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Oral Combination of Vitamin B1, B6, and B12 Supplementation on CD4+ T Cell and IFN-γ in Pulmonary Tuberculosis Patients with First-Line Opioid agonist therapy

Combinación oral de la suplementación con vitamina B1, B6 y B12 en el recuento de células T CD4+ y IFN-γ en pacientes con tuberculosis pulmonar con terapia de agonistas de opioides de primera línea

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SUMMARY

Introduction: *Tuberculosis (TB) is a wasting or consumption disease which causes metabolic changes in tuberculosis patients. The metabolic changes that can occur are decreased appetite, micronutrient malabsorption, and malnutrition. The purpose of this research was to determine the differences in the result of an oral combination of vitamins B1, B6, and B12 supplementations on Interferon Gamma (IFN-γ) levels and CD4+ T-cell counts in pulmonary tuberculosis patients receiving first-line Opioid Agonist Therapy (OAT) compared to only OAT therapy.*
Methods: *The type of research used was experimental research with a randomized pre-test and post-test control group design. The samples were obtained by purposive sampling. The sampling location was at Health Public Center in East Java from Juli – December 2019. The samples were divided into two groups, the group of TB patients who received first-line OAT supplementation with a combination of vitamins B1, B6 and B12 as the test group and the group of TB patients who only received OAT as the control group. In each group, the CD4+ T cell count and IFN-γ levels were quantified twice as pre and post-test using flow cytometry and ELISA. The data were analyzed with Mann-Whitney and Wilcoxon test using a p-value of 0.05.*
Results: *The results showed a significant decrease in IFN-γ levels and an increase in the number of CD4+ T cells in the test group compared to the control group.*
Conclusion: *Vitamins B1, B6 and B12 are recommended to be given as a complementary treatment to OAT at the public health center to increase the immunity of TB patients. Health staff can play a role in enhancing the compliance of TB patients toward medication.*

Keywords: *Supplementation vitamins B1, B6, B12, tuberculosis, OAT, CD4+ T cells and IFN γ*

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RESUMEN

Introducción: *La tuberculosis (TB) es una enfermedad de desgaste o consumo que provoca cambios metabólicos en los enfermos de tuberculosis. Los cambios metabólicos que pueden ocurrir son disminución del apetito, malabsorción de micronutrientes y desnutrición. El propósito de esta investigación fue determinar las diferencias en el resultado de la combinación oral de suplementos de vitaminas B1, B6 y B12 sobre los niveles de interferón gamma (IFN-γ) y los recuentos de células T CD4+ en pacientes con tuberculosis pulmonar que reciben terapia con agonista opioides (TAO) de primera línea en comparación con los que reciben solo terapia TAO.*
Métodos: *El tipo de investigación utilizado es la investigación experimental con un diseño de grupo de control aleatorio antes y después de la prueba. Las muestras se obtuvieron por muestreo intencional. El lugar de muestreo en el Centro Público de Salud en Java Oriental durante julio - diciembre de 2019. Las muestras se dividieron en dos grupos, el grupo de pacientes con TB que recibieron TAO de primera línea con una combinación de suplementos de vitaminas B1, B6 y B12 como el grupo de experimental y el grupo de pacientes con TB que solo recibieron TAO como grupo de control. En cada grupo, el recuento de las células T CD4+ y los niveles de IFN-γ se cuantificaron dos veces, antes y después de la prueba mediante citometría de flujo y ELISA. Los datos fueron analizados con la prueba de Mann-Whitney usando alfa 0.05.*
Resultados: *Los resultados mostraron una disminución significativa en los niveles de interferón gamma (IFN-γ) y un aumento en la cantidad de células T CD4+ en el grupo experimental en comparación con el grupo de control.*
Conclusión: *Se recomienda administrar las vitaminas B1, B6 y B12 como tratamiento suplementario del TAO en el centro de salud pública para aumentar la inmunidad de los pacientes con TB. El personal de salud puede desempeñar un papel en la mejora del cumplimiento de la medicación por parte de los pacientes con TB.*

