Vitamin D treatment improves survival and infant lung structure after intra-amniotic endotoxin exposure in rats: potential role for the prevention of bronchopulmonary dysplasia

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Mandell, E., Seedorf, G., Gien, J., and Abman, S. H. Vitamin D treatment improves survival and infant lung structure after intra-amniotic endotoxin exposure in rats: potential role for the prevention of bronchopulmonary dysplasia. Am J Physiol Lung Cell Mol Physiol 306:L420–L428, 2014. First published January 10, 2013; doi:10.1152/ajplung.00344.2013.—Vitamin D (vit D) has anti-inflammatory properties and modulates lung growth, but whether vit D can prevent lung injury after exposure to antenatal inflammation is unknown. We hypothesized that early and sustained vit D treatment could improve survival and preserve lung growth in an experimental model of bronchopulmonary dysplasia induced by antenatal exposure to endotoxin (ETX). Fetal rats (E20) were exposed to ETX (10 μg), ETX + Vit D (1 ng/ml), or saline (control) via intra-amniotic (IA) injections and delivered 2 days later. Newborn pups exposed to IA ETX received daily intraperitoneal injections of vit D (1 ng/g) or saline for 14 days. Vit D treatment improved oxygen saturations (78 vs. 87%; P < 0.001) and postnatal survival (84% vs. 57%; P < 0.001) after exposure to IA ETX compared with IA ETX alone. Postnatal vit D treatment improved alveolar and vascular growth at 14 days by 45% and 25%, respectively (P < 0.05). Vit D increased fetal sheep pulmonary artery endothelial cell (PAEC) growth and tube formation by 64% and 44%, respectively (P < 0.001), and prevented ETX-induced reductions of PAEC growth and tube formation. Vit D directly increased fetal alveolar type II cell (ATIIIC) growth by 26% (P < 0.001) and enhanced ATIIIC growth in the presence of ETX-induced growth suppression by 73% (P < 0.001). We conclude that antenatal vit D therapy improved oxygenation and survival in newborn rat pups and enhanced late lung structure after exposure to IA ETX in vivo, which may partly be due to direct effects on vascular and alveolar growth.

bronchopulmonary dysplasia; chronic lung disease; vitamin D; lung development; angiogenesis; chorioamnionitis; prematurity; disease prevention; pulmonary hypertension; persistent pulmonary hypertension of the newborn

BRONCHOPULMONARY DYSPLASIA (BPD) is the chronic lung disease of infancy that often complicates the clinical course and outcomes of preterm newborns (24). Infants with severe BPD are at high risk for death, prolonged need for ventilator or oxygen support, pulmonary hypertension, recurrent respiratory infections, abnormal lung function, exercise intolerance, and late neurodevelopmental sequelae (4). BPD is characterized by a disruption of distal lung growth with impaired alveologenesis and angiogenesis, but mechanisms that cause these sustained abnormalities of lung structure are poorly understood (11, 44).

Epidemiological and preclinical studies have suggested that chorioamnionitis with antenatal inflammation has been strongly associated with a high risk for the development of BPD after preterm birth (17, 21, 36, 38, 41). Although chorioamnionitis has been shown to increase lung inflammation, mechanisms through which early inflammation causes sustained impairment of lung structure in BPD are incompletely understood. Recent studies suggest that antenatal endotoxin (ETX) causes high neonatal mortality and inhibits lung growth in infant rats, which is thought to be in part due to early disruption of angiogenesis through endothelial cell dysfunction and down-regulation of proangiogenic factors such as vascular endothelial growth factor (VEGF) and related mechanisms (9, 16, 40, 42). However, mechanisms through which chorioamnionitis leads to sustained impairment of lung structure are poorly understood, and potential therapeutic strategies to prevent BPD are lacking.

