate nursing after switching cages to ensure nutrition for the pups. Mouse health was monitored frequently for signs of health deterioration, and dams that showed signs of poor adaptation to new pups were replaced. Neonatal pups showing overt signs of failure to thrive were euthanized and removed from the data. Neonatal pups were humanely euthanized by exposure to isoflurane followed by decapitation. Mice used in assessment of lung mechanics were humanely euthanized by lethal injection of avertin at 250 mg/kg intraperitoneally (IP).

Administration of vitamin D to pups

Calcitriol (BML-DM200, Enzo Life Sciences) was dissolved in olive oil as previously described [27–29]. All pups were gavaged daily from day 2 to day 5. Control groups received 20 µL of olive oil, low dose groups were given 5 ng (0.2 IU) of VitD and high dose groups were given 25 ng (1 IU) of VitD in 20 µL olive oil. [30–33]. Utilizing previously published methodology with wide variation in vitamin D dosing determined and given the weight of our pups at birth, we determined that 5 ng would correlate to the low dose and five times the low dose at 25 ng would correlate to the current recommendation for higher daily vitamin D supplementation [31, 34, 35]. Sample size per group was $n \geq 6$ of mixed mice sex.

Assessment of lung mechanics

Pups at 23 days ± 2 days and weight of 10 g ± 1 g were anesthetized with 250 mg/kg avertin IP [36]. To determine lung function parameters, a FlexiVent FX system (SCIREQ Inc.) equipped with the FX1 module and Flexiware v8.3 software was used. Mice were ventilated

Table 1 Primer sequences used for real-time quantitative reverse transcriptase polymerase chain reaction PCR (qRT-PCR) analysis

Mouse Target Gene	Forward Primer	Reverse Primer	Amplicon
IL-1β	5′-GCCACCTTTTGACAGTGATGAG	5′-AAGGTCCACGGGAAAGACAC	218 bp
IL-6	5′-TAGTCCTTCCTACCCCAATTTCC	5′-TTGGTCCTTAGCCACTCCTTC	75 bp
CXCL1	5′-CTGCACCCAAACCGAAGTC	5′-AGCTTCAGGGTCAAGGCAAG	66 bp
TNF-α	5′-CAGCCTCTTCTCATTCCTGC	5′-GGTCTGGGCCATAGAACTGA	132 bp
IL-10	5′-GCTCTTACTGACTGGCATGAG	5′-CGCAGCTCTAGGAA-GCATGTG	104 bp
SP-A	5′-GTGCACCTGGAGAACATGGA	5′-TGACTGCCCATTGGTGGAAA	177 bp
SP-B	5′-AGAACAGAATCCAGGGATGC	5′-CAGAGAAGTCCTGAGTGTGAG	118 bp
SP-C	5′-ACACCATCGCTACCTTTTCC	5′-CGAAAGCCTCAAGACTAGGG	146 bp
SP-D	5′-GAGAACGTGGACTAAGTGGAC	5′-GCACCTACTTCTCCTTTGGG	137 bp
18S rRNA	5′-GTAACCCGTTGAACCCCATT	5′-CCATCCAATCGGTAGTAGCG	150 bp

(tidal volume 10 ml/kg; rate 300 breaths/min; positive end-expiratory pressure 3 cm H_2O at FiO2 of 21%) as previously described [37–40]. Pulmonary mechanics parameters (respiratory system elastance (E_{RS}), resistance (R_{RS}), tissue damping (G) and tissue elastance (H)) were evaluated using phosphate buffered saline (PBS). Increasing doses of acetyl-β-methacholine chloride (Sigma-Aldrich, St. Louis, MO) were nebulized (Aeroneb, SCIREQ, Montreal, Canada) at 0.3 log unit at 0 mg/mL, 1.5 mg/mL, 6.25 mg/mL, 25 mg/mL and 100 mg/mL to assess airway responsiveness via Flexivent (SCIREQ, Montreal, Canada). Pulmonary function data was analyzed with constant phase model as previous described [23, 41, 42]. Sample size per group was $n \geq 12$ of mixed mice sex. Anesthetized mice were humanely euthanized by cervical dislocation after lung mechanics assessment was performed.

Assessment of edema

Left lungs were obtained and weighed immediately (wet lung weight). Whole lungs were then dehydrated in an oven at 65 °C for 90 s. The tissue was re-weighed (dry lung weight) after removal from the oven. Wet/dry lung ratio was calculated. Sample size per group was $n \geq 6$ of mixed mice sex.

Histology preparation of lung sections

Mice were tracheostomized, right bronchus tied off and left lungs were flushed with PBS then instilled for 3 min with 10% neutral buffered formalin at a constant pressure of 20 cm H_2O and fixed overnight at 4 °C. Paraffin-embedded tissues were cut to 5 µm thick sections using a rotary microtome and sections mounted on glass slides. Lung sections were then stained with hematoxylin and eosin according to manufacturer's protocol.

Pulmonary morphometry

Lung sections were analyzed for mean linear intercept (MLI) using previously described methodology [43] and radial alveolar count (RAC) calculated by determining the number of alveoli that were crossed by a perpendicular line placed between the conducting and respiratory bronchiole to the closest septum or pleural edge [44, 45]. Three separate observers assessing the sections were blinded to the groups. Sample size per group was $n \geq 6$ of mixed mice sex.

Assessment of cytokine and surfactant protein expression

Expression of mRNA was determined in lung lysate via real-time quantitative PCR as described previously [22, 46] using primers specific for target mRNAs (see Table 1). 18S rRNA was chosen as a normalizer for all analyses as it provided more consistent results than using β-actin or TATA binding protein (TBP) mRNA, which demonstrated greater variability regarding pup age and hyperoxic exposure. Data was calculated by the comparative C_T method (C_T, threshold cycle). mRNA expression was determined via replicated $2^{-\Delta\Delta C(t)}$ values and normalized to normoxia values = 1 [47–49].

Alveolar epithelial type II cells quantification

To visualize surfactant protein C (SP-C) positive cells (alveolar type II cells) in the parenchyma of the lung sections, fixed sections were prepared and subjected to immunohistochemistry using an antibody specific for pro SP-C (EMD Millipore; AB3786), followed by incubation with a peroxidase-conjugated mouse IgG specific for rabbit IgG labeled and the resulting complexes visualized using a chromogenic stain to stain the cells blue (Vector Laboratories; Vector® Blue Substrate Kit; SK-500). The nuclei of all cells in the lung sections were visualized after staining with nuclear fast red stain (Vector Laboratories; Nuclear Fast Red Counterstain; H-3403–500).

To quantitate type II cells and all cells in the fixed lung sections, areas of the sections predominately containing peripheral alveolar areas were digitally imaged (5 areas per section, lung lungs from each group). Quantitation of SP-C positive and all cells were performed using the KEYENCE BZ-X800 (Keyence Corporation of America; Itasca, IL) which possesses imaging and software that allows for automated cell counting for user-independent, repeatable quantification. Images were acquired using the BZ-X810 at 40X using brightfield. The images were analyzed for cell counts using the BZ-X800 Analyzer software. Two sequential color extractions were utilized, first with blue to quantify cells positive for SP-C, including those structures that were of proper hue, size, and brightness to represent a positive cell. Next an additional separate extraction was performed to quantify cells positive for red nuclei. The software output provides the number of cells positive for each target in each image, from which the percentages were calculated. The software allows for exclusion of stained blood cells or cells within the alveolar space as well as cells comprising capillary vessels or bronchiolar regions from the analysis.

