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Vitamin D improves pulmonary function in a rat model for congenital diaphragmatic hernia

Yumiko Ito [a,b], Hiroyuki Tsuda [b], Kenji Imai [a], Rika Miki [c], Mayo Miura [a], Asuka Tachi [a], Sho Tano [a], Shima Hirako-Takamura [d], Yoshinori Moriyama [a,g], Takafumi Ushida [a], Tomoko Kobayashi [a], Seiji Sumigama [e], Hiroaki Kajiyama [a], Fumitaka Kikkawa [a], Tomomi Kotani [a,f,*]

[a] Department of Obstetrics and Gynecology, Nagoya University Graduate School of Medicine, Nagoya, Aichi, 466-8550, Japan
[b] Department of Obstetrics and Gynecology, Japanese Red Cross Nagoya Daiichi Hospital, Nagoya, Aichi, 453-8511, Japan
[c] Laboratory of Bell Research Center, Department of Obstetrics and Gynecology Collaborative Research, Nagoya University Graduate School of Medicine, Nagoya, Aichi, 466-8550, Japan
[d] Department of Obstetrics and Gynecology, Kasugai Municipal Hospital, Kasugai, Aichi, 486-8510, Japan
[e] Office of International Affairs, International Medical Education, Nagoya University Graduate School of Medicine, Nagoya, Aichi, 466-8550, Japan
[f] Centre for Maternal-Neonatal Care, Nagoya University Hospital, Nagoya, Aichi, 466-8560, Japan
[g] Department of Obstetrics and Gynecology, Fujita Health University Graduate School of Medicine, Toyoake, Aichi, 470-1192, Japan

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ABSTRACT

A congenital diaphragmatic hernia (CDH) is an anomaly caused by defects in the diaphragm; the resulting limited thorax cavity in turn restricts lung growth (pulmonary hypoplasia). This condition is related to pulmonary hypertension. Despite advances in neonatal CDH therapy, the mortality for severe pulmonary hypoplasia remains high. Therefore, it is essential to establish prenatal therapeutic interventions. Vitamin D was reported to have beneficial effects on adult pulmonary hypertension. This study aims to evaluate the efficacy of prenatal vitamin D administration for CDH. First, serum 25-hydroxyvitamin D [25(OH)D] levels in umbilical cord blood were evaluated among CDH newborns. Second, Sprague Dawley rat CDH models were exposed to nitrofen on embryo day 9 (E9). Randomly selected rats in the nitrofen-treated group were infused with calcitriol from E9 to E21. Samples from CDH pups diagnosed after birth were used for lung weight measurements, blood gas analysis, and immunohistochemical analysis. Third, microarray analysis was performed to examine the effect of vitamin D on gene expression profiles in CDH pulmonary arterial tissues. Serum 25(OH)D levels in the umbilical cord blood of newborns who did not survive were significantly lower than those who were successfully discharged. Prenatal vitamin D showed no significant effect on CDH incidence or lung weight but attenuated alveolarization and pulmonary artery remodeling accompanied the improved blood gas parameters. Vitamin D inhibited several gene expression pathways in the pulmonary arteries of CDH rats. Our results suggest that prenatal vitamin D administration attenuates pulmonary vascular remodeling by influencing several gene pathways in CDH.

1. Introduction

A congenital diaphragmatic hernia (CDH) is a life-threatening anomaly characterized by the migration of the abdominal contents into the thoracic cavity due to a hole in the diaphragm. Pulmonary growth is restricted as the lungs are compressed by exposed viscera in the thorax, leading to pulmonary hypoplasia. The severity of pulmonary hypoplasia can have a critical impact on the prognosis of neonatal CDH patients, as this condition is known to cause pulmonary hypertension (PH) due to abnormal bronchial and vascular branching patterns that increase pulmonary vascular resistance [1]. Despite recent advances in PH therapy, such as extracorporeal membrane oxygenation after birth,

Abbreviations: CDH, congenital diaphragmatic hernia; CI, Confidence interval; FETO, fetal endoscopic tracheal occlusion; PH, pulmonary hypertension; OS, Overall survival; RDS, respiratory distress syndrome.

* Corresponding author. Centre for Maternal-Neonatal Care, Nagoya University Hospital, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8560, Japan.
E-mail address: itoto@med.nagoya-u.ac.jp (T. Kotani).

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the mortality of CDH associated with severe pulmonary hypoplasia remains high in neonatal patients. Therefore, prenatal intervention to treat CDH has been an important focus of research. Pulmonary hypoplasia can be evaluated from the 'observed: expected lung area to head circumference ratio' diagnosed with a prenatal ultrasound examination. The most promising fetal treatment modality to accelerate lung growth is fetal endoscopic tracheal occlusion (FETO) in patients with severe and moderate pulmonary hypoplasia; an international randomized trial to test this treatment has been conducted [2]. FETO has been reported to improve the survival rate of CDH patients, but the procedure carries a high risk of preterm birth or premature membrane rupture (e.g., during the fetoscopy insertion) [3,4]. A less invasive intervention method is hence strongly desirable.