Although vitamin D (vit D) has been extensively studied in the setting of neonatal calcium and bone homeostasis, a growing literature suggests that vit D has multiple roles in diverse physiological and pathophysiological processes (3, 5, 6). Recent epidemiological studies have linked vit D deficiency with increased severity of inflammatory chronic lung diseases, such as asthma and chronic obstructive pulmonary disease (COPD) (8, 10, 29, 33, 47, 48, 50). Low vit D levels are associated with decreased lung function in asthma and COPD after exposure to air pollution (7, 13, 19). Vit D treatment attenuates inflammation in experimental autoimmune diseases and modulates cytokine production by vascular endothelial cells and human decidual cells (20, 49). Importantly, vit D may contribute to normal development of the lung (35). Maternal vit D deficiency is associated with an increased risk for wheezing episodes during early childhood (46) and has also been linked with systemic vascular endothelial dysfunction, systemic hypertension, and an increased risk for cardiovascular disease (31, 43). In addition, vit D modulates lung and vascular function in diverse experimental settings (9, 16, 40), but whether vit D treatment can improve lung structure in preterm infants after exposure to intrauterine inflammation is unknown.

Past studies from our laboratory and others have shown that intrauterine exposure to ETX causes pronounced lung inflammation and evidence of changes in pulmonary vascular structure before birth (22, 25, 42). In rodent models, antenatal ETX causes neonatal respiratory distress with poor survival after birth and sustained impairments of lung structure after 2 wk of life (42). However, strategies to attenuate early lung injury and improve late lung structure in experimental chorioamnionitis...
remain limited, and the effects of vit D therapy in this setting remain unknown.

Therefore, we hypothesized that early or sustained vit D therapy may attenuate inflammation-induced lung injury and preserve lung growth in experimental BPD caused by antenatal ETX. Because recent studies have linked early disruption of angiogenesis and alveolar growth with BPD (1, 22), we further hypothesized that vit D can directly stimulate fetal pulmonary artery endothelial cells (PAEC) and alveolar epithelial type II cell (ATIIIC) growth and function and will restore cell growth during ETX exposure in vitro. To test these hypotheses, we studied the effects of vit D treatment on neonatal rat pup survival and lung structure after antenatal (intra-amniotic, IA) ETX in vivo and on isolated endothelial cells and ATIIIC in vitro.

MATERIALS AND METHODS

Animals

All procedures and protocols were approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center. Timed pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and maintained in room air at Denver altitude (1,600 m; barometric pressure, 630 mmHg; inspired oxygen tension, 122 mmHg) for at least 1 wk before they gave birth. Animals were fed ad libitum and exposed to day-night cycles alternatively every 12 h. Rats were killed with an intraperitoneal injection of pentobarbital sodium (0.3 mg/g body wt; Fort Dodge Animal Health, Fort Dodge, IA).

Animal Model and Study Design

IA ETX administration. We utilized an animal model of chorioamnionitis as previously described (42). At 20 days gestation (term; 22 days), pregnant rats were prepared for receiving IA injections. The timing of injection during the late canalicular stage of lung development in the rat was selected to parallel the similar stage of human lung development in 24–26-wk premature newborns, who are at the highest risk for BPD. After premedication with buprenorphine (0.01–0.05 mg/kg, subcutaneous injection), laparotomy was performed under general anesthesia with 1–2% isoflurane inhalation via facemask (anesthesia machine: Matrix by Midmark, model VIP3000). During anesthesia and laparotomy, pregnant rats were kept on a heating pad for preventing hypothermia. Pregnant rats were randomly assigned to saline control (SA), ETX, or vit D group; the SA group received 50 μl of normal saline per amniotic sac, the ETX group received 10 μg of Escherichia coli 055:B55 ETX (Sigma Chemical, St. Louis, MO) diluted to 50 μl with normal saline per sac, and the vit D group received 50 pg diluted to 50 μl with normal saline. Under sterile preparation, a midline abdominal incision of 3–4 cm in length was made to expose the amniotic sacs for IA injections. The amniotic sac closest to the right ovary was first identified and injected, and then in a counterclockwise sequence each sac was identified and injected with a maximum of 10 sacs injected per dam. Injections were limited to 10 sacs to prevent maternal mortality due to systemic toxicities from accumulating doses of IA ETX. The dose of ETX was established from previous studies in our laboratory that demonstrated ETX at lower doses (1–5 μg/sac) failed to induce abnormal lung structure at 14 days of age. The dose of vit D was established again from previous studies in our laboratory demonstrating that vit D at higher doses (500 ng/g) produced subcutaneous calcium deposits noted in rat pups. The abdominal incision was closed with nylon sutures. Bupivacaine (1–2 mg/kg, intramuscular injection) was applied over the incision wound for postoperative pain control. Pregnant rats were monitored closely to ensure arousal within 10 min after surgery, and rats were placed back to the cages and were monitored for activity and for signs of bleeding or infection.

