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ANALYSIS OF VITAMIN D LEVELS IN CHILDREN WITH THALASSEMIA BETA

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ABSTRACT

Background : Beta Thalassemia is a genetic disorder inherited by autosomal recessive and has spread throughout the world, including Indonesia. Beta thalassemia requires lifelong transfusions, which can cause an accumulation of iron in the skin, liver, and kidneys, resulting in a decrease in vitamin D synthesis.
Purpose : This study aims to analyze the levels of 25-OH-Vitamin D in beta thalassemia.
Method : This study used a cross-sectional design and was conducted at Dr. Wahidin Sudirohusodo Hospital from April to July 2021. The population of this study was patients diagnosed with beta thalassemia and non-thalassemia (controls) who met inclusion criteria. This study compared vitamin D levels in beta thalassemia and non-thalassemia patients.
Results : This study involved 60 children aged 6 months to 18 years, who were divided into 2 groups: 30 children in the beta thalassemia group and 30 in the non-thalassemia group. In this study, the levels of 25-OH-Vitamin D were lower in beta thalassemia children compared to non-thalassemic children, with a p value =0.012. Children with beta-thalassemia have a 4.33 times higher risk of vitamin D deficiency compared to non-thalassemic children. With a p value =0.023, 25-OH-Vitamin D levels were significantly lower in beta HbE thalassemia children compared to beta thalassemia major children.
Conclusion : Levels of 25-OH-Vitamin D in beta thalassemia children were lower than in non-thalassemic children. Levels of 25-OH-Vitamin D in children with beta HbE thalassemia are lower than in children with beta thalassemia major.

Keywords: 25-OH-Vitamin D, beta thalassemia, transfusion

1. INTRODUCTION

Beta Thalassemia is a genetic disorder inherited by autosomal recessive and spread throughout the world.[1] Thalassemia is genetically classified as -α, -β, -β/HbE or –δβ thalassemia based on the reduced globin chain production.[2] Beta thalassemia major, or homozygous beta thalassemia, is a severe clinical disorder that results from the inheritance of two beta thalassemia alleles, one on each copy of chromosome 11.[3] Hemoglobin E is a variant of Hb that has one point mutation in the chain. At position 26, there is a change in amino acids from glutamic acid to lysine. This mutation causes a mild deficiency of normal mRNA production with abnormal mRNA formation, which reduces production of globin chains and manifests as beta thalassemia.[2]

Indonesia is one of the countries in the world's thalassemia belt. This is evidenced by epidemiological studies in Indonesia, which found the beta thalassemia gene frequency ranged from 3-10%. Data from the thalassemia center of the Department of Pediatrics at the University of Indonesia shows that up to May 2014, there were 1,723 patients with an age range of 11-14 years. The number of new patients continues to increase to 75-100 people per year, with the oldest patient currently being 43 years old.[4] Based on data from Wahidin Sudirohusodo Hospital Makassar, thalassemia patients who received treatment from 2011-2019 were 97 patients with an age range of 3 months to 46 years, with the most common age being between 4-5 years, and 82% were under 18 years old. Data from the Department of Pediatrics at Hasanuddin University shows that until March 2021, there were 71 patients with a diagnosis of beta thalassemia.

As a consequence of reduced HbA synthesis in thalassemia, circulating red blood cells are highly hypochromic, abnormally shaped, and contain very low amounts of Hb. Anemia in thalassemia major is so severe that long-term blood transfusions are usually required for survival.[3] With regular transfusions, patients can live longer. The negative impact of regular transfusion is chronic iron burden, which can lead to growth disorders, diabetes mellitus, delayed sexual maturation, and congestive heart failure.[5] Osteoporosis and cardiac dysfunction remain frequent complications. Adequate and circulating

levels of vitamin D are essential for optimal bone health and reducing the risk of fractures. Vitamin D is essential for calcium homeostasis and for bone mineralization, especially during periods of rapid growth, namely the growth period in childhood and puberty.[6]

