

The Role of Vitamin D against to Lung Damage on Infected Mice by *Mycobacterium tuberculosis*

Hotmaida Siagian

Health Polytechnic of Surabaya, Ministry of Health, Republic of Indonesia

Abstract

Tuberculosis disease eradication efforts in Indonesia have been carried out using a strategy Directly Observed Treatment Short-Course (DOTS) to free treatment, but prevention and eradication is still not satisfactory. The sample consisted of 24 mice were divided randomly into two groups, one group was given oral vitamin D through 100ng of each mouse each day and the other group did not receive vitamin D, then each group was randomly divided into two groups were fed a vitamin D were infected with *Mycobacterium tuberculosis* and one group who did not receive vitamin D were infected with *Mycobacterium tuberculosis*. β , IL-12 and Nfk- γ at -14 in the week to check the levels of IFN-. ANOVA test was used to see the difference in vitamin D given to those not given vitamin D with $p < 0.05$, there is an increase in levels of IL-12, $p = 0.001$ but no elevated levels of IFN- β . The results showed no effect of vitamin D increased expression Nfk- and results of research into the effects of infection with *Mycobacterium tuberculosis* found no elevated levels of IL-12, $p = 0.00$, but no increase in levels of IFN- γ and- β expression Nfk. Results of research into the effects of Vitamin D and *Mycobacterium tuberculosis* infection found no elevated levels of IFN- γ $p = 0.043$, but no increase in levels of IL-12, and Nfk- β expression and decreased granuloma process, but not an increase in the expression of Nfk- β and IL-12. γ Effect of vitamin D can increase the cellular immune response by increasing IFN- probable cause is the increasing Nfk- β expression induced by vitamin D due to increased transcription in the nucleus of cells that signal the cell nucleus to increase the production of IL-12. Then IL-12 increases Th1 response to produce IFN- γ , increased IFN- γ production and activates macrophages to kill *Mycobacterium tuberculosis*.

Keywords: Vitamin D, *Mycobacterium tuberculosis*

1. Introduction

Tuberculosis (TB) is an infectious disease caused by the bacteria *Mycobacterium tuberculosis*. The germs enter the body through breathing air into the lungs, then the bacteria spreads from the lungs assigned to other bodies through the circulatory system, the lymph channel system, through the respiratory tract or direct spread to other body (P2M PLP, 1997). The incidence of TB patients in Indonesia is on the fifth rank in the world (WHO, 2010). Based SKRT MOH, 2004, found in Indonesia each year 583,000 new TB cases and 50% of them were patients with acid-fast bacilli category (BTA) significant positive as transmitters, and are reported every year 140,000 people died from TB (WHO, 2009). TB disease eradication efforts in Indonesia have been carried out using a strategy Directly Observed Treatment, Short-Course (DOTS) to free treatment, but prevention and eradication is still not satisfactory (MOH, 2004). Amount of morbidity and mortality caused by *Mycobacterium tuberculosis* while the treatment is still not satisfactory, the research problem is that the mechanism of immunity to *Mycobacterium tuberculosis* in patients given vitamin D to date has not been clear.

Vitamin D is a hormone imunoregulator beneficial for bone health that helps the absorption of calcium so that bones stay strong. In addition to vitamin D for bone health is also proven to help maintain the immune system (Sebastian, 2008). Benefits of vitamin D is important for active macrophages and dendritic cells, is not the same as the kidneys, are not regulated by homeostatic signals Ca^{+} but specifically regulated by immune especially IFN-g and TLR pattern recognition receptors. Human macrophages by signaling through heterodimers TLR1/2 were stimulated with bacterial lipopeptide will cause the CY27B-1 expression and VDR. Mice show that induction by CD-14 25D-deficient mice failed to CY27B-1. Vitamin D signaling increases the expression of TLR-2 doubled in keratinoid humans.