Palabras clave*: Suplementación de vitaminas B1, B6, B12, tuberculosis, TAO, células T CD4+, IFN γ*

INTRODUCTION

Tuberculosis (TB) is still a health problem in the world even though control efforts with the DOTS (Directly Observed Treatment Short Course Chemotherapy) strategy have been implemented in many countries (1,2). Tuberculosis is the third cause of death worldwide after HIV/AIDS and Malaria (3). Globally in 2016, there were 10.4 million incident cases of tuberculosis (CI 8.8 million – 12 million), equivalent to 120 cases per 100 000 population. The five countries with the highest incidence of cases are India, Indonesia, China, the Philippines, and Pakistan (4). TB is a wasting or consumption disease that causes metabolic changes in tuberculosis patients. The metabolic changes that occur are anabolic blocks. An anabolic block is a condition in which amino acids cannot be built into more complex proteins. Metabolic changes that can occur are decreased appetite, malabsorption of nutrients, and malabsorption of micronutrients (5,6).

There are two relationships between malnutrition and tuberculosis, namely, the effect of tuberculosis on nutritional status and the effect of malnutrition on the clinical manifestations of tuberculosis due to a weak immune system. Malnutrition is also a significant risk factor for the onset of active tuberculosis, and malnutrition can worsen the prognosis of TB disease. Malnutrition affects cell-mediated immunity (CMI). CMI is the body's primary defense against TB (7). The association between TB and undernutrition has long been known. TB worsens undernutrition and weakens immunity, thereby increasing the likelihood of latent TB developing into active disease. Based on World Health Organization (WHO) data, 9.7 million individuals with active TB in 2016 were in a catabolic state and experienced weight loss, and some showed signs of vitamin and mineral deficiencies at diagnosis (8).

Based on these conditions, comprehensive and thorough treatment of tuberculosis patients must be carried out to increase the percentage of successful treatment of tuberculosis patients (9-11). The provision of nutritional intake is also essential to note. Micronutrients are needed for the body's immune system to function normally. A deficiency of micronutrients can suppress immunity by affecting the innate immune response, T cells and adaptive immune response resulting in an imbalance (12). One of the micronutrients that can be used to improve the work and response of the body's immunity is the provision of vitamin intake (13). Inadequate vitamin intake can lead to suppression of the immune system and can increase the risk of

infection. Administering vitamins B1, B6, and B12 can increase the number of T lymphocytes and the activity of Natural Killer cells (NK cells), both of which can trigger the release of interferon-gamma (INF-γ). Giving vitamins can reduce the risk of recurrence of tuberculosis by 45 % and reduce the incidence of extrapulmonary tuberculosis (14).

Recent research in 2019 suggested that the combination of Opioid Agonist Therapy (OAT) and vitamin D supplementation may improve the success of TB treatment (15). Similarly, a study in 2017 showed that administering Vitamin B1, B6, and B12 along with probiotics could induce an immune response involving interleukin-10 (IL-10) and interferon-gamma (IFN-γ), which are important in the immune defense against infections (16). Building on these findings, novel research in 2023 would be focused on pulmonary TB patients and investigated the effects of providing vitamin B1, B6, and B12 supplements, with and without OAT, on the levels of IFN-γ and CD4+ T-cell counts. Based on this, this study aimed to analyze the effects of oral supplementation of vitamins B1, B6, and B12 with OAT and OAT alone on Interferon Gamma (IFN-γ) levels and CD4**+** T-cell counts in pulmonary tuberculosis patients.