Cesarean section. Two days after IA injections, cesarean section was performed on pregnant rats under general anesthesia with isoflurane inhalation, as described above. The fetus in the amniotic sac closest to the right ovary was first delivered, which was followed by delivery of the rest of the fetuses in a counterclockwise sequence, to identify fetuses exposed to IA injections. We performed cesarean sections instead of allowing vaginal deliveries to identify fetuses exposed to specific IA injections, based on the order of the amniotic sacs and their anatomic locations related to the ovaries. All of the rat pups in the injected amniotic sacs were delivered within 5 min after onset of anesthesia. Mother rats were then euthanized with pentobarbital sodium. Newborn rats were immediately dried and placed on a heating pad to avoid hypothermia. Pups received no supplemental oxygen or artificial ventilation at birth. Within 30 min after birth, pups were weighed and either killed for histology or placed with foster mother rats to be raised through 14 days. Rat lungs were harvested at birth and 14 days of age for Western blot analysis and histological assessment. Survival of the infant rats was monitored and recorded daily from birth throughout the study period. Survival rate was calculated as the number of pups that survived divided by the number of sacs that received IA injection in each given litter.

Study Measurements

Tissue for histological analysis. Animals were killed with intra-peritoneal pentobarbital sodium. A catheter was placed in the trachea, and the lungs were inflated with 4% paraformaldehyde and maintained at 20 cmH2O pressure for 60 min. A ligature was tightened around the trachea to maintain pressure, and the tracheal cannula was removed. Lungs were immersed in 4% paraformaldehyde at room temperature overnight for fixation. A 2-mm-thick transverse section was taken from the midplane of the right lower lobe and left lobe of the fixed lungs per animal, respectively. Two sections from each animal were processed and embedded in paraffin wax for study.

Immunohistochemistry. Slides with 5-mm paraffin sections were stained with hematoxylin and eosin for assessing alveolar structures and with von Willebrand Factor (vWF), an endothelial cell-specific marker, for assessing vascular density and vascular wall thickness.

Radial alveolar counts. Alveolarization was assessed by the radial alveolar count (RAC) method of Emery and Mithal as previously described and applied (42). Respiratory bronchioles were identified as bronchioles lined by epithelium in one part of the wall. From the center of the respiratory bronchiole, a perpendicular line was dropped to the edge of the acinus connective tissues or septum or pleura, and the number of septae intersected by this line was counted. In each animal, at least five measurements were obtained.

Pulmonary vessel density. Pulmonary vessel density (PVD) was determined by counting vWF-stained vessels with external diameter less than 100 μm per high-power field. The fields containing large airways or vessels were avoided. At least five pulmonary vessels were measured.

Right ventricular hypertrophy. Hearts were dissected and weighed at 14 days of age. The right ventricle (RV) and left ventricle plus septum (LV+S) were dissected and weighed, and the ratio of RV to LV+S weights was determined.

Oxygen saturation. Oxygen saturation (SaO2) was measured by using Nonin Pulse Oximeter (Nonin Medical, Plymouth, MN) on rats in room air. The sensor was attached to the precordial site, and animals were placed in a prone position to keep good attachment to the sensor. During measurement, animals were kept on a heating pad to avoid hypothermia. The readings of SaO2 were interpreted as valid when the heart rate, which was simultaneously monitored on Nonin Pulse Oximeter, was stably maintained. Each measurement was finished within 10–15 s to avoid desaturation secondary to prolonged...
measurement. For each animal, three to five measurements were taken, and the highest value of SaO2 was used for analysis.