Statistical analysis

Paired student t-test and/or two-way repeated measures analysis of variance (ANOVA) adjusted with Tukey's method for comparisons. Statistical significance was considered at $p < 0.05$. Values are graphed as mean ± standard deviation (SD). Paired student t-test were performed using Excel while ANOVA was performed in Jamovi (Version 1.2.27, Sydney, Australia).

Results

Impact of vitamin D administered during hyperoxia on short-term weight gain

To characterize the physiological changes in response to HALI in our neonatal hyperoxia model, we measured changes in weight after exposure to 85% oxygen for 6 days. In the results shown in Fig. 1A, neonatal pups exposed to hyperoxia were observed to have significantly decreased weight gain on day 2 through day 5 as compared to normoxia control as previously reported in the literature [31, 50–52]. Significant improvement in the rate of weight gain was observed on days 3 and 4 in mice housed under hyperoxic conditions when administered low dose VitD as compared to the hyperoxia control group (Fig. 1A). When high doses of VitD were administered, a significant increase in weight gain was observed on days 2, 3 and 4 as compared to hyperoxia control group. No significant difference in weight gain is observed between different sexes. When pups were administered vehicle, low does VitD or high does VitD in the presence of normoxia rather than hyperoxia,, no significant impact on weight gains was noted (Fig. 1A, Supplemental Data). It should be noted that the increases in weight gain in the pups administered high dose VitD in hyperoxic conditions did not reach the rate of weight gain demonstrated by pups raised in normoxic conditions, suggesting that vitamin D does not completely abrogate the negative impact of hyperoxia on weight gain in neonatal mice.

Impact of vitamin D administered during hyperoxia on long-term weight changes

To further assess the long-term physiological impact of HALI and treatment with VitD, we assessed changes in weight after the neonates and dams were switched to normoxic conditions after exposure to hyperoxia. The results, shown in Fig. 1B, demonstrate significant weight differences in hyperoxia control as compared to normoxia control throughout the experiment from day 7 through day 21 (day 7: 1.47 ± 0.12 vs 1.26 ± 0.04, $p < 0.0001$; day 14: 2.34 ± 0.16 vs 1.88 ± 0.05, $p = 0.005$; day 21: 4.39 ± 0.30 vs 3.33 ± 0.09, $p = 0.0006$). Mice in hyperoxia gavaged with low dose vitamin D had significant increase in weight as compared to hyperoxia control on days 14 and 21 (day 14: 2.25 ± 0.12 vs 1.88 ± 0.05, $p = 0.002$; day 21: 4.13 ± 0.19, $p = 0.0002$). Similarly, mice in hyperoxia gavaged with high dose vitamin D have significant increase in weight change as compared to hyperoxia control on days 7, 14 and 21 (day 7: 1.54 ± 0.13 vs 1.26 ± 0.04, $p < 0.014$; day 14: 2.24 ± 0.20 vs 1.88 ± 0.05, $p = 0.0.027$; day 21: 4.12 ± 0.23 vs 3.33 ± 0.09, $p = 0.0005$). No significant difference in weight change is observed between low dose and high dose groups exposed to hyperoxia as compared to normoxia control group. No significant difference in long term weight changes is observed between sex groups. When pups were administered vehicle, low does VitD or high does VitD in the presence of normoxia rather than hyperoxia, no significant impact on weight gains was noted (Fig. 1B, Supplemental Data). The findings indicate that while the effects of acute hyperoxia on long-term resolution of weight gain was not seen in our model of HALI, administration of both low and high dose VitD to neonatal pups during acute exposure of hyperoxia resulted in significant weight gain compared to untreated pups exposed to hyperoxia at 1–3 weeks of age.

Impact of vitamin D administered during hyperoxia on pulmonary edema

Exposure to hyperoxia is known to increase pulmonary edema in neonates (9, 11, 56). We measured the wet-to-dry weight lung ratio as described in Materials and Methods to determine the presence of edema in this study. In the results shown in Fig. 2, neonatal pups exposed to hyperoxia for 6 days were observed to have a significantly

