A Randomized Control Trial: The Role of Vitamin D, A, and Beta Carotene in Tuberculosis Patients with Vitamin D Receptor Gene Polymorphism

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Abstract. Recent studies showed that vitamin D and A has an effect in improving sputum conversion in tuberculosis. This study aims to find out the effect of vitamin D 1000 IU and A 6000 IU supplementation on Tuberculosis patients with vitamin D receptor gene polymorphism, who live in North Sumatera, Indonesia. This study is a randomized control clinical trial, with 48 tuberculosis patients with vitamin D receptor gene polymorphism which are *Taq*I and *Fok*I participating, divided into two groups, each with 24 participants, which are treatment group (I) which receives nutritional counseling, vitamin D 1000 IU, vitamin A 6000 IU, and control group (C) which only receives nutritional counseling for 28 days. Patients who participated was found to be heterozygous with *Taq*I (T>C) or *Fok*I (C>T) genotype variants. The result of this study showed that at the start, serum 25(OH)D levels in group I were lower compared to group C (19.74±6.59 ng/mL vs 25.21±7.57 ng/mL). Group I showed significant correlation between vitamin D level categories with sputum conversion (mean: standard deviation= 2.25±0.68 weeks). Supplementation of vitamin D 1000 IU provides an accelerated sputum conversion in tuberculosis patients with vitamin D receptor gene polymorphism.

1 Introduction

Prevalence of tuberculosis (TB) is rising each year and is one of the causes of mortality in Indonesia and is also becoming a health problem in the world. Governments of various countries with the highest prevalence have also performed initiation and implementation of DOTS (directly observed treatment, short course) as well as possible and Stop TB Strategy all over the world, but new cases always appear and relapse cases because of resistance emerge. [1] This condition is worsened by susceptibility from Mycobacterium tuberculosis infection. This susceptibility is caused by environmental, nutritional, and genetical factors. [2-4] Based on previous studies, it's stated that existence of vitamin D receptor gene polymorphism has a firm influence on the high risk of tuberculosis disease. [2]

Vitamin D receptor gene polymorphism is related to vitamin D deficiency and is related to an increase risk of tuberculosis disease. Several of the related genes are FokI and TaqI which are related to high vitamin D deficiency and in the end is related to the role of immune regulation in the body.[2, 4, 5] Vitamin D supports the induction of pleiotropic antimicrobial response in tuberculosis patients. [6, 7] Supplementation of vitamin D may accelerate sputum conversion and support improvement of tuberculosis patients' clinical condition and the mechanism of action is through an increase in immune cells by activating 25-hydroxyvitamin D receptor.[7, 8] Vitamin D does not work alone, it performs interaction with other vitamins or minerals [9, 10]. There is a decrease of several vitamins, for example vitamin A and carotenoid which is caused by low nutritional intake because of loss of appetite and lost from urine secretion because of severe infection [10, 11].

The role of vitamin A and beta carotene also affects the improvement of tuberculosis patients' clinical condition [9, 10, 12]. Previous studies showed that interaction between vitamin A and D in improving tuberculosis patients' clinical condition, low level of vitamin A, which is retinol, is affected by acute phase response (APR). During that APR process, processes of transcription and translation of positive acute phase response proteins (such as C-reactive protein/CRP) experience an increase [1, 13, 14]. While the process occurs, the role of protein is highly necessary, which is retinol binding protein, with the role as the primary transport protein to move vitamin A from liver storage towards peripheral tissues and during the APR process [9]. Based on this, then the role of protein in the improvement process of tuberculosis patients' clinical condition is highly necessary, tuberculosis patients' malnutrition condition with low level of protein in the body [7, 15].

Vitamin D deficiency is found in tropical areas like Indonesia, it even occurs in healthy individuals. It's predicted that lack of vitamin D does not occur because there is enough amount of sun light, although aside from sun-evading lifestyle, in addition to lack of vitamin D intake, then the prevalence of vitamin D deficiency is increasing, although several previous studies stated that vitamin D levels do not affect calcium levels. Calcium has a role in regulation of tuberculosis infection through its role in cellular activity in tuberculosis infection.

