Paediatrica Indonesiana

p-ISSN 0030-9311; e-ISSN 2338-476X; Vol.61, No.4 (2021). p.192-7; DOI: 10.14238/pi61.4.2021.192-7

Original Article

Vitamin D and T regulator cells are not independent factors for RDS in premature neonates

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Abstract

Background The high morbidity and mortality of premature neonates remain significant problem in Indonesia with respiratory distress syndrome (RDS) as one of the most common problem. Vitamin D plays an important role in lung maturity. Vitamin D deficiency causes epithelial cell inflammation, leading to a higher risk of RDS. Previous studies suggest that T regulatory cells (Treg) in inflammatory diseases, such as RDS in neonates, are possibly linked to vitamin D deficiency.

Objective To determine the role of vitamin D on RDS and Treg cells in very premature or very low birth weight neonates.

Methods A prospective cohort study conducted on premature neonates in Neonatology Division, Department of Child Health, Dr. Cipto Mangunkusumo Hospital, Jakarta, Indonesia. Umbilical cord blood samples were collected to evaluate total vitamin D 25-OH levels and Treg cells. Subjects with RDS were evaluated until the end of the observation period.

Results The mean umbilical cord vitamin D level was 15.79 (SD 6.9) ng/mL, and 53% of the subjects were found to be deficient. As much as 65.1% of neonates had RDS. The mean Treg level was 11.38 (SD 2.45)%. No significant correlation was observed between vitamin D level and the occurrence of RDS (RR 0.87; 95%CI 0.56 to 1.34; P=0.53); vitamin D level and the dysregulation of Treg cells (RR 1.30; 95%CI 0.76 to 2.21; P=0.31) as well as between Treg dysregulation and RDS (RR 1.11; 95%CI 0.70 to 1.75; P=0.64). However, we found that RDS group had a lower gestational age and higher presentation of dysregulation Treg.

Conclusion In very premature or very low birth weight neonates, no association between occurence of RDS and vitamin D deficiency as well as Treg cell dysregulation. **[Paediatr Indones. 2021;61:192-7 ; DOI: 10.14238/pi61.4.2021.192-7**].

Keywords: *vitamin D; respiratory distress syndrome; Treg*

Respiratory distress syndrome (RDS) remains one of the most frequent respiratory problems causing mortality and morbidity, particularly in very premature or very low birth weight neonates. Incidence of RDS can vary from 27% in neonates with a birth weight of 1.251 to 1.500 grams to 86% in neonates with a birth weight of 501-750 grams.[1] Studies also generally found that the younger the gestational age of the neonates, the higher the incidence of RDS.[1,2] Clinically, neonates with RDS can present with clinical manifestations such as tachypnea, chest indrawing, grunting, nasal flaring, cyanosis, and increased oxygen requirement. This can occur following an acute inflammatory process which increases vascular permeability inside the lungs, leading to the loss of air-filled lung tissues, increased lung dead space, and reduced lung compliance.[2]

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Submitted June 3, 2021. Accepted August 16, 2021.

Vitamin D levels in premature neonates could be associated with the incidence of RDS.[2-4] Vitamin D plays a role in lung maturity through the mechanism of phospholipid (surfactant) production and secretion from type II alveolar cells (ATII).[3] Incidence of RDS was higher in premature neonates, and this risk was found in severe deficiency vitamin D (28 %) compared to mild-moderate vitamin D deficiency (14 %).[5] Another study found that vitamin D deficiency causes inflammation of epithelial cells and can affect more than 600 genes, thereby increasing the risk of RDS.[6]

A previous study found that neonates with a gestational age < 32 weeks have a higher risk of vitamin D deficiency compared to term neonates.[7] The vitamin D levels of fetus and neonates greatly depend on maternal vitamin D reserve.[8] This is due to the fact that the umbilical cord contains 50-60% of the 25(OH)D concentration from the maternal circulation. Therefore, mothers with hypovitaminosis D can lead to vitamin D depletion in the fetus during its developmental period.[9] Vitamin D plays an important role in the mechanism of inflammation, and Treg are the mediating factors between the inflammatory processes and vitamin D. Several studies found that several infectious and autoimmune diseases can be linked to Treg cells and vitamin D deficiency.[10-12] The function of Treg cells include inhibiting several immune responses and maintaining immunological tolerance. Treg cells work together with effector T cells, which are very important in the elimination of pathogens. Natural Treg cells derived from the thymus plays an important role in maintaining peripheral tolerance and inhibiting immune responses from self-antigens.[13] The aim of this study was to determine the role of vitamin D on RDS and Treg cells in very premature or very low birth weight neonates.