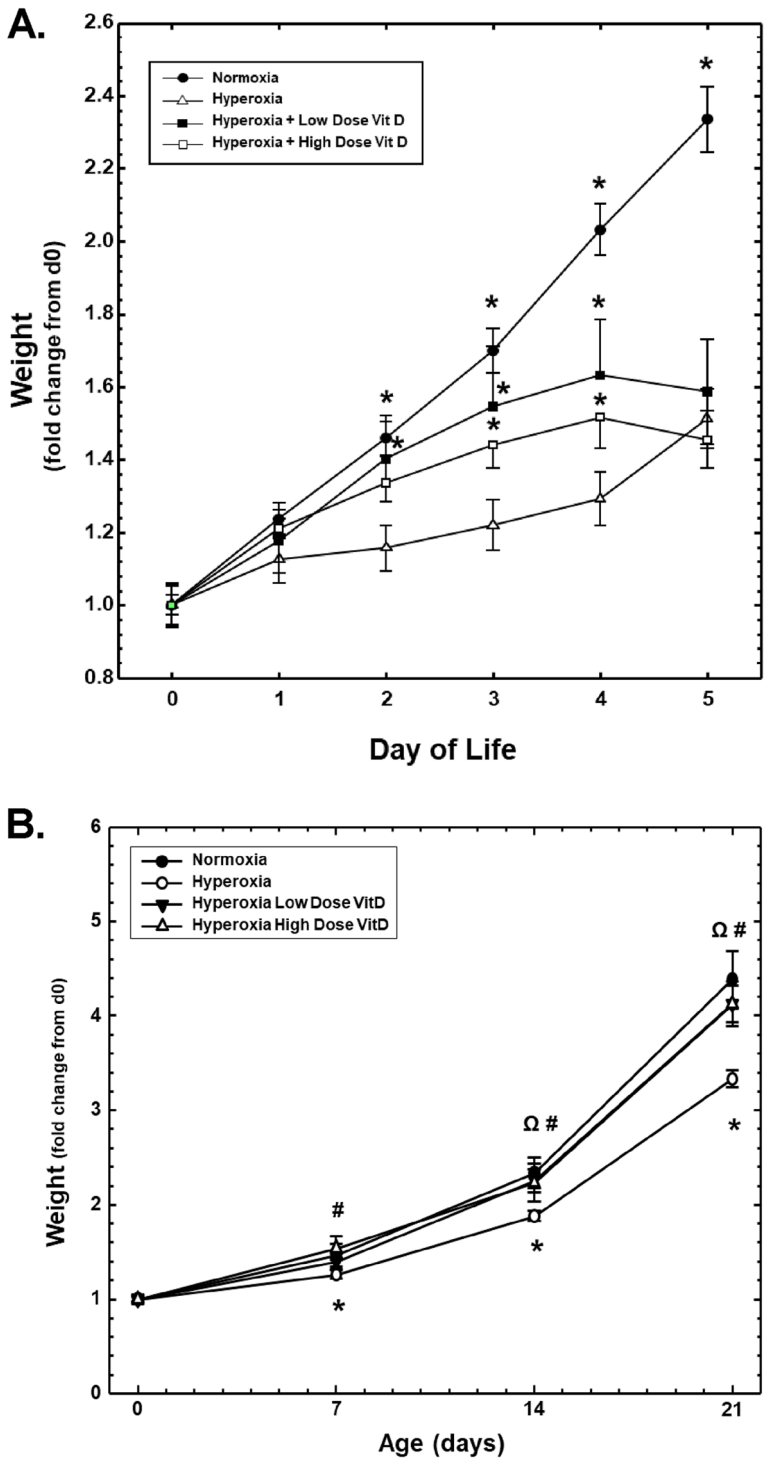

Fig. 1 Impact of vitamin D administration on growth rate in neonatal mice exposed to normoxia with administered vehicle or hyperoxia with administered vehicle, low dose vitamin D or high dose vitamin D (combined male and female data). **A** Acute (0–5 days) growth. Values are relative to average weight at DOL 0 normalized as 1. Data is average ± SEM of 2 independent experiments; $n > 10$. * denotes significant difference ($p < 0.05$) to mice in hyperoxia with administered vehicle. **B** Long term (0–21 days) growth. Neonatal mice were exposed to hyperoxia or normoxia with interventions up to DOL6, then all groups were subject to normoxia until DOL21 without interventions. Values are relative to average weight at DOL 0 normalized as 1. Data is average ± SEM of > 2 independent experiments; $n > 10$. * denotes significant difference ($p < 0.05$) of mice exposed to normoxia receiving vehicle compared to mice exposed to hyperoxia. # denotes significant difference ($p < 0.05$) of mice exposed to hyperoxia receiving low dose VitD compared to mice exposed to hyperoxia. Ω denotes significant difference ($p < 0.05$) of mice exposed to hyperoxia receiving high dose VitD compared to mice exposed to hyperoxia

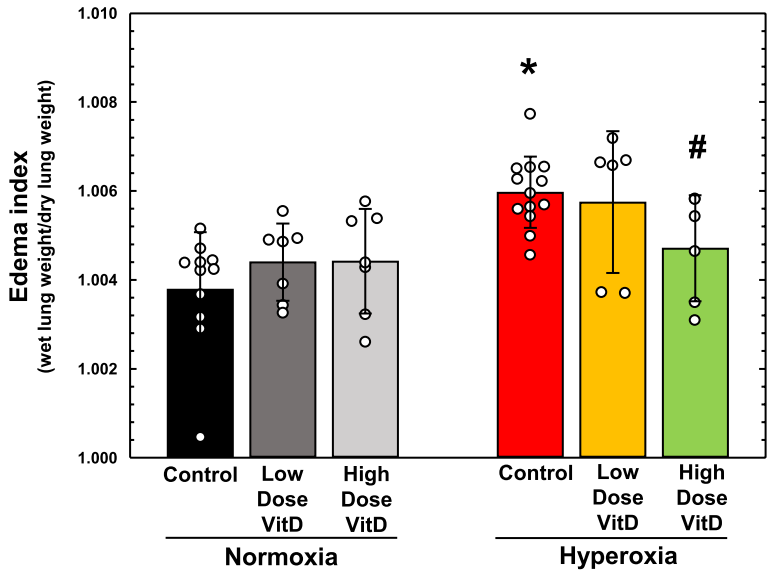

Fig. 2 Impact of vitamin D administration on pulmonary edema in neonatal mice exposed to normoxia or hyperoxia at DOL6 (combined male and female data). Values represent wet lung to dry lung ratio determined before and after drying lung in a 65 °C oven for 90 s. Data is average ± SEM of > 2 independent experiments; $n > 10$. * denotes significant difference ($p < 0.05$) to control mice in normoxia. # denotes significant difference ($p < 0.05$) to control mice in hyperoxia

increased wet to dry lung ratio as compared to neonatal pups exposed to normoxia for 6 days as previously reported in the literature. Administration of high dose VitD to pups raised in hyperoxia significantly decreased wet/dry lung ratio as compared to hyperoxia control, but no significant reduction in pulmonary edema was observed with low dose VitD treatment in hyperoxia. No significant difference was observed between sexes in relation to wet/dry lung ratio. These results indicate that administration of high dose VitD reduces the expected edema in neonatal mice under hyperoxic conditions while low dose VitD does not provide the same benefits.

Impact of vitamin D administered during hyperoxia on alveolar structure and development

Alveolar simplification with enlarged alveolar sacs is seen in hyperoxia control (Fig. 3B) as compared to animals in normoxia, which has been reported in literature (Fig. 3A) [52, 53].). In hyperoxia, the alveolar structure of pups treated with low dose VitD (Fig. 3D) appears to have less simplification of structure as compared to untreated pups in hyperoxia (Fig. 3B) but appears to be more simplified than pups receiving low dose VitD in normoxia (Fig. 3C). On the other hand, the alveolar structure of pups in hyperoxia treated with high dose VitD (Fig. 3F) is clearly less simplified than untreated hyperoxia-exposed pups (Fig. 3B) and approaching the structure seen in pups in normoxia administered high dose VitD (Fig. 3E).

In order to quantify the structural differences shown in Fig. 3A-F, morphometric analysis measuring mean linear intercept length (MLI) and radial alveolar count (RAC) of lung sections of pups exposed to normoxia and hyperoxia and administered VitD was performed. Mouse pups in the hyperoxia control group had significant increase in MLI as compared to normoxia control (73.1 ± 0.8 μm vs. 121.8 ± 1.9 μm, $p < 0.0001$) (Fig. 3G). Within the

(See figure on next page.)

Fig. 3 Hematoxylin and eosin (H&E) staining and morphometric analysis of lung sections from neonatal mice exposed to normoxia or hyperoxia and administered vehicle, low dose vitamin D or high dose vitamin D (assortment of male and female stained lung sections). H&E-stained sections visualized with 40 × objective. **A** Normoxia control. **B** Hyperoxia control. **C** Normoxia with low dose VitD. **D** Hyperoxia with low dose VitD. **E** Normoxia with high dose VitD. **F** Hyperoxia with high dose VitD. **G** Mean linear intercept was determined from histological sections of lungs from 10 lung neonatal pups as described in the Materials and Methods, shown are whisker plots of the data. **H** Radial alveolar count was determined from histological sections of lungs from 10 lung neonatal pups as described in the Materials and Methods, shown are whisker plots of the data. * denotes significant difference ($p < 0.05$) to mice in normoxia. # denotes significant difference ($p < 0.05$) of mice exposed to hyperoxia receiving low dose VitD compared to mice exposed to hyperoxia. Ω denotes significant difference ($p < 0.05$) of mice exposed to hyperoxia receiving high dose VitD compared to mice exposed to hyperoxia