Vitamin D is carried to the liver and hydroxylated to 25-OH-vitamin D, then undergoes additional hydroxylation to 1-25-OH-Vitamin D3 in the kidney. The main circulating vitamin D metabolite is serum 25-OH-vitamin D, and this is the best indicator of vitamin D status.[6] The prevalence of vitamin D deficiency is reported to be high in thalassemia patients (2-87.5%).[5] A report from North India showed the prevalence of vitamin D deficiency in about 80% of thalassemia patients. Another study from Thailand showed vitamin D deficiency in 90% of thalassemia patients.[6]

The cause of vitamin D deficiency in thalassemia is not known with certainty. However, this is thought to be the result of an increase in liver iron as a result of repeated transfusions.[7] Iron deposition in the liver causes disruption of the enzyme 25-hydroxylase in the liver, which is required for hydroxylation of vitamin D. Hyperpigmentation is also common in patients with thalassemia. Dark skin will inhibit the conversion of vitamin D by sunlight.[5] Sun-deficient individuals are susceptible to vitamin D deficiency. Sun exposure is responsible for physiological vitamin D formation, so vitamin D deficiency in thalassemic patients living in countries with abundant sunlight is not a major concern.[8] However, research in Thailand showed that the prevalence of vitamin D deficiency and insufficiency in patients with thalassemia major and thalassemia intermedia reached 90% in Thailand. This shows that sun exposure alone is not sufficient to synthesize vitamin D in children with thalassemia.[7]

Vitamin D gene receptor polymorphisms in patients with thalassemia major are also thought to play an important role in vitamin D deficiency. Most of the biological activity of vitamin D is mediated by the Vitamin D receptor (VDR), which acts as a nuclear transcription factor, regulates protein synthesis, and cell proliferation. The VDR gene is found on chromosome 12 (q12-q14), which has 11 exons. Exons 7, 8, and 9 have an important role in the binding of intracellular vitamin D. There are several

single nucleotide polymorphisms (SNPs) in the VDR gene that can affect the expression and activation of vitamin D receptors, namely FokI and BsmI. Therefore, genetic factors can affect vitamin D status in thalassemia.[9]

Research for vitamin D levels in beta thalassemia patients has never been done in Makassar, so this research is expected to increase our knowledge for better clinical applications in the future.

2. MATERIALS AND METHODS

2.1. Study Design

This study is an analytical study using a cross-sectional study design to analyze vitamin D levels in beta thalassemia children. The research variables consist of an independent variable (thalassemia), a dependent variable (vitamin D), which is a numerical variable, control variables (age, co-morbidities in the liver and kidneys), a moderator variable (gender), random variables (diet, genetic factors, and physical activity), and an intermediate variable (the biological process of disruption of vitamin D metabolism due to iron accumulation in children with thalassemia).

2.2. Research Location and Time

The study was conducted from March 2021 until August 2021 at Wahidin Sudirohusodo Hospital. Sample testing was carried out at the research laboratory unit of Hasanuddin University Hospital using the Elisa method.

2.3. Sample and Population

The population is all pediatric patients with beta thalassemia aged 6 months to 18 years. The sample for this study was the entire population that met the inclusion and exclusion criteria. The sample size in this study was 60 patients, consisting of 30 beta thalassemia patients and 30 normal children. Of the 30 beta thalassemia patients, consisting of 21 patients with beta thalassemia major and 9 patients with beta HbE thalassemia.

2.4. Inclusion and Exclusion Criteria

The inclusion criteria were children with beta thalassemia major and HbE who were treated in the hematology-oncology subdivision in one daycare, inpatient care, and outpatient clinic and non-thalassemic children who were healthy children who were controls in outpatient clinic or children who were treated with acute illness without thalassemia and comorbidities (controls), age 6 months to

18 years, and willing to be a research subject (get permission from parents) and sign an informed consent. Exclusion criteria were children that had comorbid liver and kidney disease obtained through history taking, physical examination and laboratory, and children who had received vitamin D supplementation.