Vitamin D's mechanism of action is among others by modulating the body's immune response starting from the increase of vitamin D receptor (VDR) activity, and after that it will activate monocytes as the body's immune cell [7, 16, 17]. Another mechanism is the active form of vitamin D which is $1,25(OH)_2D_3$ will hinder the proliferation of T cell and increase the abilities of pathogen-activated macrophages [15, 17].

Vitamin D receptor is a member of the nuclear receptor of transcription factors, with activation of $1.25(OH)_2D_3$, binding DNA response element and form heterodimer with retinoic acid receptor (RXR). This heterodimer will link the relation between vitamin D and vitamin A or carotenoid, including beta carotene.

Because of the big role of both vitamins and carotenoid, a clinical randomized trial will be performed with supplementation of vitamin D 1000 IU and vitamin A 6000 IU for 28 days on new cases of tuberculosis patients in Public Health Center (Pusat Kesehatan Masyarakat) as the referral center of tuberculosis cases in the City of Medan. Tested parameters are levels of 25(OH)D, retinol, beta carotene, and calcium before and after treatment.

2 Methods

The aim of this study is to find the effect of vitamin D 1000 IU and vitamin A 6000 IU supplementation along with nutritional counseling on sputum conversion, for 28 days on new cases of tuberculosis patients who visit three Public Health Centers, which are Teladan Public Health Center, Amplas Public Health Center, and Belawan Public Health Center, which are located in the City of Medan, North Sumatera, Indonesia. The location of sample collection is in Sumatera Island with latitude 3.57 N and longitude 98.65 E, with enough amount of sun light, temperature of ± 32 ^{o}C (90 ^{o}F). The study was performed during the period of May to September 2019. This study is a controlled randomized clinical trial, after going through inclusion and exclusion criteria, 48 patients participated in the study, divided into 24 patients in the treatment group (I) and the control group (C) has 24 patients (**Figure 1**).

Research subjects were males or females, new cases tuberculosis patients with AFB (+) who fulfills inclusion and exclusion criteria, chosen through purposive sampling technique. The inclusion criteria are new case lung tuberculosis patient with acid fast bacilli (AFB) result

(+), aged 18-60 years, heterozygote genotype test on one of the genes TaqI and FokI, willing to participate in the study and complete the informed consent. The exclusion criteria are pregnant, found to have comorbidities such as diabetes mellitus or Human Immunodeficiency Virus (+), and Tuberculosis on category II. Research subjects will drop out if they don't consume both supplements for more than 14 days and if they experience worsening clinical condition, although they will still be included when blood samples were drawn at the end of the study.

Randomization process

Sample sized determined by using formula based on tuberculosis prevalence in Medan City. In order to avoid bias in the study, this was double blind trial. Randomization was performed through block randomization. This action was also taken in order to remove selection bias, names of research subjects were assigned codes, and later a block randomization process was performed by using block table and divided by using closed envelope in order to divide treatment and control groups.

Treatment

The treatment group were given vitamin D 1000 IU supplementation in the form of soft gel and vitamin A 6000 IU in the form of tablet, along with nutritional counseling. Meanwhile, the control group only received nutritional counseling, but no supplementation. Supplementation were given once a day for 28 days, monitoring and administration of supplementation were given every two weeks in order to inquire adherence towards supplementation consumption and perceived side effects. All research subjects went through a clearance period of one week and were requested not to consume any supplements. Afterwards, measurements were taken before and after treatment, covering four tests, which are for 25(OH)D, calcium, retinol level, and beta carotene serum level.