**PAEC lysate quantitative real-time PCR.** Total RNA was extracted from PAEC cell lysates using an Ambion RNAqueous Phenol-free total RNA isolation kit (Invitrogen, Carlsbad, CA) and DNase I treated using the Ambion TURBO DNA-free kit (Invitrogen). RNA was assessed for purity and concentration using the NanoDrop (Thermo Scientific, San Jose, CA). cDNAs were prepared with random hexamer primers and Superscript III (Invitrogen). Real-time PCR was performed using SYBR Green I primers that were designed for VEGF (Forward: TTG CCT TGC TGC TCT ACC TT, Reverse: GGG CAC ACA CTC CAG ACT TT), and kinase domain receptor (KDR) (Forward: TTC CAC TGG GAA TAC CCT TCT TCG, Reverse: TCC ACC AAG GAT TCC ATG CCA CTA). Each gene was normalized to s15. Samples were quantified with Roche LC480II software.

**Fetal PAEC isolation.** PAECs were harvested from the proximal pulmonary arteries of late gestation control fetal sheep at day 135 (day 147 term), as previously described (12). Immunohistochemistry with standard endothelial markers confirmed the cell phenotype. Low-passage PAECs (passage 4–5) were then exposed to ETX, vit D, or ETX + vit D in the experiments below.

**Growth of PAECs while exposed to ETX and vit D.** Fetal PAECs were plated in triplicate at 50,000 cells/well in DMEM with 10% FBS into 12-well plates and allowed to adhere overnight in 21% oxygen. The following day (day 0), the cells were washed twice with PBS. DMEM with 2.5% FBS with VEGF, ETX, vit D, or ETX + vit D was then added, and cells were incubated in 21% oxygen. Experimental media was changed daily, and cells were counted on day 3 after removing cells with 0.25% trypsin and counted with a cell counter (Beckman Coulter, Fullerton, CA). Growth studies with treatment were performed in DMEM with 2.5% FBS, based on previous studies that determined that this was the lowest serum concentration that supported fetal PAEC survival with some proliferation (12).

**PAEC tube formation assay.** To assay in vitro angiogenesis, we cross-linked rat-tail collagen using 0.2% flavin mononucleotide and a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Cells (50,000 cells/well) were added in serum-free DMEM media supplemented with ETX, vit D, or ETX + vit D, and each condition was tested in triplicate for each animal. PAECs were then incubated for 12–18 h under 3% oxygen conditions based on previous studies that determined that tube formation was more robust in 3% compared with 21% oxygen (12). Branch-point counting was performed in blinded fashion under ×10 magnification from each of three wells with three to four fields of view per well, as previously described (12). Wells were imaged using an Olympus IX71 fluorescence microscope (Olympus).

**Fetal ATIIC isolation.** Distal lung was dissected free, avoiding large airways and blood vessels. Distal lung was further minced to 3–5-mm³ sections and incubated with 0.1% collagenase and 0.025%...
trypsin for 30 min at 37°C. Trypsin inhibitor solution was added to the lung digest and then subjected to low-speed homogenization for 60 s. Lung suspension was filtered through a 10-μm Nitex filter to remove undigested tissue. Cells were centrifuged at 300 relative centrifugal force for 10 min, resuspended in wash media, and plated on IgG-coated dishes for 1 h at 37°C. Nonadherent cells were recovered, centrifuged, and plated at a density of 1 × 10^5 cells/well. We confirmed the ATIIC phenotype by positive immunostaining for prosurfactant protein C and negative staining for T1-α (an ATIC marker), vWF (an endothelial marker), and desmin (a smooth muscle cell marker).

**ATIC growth assay.** Control fetal ATIICs were plated at 1 × 10^5 cells/well and allowed to adhere overnight. Cells were plated in DMEM/F12 media supplemented with 10% FBS for 24 h to allow cells to attach. Cells were washed with PBS and DMEM with 2.5% FBS with either ETX, vit D, or ETX + vit D added to the ATIICs. The cells were placed in 21% oxygen. Experimental media was changed daily. After 4 days of treatment, ATIICs were washed twice with PBS and incubated with 0.25% trypsin until all cells were detached. Cells were assayed for viability using Trypan blue exclusion and counted on a hemacytometer (viability >90%).