2.5. Ethical Clearance

In carrying out this research, a permit to the Training and Research section of Wahidin Sudirohusodo Hospital was obtained. This research also met the ethical requirements by the Ethics Committee for Biomedical Research in Humans, Faculty of Medicine, Hasanuddin University.

2.6. The Method of Collecting Data

At the time of hospital admission, children with a working diagnosis of beta thalassemia major or beta thalassemia HbE and non-thalassemic children from the control population, aged 6 months to 18 years, who met the inclusion criteria were recorded with the registration number, age, gender, and nutritional status. Blood samples were taken through the veins using a 5 cc disposable syringe after previously installing a tourniquet and disinfecting technique with 70% alcohol cotton. 3 cc of blood samples were put into blood sample tubes and then centrifuged within 30 minutes of sample collection. All blood samples were placed in a cooler box containing an ice pack with a temperature of 2-8^{o}C degrees which could last 8-72 hours, then the samples were taken to the laboratory. For the preparation of the 25-hydroxy vitamin D test kit, the kit and samples should be heated naturally at room temperature for 30 minutes. The sample was placed on a plate, then given reagents and ELISA liquid, and incubated for 60 minutes at 37^{o}C. Wash the plate 5 times. Add liquid substrates A and B. Incubate for 10 minutes at 37^{o}C until the color changes. Dry the plate for 10 minutes, then the research sample is ready for analysis.

2.7. Data Analysis

The data analysis was conducted using SPSS Statistics for Windows, version 23.90 (IBM Co., Armonk, NY, USA). Data characteristics such as frequency, distribution, mean, median (range), and standard deviation were recorded, while the Chi-square or Fisher Exact test was used to determine the significance of the incidence of vitamin D deficiency. The risk factor was determined using crude odds ratio (OR) analysis with a 95%

confidence interval (CI). Statistical significance was indicated with P≤0.05.

3. RESULT

During the study period, there were 30 patients aged 6 months to 18 years diagnosed with beta thalassemia and 30 non-thalassemia patients. Of the 30 patients diagnosed with beta thalassemia, all of them met the inclusion criteria, consisting of 21 patients with beta thalassemia major and 9 patients with beta HbE thalassemia.

Based on gender, there were 14 male patients (46.7%) and 16 female patients (53.3%) with beta thalassemia. In the non-thalassemia group, 17 male patients (56.7%) and 13 female patients (43.3%) were involved. No significant difference was found in terms of sex distribution between the two groups with p = 0.438 (p > 0.05). Based on nutritional status, age, hemoglobin level, MCV value, MCH value, RDW value, and Mentzer index, statistical analysis showed a significant difference between the two groups (p < 0.05). (Appendix Table 1.) Compared with the characteristics of the thalassemia beta mayor group to thethalassemia beta HbE group, no significant difference was found in terms of sex distribution, nutritional status, age, hemoglobin level, and ferritin level between the two groups (p > 0.05). (Appendix table 2).

A comparison of vitamin D levels in beta thalassemia and non-thalassemia patients showed a mean value of 13,547 ng/ml and 33,83 ng/ml, respectively. The results of the Mann-Whitney U test showed a significant difference between vitamin D levels in beta thalassemia and non-thalassemia patients, with a p value = 0.012 (p < 0.05) (appendix, Table 3).

Based on vitamin D status, in the beta thalassemia group, 26 patients (87%) had vitamin D deficiency and 4 (13%) had normal vitamin D status. Meanwhile, in the non-thalassemia group, there were 18 patients (60%) who had vitamin D deficiency and 12 patients (40%) who had normal vitamin D status. Statistical analysis showed a significant difference in the distribution of vitamin D status between the two groups with a p value = 0.02 (p < 0.05) and an odds ratio (OR) of 4.33 (95% CI 1.2-15.6). (Appendix table 4)

Comparison of vitamin D levels in beta thalassemia major and beta thalassemia HbE showed a mean value of

17 ng/ml and 5,3 ng/ml, respectively. The results of the Mann-Whitney U test showed a significant difference between vitamin D levels in beta thalassemia and non-thalassemia patients, with a p value = 0.023 ($p < 0.05$) (appendix, Table 5).