METHODS

This was an experimental research with quasi-experimental research with pre-and post-test control group design. The population was tuberculosis patients enrolled at Public Health Center in East Java, with 34 samples taken by purposive sampling. The inclusion criteria were new TB patients for whom OAT was never applied. The exclusion criteria are TB patients with autoimmunity and comorbidities. The samples were divided into two groups, TB patients who received first-line OAT drugs with a combination of vitamins B1, B6 and B12 as supplements as the test group, and the TB patient group who only received OAT drugs without supplementation of vitamins B1, B6 and B12, as the control group. Vitamins B1, B6 and B12 used in this study were in tablet form containing 100 mg B1, 100 mg B6 and 5000 mcg B12. Tablets for vitamin B1, B6 and B12 preparations were produced by PT PnG Health, giving the treatment group 1 tablet once a day for two months. The sample collection was performed from July – December 2019.

The samples were assessed for CD4+ T-cell counts and IFN-γ levels twice as a pre-test before the patient's administration of OAT and Post-Test after patients were treated with OAT and Vitamins B1, B6 and B12 for two months. Quantification of IFN-γ levels was carried out by using ELISA assay at the Institute for Tropical Diseases (ITD) Campus C, Airlangga University, and evaluation of the number of CD4+ T cells was carried out by using Flow Cytometry at the Pramita Laboratory Surabaya. The CD4+ T-cell counts, and IFN-γ levels test were carried out immediately after patients finished their OAT and Vitamins B1, B6 and B12 therapy for two months. The procedure is shown in Figure 1.

This research has been declared ethically feasible by the Health Research Ethical Clearance Commission, Faculty of Dental Medicine, Universitas Airlangga Number: 239/HRECC. FODM/V/202. Before enrolling in the study, patients were provided with detailed information about the experiment and procedures, and they were required to present signed informed consent indicating their voluntary participation and

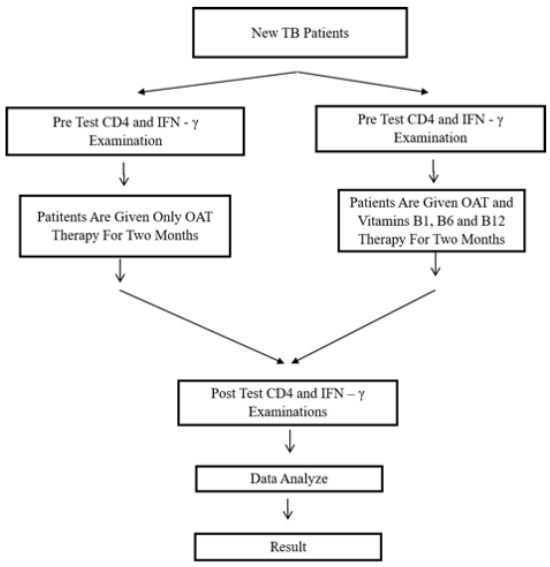

Figure 1. Research Procedure

understanding of the study's objectives, potential risks, and their right to withdraw at any time.

IFN-γ ELISA assay

We prepared all reagents, working standards, samples, and controls for the IFN-γ assay, which was performed using an enzyme-linked immunosorbent assay (ELISA) development kit designed for the quantitative detection of IFN Gamma / Interferon Gamma (17). Briefly, 100 µL of Assay Diluent RD1-51 was added to each well, followed by the addition of 100 µL of standard, control, or sample into each well. The plate was thoroughly mixed and then covered with the provided sealer, incubating at room temperature for 2 hours. After incubation, the contents of each well were discarded, and washing was performed by adding 400 µL of wash buffer into each well, repeating the process three times for a total of four washes. Following the final wash, the excess wash buffer was removed by tapping the plate upside down against a clean paper towel. Next, 200 µL of the conjugate was added into each well, and the plate was once again covered with a new sealer and incubated at room temperature for 2 hours. Another round of washing was performed. Subsequently, 200 µL of substrate solution was added to each well, and the plate was covered and incubated at room temperature for 30 minutes, protected from sunlight. Finally, 50 µL of stop solution was added to each well, leading to a color change from blue to yellow. In cases where the color produced was green or the color change appeared non-uniform, gentle shaking was employed to ensure thorough mixing. The optical density of each well was determined within 30 minutes using the ELISA reader at 450 nm. The examination results were validated by a clinical pathology specialist.