Laboratory examinations for vitamin D, calcium, vitamin A, and beta carotene

At the end of the study, analysis was performed on the whole sample data, storage of samples was done at a temperature of -80^{o}C beforehand. Laboratory examiners did not know which specimens were case or control samples, samples were assigned codes and they were given randomly. Measurement of 25(OH)D serum level by using chemiluminescent immunoassay (CLIA) technology (Diasorin, Stillwater, MN) device, the measurement range was 4.0 and 150 ng/mL. The lowest level was 4.0 ng/mL based on inter assay precision of 3.90% CV. Range of categories of vitamin D levels are <20 ng/mL considered deficiency, 20-30ng/mL (insufficiency), 30-100 ng/mL (sufficiency).[18] Serum calcium level was measured by using ADVIA Bayer Assayed Chemistry Controls with reaction measurement 545/658 nm, and normal calcium serum level was 8.3-10.6mg/dL. Measurement of retinol and β-carotene serum levels in this study was performed by using high-performance liquid chromatography (HPLC) device, with interassay coefficient variation for retinol and beta carotene was <5%. The normal range of retinol level was >0.7μmol/L, while the normal range for beta carotene serum level was 0.3–0.6 μmol/L.

Patient sputum tests were performed in the laboratories of Teladan, Amplas, and Belawan Public Health Centers by using the *Ziehl Neelsen* method. Sputum collection was performed using the SPS (*Sewaktu-Pagi-Sewaktu*/Anytime-Morning-Anytime) 3 times method. Administered Anti Tuberculosis medications were Rifampicin, INH, Pyrazinamide, Streptomycin, Ethambutol with dosages adjusted to the patients' body weight. AFB tests were performed before treatment, on week 2, 4, 6 and week 8 during the intensive phase treatment, afterwards data was collected and noted.

Continuous variables were expressed as continuous variables using means±SDs. In order to find differences of vitamin D, retinol, beta carotene, and calcium levels between the two groups before and after the treatment, analysis was performed by using the t test independent. Categorical variables were expressed as percentage proportions and in order to show the relationships between vitamin D deficiency and sputum conversion, chi-square test was used, and Fischer test if the data did not meet the criteria. The p values <0.05 were considered statistically significant. We used SPSS program (version 11.5; SPSS Inc, Chicago, IL) to perform the analysis. This study was carried out after ethical approval was obtained from the Health Research Ethics. Committee of Sumatera Utara University Medical School (No. 135/TGL/KEPK FK USU-RSUP HAM/2019) and all participants were given written informed consent to the study procedures.

3 Results and Discussions

This study has 48 research subjects taking part, after completing the screening process, through inclusion and exclusion criteria (Figure 1), from a total of 63 research subjects. After signing informed consent and going through block randomization, they were divided into two groups, which are the treatment and control groups each with 24 research subjects. During the study period, two research subjects couldn't participate until the end of the period because they experienced nausea and didn't routinely consume the supplements, although both research subjects participated in having their blood drawn at the end of the study as part of the research (intention to treat).

Baseline characteristic

Baseline characteristic of all research subjects showed that most of the tuberculosis prevalence was experienced by the Batak Tribe (this is probably because the location of the study is dominated by this tribe), married, male, younger age group, and low body weight (Table 1). Statistic testing did not show any difference between the groups, did not indicate any significant difference between the treatment and the control groups. For baseline characteristic, both groups are homogenous in nature.

Polymorphism of vitamin D receptor gene influences 25(OH)D serum level in the body. Two widely studied vitamin D receptor genes were TaqI and FokI, especially in relation to tuberculosis. This study included vitamin D receptor gene polymorphism for all research subjects, with heterozygote genotype on vitamin D receptor gene which are TaqI (rs731236) or FokI (rs10735810). Hopefully, with vitamin D and A supplementation, both vitamins would synergize in improving clinical condition of tuberculosis patients aside from providing routine anti-tuberculosis regimens. In previous studies, FokI receptor gene polymorphism was related to increased risk of tuberculosis [2, 4, 5, 19-21]. This study showed that patients with VDR gene polymorphism would exhibit vitamin D deficiency and insufficiency. Administration of vitamin D supplements showed an increase in vitamin D level, but it still did not show significant differences of 25(OH)D serum levels in both groups. The differences in vitamin D levels in both groups were significant, therefore administration of vitamin D supplementation was highly necessary in tuberculosis patients with VDR gene polymorphism. At the end of this study, significant result in sputum conversion was shown.

