

THE EFFECT OF 1,25-DIHYDROXYVITAMIN D TO THE PHAGOSITOSIS ACTIVITY OF CHILDREN MACROPHAGE IN TUBERCULOSIS INFECTION

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ABSTRACT

Introduction: Children infected with TB do not always experience TB pain, depending on the number of bacteria that enter and the amount of endurance the child has. 1,25-dihydroxyvitamin D (1,25 (OH) 2D) is a function modulator for macrophages. **Objective:** This study aims to determine the effect of giving 1,25 (OH) 2D on the phagocytic activity of macrophages in children infected with TB. **Methods:** This study was an experimental Post Test Only Control Group Design study of 27 samples of children infected with TB. The 1,25 (OH) 2D concentration used was $10^{-8}M$ and the concentration of $10^{-7}M$. Examination of phagocytic activity is carried out using latex beads. **Results:** One Way ANOVA analysis showed a significant difference in the percentage of macrophages that phagocyte latex and the average number of latex phagocytes by each macrophage between the control group and the treatment group ($p < 0.05$). In the Post Hoc Bonferroni analysis, the mean percentage of macrophages that phagocytized latex was significant in group K with P2 ($p = 0.01$) and group P1 with P2 ($p = 0.03$), while the mean percentage of macrophages that phagocytized latex was not significant in group K with group P1 ($p = 0.13$). The mean number of latex particles phagocytized by each macrophage was significantly found in group K with P1 ($p = 0.01$) and group K with P2 ($p = 0.01$), while the mean number of latex particles phagocytized by each macrophage was not significantly found in group P1 with group P2 ($p = 0.91$).

Keywords: 1,25 dihydroxyvitamin D, Phagocytosis, Tuberculosis

ABSTRAK

Pendahuluan: Anak yang terinfeksi TB tidak selalu mengalami sakit TB, tergantung jumlah bakteri yang masuk dan besarnya daya tahan tubuh anak. 1,25-dihydroxyvitamin D (1,25(OH)₂D) merupakan modulator fungsi makrofag. **Tujuan:** Penelitian ini bertujuan untuk mengetahui pengaruh pemberian 1,25(OH)₂D terhadap aktivitas fagositosis makrofag anak yang terinfeksi TB. **Metode:** Penelitian ini merupakan penelitian eksperimental Post Test Only Control Group Design pada 27 sampel anak yang terinfeksi TB. Konsentrasi 1,25(OH)₂D yang digunakan adalah $10^{-8}M$ dan konsentrasi $10^{-7}M$. Pemeriksaan aktivitas fagositosis dilakukan menggunakan latex beads. **Hasil:** Hasil analisis One Way ANOVA menunjukkan perbedaan yang bermakna rerata persentase makrofag yang memfagosit latex

dan rerata jumlah *latex* yang difagosit oleh setiap makrofag antara kelompok kontrol dengan kelompok perlakuan ($p < 0,05$). Pada analisa *Post Hoc Bonferroni* diperoleh rerata persentase makrofag yang memfagosit *latex* bermakna terdapat pada kelompok K dengan P2 ($p = 0,01$) dan kelompok P1 dengan P2 ($p = 0,03$), sedangkan rerata persentase makrofag yang memfagosit *latex* tidak bermakna terdapat pada kelompok K dengan kelompok P1 ($p = 0,13$). Rerata jumlah partikel *latex* yang difagosit oleh setiap makrofag bermakna terdapat pada kelompok K dengan P1 ($p = 0,01$) dan kelompok K dengan P2 ($p = 0,01$), sedangkan rerata jumlah partikel *latex* yang difagosit oleh setiap makrofag tidak bermakna terdapat pada kelompok P1 dengan kelompok P2 ($p = 0,91$).

Kata Kunci: *1,25-dihydroxyvitamin D*, Fagositosis, Tuberkulosis.

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INTRODUCTION

Tuberculosis (TB) is a global health problem that causes health problems among millions of people each year and ranks second as a cause of death in the category of infectious diseases after the human immunodeficiency virus (HIV). Based on Global Tuberculosis Report data, in 2012, there were an estimated 2.9 million new TB cases and 410,000 deaths due to TB among women, as well as 530,000 TB cases and 74,000 deaths in children. Indonesia ranks fourth in the number of TB cases in the world, which is around 0.4-0.5 million cases in 2012.¹ According to pediatric TB data in Indonesia, the proportion of pediatric TB cases among TB cases in 2012 was 8.2%. When viewed from the data per province, shows variations in the proportion from 1.8 to 15.9%.²

Tuberculosis is a directly infectious disease caused by *Mycobacterium*

tuberculosis (*M. tuberculosis*). Children infected with TB do not always experience TB pain; the continuation after infection depends on the number of germs that enter and the amount of immune response (cellular immunity) of children.³ In individuals who have an infection, if the body's resistance is normal, 90% will heal by itself, but in those who are low endurance high risk of becoming ill TB from mild to severe, can even spread throughout the body.⁴

Mycobacterium tuberculosis is an intracellular pathogen that mainly resides in macrophages. The ability of macrophages to kill intracellular pathogens will determine the outcome of infection because macrophage activity is the first type of defense in TB infection.⁵ One mechanism for promoting macrophage function in eliminating *M. tuberculosis* is by using immunomodulators.