CD4+ T Cell Count

The number of CD4+ T cells was performed by using Flow Cytometry (18). Trucount™ tubes were used for the assay. Briefly, the method consisted, Add 50 µL of blood with EDTA to the tube. Add 20 µL of CD4+ reagent. The tube containing blood with EDTA, and reagents was vortexed for 5 seconds and then incubated for 15 minutes at room temperature. After 15 minutes, add 450 µL Facslyse, which has been diluted with a ratio of 1:10. Vortex for 5 seconds, then incubate for 15 minutes at room temperature. The sample was ready to be analyzed with Fluorescence-Activated Cell Sorting (FACS), which is a technique used in flow cytometry to physically separate cells based on their fluorescent properties. During FACS, the cells are first stained with fluorescent dyes or antibodies that bind to specific molecules on the cell surface. A clinical pathology specialist validated the examination results.

Data Analysis

The data were analyzed as parametric data. Data analysis was performed using the Wilcoxon and Mann-Whitney statistical tests with a p-value < 0.05.

RESULTS

The total respondents were 34 patients. Table 1 shows that patient distribution according to age was in the range of 12-25 years one patient (2.9 %), ages 26-45 years 13 patients (38.4 %), ages 46-65 years 19 patients (55, 8 %) and age> 65 years one patient (2.9 %). The respondents who received the first-line OAT at the Arosbaya Health Center that were male were 19 patients (55.8 %) and female patients 15 (44.2 %). The distribution of patients based on the results of smears before giving vitamins shows that patients with smear +1 results were two patients (5.88 %), smear +2 were 14 patients (41.1 %), and smear +3 were 18 patients (53.02 %).

Table 2 presents a comparison of IFN-γ levels and CD4+ cell counts between the control group and the experimental group in both the pre-test and post-test conditions. In the pre-test phase, there was no statistically significant difference in IFN-γ levels between the control group (99.46 ng/mL) and the experimental group (110.28 ng/mL) with a p-value of 0.07. Similarly, the CD4+ cell counts did not show a statistically significant difference in the pre-test, with the control group at 257.18 cells/mm^3 and the experimental group at 301.41 cells/mm^3 (p-value = 0.12). However, after the intervention, significant changes were

observed. The control group's IFN-γ levels decreased to 95.76 ng/mL (post-test) with a p-value of 0.001, while the experimental group's IFN-γ levels decreased to 90.44 ng/mL (post-test) with a p-value of 0.005, both indicating statistical significance. Moreover, the CD4+ cell counts increased significantly in the post-test for both groups. The control group showed a rise to 260.95 cells/mm^3 with a p-value of 0.003, and the experimental group showed an increase to 332.71 cells/mm^3 with a p-value of 0.002. These findings suggest that the intervention had a significant impact on both IFN-γ levels and CD4+ cell counts in the experimental group, compared to the control group.

Table 1. Patients Distributions characteristics

Characteristics	Frequency	Percentage (%)
Age (Year)		
12-25	1	2.9
26-45	13	38.4
46-65	19	55.8
>65	1	2.9
Total	34	100
Gender		
Male	19	55.8
Female	15	44.2
Total	34	100
BTA test		
+1	2	5.88
+2	14	41.1
+3	18	53.02
Total	34	100

Table 2. IFN-γ levels CD4+ Cell Counts in the control group and the test group

IFN-γ Levels (ng/mL)	Pre -Test	Post-Test	p-value*
Control	99.46	95.76	0.001
Experimental	110.28	90.44	0.005
P-value*	0.077	-	
CD4+ Cell Counts (Cell/mm^3)	Pre-Test	Post-Test	p-value*
Control	257.18	260.95	0.003
Experimental	301.41	332.71	0.002
P-Value*	0.12	-	

*Alpha = 0.05

DISCUSSION

Vitamins are organic compounds and essential nutrients required by an organism in limited amounts. An increasing number of studies have begun to explore the mechanisms by which vitamins regulate immunity and their effects as adjuvant to treat tuberculosis. Vitamin A, D, and E are the most widely studied, and the mechanisms by which they regulate immunity have been partly elucidated (15,16).