Categories and laboratory parameter levels

Table 2 shows that before the study, the percentage of vitamin D deficiency and insufficiency were found in higher numbers from all research subjects. None of the research subjects was categorized as normal, only reaching the sufficient category. Although, when compared to the parameter categories from before the study, then vitamin D and calcium exhibited homogenous data. Meanwhile, study results for vitamin A and beta carotene categories showed significant differences between both groups for vitamin A (p=0.001) and beta carotene (p=0.001) before the start of the study. These results showed that from the laboratory test category before the study, vitamin D and calcium indicated no difference in both groups categories, but for vitamin A and beta carotene categories, there were differences.

Other factors were body fat mass and profession. In this study, the body fat mass of tuberculosis patients was mostly low-fat level. Although high level of fat was still found, the percentage of vitamin D level categories were still considered as vitamin D deficiency and insufficiency. Low vitamin D levels at the start of the study was probably caused by the vitamin D receptor gene polymorphism, aside from other possibilities such as disease progressivity, sun-evading lifestyle, and lack of high vitamin D and vitamin A nutrition intake [9, 22, 23]. Types of profession also affected vitamin D levels. Jobs in which the person is mostly protected from sunlight such as indoor jobs would cause more pronounced vitamin D deficiency [24-27].

The roles of vitamin A and beta carotene towards vitamin D activities hopefully would help with clinical improvement of tuberculosis patients [13, 14]. The mechanism of action of vitamin A and beta carotene were through the role of retinoic acid, in which retinoic acid would be converted into 9-*cis* retinoic acid and into ligand which could activate RXR. RXR would then interact with VDR. That heterodimer would change protein conformation so that it may activate or repress molecules through gene transcription [9].

The dimer forms of VDR-RXR would then bind on a specific sequence in the target promoter region called vitamin D response elements (VDRE). Several genes involved in the regulation of calcium, phosphor homeostasis, and vitamin D metabolism were related to VDRE. This transcription process would affect enzyme production involved in vitamin D metabolism such as enzymes involved in vitamin D synthesis. Increased vitamin D level in circulation would affect VDR activities in T lymphocytes, macrophages, and thymus tissues.

Table 2 showed that after treatment, there was also no categorical difference in all groups, but not for laboratory parameter levels, which are for vitamin D, calcium, vitamin A, and beta carotene levels. This indicated that there was an increase in laboratory parameter levels though still not reaching normal values (Table 3). Table 3 showed that before the treatment, the data was inhomogeneous, which are for 25(OH)D serum level and beta carotene level, there were significant differences between both groups. In 25(OH)D serum level, it was seen that average 25(OH)D serum level was very low in the treatment group, while in the control group the average was high. Table 4 showed significant difference for the delta or difference of 25(OH)D serum levels during treatment. This indicated that with supplementation of vitamin D 1000 IU showed a significant increase in the treatment group compared to the control group, although there were other factors which made the levels unable to reach normal vitamin D category. This is probably caused by vitamin D receptor gene polymorphism.

In this study, the low retinol levels found did not make way for a change in retinol serum levels compared to vitamin D levels after treatment. This was probably caused by the preparation of vitamin A supplements provided for the treatment group. Vitamin D administration in gel preparation provided good absorption. Bioavailability of the supplement's preparation showed an increase of 25(OH)D serum levels, but the preparation of vitamin A supplement as tablets did not exhibit optimum bioavailability. The result of this study indicated that there was no significant increase in serum retinol. Whether or not this was caused by the tablet preparation or lack of food sources of vitamin A intake, which mostly came from animal produce such as liver and fish oil not being a frequently consumed food products in this study.

In this study, nutritional counseling was also performed, in which the benefits of vitamin A and provitamin A were also explained. Therefore, intake of food sources of provitamin A which contained a lot of carotenoid was increased. Significant improvement was shown on beta carotene levels in group C, although they didn't receive beta carotene supplements, beta carotene intake probably may have been gained from easily acquired and affordable food sources. Food sources of beta carotene such as carrots, sweet potatoes, mango, sources from yellow-orange colored fruits, and dark green colored vegetables. This study did not find a significant difference between both groups, but it was shown through the increased levels, there was possibly a mechanism in which beta carotene held a role in sputum improvement.