4. DISCUSION

This study shows that the levels of vitamin D in beta thalassemia are lower than those in non-thalassemia. Vitamin D deficiency was also found in the non-thalassemic group.

In this study, the mean serum level of vitamin D in beta thalassemia patients was 13.547 ng/ml and the mean value in control patients was 33.83 ng/ml. Statistical analysis showed that there was a significant difference between serum levels of 25-OH-vitamin D in the non-thalassemia group and the beta thalassemia group with a p value = 0.012 ($p < 0.05$). This is in line with a study conducted by Agrawal et al. who found a significantly lower mean vitamin D level in the beta thalassemia major group (8,85 ng/ml) than the non-thalassemia group (16 ng/ml).[6] Research conducted by Gombar et al. also found a lower level of vitamin D in the thalassemia population (17.15 ng/ml) compared to the control group (28.85 ng/ml).[10] In Indonesia, similar research was conducted by Caroline et al. in Bali and obtained different results from our study. In that study, the mean value of vitamin D in the thalassemia group was 25.96 ng/ml and in the non-thalassemia group it was 27.54 ng/ml and there was no significant difference between the two groups with a p value = 0.45.[11]

The cause of vitamin D deficiency in thalassemia is not known with certainty. This is thought to be the result of an increase in liver iron concentration (liver iron concentration) as a result of repeated transfusions.[7] Iron deposition in the liver causes disruption of the enzyme 25-hydroxylase in the liver, which is required for the hydroxylation of vitamin D to 25-hydroxy-Vitamin D. Hyperpigmentation is also common in patients with thalassemia. Dark skin will inhibit the conversion of vitamin D by sunlight.[5] Impaired absorption of vitamin D in children with thalassemia is also thought to be the cause of vitamin D deficiency.[8] Vitamin D deficiency is generally rare in children who live in countries with abundant sun exposure. However, a study in Thailand showed that the prevalence of vitamin D

deficiency and insufficiency in patients with thalassemia major and thalassemia intermedia reached 90%. This shows that sun exposure alone is not sufficient to synthesize vitamin D in children with thalassemia.[7]

In our study, we compared vitamin D between the beta thalassemia major group (21 people), and the beta HbE thalassemia group (9 people) and the mean value in the beta thalassemia major group was 17 ng/ml (4-74 ng/ml) and in the beta thalassemia HbE group was 5.3 ng/ml (1-9 ng/ml). From the results of statistical analysis obtained p value = 0.023 ($p < 0.05$). To the researcher's knowledge, there has been no study that has compared the beta thalassemia major group with the beta thalassemia HbE group. A study in Thailand by Nakavachara et al compared transfusion-dependent and non-transfusion-dependent beta HbE thalassemia patients and found that non-transfusion-dependent patients were more likely to develop vitamin D deficiency (33.3% vs 12.2%; $p = 0.01$) and the non-transfusion dependent group had a lower mean (22.7 ng/ml vs 25ng/ml; $p = 0.043$). Still, in the same study, in the non-transfusion-dependent group, they found no association between iron overload and vitamin D deficiency and reported that those in this group had lower hemoglobin and serum ferritin levels than the transfusion-dependent group. However, they could not find a significant relationship between vitamin D and the degree of anemia.[8] Fung et al. also reported a high prevalence of vitamin D deficiency in the non-transfusion dependent group. They concluded that this group had fewer hospital visits and nutritional counseling, combined with poor dietary intake and supplementation, darker skin tone, and less sun exposure, that contributed to the risk of developing vitamin D deficiency.[12]