Vitamin D is known to maintain calcium and phosphorus homeostasis, whereas it has been suggested that the active form of vitamin D—1,25-dihydroxyvitamin D [1,25$(OH)_2D_3$]—is found in the immune, cardiovascular, and neurological systems and is also involved in glucose regulation and placental function [5]. Moreover, several studies have observed correlations between PH and vitamin D deficiency [6–8], with some reporting that vitamin D supplementation improved PH symptoms in rat models [9,10]. Vitamin D has also been shown to have therapeutic effects on infant PH rat models after intra-amniotic exposure to endotoxin [11]. Vitamin D supplementation has been shown to reduce the risk of pre-eclampsia, gestational diabetes, and low birth weight [12,13]; even a high dose regimen is considered to be a safe intervention during pregnancy [14]. A recent study reported that the maternal serum vitamin D level of women pregnant with a CDH fetus is lower than in healthy controls [15]. On the basis of these findings, we hypothesized that vitamin D could be a suitable noninvasive prenatal therapy for CDH; however, there is no published literature testing this hypothesis to our knowledge. In this study, we investigated the efficacy of prenatal vitamin D administration in a CDH rat model, aiming to gather relevant data before proceeding to clinical trials.

2. Materials and methods

2.1. 25-hydroxyvitamin D [25(OH)D] measurement in human umbilical cord serum

All fetal subjects were delivered at term by healthy women at the Nagoya University Hospital. CDH cases (n = 44) and normal healthy controls (n = 86) were matched by gestational age at delivery, season at delivery, birth weight, and sex. The umbilical cord serum was collected immediately after birth. Vacuum blood collection tubes containing specimen samples were kept at 4 °C for 16–24 h and then isolated. Finally, samples were stored at −80 °C until further analysis. The subjects' clinical data are presented in Table 1. The concentration of 25(OH)D was measured using an ELISA Kit (Enzo Life Sciences Inc., NY, USA). CDH newborns were divided into survival (n = 37) and death (n = 7) groups, and the 25(OH)D levels were compared between the two groups. Patients who could leave the hospital after birth were included in the survival group, and only one patient died after leaving the hospital. The median concentration of 25(OH)D was 15.6 ng/mL in the CDH group. The CDH groups were further subdivided into those with umbilical cord serum 25(OH)D levels of ≥ 15 ng/mL (Group 1), and those whose levels were < 15 ng/mL (Group 2). The subjects' clinical characteristics (Group 1 vs. Group 2) are presented in Table 2. Overall survival (OS) was determined as the interval between the birth date and the last date of follow-up or death from any cause. Kaplan–Meier survival curves were generated within the CDH groups and were compared using the log-rank test. A Cox proportional hazards regression model was employed to investigate associations between the concentrations of 25(OH)D (Group 1 vs. Group 2) and OS.

Table 1
Clinical characteristics of the study population.

	Control (N = 86)	CDH (N = 44)	*p* value
Maternal age (years)	33.8 ± 4.7	31.8 ± 5.9	0.093
Nulliparity	48 (55.8)	26 (59.1)	0.852
Fetal number			1.000
Singleton	86 (100.0)	42 (95.5)	
Twin	0 (0.0)	2 (4.5)	
Delivery mode			1.000
Cesarean section	85 (98.8)	44 (100.0)	
Vaginal delivery	1 (1.16)	0 (0.0)	
Gestational age at birth (weeks)	37.6 ± 1.5	37.1 ± 2.1	0.100
Birth weight (g)	2819.2 ± 400.9	2683.4 ± 500.7	0.193
Male newborns	49 (57.0)	28 (63.6)	0.572
Cord blood pH	7.31 ± 0.04	7.32 ± 0.04	0.067
1-min Apgar score	7.8 ± 1.7	4.5 ± 2.2	< 0.001
5-min Apgar score	8.7 ± 1.3	4.4 ± 2.1	< 0.001
Seasons at birth			0.943
March to June	36 (41.9)	19 (43.2)	
July to October	30 (34.9)	16 (36.4)	
November to February	20 (23.3)	9 (20.5)	
Diabetes mellites	5 (5.8)	1 (2.3)	0.663
Hypertensive disorders of pregnancy	0 (0.0)	2 (4.6)	0.113

Continuous variables are presented as mean ± SD and *p* values were calculated by Wilcoxon rank-sum test. Categorical variables are presented as number (percentage) and *p* values were calculated by Fisher's exact test.

Table 2
Clinical characteristics of the Group 1 vs. Group 2.

	Group1 (N = 24)	Group2 (N = 20)	*p* value
Maternal age (years)	31.8 ± 5.7	31.9 ± 6.3	0.841
Nulliparity	13 (54.2)	13 (65.0)	0.547
Singleton	23 (95.8)	19 (95.0)	1.000
Right-sided CDH	2 (8.3)	1 (5.0)	1.000
FETO	1 (4.2)	1 (5.0)	1.000
Liver up	8 (33.3)	6 (30.0)	1.000
Gestational age at birth (weeks)	37.1 ± 2.4	37.1 ± 1.8	0.272
Birth weight (g)	2633.0 ± 507.0	2743.9 ± 499.2	0.517
Male newborns	16 (66.7)	12 (60.0)	0.757
Seasons at birth			0.337
March to June	8 (33.3)	8 (40.0)	
July to October	7 (29.1)	2 (10.0)	
November to February	9 (37.5)	10 (50.0)	
Complications			
Genetic anomaly[a]	3 (12.5)	2 (10.0)	1.000
Cardiac anomaly[b]	2 (8.3)	2 (10.0)	1.000
Other anomaly[c]	3 (12.5)	1 (5.0)	0.614
ECMO support	4/24 (16.7)	5/19 (25.0)	0.360

Continuous variables are presented as mean ± SD and *p* values were calculated by Wilcoxon rank-sum test. Categorical variables are presented as number (percentage) and *p* values were calculated by Fisher's exact test.
ECMO, extracorporeal membrane oxygenation; FETO, fetal endoscopic tracheal occlusion.
[a] Genetic anomaly included as follows: chromosome deletion (n = 2), mutation (n = 1), deletion variant (n = 1) and a suspected case for multiple anomalies (n = 1).
[b] Cardiac anomaly included as follows: double-outlet right ventricle (n = 2), transposition of great arteries (n = 1), and ventricular septal defect (n = 1).
[c] Other anomaly included as follows: agenesis of the corpus callosum (n = 1), bronchopulmonary sequestration (n = 2), hiatal hernia (n = 1) and right renal agenesis with left double kidney (n = 1).