Methods

This was a cohort study conducted in the Neonatology Division, Department of Child Health/Dr. Cipto Mangunkusumo Hospital, Jakarta, from November 2019 to January 2021. The inclusion criteria were very premature neonates (gestational age < 32 weeks) or birth weight of < 1500 grams, hospitalized in the neonatology unit along with parental consent to participate in the study. Meanwhile, the exclusion criteria included neonates with lethal congenital abnormalities and incomplete maternal data.

The assessment for vitamin D involved 5 mL of blood taken from umbilical cord after birth. Blood was taken using a serum separator tube (SST) with a yellow cap using the direct competitive chemiluminescence immunoassay (CLIA) at *Prodia Laboratory*. The serum was separated and inserted into *DiaSorin* (*Liaison®*, Saluggia, Italy) analyzer. During the first incubation, 25-OHD was dissociated from the binding protein and was binded to the specific antibody on the solid phase. After 10 minutes, vitamin D that linked to the tracer was added and incubated for another 10 minutes. The unbound tracer was washed and the starter reagents were added to initiate the chemiluminescence reaction. The light signal was measured by photomultiplier as relative light units (RLU) and was inversely proportional to the concentration of 25OHD present in calibrator control, or study sample. Chemiluminescence immunoassay was a direct competitive CIA for quantitative determination of total 25-OHD in serum. For our analysis, we classified neonates into two groups that defined vitamin D status; vitamin D deficiency [25(OH)D ≤ 15 ng/mL] and vitamin D non deficiency [25(OH)D > 15 ng/mL].[14]

Three to five mL blood for evaluation of Treg cells was taken from umbilical cord blood samples at birth. The EDTA cord blood samples were stored at room temperature and processed within 24 hours after withdrawal. First, CD4+CD127lowCD25+ Tregs were enriched using the complete kit for human CD4+CD127lowCD25+ regulatory T cells (*Human Regulatory T Cell Cocktail, BD Pharmingen™*, USA). One to four milliliter of cord blood was used for the Treg isolation and purity was assessed using the flow cytometry Treg staining protocol. The Treg purity after isolation was 85-95% gated on living CD4+ singlet cells. Isolated Tregs were then activated for 3 days in vitro. Therefore, 1 to 5 × 104 Tregs were seeded per round-bottom 96-well in 200 μL *ImmunoCult™-XF T Cell Expansion Medium (Human Regulatory T Cell Cocktail, BD Pharmingen™*, USA) supplemented with 5% autologous heat-inactivated serum, 500 U/mL interleukin (IL)-2 (*eBioscience, Thermo Fisher Scientific*, Waltham, MA, USA), 5 ng/mL transforming growth factor (TGF)β (*eBioscience, Thermo Fisher Scientific*, Waltham, MA, USA). Additionally, Treg inspector beads (*MiltenyiBiotec, BergischGladbach*, Germany)

were added in a 1:1 bead to cell ratio according to manufacturer's instructions for activation and proliferation of isolated Tregs. After an overnight incubation, the positive and negative controls were stained with CD4/PE and a 1:2000 dilution of the fixable viability dye/*eFluor®780* for 20 min at room temperature in the dark, washed and resuspended in 200 μl FACS buffer for flow cytometry analysis at the *BD FACSCanto™ II* cytometer to assess the CFSE staining. After 4 days of co-culture, all samples were stained with CD4/PE and fixable viability dye/*eFluor®780* for 20 min at room temperature in the dark as described above. Proliferation of CD4- CD25-T cells was determined of single, living CD4+ cells in the CFSE+ gate. which was determined using the negative controls. Single antibody stainings were used to calculate the compensations. Cell viability was always above 90%. Treg cells were expressed in percentage and amount in microliters. Cut off of Treg level in normal preterm was 8.97 (range 7.25-10.07) and outside the range considered dysregulation.[15]

The diagnosis of RDS was established based on the existing signs and symptoms, including tachypnea (respiratory rate of > 60 times/minute), nasal flaring, central cyanosis, and radiological findings of ground glass appearance. Neonates with RDS were evaluated until the end of the observation period. The diagnosis of preeclampsia and maternal infection was determined by the obstetrician and data was obtained from medical record. Based on the history to the mother, the mother's consumption was only seen by the mother taking vitamin D or not, regardless of how much was consumed.

Data entry and analysis was performed with the *Statistical Package for the Social Sciences (SPSS) program version 20.0 for Windows*. Data that was not compatible with normal distribution was presented in median (range). Chi-square test was used to analyze the association between two categorical variables, and the independent-samples T-test was used to analyze the association between a categorical variable and a numerical variable for data with normal distribution. P value of ≤ 0.05 was considered significant for all hypotheses, and data was presented with a 95% confidence interval. This study has been reviewed and approved by the Health Research Ethics Committee Universitas Indonesia Medical School/Dr. Cipto Mangunkusumo General Hospital.