IL-12 is a major mediator of early nonspecific immunity against intracellular microbes and is a key indicator in specific cellular immunity against microbes. The main source of IL-12 is a mononuclear phagocytes and dendritic cells are activated. Biological effects of IL-12 is stimulated IFN-g production by NK cells and T cells, CD4 T cells differentiate into Th1 cells that produce IFN-g. IL-12 also increases sitolitik function of NK cells and CD8 cells (Karnen, 2009). The degree of damage to lung tissue can be used to assess the effectiveness of the drugs being tested in experimental animals (Bast, 2004; Koendhori, 2008), but it also can be used to assess the virulence (Dormans et al., 2004). Lung tissue damage in *Mycobacterium tuberculosis* infection began to form neutrophil aggregation, followed by proliferation and exudation in the lung parenchyma (Robbins, 1974), although the so granuloma is a sign of chronic stage of infection with *Mycobacterium tuberculosis* host immune system in an attempt to localize the multiplication and spread further to the other organs (Ordway et al., 2005).

The first response that appears after granting bacterial infection is the accumulation of macrophages and neutrophils, either abnormalities alveolitis, andperibronkiolitisperivaskulitis on day 3 (Dormans,

2004). Granuloma formation occurred some time later, most often began to appear on day 20 (Flynn, 2004). Initial trip *Mycobacterium tuberculosis* infection varies widely, depending on factors of immunity, the sensitivity of the host and the virulence or aggressiveness of the germs (Dormans, 2004). Tuberculosis lesions in the early course of the disease is proliferative and exudative. In individuals who are resistant or have endurance, reaction will be adequate phagocytosis fibroblastic wall boundary formation and scarring. In susceptible individuals, exsudatif lesions will be more extensive, involving many inflammatory cells and a marked ability to locate the bad (Robbins, 1974). Furthermore, in response to growing bacteria to grow the acquired immune system (adaptive/acquired) with granuloma formation. Acquired immune system in tuberculosis there are 2 of cell mediated immunity (CMI) and delayed type hyper sensitivity (DTH). Cell mediated immunity (CMI) shows the number of processes that lead to the accumulation of macrophages that are mikrobisidactivation, while DTH showed cytotoxic immune process that kills immature and activated macrophages that cause tuberculosis bacilli multiplication (Piessens&Naedell, 2000).

Granuloma formation starts from the introduction of CD4 + Th1 cells that recognize antigen receptor peptide fragments via major histocompatibility MHC) class II. Antigen peptide fragments formed by proteolytic enzyme digestion generated by antigen presenting cells such as macrophages and dendritic cells. to *Mycobacterium tuberculosis*-infected macrophages to enhance the effector function in macrophages. β/γ , GMCSF, TNF- γ Some cytokines produced by CD4 Th1 cells is IFN- Other cytokines, namely IL-2 also produced by CD4 + Th1 cells to stimulate CD8+ T cells as cytotoxic, further destroying acts immature macrophages infected apoptosis, chemotractant monocyte protein 1 (MCP-1), and IL-8. γ Activated macrophages surrounding infected with *Mycobacterium tuberculosis* lesions by secreting chemotactic cytokines such as IFN- Similarly, aggregation occurs at the lesion, monocytes move from the circulation into the lesion. The number of monocytes into alveolar macrophages, subsequently changed to histosit palisade or epithelial cells. Some of these cells serves to form Langhans giant cells, which is a typical sign of granuloma (Piessens&Naedell, 2000).

Granulomas in mice in contrast to granulomas in humans. Granulomas in mice composed by neutrophils, macrophages and lymphocytes by activated macrophages and lymphocytes structure that surrounds a collection of infected macrophages. At the granuloma there is necrosis and Langhans cell type that is different histopathology (Cardona et al., 2000). Although different structures but the same function is to control the infection and prevent the spread of infection (Flynn, 2004). No appearance of granuloma necrosis in mice showed that the immune response generated in mice is stronger than in humans, so the degree of spread of infection is lower (Cardona et al., 2000).