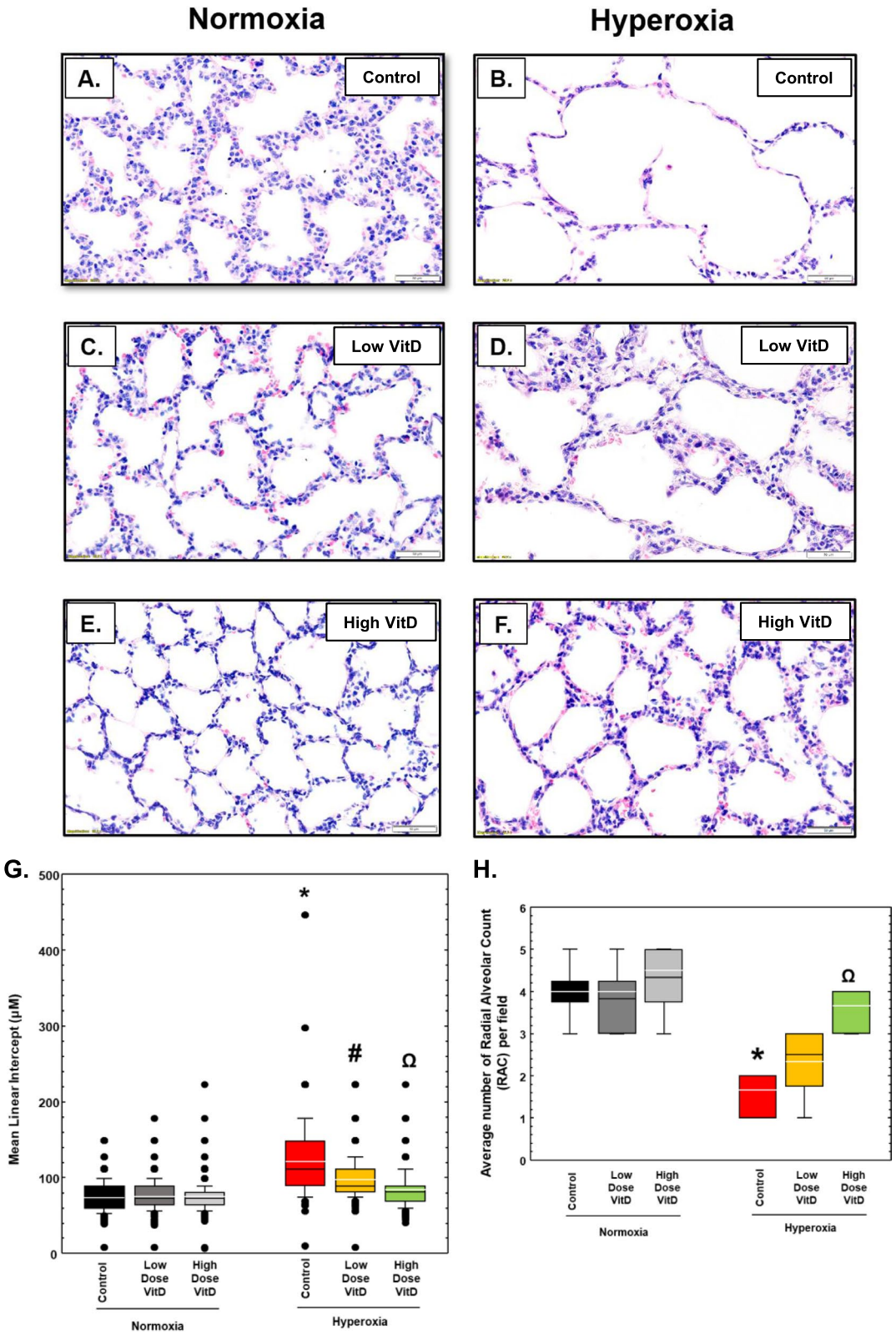

Fig. 3 (See legend on previous page.)

hyperoxia-exposed groups, pups given low dose VitD had significantly decreased MLI as compared to hyperoxia control (98.7 ± 1.1 μm vs 121.8 ± 1.9 μm, $p < 0.0001$) as well as pups given high dose VitD (83.3 ± 0.8 μm vs 121.8 ± 1.9 μm, $p < 0.0001$) (Fig. 3G). Significant reduction in RAC is observed in hyperoxia control as compared to normoxia control as well (4 ± 0.0 vs 1.7 ± 0.2, $p < 0.0001$) (Fig. 3H). Groups in hyperoxia gavaged VitD showed some preservation of radial alveolar count (low dose 2.3 ± 0.3 vs 1.7 ± 0.2, $p = 0.12$; high dose 3.7 ± 0.2 vs 1.7 ± 0.2, $p < 0.0001$). Pups gavaged low dose VitD in hyperoxia had significantly decreased RAC as compared to normoxia control (low dose 2.3 ± 0.3 vs 4 ± 0.0, $p = 0.0005$) while interestingly, pups gavaged with high dose VitD in hyperoxia showed no significant RAC reduction as compared to normoxia control.

Males and females show no difference in observed RAC but MLI of male control mice was statistically more affected by hyperoxia as compared to their female counterparts (122.1 ± 2.6 vs 121.5 ± 2.8) (data not shown). Males also had significantly lower MLI than females when treated with high dose VitD in hyperoxia (80.9 ± 1.0 vs 85.6 ± 1.3). These results indicate that administration of low or high dose VitD to neonatal pups reverses oversimplification of alveolar structure induced by exposure to hyperoxia, and high dose VitD proved to be more protective of developmental alveolarization. This effect seems to be amplified in males as compared to females.

Impact of vitamin D administered during acute hyperoxic exposure in the neonatal period on pulmonary function at 3 weeks of age

Airway responsiveness to increasing concentrations of aerosolized methacholine, a bronchoconstrictor, was measured to investigate the possibility that VitD administration could improve pulmonary function of older mice that were subjected to our neonatal HALI model. In Fig. 4A, total respiratory system elastance (E_{RS}) shows significant difference at baseline and all methacholine dosages between normoxia control and hyperoxia control ($p \leq 0.008$ for all comparisons). Low and high dose VitD intervention in the hyperoxia arm showed significant reduction in E_{RS} for all values when compared to placebo control (low dose: $p \leq 0.001$; high dose: $p \leq 0.0008$). On the other hand, total respiratory system resistance (R_{RS}) did not show any significant difference between groups (Fig. 4B). As seen in Fig. 4C, G (tissue dampening), which closely represents resistance of the small airway, is significantly increased in hyperoxia control as compared to normoxia control for all values ($p \leq 0.007$). Groups exposed to hyperoxia and gavaged with low and high dose vitamin D both had significant reduction of G in hyperoxia as compared to hyperoxia control (low dose: $p \leq 0.01$; high dose: $p \leq 0.001$). As seen in Fig. 4D, hyperoxia control also had significantly higher H (tissue elastance) values as compared to normoxia controls at baseline and for all methacholine doses ($p \leq 0.008$). Mice in hyperoxia given low and high dose vitamin D also showed significantly decreased H values as compared to hyperoxia control (low dose: $p \leq 0.002$; high dose: $p \leq 0.001$). Surprisingly, no significant difference is observed in any of the constant phase models (Fig. 4G, 4H) and single compartment model (E_{RS}, R_{RS}) when low and high dose intervention groups in hyperoxia are compared to normoxia controls. Again, no difference is observed between sexes. When pups were administered vehicle, low does VitD or high does VitD in the presence of normoxia rather than hyperoxia, no significant impact on pulmonary function was noted (Fig. 2, Supplemental Data).

Impact of vitamin D administered during hyperoxia on inflammatory cytokine expression

Since pulmonary inflammation has been shown to increase in neonates after hyperoxic exposure, we assessed the expression of pulmonary cytokine mRNA in lung lysate of neonatal pups exposed to hyperoxia and administered VitD from days 2 through 5. As can be seen in Fig. 5, exposure to hyperoxia resulted in significant increases in expression of pro-inflammatory Il-1β, Il-6, CXCL1 and TNF-α mRNAs when compared to their expression under normoxic conditions. No significant differences in cytokine expression were observed with VitD intervention when hyperoxia controls were compared with both low and high dose VitD intervention in hyperoxia groups (Fig. 5). The only significant difference observed between sex groups is a more robust expression of IL-1β in females as compared to males (2.6 ± 0.4 vs 1.2 ± 0.4, $p < 0.05$) when exposed to hyperoxia with no VitD treatment (Data not shown). No change in IL-10 was found in hyperoxia environment or with VitD administration (Data not shown). We did not observe VitD to have any impact on inflammatory cytokine expression in HALI as previously described [31, 32, 54]. When pups were administered vehicle, low does VitD or high does VitD in the presence of normoxia rather than hyperoxia, no significant impact on expression of inflammatory cytokines was noted (Fig. 3, Supplemental Data).