Another factor that can affect vitamin D status is nutritional intake. Herawati et al. reported that nutrient intake, especially energy, protein, and fat, affects vitamin D levels. Ineffective erythropoiesis occurs in thalassemia and causes an increase in energy and protein use. Protein is needed for the formation of enzymes, vitamin D receptors, and vitamin D binding proteins that carry 95-99% of the total 25 (OH) vitamin D. Fish, fortified milk, and meat are the main sources of vitamin D3 (cholecalciferol). Fat intake is also known

to be positively correlated with vitamin D levels, especially in terms of helping the absorption of vitamin D.[13] The main source of vitamin D is through the synthesis in the skin derived from cholesterol after exposure to UV-B rays. Full body exposure for 10 to 15 minutes in adults with fair skin pigmentation will produce between 10,000-20,000 IU of vitamin D3 within 24 hours; individuals with darker pigmentation need 5 to 10 times more exposure to produce the same amount of vitamin D. The amount of UV exposure available for vitamin D synthesis depends on many factors apart from time spent outdoors. These factors include the amount of skin pigmentation, body mass, location of residence, season, level of air pollution, area of skin exposed, and level of UV protection, including clothing and sunscreen.[14]

In our study, it was found that the mean value of vitamin D levels was lower in the beta thalassemia HBE group compared to the beta thalassemia major group because the 9 samples of beta thalassemia HbE in this study were transfusion dependent samples who received routine transfusions every month for several years. This means the frequency of transfusions received by HbE beta thalassemia patients is the same as beta thalassemia major patients. Another factor that caused the lower mean value in the beta thalassemia HbE group was the presence of normal vitamin D levels in 4 samples of beta thalassemia major and no normal vitamin D levels in the beta thalassemia HBE group. This may be due to the intake of foods containing vitamin D and adequate sun exposure received by the four patients, which may affect the production of vitamin D in the body, although there is iron deposition in the liver and skin. The vitamin D receptor gene also influences the expression and activation of the vitamin D receptor in children with thalassemia, and this may be associated with lower vitamin D levels. Transfusion frequency and duration of diagnosis also affect iron loading in patients with beta thalassemia, and this may also affect vitamin D levels. Researchers have not been able to rule out these variables, and this is a limitation of this study.

The Indonesian Minister of Health's regulation regarding the national guidelines for medical services for the management of thalassemia, which was

passed in 2018, has recommended the provision of vitamin D supplementation in thalassemia patients who have vitamin D levels below 20 ng/dL. The recommended dose is 50,000 IU once a week and given until the child reaches normal levels.[4] This can have a positive impact on preventing morbidity due to vitamin D deficiency that occurs in beta thalassemia patients.

The strengths of this study are that 1) this study was conducted in a hospital. Wahidin Sudirohusodo, which is the largest referral hospital in Eastern Indonesia, means that thalassemia patients who are treated at the hospital Wahidin Sudirohusodo come from most of eastern Indonesia, so it can be used as a reference to get an overview of vitamin D levels in beta thalassemia patients in eastern Indonesia. 2) Comparison with the control population can provide an illustration that vitamin D deficiency occurs in the normal population and can aggravate vitamin D deficiency that occurs in beta thalassemia patients. The weaknesses of this study are: 1) it is still not able to rule out factors that can affect the results (moderator factors), namely duration of exposure to sunlight, diet, and genetics. 2) a small and unbalanced sample population of beta thalassemia major and beta HbE patients.

Levels of 25-OH-vitamin D in beta thalassemia children are lower than in non-thalassemic children. Comparing levels of 25 (OH) vitamin D in children with beta thalassemia major and beta thalassemia HbE found higher level in beta thalassemia major than in beta thalassemia HbE group and children with beta thalassemia have a risk of 4.33 times to have vitamin D deficiency compared to non-thalassemia children with 95% (CI 1.2-15.6).