In **Table 2**, vitamin A categories showed significant differences between both groups before the study, but vitamin A levels did not exhibit differences before and after the study on both groups (**Table 3**). During the study, vitamin A levels did not show significant differences between both groups, this indicated that vitamin A supplementation did not show a difference on both groups (**Table 4**). This also applied for calcium and beta carotene levels which did not have significant differences on both parameters (**Table 3 and 4**). Beta carotene categories before the study showed significant differences on both groups, although not showing significant differences during treatment between both groups.

Relationship of vitamin D status and sputum conversion

Using chi-square analysis, conclusion was drawn that there was significant relationship between vitamin D categories and the week of sputum conversion (**Table 5**). It can be concluded that the fastest sputum conversion rely on the highest vitamin D level in the blood.

Provision of counseling regarding increase of vitamin D food sources was also given. Although, vitamin D food sources belonged to expensive food products, such as codfish oil, salmon, or mushrooms. Fortification of vitamin D in milk is a good choice in fulfilling vitamin D intake. Sunlight is a renewable source in the tropical area, although jobs in which the workers are mostly covered from sunlight might make tuberculosis patients could not acquire adequate sunlight exposure in order to activate vitamin D from below the skin. Therefore, vitamin D supplementation in tuberculosis patients may provide more optimum synergistic effects by increasing beta carotene food sources[18, 28-30].

This study did not show any relations between calcium and vitamin D. Calcium intake also did not exhibit an increase in this study. Dietary sources of calcium such as milk, sour milk, or cheese are food products not commonly a part of daily meals for tuberculosis patients. This lack of calcium may be caused by the lack of consumption of such products by tuberculosis patients. This may be caused by high prices of those food or milk not being a commonly consumed food product. Other forms of calcium food sources are fish consumed along with their bones, such as anchovies[31, 32]. This form is a kind of food product which may be provided to tuberculosis patients in order to fulfill calcium requirements.

This study did not show low serum calcium levels in the blood, even though the mean of serum 25(OH)D serum levels were low. In tuberculosis patients, there were found low levels of 25(OH)D, which should have an interaction between vitamin D and calcium. If the level of vitamin D in the blood is high, calcium absorption will increase. However, this mechanism did not occur in this study. Although 1000 IU of vitamin D had given per day for 28 days, and there had been an increased in vitamin D levels, but it has not reached normal range, and this did not affect calcium levels in the blood. Calcium which has a vital role in bone remodeling, support by vitamin D, which converted to calcitriol, would help the absorption of calcium in the intestine.

About 300-500 mg of calcium derived from extracellular calcium as much as 900 mg, meaning that in the process of calcium, especially for bone remodeling, required 300-500 mg calcium. This amount will add to calcium intake from food sources, so it ranges from 1000-

1500 mg[33-35]. However, this condition put the serum calcium in a homeostatic (balanced) state. Phosphorus also plays a role in the balance of calcium levels in the blood and the rate of calcium storage in the intestine. The process of calcium absorption with the support of vitamin D is significant for the metabolism of calcium and phosphorus[36]. Calcium absorption by the digestive tract will be increased, calcium and phosphorus from the bone mobilized, vitamin D controls expenditure and balance of minerals in the blood[35]. Although the calcium and vitamin D metabolism appeared to be altered in tuberculosis, there was no association between vitamin D and calcium categorization in this study. Previous study confirmed that serum calcium is raised in tuberculosis but the effect may be reduced by a low calcium intake[37].

However, with the length of this study, administration of vitamin D along with nutritional counseling, especially high intake of beta carotene food would show improved sputum conversion in the treatment and control group. Significant increase in vitamin D levels, although not exhibiting a difference between treatment and control group at the end of this study, showed clinical improvement of tuberculosis patients.