2.2. Animals

This study was performed following Nagoya University's institutional guidelines for animal care (permit number: 31380), which are conform to the Guide for Care and Use of Laboratory Animals of the

National Institutes of Health. All surgery was performed under anesthesia with isoflurane inhalation, using every effort possible to minimize pain and suffering.

2.3. Experimental design

Nitrofen-induced rat CDH models were prepared as previously reported [16,17]. Timed pregnant Sprague Dawley rats were purchased from Chubu Kagaku Shizai (Japan) and randomly divided into three groups: i) Control: oral administration of 1 mL of olive oil through a stomach tube on E9. ii) Nitrofen: oral administration of 1 mL of olive oil and 100 mg of dissolved nitrofen (Sigma-Aldrich, Japan) delivered through a stomach tube on E9. iii) Nitrofen + vitamin D (VD): administration of 1 mL of olive oil with 100 mg of nitrofen, coupled with the continuous administration of calcitriol (0.03 μg/kg/day, Cayman Chemical, MI) through a subcutaneous osmotic mini-pump (model 2002, ALZET, CA) from E9 to E21. For Sprague Dawley rats, full-term pregnancy was considered to be reached at E21, as previously reported [16].

To quantify the concentration of $1,25(OH)_2D_3$ in this model, blood samples were collected from maternal rats (E21). Sample plasma was isolated by centrifugation at 10000 rpm for 10 min, and the $1,25(OH)_2D_3$ plasma concentration was measured via a radioimmunoassay conducted by the inspection agency (BML, INC., Japan).

2.4. Tissue collection

On E21, fetuses were collected via cesarean section. A thoracic-laparotomy was then performed to confirm CDH; only those with a positive diagnosis were included in further analyses. For these analyses, the samples were split into three groups: the Control, the CDH pups treated with nitrofen (CDH), and the CDH pups treated with both Nitrofen and vitamin D (CDH + VD). Fetal lungs were collected and weighed to calculate the lung/body ratio.

2.5. Gasometric evaluation

The measurements were performed as previously reported [16,18]. Pups collected on E21 were euthanized by decapitation 5 min after birth. Neonatal blood was collected from the neck for gasometric analysis, using an i-STAT analyzer (Fuso, Japan). Pups were then dissected to diagnose CDH. The number of gasometric evaluations performed in each group was as follows: Control, n = 25; CDH, n = 25; and CDH + VD, n = 24.

2.6. Lung morphometry

Fetal lungs from each group were fixed by tracheal instillation of 10% formalin under a continuous pressure of 20 cm H_2O and then embedded in paraffin. Serial 4 μm sections were stained with hematoxylin and eosin (HE) or Elastica Van Gieson (EVG). The stained sections were then analyzed using a Biorevo BZ-9000 system (Keyence, Japan). The mean linear intercept was measured to assess the pulmonary airspace in the HE-stained lung sections (n = 10 pups/group, one section per pup) using a semi-automated method as previously reported [19]. Five representative fields per section were measured, and their values were averaged. Pulmonary artery remodeling was quantified using artery wall thickness in EVG-stained lung sections at 400× magnification. Only pulmonary arteries with an external diameter ranging from 30 to 100 μm were included in these measurements (five fetuses per group). Two sections per fetus were evaluated, and a mean of 38 arteries (min−max: 22−57) per section were measured.

2.7. Microarray for fetal left pulmonary artery

Since the model showed a left-sided CDH, the left fetal pulmonary artery was isolated using a stereomicroscope before removal of the fetal lung. The left pulmonary artery was distinguished from the left pulmonary vein and the left main bronchus based on the position and structure of these anatomical features. Total RNA was prepared from the fetal left pulmonary artery using the ReliaPrep™ RNA Cell Miniprep System (Promega, WI, USA). A microarray analysis of gene expression was conducted using Affymetrix GeneChip® Rat Clariom™ S Assay (Affymetrix, CA, USA). The intensity of each spot was normalized by quantile using the Microarray Data Analysis Tool (Filgen Inc., Japan). Enrichment analysis was performed using MetaScape (http://metascape.org) [20]. Data with a z-score of > 0 and a p value of < 0.01 were considered to represent significantly upregulated and downregulated gene ontology (GO) in the enrichment analysis. Genes in molecular interaction networks and biological pathways were analyzed using Cytoscape (https://cytoscape.org/index.html) in the following groups: i.) upregulated in CDH compared with Control; ii.) downregulated in CDH + VD compared with CDH; iii.) downregulated in CDH compared with Control; and iv.) upregulated in CDH + VD compared with CDH.

2.8. Statistical analysis

Statistical analysis was performed using JMP Pro 14 (Japan). The Fisher's exact test was used to compare categorical variables. The Wilcoxon rank-sum test and Student's *t*-test were used to compare continuous variables with a non-normal and normal distribution, respectively. For multiple comparisons with normal distributions, Tukey's post hoc test and Games-Howell test were used with and without variance of homogeneity, respectively. The Steel-Dwass test was used for multiple comparisons with a non-normal distribution. The statistical significance level was set at $p < 0.05$.