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APPENDIX

Table 1. Characteristics of the research sample

Variable	Non-Thalassemia n=30(%)	Thalassemia beta n=30(%)	P value
Sex			
Male	17(56,7%)	14(46,7%)	0,605*
Female	13 (43,3%)	16(53,3%)	
Nutritional status			
Malnourished	0	1 (3,3%)	
Undernourished	0	6 (20%)	0.019*
Normal	30(100%)	23 (76,7%)	
Age (Year), mean (min-max)	6,5 (1,4-17,6)	10,5 (1,6-17,8)	0,001**
Hb (gr/dl), mean (min-max)	12,2 (11-14,2)	7,6 (4,4-10,6)	0,000***
MCV (fl), mean (min-max)	78,5(68-91)	71,5 (55-83)	0,000***
MCH (pg), mean (min-max)	26.7 (20-31)	24 (17-29)	0,001***
RDW (fl), mean (min-max)	13,57 (12-17)	22,8 (12,6-35,2)	0,000***
Mentzer Index, mean (min-max)	17,5 (12,9-22,3)	23,81 (12-46)	0,000***

* Chi^2 test
** *Independent* t-test
*** Mann-Whitney Test

Table 2. Characteristics of beta thalassemia major and beta thalassemia HbE samples

Variable	Beta thalassemia major n=21(%)	Beta thalassemia HbE n=9(%)	P value
Sex			
Male	10(47,6%)	4 (44,4%)	0,596*
Female	11(52,4%)	5(55,6%)	
Nutritional status			
Malnourished	1(4,8%)	0 (0%)	
Undernourished	3(14,3%)	3 (33,3%)	0,419*
Normal	17(80,9%)	6 (66,7%)	
Age (Year), mean (min-max)	10,1(1,6-17,8)	11,5 (6,5-16,7)	0,418**
Hb (gr/dl), mean (min-max)	7,5 (4,4-10,4)	7,9 (6-10,6)	0,418**
MCV (fl), mean (min-max)	73,2(64-83)	67,4 (55-79)	0,033**
MCH (pg), mean (min-max)	24,9 (20-29)	22,2 (17-28)	0,033**
RDW (fl), mean (min-max)	21,5 (12,6-30,7)	25,9 (15-35,2)	0,078**
Mentzer index, mean (min-max)	25,8 (17-46)	19,2 (12-26,7)	0,022***
Ferritin (ng/ml), mean (min-max)	3695 (137-11900)	1801 (456-9088)	0,213***
Fe (ug/dl), mean (min-max)	177,7 (39-371)	198,9 (80-300)	0,564**
TIBC (ug/dl), mean (min-max)	175,9 (88-337)	150,6 (81-258)	0,315**
Transferrin saturation (%), mean (min-max)	78,5 (18-100)	96 (73-100)	0,246***

* Chi^2 test
** *Independent* t-test
*** Mann-Whitney Test

Table 3. Comparison of levels of vitamin D (25 (OH) vitamin D) in the non-thalassemia group and the beta thalassemia group

25 (OH) Vitamin D level (ng/ml)	Non-thalassemia n=30	Thalassemia beta n=30
Mean (SD)	33,83 (35)	13,54 (16,3)
Median	15	8
Minimum-Maximum	3-132	1-74

Mann-Whitney U test, p value = 0,012 (p < 0,05)

Table 4. Comparison of vitamin D (25(OH)Vitamin D) status in the non-thalassemia group and the beta thalassemia group

Group	Vitamin D status			
	Deficiency	Normal	Total	OR (IK 95%)
Beta thalassemia	26 (87%)	4 (13%)	30(100%)	4,33 (1,2-15,6)
Non-thalassemia	18 (60%)	12 (40%)	30 (100%)	
	44 (73%)	16 (27%)	60 (100%)	

Chi^2 test, Nilai p = 0,041 (p < 0,05)

Table 5. Comparison of 25 (OH) Vitamin D levels in beta thalassemia major and beta thalassemia HbE groups

25 (OH) Vitamin D (ng/ml) level	Beta thalassemia major n=21	Beta thalassemia HbE n=9
Mean (SD)	17 (18,4)	5,3 (2,6)
Median	8	5
Minimum-Maximum	4-74	1-9

Mann-Whitney U test, p = 0,023 (p < 0,05)