Surfactant protein gene expression has been shown to be impacted by hyperoxic exposure

Hyperoxia and exposure to vitamin D has been shown to alter surfactant gene expression in a number of models [55–57], but the impact of both hyperoxia and vitamin D exposure together has not been investigated. When pups were treated with VitD under normoxic conditions,

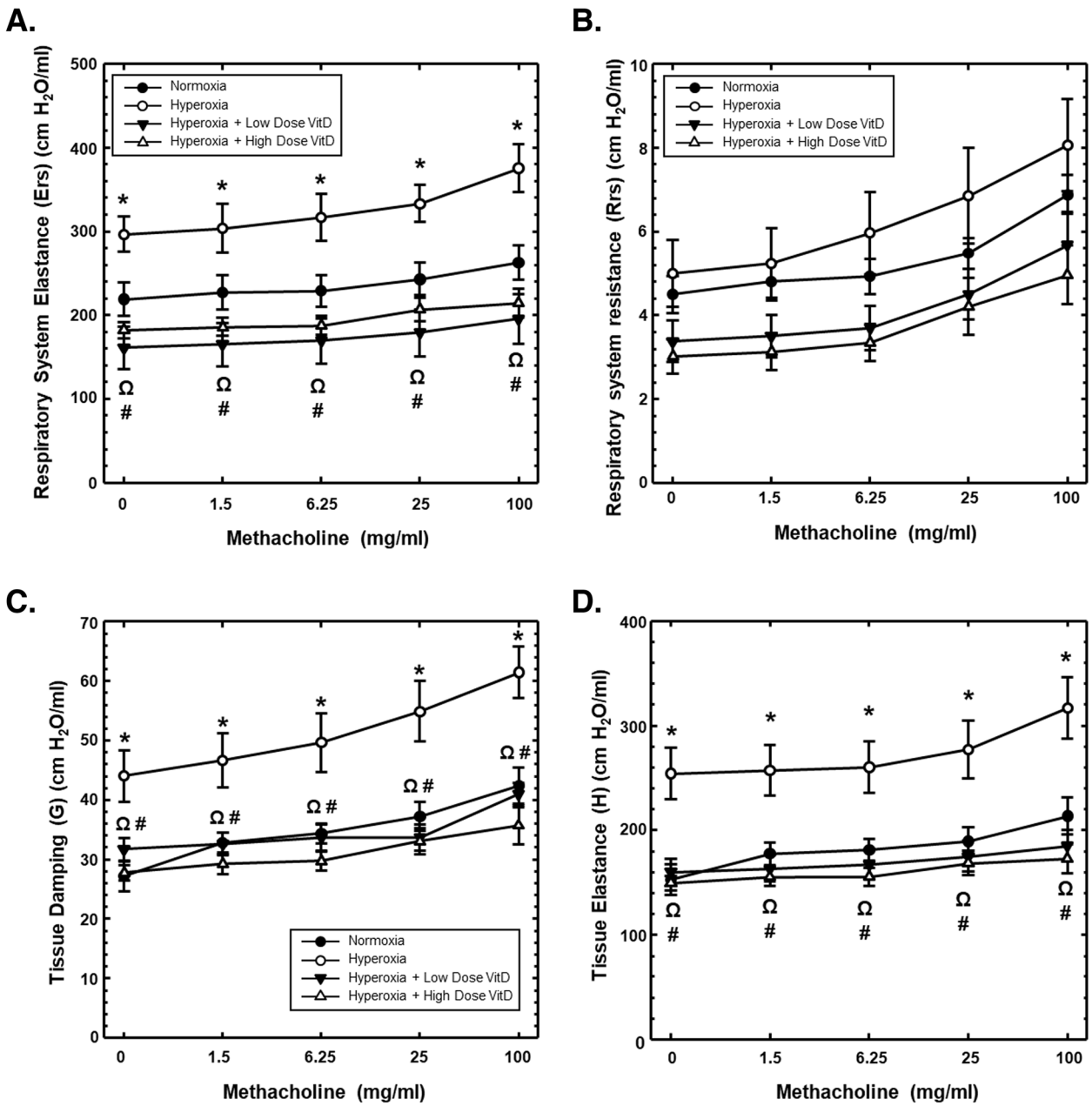

Fig. 4 Impact of vitamin D treatment in mice subjected to the HALI model on pulmonary mechanics measured at baseline and in response to aerosolized methacholine (combined male and female data). Neonatal mice were exposed to hyperoxia or normoxia with interventions up to DOL 6, then all groups were subject to normoxia until DOL 21 without interventions. Pulmonary mechanics were measured as described in the Materials and Methods. **A.** Respiratory System Elastance (Ers) **B**. Respiratory System Resistance (Rrs) **C**. Tissue damping (G) **D**. Tissue elastance (H). Data is average ± SEM of $n > 10$ subjects. * denotes significant difference ($p < 0.05$) of mice exposed to normoxia compared to mice exposed to hyperoxia. # denotes significant difference ($p < 0.05$) of mice exposed to hyperoxia receiving low dose VitD compared to mice exposed to hyperoxia. Ω denotes significant difference ($p < 0.05$) of mice exposed to hyperoxia receiving high dose VitD compared to mice exposed to hyperoxia

expression of all surfactant proteins genes increased, but significant increases in expression were only observed with SP-A (low and high dose VitD) and SP-D (low dose VitD). In our model of HALI, we found that expression of all surfactant protein genes were significantly increased in hyperoxia control as compared to normoxia control (Fig. 6)(SP-A: 3.1 ± 0.58 vs. 1 ± 0.07, $p = 0.0002$, SP-B: 4.2 ± 0.6 vs. 1 ± 0.08, $p < 0.0001$, SP-C: 4.1 ± 0.6 vs. 1 ± 0.09,

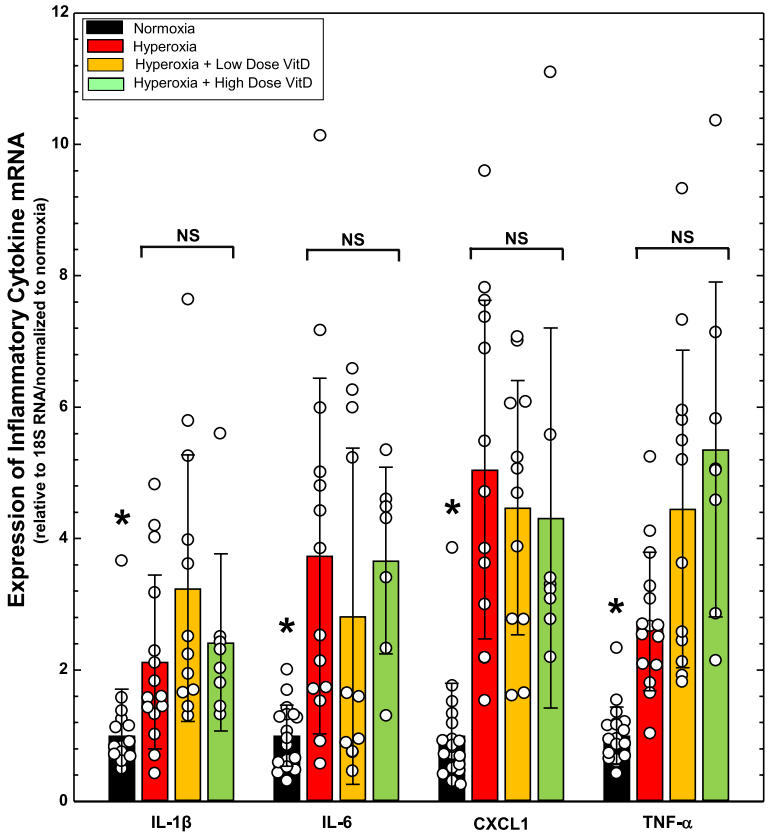

Fig. 5 Expression of cytokine mRNA expression in neonatal mice with administered vehicle, low dose vitamin D or high dose vitamin D and exposed to normoxia or hyperoxia at DOL6 (combined male and female data). RNA was isolated from lungs and levels of cytokine mRNA assessed by quantitative real-time RT-PCR analysis. Shown are levels of expression relative to levels in mice exposed to normoxia (set as 1). Data is average ± SEM of 2 independent experiments; $n > 10$. * denotes significant difference ($p < 0.05$) to control mice in hyperoxia, mice treated with Low Dose VitD in hyperoxia and mice treated with High Dose VitD in hyperoxia. There were no significant differences in any of the treated or untreated groups in hyperoxia