**Statistical Analysis**

Data are presented as means ± SE. Statistical analysis was performed with the Prism software package (v. 5.0a, GraphPad, La Jolla, CA). Repeated-measures one-way analysis of variance (ANOVA) was formed with the Prism software package (v. 5.0a, GraphPad, La Jolla, CA). Statistical analysis was performed with Bonferroni posttest analysis was performed. P values <0.05 were considered significant.

**RESULTS**

A total of 12 litters of rat pups received IA injections of saline, ETX, or ETX + vit D; 20 pups received IA saline, 54 pups received IA ETX, 27 received IA ETX and vit D, and 28 of the IA ETX-exposed pups went on to receive postnatal daily intraperitoneal vit D injections. Newborn rats exposed to antenatal ETX were cyanotic with respiratory distress after birth. In contrast, animals that received antenatal ETX and vit D treatment were pink without signs of distress and appeared similar to saline controls. Oxygen saturations after birth were lower in the ETX-exposed rat pups when compared with control pups (77% vs. 87% P < 0.001), which was improved in the antenatal ETX and vit D group. Antenatal vit D treatment improved oxygen saturations at delivery compared with pups that received IA ETX alone (85% vs. 77% P < 0.001; Fig. 1A).

Birth weights were slightly lower in the ETX-exposed rats (average weight 5.1 ± 0.4 g) compared with saline controls (average weight 5.4 ± 0.7 g) (P = ns vs. controls). Pups that received antenatal ETX and vit D had birth weight averages of 5.2 ± 0.6 g (P = ns). At day 14, the body weights for saline controls were 32.9 ± 1.2 g; pups that received IA ETX had average weights of 30.9 ± 1.8 g, whereas the pups that received IA ETX and vit D had weights of 31.2 ± 2.1 g. The IA ETX-exposed pups that went on to receive IP vit D had body weights of 30.3 ± 1.4 g. There were no significant body weight differences among study groups at birth or at 14 days.

In comparison with control rat pups, the ETX group had markedly worse survival (Fig. 1B). Antenatal vit D treatment of ETX-exposed pups improved survival from 52 to 84% and from 42 to 84% at days 1 and 3, respectively (P < 0.001 for each comparison). Survival was similar between the vit D treatment group and saline control pups. No mortality was found in surviving rats within any group beyond day 3.

Antenatal vit D improved lung histology at birth in pups exposed to ETX. As illustrated in the histology from ETX-exposed rats pups, lung structural abnormalities include thickened septae, a hypercellular interstitial space, and enlarged distal airspaces compared with both saline controls and IA vit D-treated ETX-exposed pups (Fig. 1C). Lung histology from IA vit D-treated ETX-exposed pups more closely resembled controls.

![Image](http://ajplung.physiology.org/)

**Fig. 2.** Effects of vit D treatment on distal lung structure at day 14. Postnatal vit D treatment improves lung structure in pups after ETX exposure. Lung histology from IA ETX-exposed pups (center) demonstrates alveolar simplification compared with saline control (left). Lung histology of pups that received vit D treatment shows lung structure that appears similar to saline control pups (right).
Lung histology from ETX-exposed pups demonstrated alveolar simplification compared with saline controls, which were markedly improved with vit D treatment (Fig. 2). Lung structure from pups that received daily IP vit D injections were similar to the lung histology from saline control pups. RAC were decreased at day 14 from animals exposed to IA ETX by 35% when compared with saline controls (Fig. 3A, \( P < 0.05 \)). Pups that received IA or daily IP vit D-treated rat pups had 45 and 42% higher RAC than IA ETX-exposed pups at day 14, respectively (\( P < 0.01 \) for each comparison).

Vit D treatment in ETX-exposed pups improved distal lung histology at birth, including increased PVD compared with ETX-alone pups (Fig. 3B, \( P < 0.05 \)). IA ETX-exposed pups had significantly lower PVD compared with saline controls at birth by 37% (\( P < 0.01 \)). Postnatal vit D treatment improved PVD at 14 days by 20% above controls (\( P < 0.05 \)). RV hypertrophy (RVH), as assessed by the RV/LV+S ratio, was increased in ETX-exposed rats at birth compared with saline controls (\( P < 0.01 \)). IA vit D decreased RVH in ETX rats at day 0 (\( P < 0.01 \)). At day 14, ETX pups that received daily IP vit D had less RVH compared with ETX-exposed rats (\( P < 0.05 \)).