Based on the description obtained above problem formulation as follows: Is there any effect of vitamin D on lung tissue damage in mice infected with *Mycobacterium tuberculosis*. Common research goal is to explain the effect of vitamin D on lung tissue damage in mice infected with *Mycobacterium tuberculosis*, specific objectives are 1) to explain the effect of vitamin D on lung tissue damage in mice infected with *Mycobacterium tuberculosis* and 2) describe the damage to lung tissue in mice infected with *Mycobacterium tuberculosis*

2. Materials and Methods

This study is purely experimental with post-test control group design. The study sample was male mice Balb / c 8-10 weeks old weighing about 20 grams. Mice were given ad libitum food and drinks before and after infected with *Mycobacterium tuberculosis*. Until large replication as determined by the formula 6 mice per group. Sample collection techniques that meet the inclusion and exclusion criteria were taken. Research variables namely: Vitamin D is the Independent variabeldehydroxycholecalciferol and *Mycobacterium tuberculosis*, with variable depending damage lung tissue of mice was.

2.1. Research Procedures

1). Culture of *Mycobacterium*

- a) Breeding is done on Lowenstein-Jensen medium
- b) Prepared tubes were still fresh media (age <6 weeks)
- c) Sputum of 1.5 ml, to put into a sterile tube and mixed with 1.5 ml of 4% -8% NaOH
- d) The mixture is stirred 1 minute and taken with a sterile loop and then painted onto the surface of the media tube
- e) Each tube was closed with cotton paper and sealed with paraffin.
- f) Tube dijamin seeding media in the incubator at 37°C for 4-8 weeks. The emergence of colony growth revealed positive culture (Koneman, 2007).

2) Provision of Vitamin D

- a) Vitamin D weighed around 1mg
- b) Further diluted with 0.9% NaCl sebanyak 1ml/100ml
- c) From the above solution, 10 ml taken for dissolved again with 190 ml of 0.9% NaCl.
- d) Given 0.2 ml (100ng) Oral (Lemire& Archer, 1991).

3) Infection in Mice

- a) How the infection in mice was approved by the ethical viability of the Research Ethics Committee of the Faculty of Veterinary Medicine, Airlangga University
- b) Initially *Mycobacterium tuberculosis* colonies grown on Lowenstein-Jensen medium suspension was made by adding NaCl to obtain a concentration of 1 Mc raw. Farland.
- c) Further diluted with saline to obtain a suspension of 0.2 mg/ml.
- d) Mice type Balb/c with a weight of 20-30 g, age 8-10 weeks of acclimatization to the conditions of a controlled room temperature of 22-25°C for 7 days.
- e) Do phenobarbital anesthesia in mice with 0.6 mg/kg intraperitoneally, then made a small incision in the linea median so it looks trachea.
- f) A total of 100µl suspension is introduced into the trachea of mice with tuberculin needle in a vertical position (Dormans, 2004)
- g) Mice were maintained with controlled ventilation and temperature to get the disease manifestations of tuberculosis, mice were sacrificed at week 14 to see the levels of cytokines and histopathology

2.2. Data Analysis Techniques

All data were tested with Kolomogorov-Smirnov normality Z, followed by ANOVA (Analysis of Varians) or t test.

3. Results and Discussion

3.1. Results

Examination of lung tissue of mice by scores of granulomas. Examination of lung tissue in the group given vitamin D and infected with *Mycobacterium tuberculosis* (D +), the group given vitamin D but not infected with *Mycobacterium tuberculosis* (D-), a group that did not receive vitamin D but infected with *Mycobacterium tuberculosis* (K +), the group that did not given vitamin D and not infected with *Mycobacterium tuberculosis* (D-) is in the following Table 1.

Table 1. Examination of lung tissue of mice based on score damage lung tissue of mice

	Group			
	D+	D-	K+	K-
1	0,83	0,47	1,45	0,25
2	0,43	0,51	1,45	0,43
3	0,74	0,54	1,02	0,23
4	0,14	0,68	1,02	0,14
5	0,19	0,20	1,08	0,08
6	0,91	0,54	1,14	0,77

Description:

D += group given vitamin D and infected with MTB

D-= group given vitamin D but not infected with MTB

K += group that did not receive vitamin D and infected with MTB

K-= group that did not receive vitamin D and not infected by MTB

In the second lung of each mouse was made pieces for histopathological examination. On the lung pieces were fixed with 10% formaldehyde solution in PBS and then soaked in paraffin. Cuts made to all examined lung tissue. The parameters used are the degeneration, necrosis, congestion, edema, inflammation, fibrosis, epithelial hyperplasia and granulation, then scored as shown in Table 2.