$p < 0.0001$ and SP-D: 4.7 ± 0.6 vs. 1 ± 0.07, $p < 0.0001$) as reported previously. VitD administration under hyperoxic conditions generally decreased surfactant protein gene expression compared to untreated pups. Only high dose VitD administration under hyperoxic conditions significantly impacted surfactant gene expression by decreasing expression of SP-A, SP-C and SP-D mRNA levels relative to untreated pups (SP-A: 3.1 ± 0.58 vs. 0.99 ± 0.25, $p = 0.01$, SP-C: 4.1 ± 0.6 vs. 2.1 ± 0.21, $p = 0.04$, SP-D: 4.7 ± 0.6 vs. 1.7 ± 0.4, $p = 0.003$), depicted in Fig. 6.

Impact of vitamin D on alveolar type II cell numbers during hyperoxic exposure

It has been reported that VitD exposure increases the number of type II cells, which are responsible for surfactant gene expression, in neonatal lung [9–11]. We then assessed the impact of VitD exposure on the numbers of type II cells in the distal parenchyma of neonatal mice subjected to our model of HALI. Sections of lungs that prepared from neonatal mice that were exposed to normoxia or hyperoxia for 6 days and administered low dose or high dose VitD were subjected to immunohistochemistry for SP-C, and the nuclei of all cells were stained as described in Methods and Materials (Fig. 7A). The percentages of alveolar type II cells were determined using software that allowed for unbiased counting of cells while allowing for the elimination of stained cells present in the alveolar space. The results of the analysis, shown in Fig. 7B, indicate that in normoxia, type II cells comprised about 30% of the total cells in each section. Exposure to hyperoxia or exposure to low and high dose VitD had no significant impact on the percentage of type II cells.

Discussion

There are several mouse models of HALI, which have been used to demonstrate the mechanisms of oxygen toxicity [26, 58–62]. Mechanisms to explain observed lung injury due to hyperoxia include reactive oxygen species accumulation, alveolar-capillary membrane permeability, chronic pulmonary inflammation

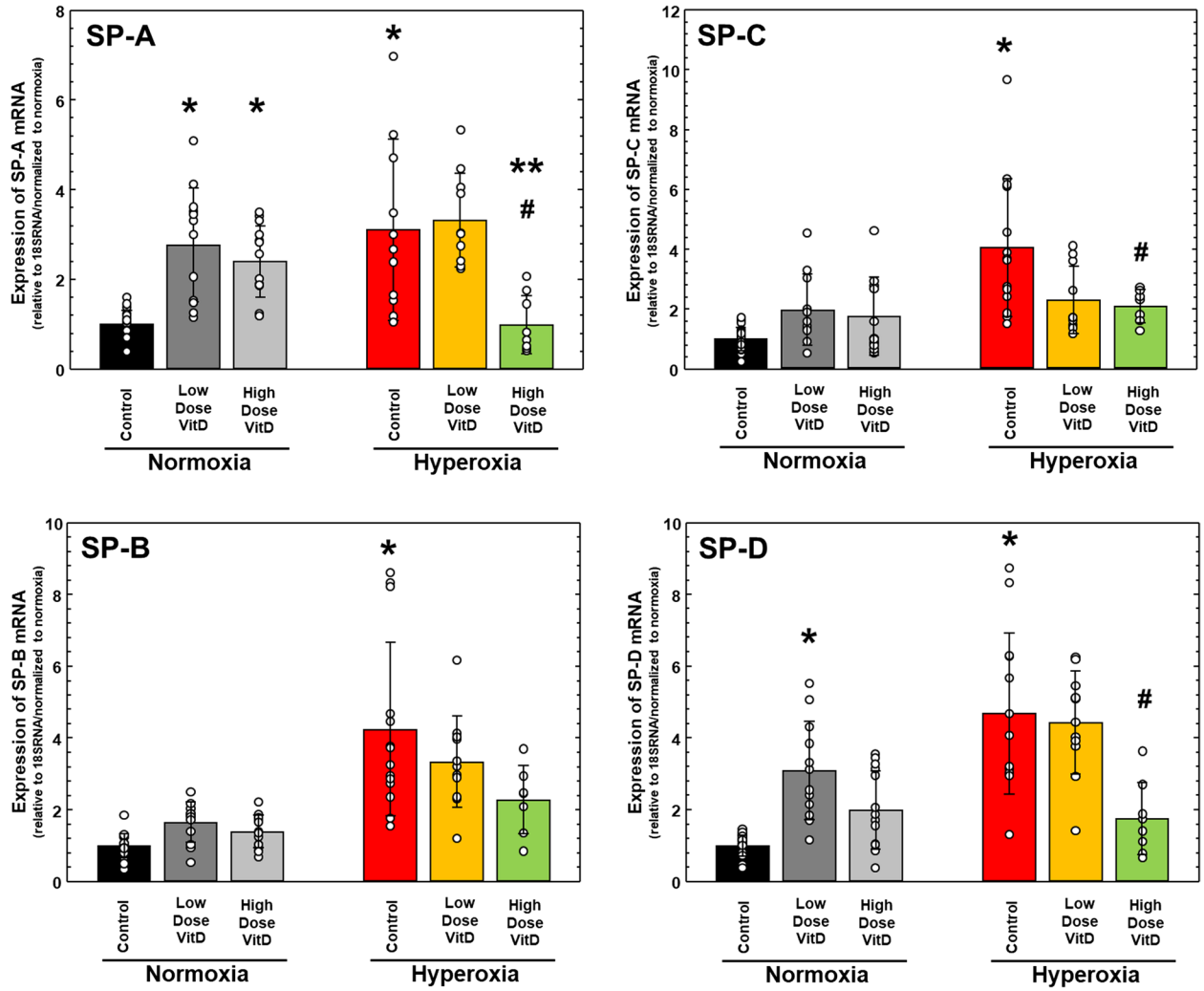

Fig. 6 Gene expression of surfactant proteins A through D in neonatal mice in normoxia and hyperoxia with administration of vehicle, low dose vitamin D and high dose vitamin D (combined male and female data). mRNA is isolated at DOL 6 and levels of cytokine mRNA assessed by quantitative real-time RT-PCR analysis. Shown are levels of expression relative to levels in mice exposed to normoxia (set as 1). Data is average ± SEM of 2 independent experiments; $n > 10$. * denotes significant difference ($p < 0.05$) to mice in normoxia. # denotes significant difference ($p < 0.05$) to mice in hyperoxia

and cellular apoptosis and necrosis [4, 63]. Our study showed that exposure to 85% hyperoxia led to significant detrimental physiologic effects in neonatal mice, and VitD ameliorated these effects. In hyperoxia exposed pups, we observed a significant increase in pulmonary edema and disruption of alveologenesis. We also found significant increases of cytokine expression (CXCL1, IL-1β, IL-6 and TNF-α). These findings are in line with previous studies of mice subjected to hyperoxic conditions that found poor growth, pulmonary edema, decreased alveolar development and significant elevations in mRNA expression of similar cytokines at 7 days [6, 60]. Treatment with VitD demonstrated improvement in weight gain, pulmonary edema, and alveolarization, but no change in cytokine expression.