3. Results

3.1. Decreased cord serum level of 25(OH)D in CDH human newborns

The measured concentrations of 25(OH)D in the umbilical cord serum of patients with CDH were significantly lower than those of healthy controls in humans ($p < 0.01$; Fig. 1A). The clinical characteristics were not significantly different between the two groups, except for the 1- and 5 min Apgar scores, which are assessment of the cardiovascular and respiratory system in neonates after birth (Table 1). Additionally, the concentration of 25(OH)D in neonatal CDH-related fatalities was significantly lower than that in infants who survived to hospital discharge ($p < 0.01$; Fig. 1B). During the follow-up of 44 patients with CDH, the 5-year OS in Group 2 (25(OH)D levels of < 15 ng/mL) was 59.2% (95%CI: 37.0–78.2), which was significantly lower than that in Group 1 (25(OH)D levels of ≥ 15 ng/mL), 100.0% (95%CI: 87.5–100.0) ($p < 0.01$, Fig. 1C). The clinical characteristics were not significantly different between the Group1 and Group 2 (Table 2).

3.2. Positive effects of prenatal vitamin D administration on blood gas analysis in CDH rats

First, maternal $1,25(OH)_2D_3$ levels were found to be significantly higher in subjects that received vitamin D infusion from E9 to E21 (174.9 ± 52.3 vs. 131.6 ± 30.5 pg/mL, $p < 0.05$). The incidence of CDH in the Nitrofen group (35.7%; 71/199 pups in 15 dams) was not significantly different from that in the Nitrofen + VD group (34.8%; 55/158 pups in 13 dams) ($p = 0.98$).

Pups that were not diagnosed with CDH in the Nitrofen and Nitrofen + VD groups were excluded from the following experiments. The following comparisons were conducted in the reconstructed Control, CDH, and CDH+VD groups. The lung weight/body weight ratio in the CDH pups was significantly lower than that in the Control ($p < 0.001$) but was similar to CDH pups in CDH + VD specimens (Fig. 2A). However, the blood gas analysis for pulmonary function showed that the pH

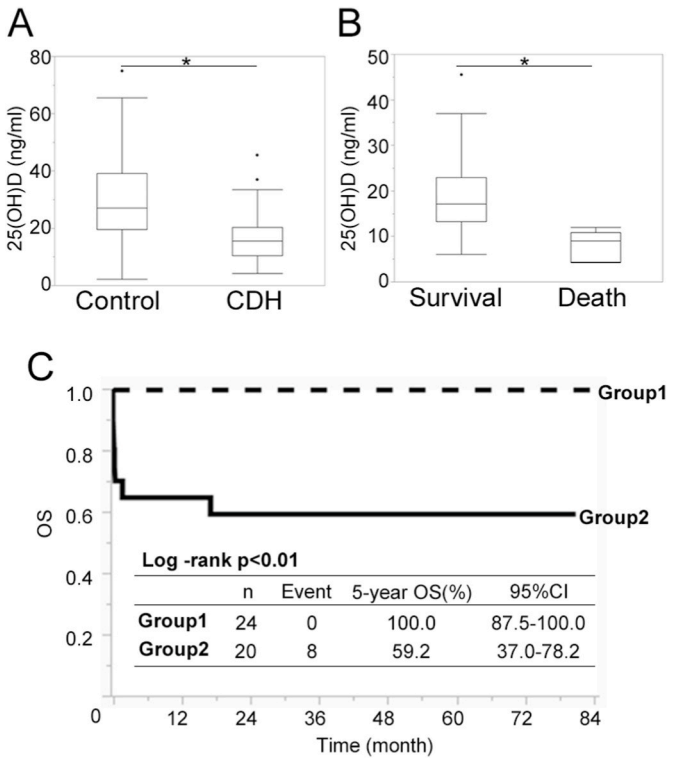

Fig. 1. 25(OH)D concentrations in human umbilical cord blood. **A.** Comparison of 25(OH)D concentrations in umbilical cord blood between Control (n = 86) and congenital diaphragmatic hernia (CDH) newborns (n = 44; $*p < 0.01$). **B.** Comparison of 25(OH)D concentrations in umbilical cord blood between survival (n = 37) and death groups (n = 7) among CDH newborns. Patients who could leave the hospital after birth were included in the survival group, and only one patient died after leaving the hospital. $*p < 0.01$. **C.** Kaplan–Meier–estimated survival curves on stratified by 25(OH)D concentrations (Group 1 vs. Group 2). Group 1 (n = 24) included CDH patients whose umbilical cord serum 25(OH)D levels were ≥ 15 ng/mL and Group 2 (n = 20) included CDH patients whose umbilical cord serum 25(OH)D levels were < 15 ng/mL. CI: Confidence interval, OS: Overall survival.