Table 2. Data rate histopathological damage lung tissue in lung tissue damage score on each study group

Group	Mean	SD	Minimum	Maximum
D+	0,5400	0,3333	0,14	0,91
D-	0,4900	0,1581	0,20	0,68
K+	1,1850	0,1918	1,02	1,45
K-	0,3167	0,2520	0,8	0,77

In this study, tissue damage for all histopathological parameters, namely degeneration, necrosis, congestion, edema, inflammation, fibrosis, epithelial hyperplasia and granulation. Level minor tissue damage occurred in the group that did not receive vitamin D but infected with *Mycobacterium tuberculosis* (K +), while in others such as

K-, D + and D-no tissue damage.

3.2. Data Analysis

Homogeneity and Normality Test Data. Homogeneity and normality test data obtained from the results of ELISA using the Kolmogorov-Smirnov test. IIDN (identically, Independency and normality), the test is to determine the resulting data has a similar variation (identical), were not affected previously observed data (independence) and normal distribution (normality) conducted by time sequence Estimated autocorrelation plot and the normal probability plot. The results turned out identical data, independent and normally distributed. IIDN of test results, the data generated is eligible for subsequent statistical analysis is carried out different tests to assess differences in the immune response that is given vitamin D group and in the group not given vitamin D. Data lung tissue damage can be seen in the following Table 3.

Table 3. Differences damage lung tissue of mice by lung tissue damage score in group D-and K-by using ANOVA

	D-		K-		Sig
	Mean	SD	Mean	SD	
Lung tissue	0,490	0,158	0,316	0,252	0,681

The mean lung tissue damage in mice given vitamin D and not infected with *Mycobacterium tuberculosis* (D-) is equal to 0.490. While the average damage of lung tissue in mice that did not receive vitamin D and not infected with *Mycobacterium tuberculosis* (K-) is equal to 0.316 with a statistical test $p > 0.681$.

1. Lung tissue damage due to the effects of vitamin D and *Mycobacterium tuberculosis* infection based on score damage lung tissue.

The mean lung tissue damage in mice given vitamin D and infected with *Mycobacterium tuberculosis* (D+) is equal to 0.540. While the average damage of lung tissue in mice given vitamin D and not infected with *Mycobacterium tuberculosis* (D-) is equal to 0.490 with a statistical test $p > 0.988$.

Table 4. Differences damage lung tissue of mice by lung tissue damage score in group D + and D-using ANOVA

	D+		D-		Sig
	Mean	SD	Mean	SD	
Lung tissue	0,540	0,333	0,490	0,158	0,988

2. Lung tissue damage due to the effects of vitamin D and *Mycobacterium tuberculosis* infection based on score damage lung tissue.

The mean lung tissue damage in mice given vitamin D and infected with *Mycobacterium tuberculosis* (D+) is equal to 0.540. While the average damage of lung tissue in mice that did not receive vitamin D and infected with *Mycobacterium tuberculosis* (K +) is equal to 1.185 with a statistical test $p < 0.002$.

Table 5. Differences damage lung tissue of mice lung tissue damage score based on the D + and K + using ANOVA

	D+		K ⁺		Sig
	Mean	SD	Mean	SD	
Lung tissue	0,540	0,333	1,185	1,191	0,002

3. Lung tissue damage due to the effects of infection with *Mycobacterium tuberculosis* based on score damage lung tissue.

Table 6. Differences damage lung tissue of mice lung tissue damage on the K + and K-by using ANOVA

	K+		K-		Sig
	Mean	SD	Mean	SD	
Lung tissue	1,185	0,191	0,316	0,252	0,000

The mean lung tissue damage in mice that did not receive vitamin D and infected with *Mycobacterium tuberculosis* (K+) is equal to 1.185. While the average damage of lung tissue in mice that did not receive vitamin D and not infected with *Mycobacterium tuberculosis* (K-) is a statistical test of 0.316 with $p = 0.000$.