Dose responsiveness of VitD Treatment

Protective effects of VitD have been described at length in the literature and a wide range of dosages and form of administration have been utilized [9, 11, 29, 31, 64–66]. To closely mimic the differences in dosages as recommended by AAP and ESPGHAN, we used 5 ng/g of VitD as our low dose group versus 25 ng/g of VitD as our high dose group. We found VitD improved alveolar development with improvement in both MLI and RAC in a dose dependent manner. This finding differs from previous study by Chen et. al. who found that lower

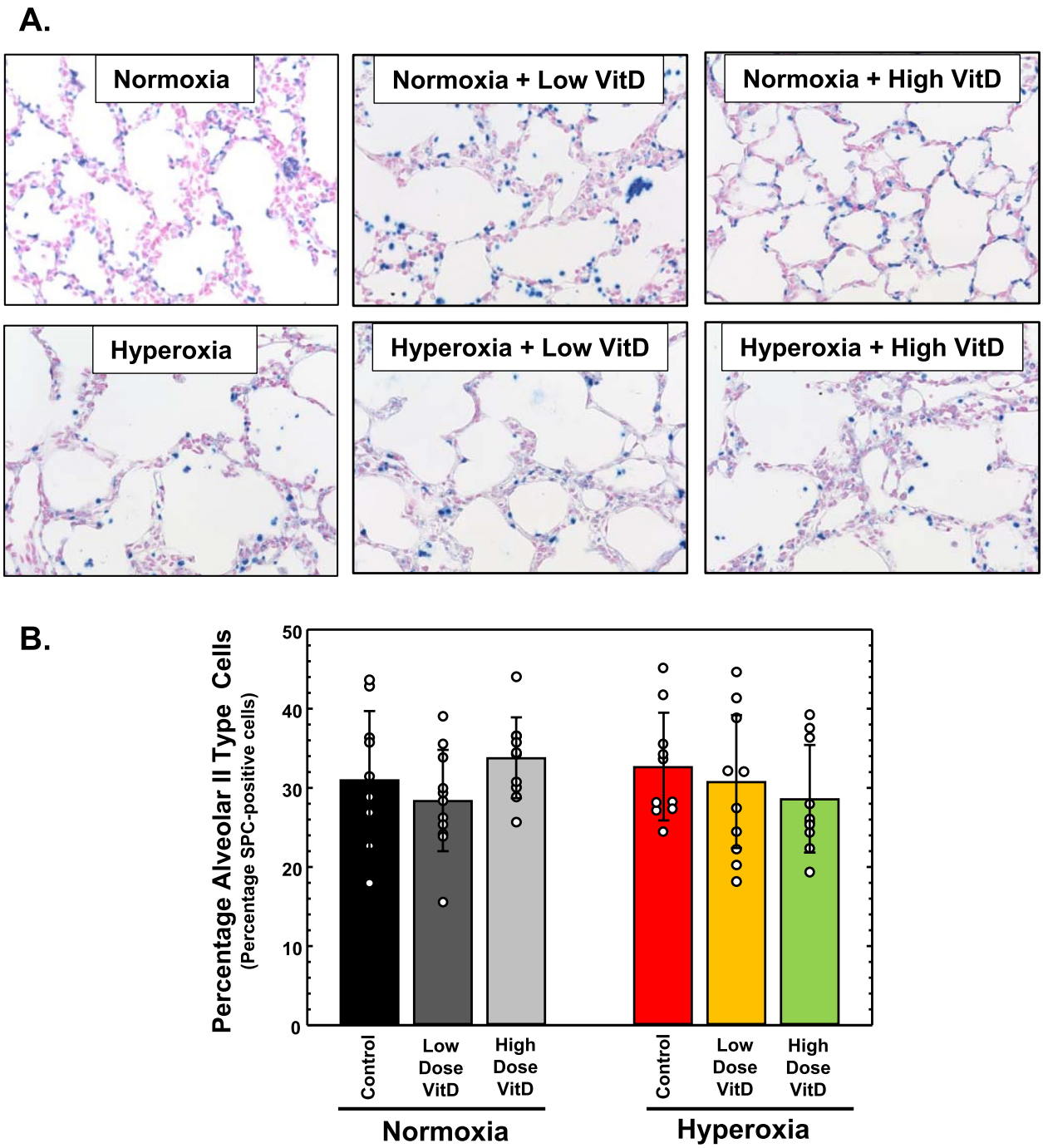

Fig. 7 Presence of alveolar type II cells in neonatal mouse lung after exposure to normoxia and hyperoxia after DOL6 (combined male and female data). **A** Representative image of SP-C positive type II cells (Blue) and nuclei of all cells (Red) in lung sections from neonates administered vehicle, low dose VitD, or high dose VitD and exposed to normoxia or hypoxia. B Representative image of SP-C positive type II cells (Blue) and nuclei of all cells (Red) in lung section from a mouse exposed to hyperoxia. **B** Quantitation of the percentage of SP-C positive (type II cells) in sections from the indicated mouse lungs. Data is average ± SD of 2 lungs, 5 areas per lung; $n = 10$

dosages of VitD at 0.5 ng/g rather than 3 ng/g was more beneficial for weight gain, alveolarization and cytokine expression [31]. These different findings may owe to different methodology and animal model including the use of neonatal rats rather than C57BL/6 neonatal mice, intraperitoneal injection versus oral gavage as well as hyperoxia exposure with 90% O_2 for 7 days rather than 6 days.

Surfactant expression in hyperoxia

White *et. al* found increased mRNA expression of all surfactant proteins in newborn rats exposed to hyperoxia which agreed with our findings [67]. The increased expression of surfactant protein can owe to the body's adaptive response to oxidative stress and inflammation [13, 67, 68]. Direct oxidation and altered metabolism of pulmonary surfactant contributing to worsened lung injury in hyperoxia could explain its loss of function while still maintaining normal to high levels [69, 70]. Extrapulmonary immune and chemotactic function of surfactant protein could also play a role in pulmonary dysfunction in hyperoxia [22, 70–72]. Surfactant synthesis could be upregulated by VitD through acceleration and maturation of pulmonary artery endothelial cell (PAEC) alveolar type II (ATII) cells [66, 73].

Prolonged effects

Weight gain and pulmonary function tests were improved at 21 days with VitD treatment. We speculate the improvement in weight gain for mice given VitD in hyperoxia as compared to hyperoxia control can be due VitD ability to enhance host cell defense, optimize bone mineralization as well as its positive interaction with insulin-like growth factor 1 (IGF-1) hepatic secretion [74, 75]. Hyperoxia significantly increased E_{RS}, G and H, but had no effect on R_{RS} as compared to normoxia control at all methacholine dosages suggesting that hyperoxia continues to impair airway dynamic response even after hyperoxic insult has ceased. Mice treated with VitD during hyperoxic exposure showed significant reduction in E_{RS}, G and H as compared to hyperoxia control. Both groups showed no significant changes from the normoxia control which suggests that VitD may continue to exert protective effects against HALI into later years if provided sufficient dosages during when the injury occurs.