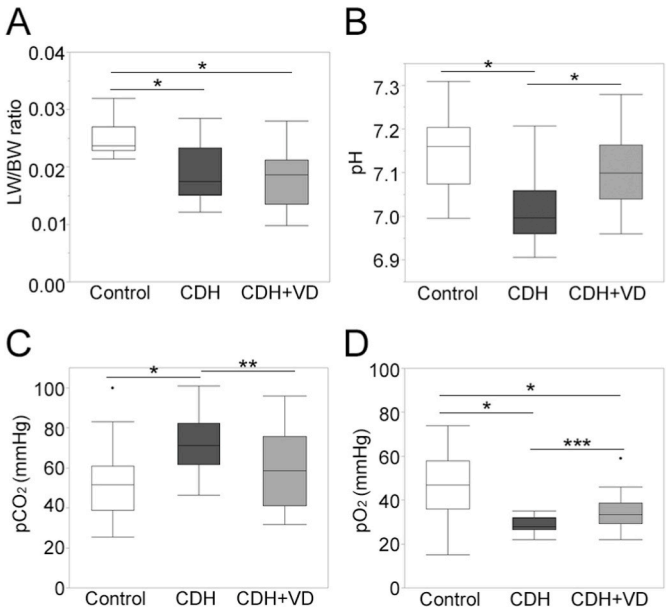

Fig. 2. Effects of prenatal vitamin D administration on oxygenation and ventilation failure in CDH pups. **A.** Effect of vitamin D on fetal lung/body weight ratio (LW/BW ratio) (Control: n = 43, CDH; n = 32, CDH + VD; n = 24). **B–D.** Fetal blood gas analysis of pH (B), pCO_2 (C), and pO_2 (D) 5 min after birth (Control: n = 25, CDH; n = 25, CDH + VD; n = 24). $*p < 0.001$, $**p < 0.05$, $***p < 0.01$.

and pCO_2 values in the CDH + VD group were significantly higher and lower, respectively, than those in the CDH group and similar to those in the Control group ($p < 0.001$ and $p < 0.05$, respectively; Fig. 2B and 2C). Furthermore, the pO_2 values in the CDH + VD group were significantly higher than those in the CDH group ($p < 0.01$) but were significantly lower than those in the Control group ($p < 0.001$; Fig. 2D).

3.3. Positive effects of prenatal vitamin D administration on alveolarization and pulmonary artery remodeling in CDH

Our observations of the histological lung structure in specimens of the CDH group revealed less alveolar airspaces compared with the Control and CDH + VD groups (Fig. 3A). The mean linear intercept in the CDH group was significantly lower than that in the Control group ($p < 0.01$), while that in the CDH + VD group was significantly increased compared with that in the CDH group ($p < 0.05$; Fig. 3C).

To determine the effect of vitamin D on pulmonary artery remodeling, the artery wall thickness was compared between the Control, CDH, and CDH + VD groups (Fig. 3B). The artery wall thickness in the CDH + VD group was significantly lower than that in the CDH group ($p < 0.05$; Fig. 3D).

3.4. Attenuation of gene expression in the pulmonary arteries of CDH rats by prenatal vitamin D administration

We detected a ≥ 2-fold increase in 655 genes (Supplementary Table 1) and a ≤ 2-fold decrease in 344 genes (Supplementary Table 2) in CDH rats compared with the Control group. In CDH + VD rats, 393 genes showed a ≥ 2-fold increase and 532 genes a ≤ 2-fold decrease compared with CDH rats. The identified genes were analyzed by enrichment analysis of the GO terms for a given biological process. The top 20 pathways that displayed a significant change are shown in Fig. 4A–4D. Eleven gene expression pathways were found to be upregulated in the CDH group compared with the Control group (Fig. 4A) and downregulated in the CDH + VD group compared with the CDH group (Fig. 4B). These pathways were specifically involved in the complement and coagulation cascades, acute inflammatory response, and cofactor metabolic processes. Other detected pathways included the muscle structure pathway, which was significantly downregulated in the CDH group compared with the Control group (Fig. 4C) and significantly upregulated in the CDH + VD group compared with the CDH group (Fig. 4D).

Furthermore, we identified an additional 386 genes that were significantly upregulated in the CDH group compared with those in the Control group and downregulated in the CDH + VD group compared with those in the CDH group. We also identified 66 genes that were significantly downregulated in the CDH group compared with those in the Control group and upregulated in the CDH + VD group compared with those in the CDH group. These genes were subject to molecular interaction networks and biological pathway analysis, the results of which are shown in Supplementary Fig. 1. The expressions of genes encoding hepcidin antimicrobial peptide (*HAMP*), fetuin B (*FETUB*), GC vitamin D binding protein (*GC*), and S100 calcium binding protein A8 (*S100A8*) were evaluated by qRT-PCR (Supplemental methods). These genes were significantly increased in CDH compared with Control and CDH + VD and were not significantly decreased in CDH + VD compared with Control in the result of microarray. In addition, no difference in E21 rat lungs is detected between CDH and Control in the previous report [21] and their changes were assumed to be specific to pulmonary artery. *HAMP* was the most increased gene in CDH compared Control and the most decreased gene in CDH + VD compared with CDH. The other three genes were included in the top 80 lists of both increased gene in CDH