3.3. Discussion

In this study, using the histopathological score Dormans looked degree of damage is only found in the control group were infected with *Mycobacterium tuberculosis*, whereas the group given vitamin D and infected with *Mycobacterium tuberculosis* there is no tissue damage. Results of this study showed that administration of vitamin D was not significant $p > 0.68$, This shows that there is no difference in tissue damage that is given vitamin D to those not given vitamin D. Tissue damage characterized by granulomas based on the DTH reaction (delayed type hypersensitivity) reactions caused tuberculin the person's reaction to tubercle bacilli. This process begins with DTH Th-1 cells and giving antigen synthesis by macrophages. The reaction appears at 8-12 hours after infection. Redness occurs due to vasodilation with perivascular of CD4 T lymphocytes (Juanita & Johann, 2006). Very important in activating macrophages (Davies, 2006). γ T cells to produce cytokines such as IL-12, which would then activate other T cells and IFN- is the main cytokine that play a dominant role in the DTH reaction and the main activator of the macrophage-monocyte cells, improve the function of phagocytes and production of reactive oxygen intermediates (Rius et al., 2008). γ IFN- produced by NK cells and Th1 cells. γ IFN- Function is to increase T cell activation markers such as IL-2 receptor, triggering the differentiation of Th1 cells and suppress Th2 cell differentiation through suppression of production of Th2 cytokines such as IL-4 and IL-15. production by NK cells and T cells and trigger differentiation of Th1 cells. γ Another major cytokines that play a role in DTH is the role of IL-12 increases IFN- IL-12 also increases sitolitik function of NK cells and CD8 + T (Ordway et al., 2006).

Effect of vitamin D and *Mycobacterium tuberculosis* infection against histopathological damage. Histopathologic examination of the results obtained there was no difference in lung tissue damage in mice given vitamin D and infected with *Mycobacterium tuberculosis* mice given vitamin D and not infected with *Mycobacterium tuberculosis* with a significant value of $p > 0.988$. It showed no granulomas formed. However, the study mice were given vitamin D and mice infected with *Mycobacterium tuberculosis* that did not receive vitamin D and not infected by *Mycobacterium tuberculosis* significant difference with the results of significant $p < 0.002$. This suggests there is formed granulomas in mice that did not receive vitamin D and infected with *Mycobacterium tuberculosis*. Characteristic of tuberculous granulomas are characterized by necrotic areas in the middle period of granuloma (Central Necrosis/CN) surrounded by epithelioid cells (SE), multiple Langhans type giant cells and lymphocytes evident in the control group were infected with *Mycobacterium tuberculosis*, while in the group given vitamin D and there is infected with *Mycobacterium tuberculosis* granuloma (Robbins, 1974). Granuloma consists of a collection of macrophages and epithelial lymphocytes are usually surrounded by a circle. Lymphocytes are always there both in bulk or small. Granulomas form there are 3 groups: primary granuloma is a granuloma characterized by the accumulation of macrophages in the central surrounded by lymphocytes. Granuloma secondary granulomas are located near primary granuloma is an extension of the primary granuloma. Secondary granuloma lymphocytes characterized by a thick sheath around the central part of the granuloma and beyond a lot of foamy macrophages. Tertiary granuloma is a further development of the secondary granuloma granuloma lesions which are combined with the most severe grading (Cardona et al., 2000). Macrophages have a central role in the immune response to *Mycobacterium tuberculosis* infection. going to activate macrophages so as to kill the tuberculosis bacillus. γ Once an infection with *Mycobacterium tuberculosis*, macrophages will mempresentasikan antigen, both MHC class I and class II to CD4 + Th1 cells, subsequent CD4 + Th1 cells secrete IFN-) is a cytokine secreted by activated Th1 cells + CD have immunomodulatory effects to some cells such as macrophages (Yamada et al., 2007). γ (IFN- γ Interferon Granuloma is the primary response of the chronic stage of infection with *Mycobacterium tuberculosis* that reflects efforts to localize the immune response system and spread further multiplication of the bacilli to other organs and cells. There is evidence that the granuloma with a small diameter and an increase in macrophages showed that the immune response is able to control the growth of *Mycobacterium tuberculosis*. In contrast to granuloma with a large diameter and a decrease in macrophages showed no effective control of basil (Ordway et al., 2005). Foamy macrophage function phagocytosing necrotic debris from the granuloma central place cell destruction occurs. After working as a cleaner next granuloma Foamy alveolar macrophages would leave the room and encouraged to coughed up or swallowed. Foamy macrophages are inflammatory cells that element is very important. Growth depending on the cell granuloma is due to the proliferation of these cells is responsible for the merger to form granuloma lesions greater. Foamy macrophages contain a proportion of the amount of BTA that much that it would cause the formation of secondary granulomas (Cardona et al., 2000), levels increased in the group given vitamin D and infected with *Mycobacterium tuberculosis*. γ This finding is consistent with the measurement of IFN- Metabolite of vitamin D can increase the ability of monocytes to restrict the growth of intracellular *Mycobacterium tuberculosis*, alveolar macrophages in the lung Tuberculosis sufferers have been known to produce 1,25-vitamin D3 dehydroxycholecalciferon involved in the immune response to *Mycobacterium tuberculosis* (Liu et al., 2004). Vitamin D is beneficial for active macrophages and dendritic cells will ekspresikan CY27B-1, is not the same as the kidneys, are not regulated by homeostatic signals Ca^{+} but