Dylag et. al also evaluated pulmonary function at 4 weeks in C57BL/6 mouse pups exposed to 80% O_2 for 8 days after birth. Pulmonary function testing was analyzed at 4 weeks, and mice exposed to hyperoxia in the neonatal period exhibited alveolar oversimplification with increased MLI and no change in $R_{RS.}$ This is similar to our findings, but they observed decreased G and H values in their respiratory function test which is contrary to our findings [23]. Possible explanation for the difference in findings could be due to the lower oxygen concentration used, longer duration of oxygen exposure as well as delayed pulmonary function testing at 4 weeks rather than 3 weeks which was done in our study. We speculate that exposure to hyperoxia at 85% O_2 for 6 days in neonatal period could have promoted increased collagen synthesis and induced destructive architectural remodeling leading to pulmonary fibrosis which may explain why we found significantly increased G and H values at 3 weeks reflecting increased small airway resistance, parenchymal stiffness and decreased airspace recruitment [76, 77]. Similarly, Wang et. al found that mice exposed to hyperoxia at 40% and 70% FiO2 had significantly increased R_{RS} and significantly decreased compliance in the 40% group versus the 70% group and attributed this puzzling difference due to increased effect of hyperoxia on smaller airways associated with collagen deposition[78]. We also found increased interstitial pulmonary edema as well as disruption of normal lung architecture which could explain the increased heat loss in the alveolar walls as noted by G leading to functional pulmonary dysregulation. The significant elevation in H may be due to increased airway closure due to heightened alveolar tension, thickening of alveolar walls, and reduced lung volume resulting in impaired tissue elastic recoil. One possible explanation for the lack of significant changes in R_{RS} could be hyperoxia exhibiting the majority of its effect on progressive alveolar destruction rather than on the larger conducting airways which is responsible for the majority of the calculated R_{RS}.

Absence of cytokine changes

Surprisingly, although VitD ameliorated the detrimental effects of hyperoxia on lung mechanics, VitD had no effect on inflammatory cytokine expression induced by hyperoxia. This study suggests that cytokine involvement in inflammatory response does not have a significant role in pulmonary outcomes when evaluating VitD effects on HALI as previously predicted. It may be possible that we did not detect significant changes in cytokine levels with VitD treatment because VitD may have a more active role in the integrity of pulmonary epithelial tight junction and regulation of innate immunity rather than via cytokine release or the timing of whole lung harvesting and inability to isolate serum for evaluation of cytokine expression was not optimal for cytokine assessment [24, 79–83]. Other considerations include upregulation of VitD receptor (VDR) expression in response to active VitD treatment in fetal lung development and maturation leading to alveolar thinning, proliferation of alveolar macrophages as well as inhibition of NF-κβ activity, critical in inflammatory signaling [73, 84]. Studies on effects of postnatal hyperoxia delivered to mothers with vitamin D deficiency in murine models also showed impairment in lung growth and persistent defects with depression of HIF-1α protein and VEGF gene expression affecting airway and microvascular development [85, 86].

Sex differences

We found that male mice had significantly higher MLI when exposed to hyperoxia as compared to female mice, but no other parameters were significantly different. This is in contrast to Lingappan et. al. who found that male mice had lower body weights, increased alveolar oversimplification and cytokine upregulation as compared to their female counterparts [62].

Limitations

Our study is limited by several factors. The most important was the rearing of pups were entirely dependent on dams' health and nurture. Many different methods were applied to enhance the nurturing of newborn pups such as using "experienced dams" that previously reared pups. However, it was difficult to ensure that the pups were properly nourished. Pups in hyperoxia chambers or those that became ill appeared to be quickly neglected by the dams, which could have affected our results. Second, there is no literature to assess if mother's milk composition is affected by hyperoxia and it is unknown if the pups' nutrition has changed significantly throughout the course of the experiment. Lastly, our results reflect combined data of both sexes. We do not have a significant sample size to fully analyze all parts of the experiment with enough power to show significance by sex.

Possible mechanisms

From the results of this study, concurrent treatment of mice exposed to VitD can mitigate the harmful impact of acute hyperoxic exposure. However, treatment does not impact the percentage of type II cells in the lung and, for the most part, surfactant gene expression is unaffected or reduced when VitD is administered during hyperoxia, negating the possibility that increased surfactant production is a beneficial impact of vitamin D. Alternatively, VitD may decrease activation of the TGF-β pathway leading to decreased monocyte and macrophage infiltration and therefore decreased lung fibroblast and remodeling [87]. Supplementation of VitD has also been found to be associated with variable expression of VDR which may affect the expression of claudin in airway epithelial cells during inflammation [88, 89]. VitD has been implicated in direct enhancement of VEGF and its receptor which is critical in alveolarization. This may play a key role in reduction alveolar simplification in pups afflicted by hyperoxia but supplemented with high dose VitD [90]. As our results found that there are significant differences in surfactant load in pups given hyperoxia and VitD, we believe it is possible that surfactant may have an extrapulmonary roles in monocyte and macrophage recruitment leading to decreased inflammation. The reduction in surfactant protein may play a role in reduction of oxygen toxicity to decreased permissible oxygen diffusion through alveolar barrier [91, 92].

Conclusions

In conclusion, our study showed that HALI has significant detrimental effects on weight gain, pulmonary edema, alveolar development as well as airway mechanics in neonatal mice. Vitamin D can provide significant protection against HALI, in a dose dependent manner. These findings lend support to usage of high dose vitamin D to those at risk of HALI and may provide an important potential strategy in the treatment of the most vulnerable population – the preterm neonate.

Abbreviations

AAP	American Academy of Pediatrics
C_T	Threshold cycle
DOL	Day of life
E_{RS}	Respiratory system elastance
G	Tissue damping
ESPGHAN	European Society for Paediatric Gastroenterology Hepatology and Nutrition
H	Tissue elastance
HALI	Hyperoxic acute lung injury
MLI	Mean linear intercept
RAC	Radial alveolar count
RDS	Respiratory distress syndrome
ROS	Reactive oxygen species
R_{RS}	Respiratory system resistance
SD	Standard deviation
SP-A	Surfactant protein A
SP-B	Surfactant protein B
SP-C	Surfactant protein C
SP-D	Surfactant protein D
VDR	Vitamin D receptor
TLR4	Toll-like receptor 4
VitD	Vitamin D
WT	Wildtype

Supplementary Information

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Supplementary Material 1.

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BioRender was used to create graphical abstract.

Authors' contributions

T.T.T. contributed to conceptualization, methodology, investigation, data curation, analysis, writing and visualization. J.D. contributed to resources, visualization, review and editing. RAJ contributed to conceptualization, methodology, review and editing. H.K.-Q. contributed to resources, review and editing. H.L. contributed to methodology and resources. C.E.C contributed to review and editing. A.M.K. contributed to conceptualization, review, editing and visualization. J.L.A. contributed to conceptualization, methodology, validation, resources, visualization, supervision. All authors reviewed the manuscript.

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Data availability
Data is provided within the manuscript or supplementary information files. Anyone wishing to get the original data can email me at Joseph.L.Alcorn@uth.tmc.edu.

Declarations

Ethics approval and consent to participate
All protocols were certified by the Animal Welfare Committee (AWC) of McGovern Medical School at the University of Texas Health Science Center at Houston (UTHealth), which is the Institutional Animal Care and Use Committee (IACUC) for UTHealth (AWC-20–0067, Houston, TX).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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