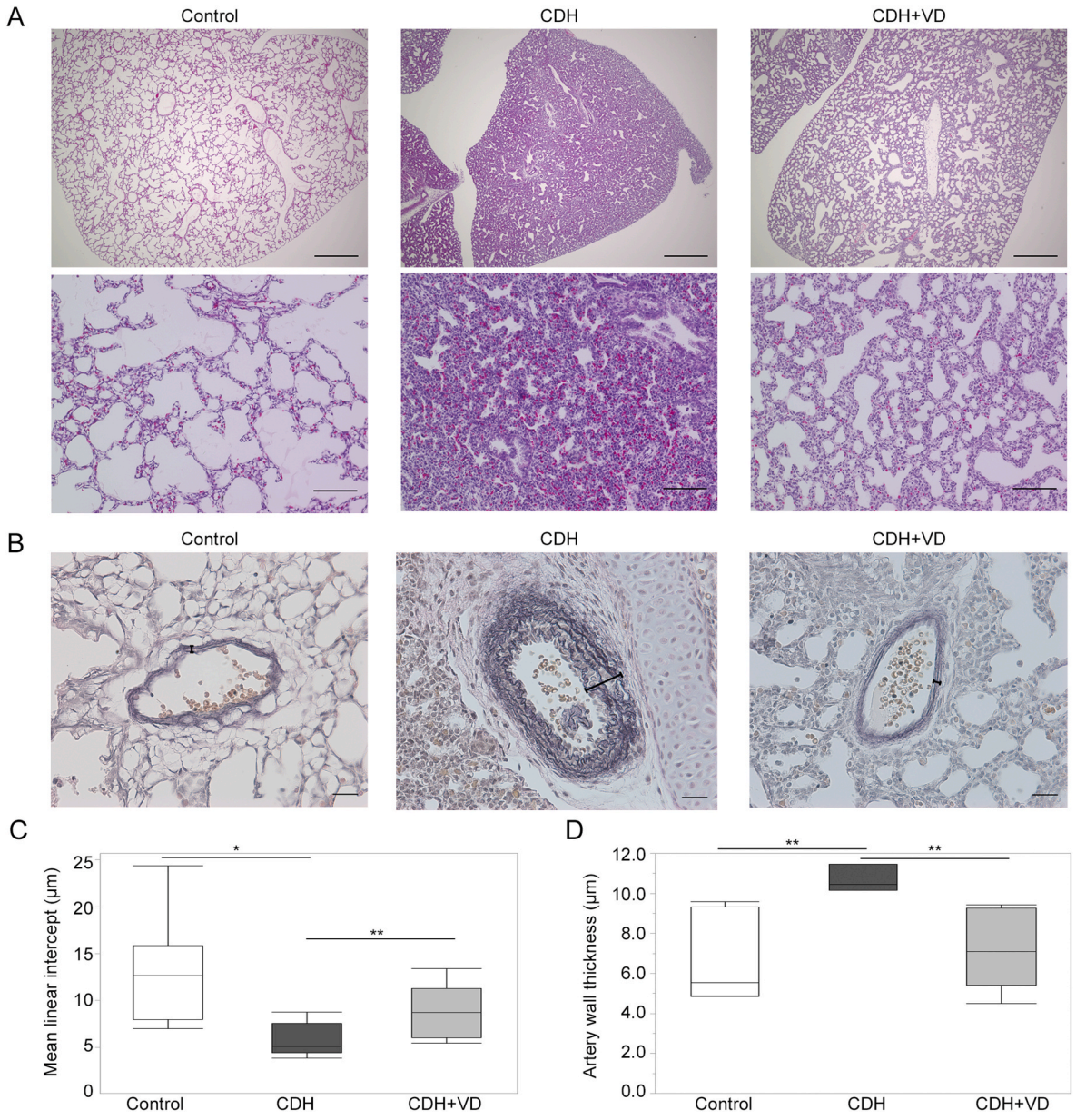

Fig. 3. Effect of prenatal vitamin D administration on lung histological structure and vascular thickness in CDH pups. **A.** Representative hematoxylin and eosin-stained lung sections. Upper panels; magnification = 40×, scale bar = 500 μm. Lower panels; magnification = 200×, scale bar = 100 μm. **B.** Representative elastic Van Gieson-stained lung sections. The bar indicates the measurement of wall thickness in the artery. Magnification = 400×, scale bar = 50 μm. **C.** Mean linear intercept (μm) in the CDH group compared with the Control and CDH + VD groups (n = 10 pups/group). **D.** Artery wall thickness (μm) of pulmonary arteries in the CDH group compared with the Control and CDH + VD groups (n = 5 pups/group). *$p < 0.01$, **$p < 0.05$.

compared with Control and decreased gene in CDH + VD and selected by reviewing the previous reports. *GC*, transport vitamin D-metabolites, is correlated with pulmonary disease [22]. *FETUB*, a biomarker for the severity of lung function [23] and *S100A8*, which is increased in pulmonary hypertension [24], were also reported to be repressed by vitamin D [25,26]. The results showed similar trends to those of the microarray analysis, but no significant differences were detected in the Control, CDH, and CDH + VD groups (data not shown).

4. Discussion

This study showed that prenatal vitamin D administration improved blood gas parameters, did not affect CDH occurrence, and limited lung growth in nitrofen-induced CDH rats. Immunohistochemical analysis revealed that prenatal vitamin D administration increased alveolarization and pulmonary artery remodeling, which was able to improve the respiratory function of CDH rats. To the best of our knowledge, this is the first study to isolate pulmonary arteries from CDH-diagnosed rat lungs and demonstrate the specific gene expression pattern compared with normal controls.

Our results showed that the vitamin D concentration in the umbilical cord blood of human CDH newborns was significantly lower than that in healthy newborns. A previous study reported that the serum vitamin D concentration in mothers with a CDH fetus is significantly lower than in healthy controls [15], suggesting that vitamin D concentration is lower in both fetuses and their mothers. Moreover, our data indicated that decreased vitamin D concentration in the umbilical cord blood was associated with high mortality in CDH patients. Given these results, we hypothesized that prenatal vitamin D administration would be a suitable treatment for CDH-diagnosed fetuses.