Vitamin B1 (thiamine) can produce a protective immune response to limit the survival of *Mycobacterium tuberculosis* in macrophages (19). Thiamine can activate peroxisome proliferator-activated receptor γ (PPAR-γ), part of the lipid-activated nuclear receptor involved in innate immune cells' differentiation and lipid metabolism, including macrophages involved in the inflammatory response (20). Within macrophages, PPAR -γ will integrate signals of metabolic and inflammatory functions that play an essential role in regulating the immune response and nutrient metabolism during infection by *M. tuberculosis* (21). In the regulation of receptors activated by PPAR-γ, thiamine promotes macrophage proliferation to a classically activated phenotype via microbicidal solid activity and increased TNF-alpha and IL-6. In addition, thiamine can enhance mitochondrial respiration and lipid metabolism to integrate metabolic signals (20,22). PPAR-γ is expressed in large quantities in alveolar macrophages and is essential for differentiation. The function of PPAR-γ activation against mycobacterial infection was shown to positively regulate prostaglandin (PG) E2 production in infected macrophages (23). Thus, PPAR-γ activation will increase cyclooxygenase (COX2) expression and PGE2 production in macrophages infected with *M. tuberculosis* so that macrophages will become activated macrophages and can kill *M. tuberculosis more* strongly (24). When macrophages can kill bacteria, the infection will gradually improve, and gamma interferon levels will decrease (25,26).

Vitamin B6 is essential in synthesizing proteins and nucleic acids so that it can affect the immune system because antibodies and cytokines - cytokines are formed from amino acids. Hence, they require vitamin B6 as a coenzyme in their metabolism (16,27). Studies conducted on human subjects it was shown that a deficiency of vitamin B6 could interfere with lymphocyte maturation, growth, antibody production, and T-cell activation. Gamma Interferon levels (22). So, when the intake of B6 is sufficient, the number of immune cells will increase.

Vitamin B12 also plays a role in the metabolism and proliferation of lymphocyte cells. Studies conducted on human subjects found that a lack of vitamin B12 significantly decreased the number of CD4+ T lymphocytes (28,29). In addition, an abnormal ratio of CD4+ T lymphocytes and suppression of NK cells were found. In contrast, other studies have shown that intramuscular supplementation of vitamin B12 (500 μg daily for two weeks) in humans improves the production of CD4+ T lymphocytes and CD8+ T lymphocytes and increases NK cell activity (30,31).

This study had limitations; the researchers did not know about the nutritional status and nutrition of the patients before. Researchers also did not measure vitamin B levels in serum before supplementing with vitamin B. Vitamin B administration using only one dose does not compare with several other doses and does not compare with variations in the duration of vitamin B supplementation. Researchers suggest giving TB patients vitamins as a support for treatment in addition to administering OAT. Administration of vitamins can increase the body's immunity in TB patients, making TB treatment more optimal.

CONCLUSION

According to the findings, adding vitamins B1, B6, and B12 to standard OAT can considerably reduce IFN-γ levels and raise CD4+ T-cell counts in patients. Vitamin supplementation can improve tuberculosis treatment outcomes by increasing immunological response. Tuberculosis patients receiving OAT were advised to include these vitamins as supplemental therapy. Vitamin supplementation should be considered by healthcare providers to improve immune function and aid patient recovery. More research is required to determine the appropriate quantity and duration of vitamin supplementation and any potential interactions or adverse effects. Raising awareness among healthcare providers about the benefits of vitamin supplementation in tuberculosis management is also critical.

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