specifically regulated by immune infut especially IFN-g and TLR pattern recognition receptors. This gives the immune system is responsive to circulating levels of 25D (Bid et al., 2005).

Stimulation of macrophages or monocytes through heterodimer raises TLR-2/TLR-1 regulation of 1- α -hydroxylase, but also in the vitamin D3 receptor (VDR) and VDR generated from the serum 25-hydroxyvitamin are signaled through specific VDR to induce antimicrobial peptide chatelicidin and kill intracellular *Mycobacterium tuberculosis* (Davies, 2006). Nfk-active to the cell nucleus (George, 2002), β activation leads to the activation of transcription factors and become involved in the translocation of β TLR signaling is essential for the manufacture of a signaling complex containing protein kinase (IRAQ members) who raises Nfk- In essence Nfk- β stimulates transcription process that produces interferon gamma (IFN-g) and interleukin 12 (IL-12) (Neil, 1997).

Granuloma is a chronic inflammatory focus consisting of a collection of modified macrophages mikroskofis into epithelial cells and surrounded by a circle of leukocytes mononukleat generally a lymphocyte but sometimes in the form of plasma cells (Ordway et al., 2006).Granuloma has an important role in regulating the interaction between the immune cells that cause the effective response blocking and kill *Mycobacterium tuberculosis*, resist infection, preventing the spread of the organism, inflammation and tissue damage (Cardona et al., 2000). Macrophages would be able to kill bacillus *Mycobacterium*Granulomas are formed because there are intracellular *Mycobacterium tuberculosis*-infected macrophages or inside but unable to be killed by the macrophages, so that CMI will localize the limited tuberculosis bacilli by T lymphocytes (Cardona et al, 2000), but with increased levels of IFN- tuberculosis, so there is no further processing of tissue damage. In contrast to the group infected with *Mycobacterium tuberculosis* dick granuloma because macrophages are unable to kill the tuberculosis bacilli or infected macrophages occurs. withNfk-impact repair of lung tissue (Dormans et al., 2004). β and γ Damage to lung tissue is an increasing role of the cellular immune response biomarker IL-12, IFN-. Results of this study provide information that the effect of increased cellular immunity to the effects of vitamin D resistance effect granuloma formation and prevent further pathological processes of the granuloma.

4. Conclusion

Conclusions is research vitamin D acts to increase body resistance against tuberculosis and lung tissue damage caused inhibit tuberculosis and minimize process granuloma. γ Mechanism of vitamin D against infection with *Mycobacterium tuberculosis* immunity in patients with TB who were given vitamin D is through increased levels of IFN-. Suggestion of this research is to conduct further clinical trials for the use of vitamin D as adjuvans in supporting prevention in patients with tuberculosis.

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