Results

Fourty three subjects were considered eligible for analysis. Technical reasons for exclusion included small veins or insufficient blood samples, refusal of participation in the study, death before the end of the observation period, and constraints due to the COVID-19 pandemic era. This study found that, the majority of neonates who experienced RDS have lower gestational age. The mean umbilical cord vitamin D level was 15.79 (SD 6.9) ng/mL, among which 53% of the subjects were found to have a deficiency (**Table 1**). We didn't found any difference between maternal vitamin D comsumption, pre-eclampsia, maternal infection, and delivery method in both groups.

Table 1. Characteristics of subjects

Characteristics	RDS n=28	None-RDS n=15	P value
Mean gestational age (SD), weeks	28.82 (2.38)	30.93 (2.71)	0.01
Mean birth weight (SD), grams	1105.82 (251.47)	1198.66 (281.16)	0.27
Maternal vitamin D consumption, n			
Yes	23	15	0.10
No	5	0	
Pre-eclampsia			
Yes	11	6	0.96
No	17	9	
Maternal infection			
Yes	18	9	0.78
No	10	6	
Delivery method			
Caesarean	27	12	0.11
Spontaneous	1	3	

Table 2. The correlations between vitamin D, Treg dysregulation, and RDS

Variables	RDS		RR (95%CI)	P value
	Yes n=28	No n=15		
Vitamin D, n				
Deficiency	14	9	0.87 (0.56 to 1.34)	0.53
No deficiency	14	6		
Treg, n				
Dysregulation	17	8	1.11 (0.70 to 1.75)	0.64
Normal	11	7		

The mean Treg value was 11.38 (SD 2.45)%. Among the participants, 28/43 neonates were found to have RDS. No significant correlations were observed between vitamin D level and the incidence of RDS (RR 0.87; 95%CI 0.56 to 1.34; P=0.53) as well as between Treg dysregulation and RDS (RR 1.11; 95%CI 0.70 to 1.75; P=0.64).

Our study found that presentation of dysregulation Treg were higher in RDS group (**Table 2**). There was no correlation between vitamin D level and the dysregulation of Treg cells (RR 1.30; 95%CI 0.76 to 2.21); P=0.31).

Discussion

This study found vitamin D deficiency and dysregulation of Treg is not associated with RDS. Several studies showed that incidence of RDS is associated to the gestational age of the infant, with more severe disease in the smaller and younger neonates because of surfactant deficiency and pulmonary immaturity.[4,5,20] Vitamin D plays a role in embryogenesis, cellular growth and differentiation, as well as lung development and regulation in the fetus. Hypovitaminosis D is estimated to aggravate lung disease in premature neonates.[16] A study found that vitamin D levels in the umbilical cord did not increase or decrease the risk of RDS (OR 1.044; 95%CI 0.349 to 0.88; P=0.0771).[17] Our study did not find a correlation between vitamin D deficiency and RDS. The RDS group had younger gestational age therefore leading this group to a higher risk of RDS.

Vitamin D stimulates the development of Treg cells and T cell function to suppress inappropriate response from Th1 and Th2 following environmental exposure to allergens or infections, leading to a more balanced immune response in inhibiting autoimmune diseases (Th1 dominance) and allergies (Th2 dominance).[18] Our study found that the mean value of Treg cells was 11.38 (SD 2.45)%. Meanwhile, another study found a mean Treg cell value of 8.97% (7.25-10.07) in premature neonates.[19] Another study found a strong correlation between Treg phenotype percentage and vitamin D level in full-term and premature neonates from mothers with vitamin D deficiency.[20] Our study was unable to find a correlation between vitamin D levels and Treg cells. Similar results were also reported in a previous study which found that 25-hydroxyvitamin D levels did not play an important role in the correlation between vitamin D levels and regulatory T cells from the umbilical cord.[10]

Our study also did not find a correlation between Treg cells and RDS. Respiratory distress syndrome occurs due to surfactant deficiency, and surfactant is known to comprise of four specific proteins, which are SP-A, SP-B, SP-C, and SP-D. It is known that SP-A and SP-B act in regulating the inflammatory process in the lungs, and SP-B is required to form normal lamellar bodies in type 2 cells and is involved in the formation of SP-C. Meanwhile, SP-C is a protein that collaborates with SP-B to repair surfactant build-up and its function in the alveolar cells to reduce surface tension.[21] In addition, a kinetic suppression test revealed that SP-A increases the functional number of Foxp3+Tregs in T-cell population responders that depend on TGF-β.[22] This is might be the reason, why Treg cells did not directly affect the incidence of RDS. A limitation of this study that we did not assessed surfactant protein that may give more information regarding surfactant function.

In conclusion, in very premature or very low birth weight neonates, there is no association between incidence of RDS and vitamin D deficiency as well as Treg cell dysregulation.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Funding Acknowledgement

This work was supported by Ministry of Research and Technology/National Research and Innovation Agency through Doctoral Dissertation Research Grant (NKB-388/UN2.RST/HKP.05.00/2020).

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