This study has several limitations, which are not measuring parathyroid hormone levels and other clinical parameters of tuberculosis improvement, such as chest X-ray and blood inflammation parameters in the blood.

4 CONCLUSION

Based on the result of this study, it can be concluded that tuberculosis patients with vitamin D receptor gene polymorphism (FokI and TaqI), may experience an increase in vitamin D level from supplementation of vitamin D 1000 IU and vitamin A 6000 IU along with nutritional counseling, which affected the acceleration of sputum conversion.

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COMPETING INTERESTS

There are no funding or other conflicts of interest to declare for this research

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Table 1. Demographical characteristic description of all research subjects before treatment (n=48)

Variable	n	Percentage
Tribe		
Batak	28	58.3
Javanese	12	25.0
Mandailing	2	4.2
Malay	2	4.2
Other (Acehnese, Minang)	4	8.4
Marita status		
Single	17	35.4
Married	31	64.6
Sex		
Male	37	77.1
Female	11	22.9
Age Categories (years)		
20-30	19	39.6
30-40	16	33.3
40-50	5	10.4
50-60	6	12.5
>60	2	4.2
Body mass index		
Underweight	16	33.4
Normal	8	16.7
Overweight	15	31.3
Obese	9	18.8

Table 2. Categories of vitamin D, calcium, vitamin A, and beta carotene levels of all research subjects before and after the study (n=48)

Categories	n	Percentage
Categories of vitamin D levels		
Before		
Deficiency (<20)	19	39.6
Insufficiency (20-32)	23	47.9
Sufficiency (32-54)	6	12.5
After		
Deficiency (<20)	7	14.6
Insufficiency (20-32)	29	60.4
Sufficiency (32-54)	12	25.0
Categories of Calcium		
Before		
Low	9	18.8
Normal	39	81.3
After		
Low	11	22.9
Normal	37	77.1
Categories of Vitamin A		
Before		
Low	13	27.1
Normal	35	72.9
After		
Low	8	16.7
Normal	40	83.3
Categories of Beta Carotene		
Before		
Low	6	12.5
Normal	9	18.8
High	33	68.8
After		
Low	1	2.1
Normal	6	12.5
High	41	85.4

Table 3. Differences of vitamin D, calcium, vitamin A, and beta carotene levels in both groups before and after the study

Parameter	I (n=24)	C (n=24)	*p*
Before			
Vitamin D	19.75 ± 6.59	25.20 ± 7.57	0.01
Calcium	9.05 ± 0.51	9.18 ± 0.49	0.35
Vitamin A	1.29 ± 0.81	1.44 ± 0.69	0.49
Beta Carotene	1.20 ± 1.27	3.67 ± 2.06	0.001
After			
Vitamin D	25.91 ± 4.96	27.52 ± 6.44	0.34
Calcium	8.87 ± 0.54	9.26 ± 0.56	0.17
Vitamin A	1.53 ± 0.77	1.59 ± 0.75	0.78
Beta Carotene	1.47 ± 1.22	4.04 ± 2.04	0.001

Note: data presented in the form of mean±standard deviation
Analysis of differences between groups using t independent test.

Table 4. Change of vitamin D, calcium, vitamin A, and beta carotene levels in both groups

Delta during the study	I (n=24)	C (n=24)	*p*
Delta Vitamin D	6.16 ± 3.27	2.31 ± 2.62	0.001
Delta Calcium	-0.07 ± 0.44	0.07 ± 0.23	0.14
Delta Vitamin A	0.24 ± 0.15	0.15 ± 0.16	0.55
Delta Beta Carotene	0.26± 0.22	0.36 ± 0.23	0.16

Data shown are means ± standard deviation
[1]Analysed using independent *t*-test

Table 5. Relationship of vitamin D status and sputum conversion in the treatment group

Vitamin D category	Sputum conversion		*p* value
	Second n (%)	Fourth n (%)	
Deficiency	2 (8.3)	2 (8.3)	
Insufficiency	9 (37.5)	1 (4.2)	0.03
Sufficiency	10 (41.7)	0 (0)	

Note: analyzed using chi square test