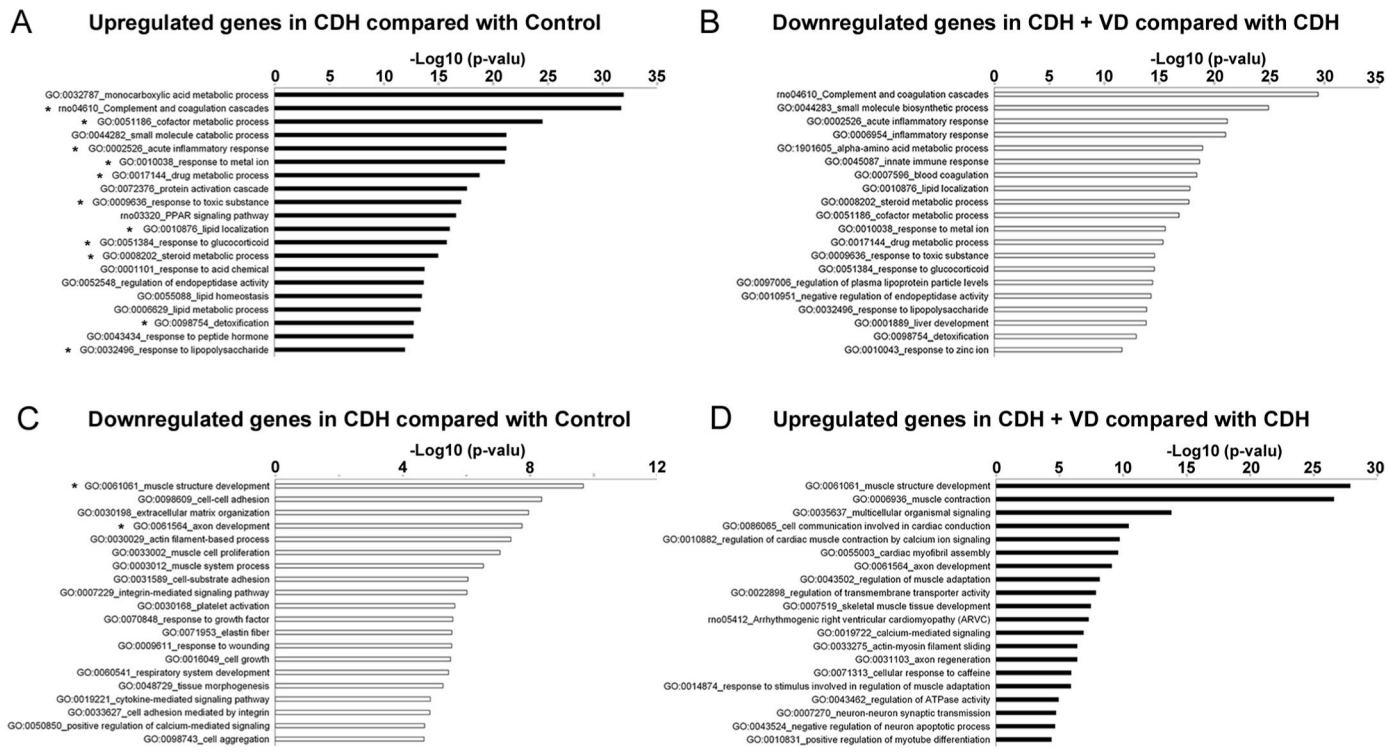

Fig. 4. Gene ontology (GO) biological process classification of differentially expressed genes among three experimental groups: Control, CDH, and CDH + VD. The top 20 enriched GO biological process categories and pathways are shown. **A.** Upregulated genes in the CDH group compared with those in the Control group. *Eleven pathways also showed significant decreases in CDH + VD compared with those in CDH. **B.** Downregulated genes in the CDH + VD group compared with those in the CDH group. **C.** Downregulated genes in the CDH group compared with those in the Control group. *Two pathways also showed significant increases in the CDH + VD group compared with those in the CDH group. **D.** Upregulated genes in the CDH + VD group compared with those in the CDH group. Black and white bars indicate up- and downregulated processes, respectively.

In our rat model, vitamin D increased alveolarization in the lungs of CDH rats. Previous research also reported that vitamin D regulated fetal lung maturation by stimulating phospholipid synthesis and surfactant release from alveolar type II cells [27]. Furthermore, prenatal vitamin D administration strengthened the lung structure and increased alveolar type II cell growth, which in turn improved oxygenation [8]. Moreover, a previous systematic review suggested that vitamin D deficiency in humans is associated with higher risks of respiratory distress syndrome (RDS) and bronchopulmonary dysplasia in preterm neonates [28]. Furthermore, a recent study [29] showed that vitamin D administration had a positive effect on RDS. Our study also showed that vitamin D attenuated delayed alveolarization in nitrofen-induced CDH rat models. However, delayed alveolarization has yet to be conclusively proven in human CDH [30,31], despite a consensus in the nitrofen-induced CDH rat model. Therefore, it is important to investigate these aspects in future translational research.

Pulmonary vascular changes caused by CDH are thought to appear during the embryonic stage, although PH develops after birth [32,33]. Therefore, the prevention of pulmonary artery remodeling is likely to reduce the severity of PH after birth in patients with CDH. Prenatal administration of sildenafil and simvastatin has been reported to attenuate pulmonary artery remodeling [34,35], with reducing arterial wall thickness in a rat CDH model [36]. In this study, prenatal vitamin D also attenuated the extent of pulmonary artery remodeling, although a previous report suggested that vitamin D administration could not reduce the medial thickness of muscular pulmonary arteries in "adult" PH rat models [9]. This inconsistency might be due to the timing of administration. That study also found a limited ability of vitamin D to ameliorate pathological right ventricular hypertrophy [9], which supports the validation of prenatal intervention strategies to prevent PH in CDH.