In control PAEC, vit D increased in cell growth above basal conditions (Fig. 4A, \( P < 0.001 \)). VEGF (10 ng/ml), a potent angiogenic factor, was used for comparison to illustrate the relative effects of vit D on cell growth. As shown, vit D treatment under basal conditions stimulated PAEC cell growth to similar values achieved with VEGF treatment. Cell growth was reduced in PAECs exposed to ETX by 50% at baseline (\( P < 0.001 \)), but growth was augmented with vit D coadministration to rates observed in control PAEC (\( P < 0.001 \)). Vit D treatment also increased PAEC tube formation in control cells under basal conditions by 33% (Fig. 4B, \( P < 0.05 \)). ETX exposure decreased tube formation by 50% (\( P < 0.001 \)); this effect was attenuated by 63% with vit D coadministration (\( P < 0.001 \)).

Vit D treatment also stimulated fetal ATIIC growth by 1.3-fold above basal conditions (Fig. 5; \( P < 0.001 \)). Although ETX exposure reduced ATIIC growth by 50% (\( P < 0.001 \)), vit D restored ATIIC growth to control values vs. ETX alone (\( P < 0.001 \)). Although vit D treatment and ETX exposure independently had no effect on basal VEGF mRNA expression in normal fetal PAEC, vit D augmented VEGF expression administered with ETX by 85% (Fig. 6). Interestingly, vit D increased PAEC KDR mRNA expression by 54% (\( P < 0.001 \)).

**DISCUSSION**

We found that antenatal vit D treatment improved oxygenation and survival of neonatal rat pups after exposure to IA ETX and that early vit D treatment prevented RVH and improved lung vascular and alveolar growth through the first 14 days of life. In addition, vit D directly stimulated fetal PAEC growth and tube formation and attenuated the inhibitory effects of ETX in vitro, suggesting that vit D has proangiogenic effects in the fetal lung. We also found that vit D upregulated VEGF and KDR mRNA expression in fetal PAEC with ETX exposure. Vit D also increased fetal ATIIC proliferation in vitro. Overall, these findings suggest that vit D has direct proliferative effects on fetal PAEC and ATIICs and enhances the transition of the lung at birth and causes sustained improvement in lung structure after antenatal ETX exposure. We speculate that vit D treatment may be an effective preventive strategy toward reducing the risk for acute respiratory distress, pulmonary hypertension, and chronic lung disease in the clinical setting of chorioamnionitis.

These findings are the first to demonstrate a potential role of vit D in preventing BPD in an experimental model of chorioamnionitis. Previous studies have shown that low vit D levels in premature neonates correlate with predisposition for a variety of long-term sequelae (13, 46). Our data demonstrate a potential proangiogenic mechanism for vit D, which acts directly on endothelial cells by improving growth and tube
formation and rescues endothelial dysfunction after ETX exposure. In addition, vit D has proproliferative and protective effects on isolated ATIIC.

Previous studies have shown that IA ETX administration during the late canalicular stage of lung development caused sustained abnormalities of alveolar and vascular growth that persist during infancy (25, 42). ETX-exposed rat pups have marked reductions in alveolarization, vascular growth, and pulmonary hypertension, which mimic features of human BPD (11, 42). In addition, past studies suggest that vit D modulates fetal rat lung maturation and may be protective for respiratory distress syndrome in preterm neonates (35). Vit D also inhibits lung fibroblast proliferation and myofibroblast transdifferentiation in an adult model of lung fibrosis (39). The theory that vit D regulates lung development has gained support from animal studies that show offspring mice from vit D-deficient mothers have reduced lung volumes and a small reduction in alveoli compared with mice born from vit D-sufficient mothers (46, 50). These data are also consistent with clinical studies that vit D-deficient preterm infants are at high risk for severe lung disease, termed “rachitic respiratory distress” (15, 37). This report further implicates possible roles of vit D in promoting normal lung development and protecting against lung injury caused by antenatal inflammation.