We exhaustively investigated the patterns of gene expression in CDH pulmonary arteries. We found that some altered pathways, including those related to the complement and coagulation cascades, the acute inflammatory response, and cofactor metabolic process in CDH rats, were downregulated by prenatal vitamin D administration. These pathways might be regulated by microRNAs, although most of their functions remain largely unknown. The expression of miR-10a, a precursor known to be involved in inflammation pathways [37], was higher in the tracheal fluid of human CDH newborns compared with controls [38]. Interestingly, in a previous study, we found that the proteins related to complement and coagulation cascades, the acute inflammatory response, and lipid localization were all higher in the umbilical cord blood of human CDH cases compared with controls [39]. In CDH ovine models, complement and coagulation cascade proteins were also higher in the tracheal fluid than in the control specimens [40]. Therefore, complement and coagulation cascades might be appropriate targets for the treatment of PH in CDH, although further research is required for confirmation. Iron has been reported to play an important role in the pathogenesis of adult PH and pulmonary artery remodeling [41–43]. The microarray data in the present study indicated that *HAMP* expression was significantly increased in the CDH group compared to the Control and CDH + VD groups, although the result could not be validated by qRT-PCR. HAMP is implicated in iron homeostasis by regulating the solute carrier family 11, member 2, the iron exporter from cells [44]. HAMP has been reported to contribute to the proliferation of pulmonary artery smooth muscle cells by iron accumulation [42], which causes pulmonary artery remodeling. Moreover, it has been reported that vitamin D deficiency is correlated with increased HAMP [45], and vitamin D suppresses *HAMP* expression [46]. Recently, vitamin D treatment has been also shown to decrease HAMP in patients with inflammatory bowel disease [47]. Those findings suggest that pulmonary

artery remodeling in CDH might be modulated by HAMP and iron, and that vitamin D treatment has a positive effect on pulmonary artery remodeling by regulating *HAMP* expression and iron metabolism. We have also previously reported that catalytic iron-dependent oxidative stress is related to the pathogenesis of CDH [48]. However, the study of iron metabolism in CDH is limited, and further research is required.

We have previously demonstrated that antenatal Saireito administration improved alveolarization, pulmonary artery remodeling, and respiratory function in a rat CDH model [16], as well as vitamin D. However, since the dose of Saireito is too high in comparison with clinical doses when applied in pregnant women, it could not proceed to clinical trials. Although evidence for the effects of sildenafil treatment on CDH has been increasing, it has potential side effects, such as an increased incidence of persistent PH in newborns, as was reported in the Dutch STRIDER trial [49]. Vitamin D supplementation during pregnancy has been shown to be safe [14] and seems to be easily translated into clinical settings. The dose of vitamin D administration was determined according to the lower limit of the safety study performed by the pharmaceutical company (Fuji Pharma Co., Ltd., Japan, https://www.info.pmda.go.jp/go/pack/3112402A1039_1_02/). In this study, maternal serum concentrations of $1,25(OH)_2D_3$ significantly increased following vitamin D administration. Maternal and fetal $1,25(OH)_2D_3$ concentrations are known to be correlated in rats [50], and hence, the fetal concentration increases with that of the mother's. We repeated this experiment with a higher dose of vitamin D, but no significant effect on pulmonary arteries was observed (data not shown).

There are several limitations to this study that must be considered. First, we did not use a vitamin D-deficient mouse model. In vitamin D-deficient CDH mice, we may have detected a larger effect from the administration of vitamin D. Second, the molecular mechanisms of the effects of vitamin D on pulmonary arteries are not fully understood, although we identified the related pathways in this study. Further research is required to determine the exact role of these pathways. Finally, we started the administration of vitamin D from E9, when lung development is still at an embryonic stage. Several studies started treatment after E14 [35,51,52], much later than we did; however, the total dose of vitamin D administered from E9 to E21 did not exceed the safety levels, and no adverse effects were observed. Further studies are needed to evaluate the variation in the effectiveness of vitamin D with the timing of its administration during the prenatal period in animal models, prior to any application in a clinical setting.

In conclusion, this is the first study to demonstrate the effects of prenatal vitamin D administration in a CDH rat model. Although vitamin D could not prevent the occurrence of CDH and increase lung volume, but it improved respiratory function with attenuated lung morphology and remodeling of the pulmonary artery in CDH rat models. In human CDH newborns, lower vitamin D concentrations in the umbilical cord blood are correlated with poor prognosis. Vitamin D supplementation in pregnant women with a diagnosed CDH fetus should be utilized as a new antenatal intervention strategy to reduce the mortality of CDH newborns, although further research is required to determine the exact molecular and genetic mechanisms through which these effects operate.

Statement of ethics

The study was approved by the Research Ethics Committee at Nagoya University Hospital (approval number: 2015–0315). Umbilical cord blood samples and clinical information from both CDH and Control groups were obtained with written informed consent.

Author contributions

YI, HT, and TK contributed to the conception and design of the study. YI, HT, KI, RM, MM, AT, ST, TU, YM, T Kobayahi, SS and TK performed the acquisition and interpretation of data and revised it critically for important intellectual content. YI and TK drafted the first version of the manuscript. HK and FK contributed to interpreting the data and revising it critically for important intellectual content. All authors gave their approval for the final version of the manuscript.

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Declaration of competing interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

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