Mechanisms responsible for the improved survival rate after a single IA injection of vit D in the antenatal ETX rat model may include preservation of endothelial function and angiogenesis, which may be related to enhanced VEGF signaling. Previous studies have shown that fetal ETX exposure decreases lung vascular density and downregulates VEGF and VEGF receptor 2 (VEGFR2), also known as KDR (25, 42). Our data support these observations and additionally show that vit D treatment increases VEGF mRNA expression in PAECs coexposed to ETX. Our studies demonstrate a direct role for vit D in protecting or augmenting resident cells, such as ATII and PAECs, and may prevent lung injury from antenatal ETX exposure. Macrophages are also known to express the vit D receptor and 1α-hydroxylase (CYP27B1), the vitamin D conversion enzyme (2), but whether the lung-protective effects of vit D are partly mediated through altered macrophage function remains unknown. Future studies are needed to delineate potential anti-inflammatory mechanisms underlying the effects of vit D in this model in addition to the direct effects of vit D on lung endothelial and epithelial cells noted in this report.
In contrast to our findings, some previous studies have suggested that vit D may actually inhibit endothelial cell growth and differentiation (18, 30, 34). However, these studies were performed with adult bovine aortic endothelial cells (34) or human microvascular endothelial cells from foreskin (30). These latter findings differ from our studies that examined the effects of vit D on developmentally relevant and lung-specific endothelial cells that are more relevant to understanding the pulmonary effects of vit D after fetal exposure to ETX. Data from our study strongly support a proangiogenic role for vit D during lung development.

Previous studies have shown that disruption of VEGF signaling during late gestation in the ovine fetus impairs lung endothelial function and growth in the distal airspaces and vessels and causes perinatal pulmonary hypertension (32). In addition, exogenous VEGF improves alveolar and lung vascular growth in rodent models of BPD (27, 28, 45). Our laboratory has previously demonstrated that lung VEGF and KDR protein contents are decreased at birth in ETX-exposed rats (42). We demonstrate an increase in VEGF mRNA expression in PAEC exposed to ETX with cotreatment of vit D and speculate that increased VEGF improves the transition shortly after birth and sustains improvements in lung structure, pulmonary hypertension, and improved pulmonary vessel density throughout infancy. In addition to the effects of vit D on PAEC, we also found that vit D enhances ATIIC growth and preserves ATIIC growth during ETX exposure. Preservation of ATIIC survival, growth, and function may partially explain vit D-mediated effects on improved lung structure and gas exchange at delivery after ETX exposure.

This study has several potential limitations. This antenatal ETX model of BPD relies on late-gestation injections directly into the amniotic fluid. Past investigators using maternal systemic injections of ETX have shown significant maternal morbidity, and we have selected our model to more closely mimic the clinical problem of chorioamnionitis, which is generally characterized by localized inflammation. It should also be noted that, although this model is excellent to simulate an in utero process, a single IA dose of ETX may not definitively represent human chorioamnionitis that occurs with preterm infants. In addition, BPD represents a complex, multifactorial disease that may only be partially explained by the effects of antenatal ETX exposure. In addition, further work is needed to determine whether maternal vit D deficiency contributes to increased susceptibility to impaired lung structure in this model or in the clinical setting. Finally, although we recognize the potential limitation of comparing in vitro studies of fetal cells from sheep with in vivo rodent experiments, we have used fetal sheep PAEC and ATIIC to more closely mimic the developmental timing and lung-specific source. Future studies are needed to further address the effects of vit D exposure on lung microvascular endothelial cells, as these cells may differ from proximal PAEC and may play an important role during lung development and the response to injury.

In summary, we report that antenatal vit D improves survival and gas exchange and preserves lung structure in neonatal rats exposed to IA ETX. Vit D treatment prevented antenatal ETX-induced RVH and preserved lung vascular density in infant rats, which may be due to its proangiogenic or other protective effects on the developing endothelium after ETX exposure. Vit D also increased ATIIC growth under basal conditions and attenuated ETX induced growth suppression. We conclude that vit D has proliferative and protective effects on fetal PAEC and ATIIC and prevents abnormal infant lung structure in an experimental model of chorioamnionitis. We
speculate that decreased vit D activity may contribute to the pathogenesis of BPD and that early vit D therapy may provide a potential strategy for the prevention of BPD in at risk preterm infants.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