The biological effects of vitamin D have long been known and developed rapidly in recent years. Vitamin D is not just a micronutrient that plays a role in calcium homeostasis but is a pluripotent hormone with extensive immunomodulatory functions.⁶ Vitamin D can increase the body's immune response, both innate and adaptive immunity, two systems that work synergistically together to fight infection. In the innate immunity of vitamin D needed to overcome the ability of intracellular pathogens in avoiding antimicrobial responses mediated by macrophages, vitamin D is a modulator of macrophage function and can activate the host's antimycobacterial activity.⁷

The active form of vitamin D, 1,25 (OH) 2D, has long been known to induce the antimycobacterial activity of mononuclear phagocytes in vitro, the cells that control the growth of *M. tuberculosis*. 1,25-dihydroxyvitamin D can help macrophages suppress the growth of *M. tuberculosis* through increased levels of cathelicidin and defensins, both of which are strong antimicrobial peptides.⁸ Besides their ability to increase cathelicidin and defensins, 1,25 (OH) 2D is a powerful stimulus for the production of NO. 1,25-dihydroxyvitamin D plays a role with IFN- γ in increasing NO synthesis and iNOS expression, thereby increasing oxidative potential in the eradication of *M.*

Tuberculosis.⁹ 1,25-dihydroxyvitamin D also impacts on innate immune responses through the promotion of autophagy. *Mycobacterium tuberculosis* in the phagosome is able to inhibit phagosome maturation and fusion with lysosomes, but the host can overcome this inhibitory mechanism through the induction of autophagy.¹⁰⁻¹²

So overall, 1,25 (OH) 2D will double the macrophage cell response and increase the activity of macrophage phagocytosis in inhibiting the replication of *M. tuberculosis*. The immunomodulatory role of 1,25 (OH) 2D is expected to increase the immunity of children, by increasing the ability of macrophage phagocytosis in killing / destroying *M. tuberculosis*. Based on the above, the researchers are interested in conducting research to see the effect of giving 1,25 (OH) 2D on the phagocytic activity of macrophages in children infected with TB.

RESEARCH METHOD

This type of research is an experimental laboratory with The Post Test Only Control Group approach. Sampling was conducted at the Community Health Center, located in the city of Padang. At the same time, the examination of the research variables was carried out at the Biomedical Laboratory of Andalas University, Padang. The study population was TB-infected

children who were in close contact with adult smear-positive TB patients in community health centers throughout Padang. The number of samples in the study was 27 people.

Research Procedure: Blood collection is performed on children after parents get informed consent. Aseptic blood was drawn in the upper arm volar of 2.5 mL. Blood is put into the EDTA vacutainer and immediately taken to the laboratory using a cool box for examination.

Macrophage Isolation and Culture

As much as 2.5 ml of blood is put into a 15 ml tube, then the percoll working solution is put into the tube slowly through a wall with a volume of 1: 1, then centrifuged at a speed of 500 G for 30 minutes.

The interface layer (buffy coat) is taken and centrifuged at 1200 RPM, at 4-5°C for 10 minutes. The pellets obtained were washed with 1 ml PBS, then resuspended with 1 ml complete RPMI and put into 25 cm² culture flask with the addition of 7 ml complete RPMI, then incubated for three days.

Monocytes attach to culture flask; non-stick cells are washed (2x) with the addition of 7 ml PBS, then proceed with trypsinization. A total of 10 mL of complete RPMI was added to the culture flask, then

the liquid was transferred into a 15 ml centrifuge tube and centrifuged at 5000 RPM for 10 minutes, the supernatant was removed. Pellets are washed (1x) with the addition of 10 ml PBS, then resuspended with 1 ml complete RPMI, then followed by cell concentration calculation.

Cell Concentration Calculation

The number of macrophages was calculated using a haemocytometer, then resuspended using complete RPMI medium, making cells with 2.5×10^5 cells/ml concentration. The calculated cell suspensions were then cultured on 24 well-culture plates that were given a round coverslip, every well contained 100 μ L (1×10^4 cells/well) and added with 100 μ L complete RPMI medium, then incubated in a 5% CO₂ incubator, 37 °C for 24 hours.

Macrophage Induction

1,25-dihydroxyvitamin D was added in concentrations of 10^{-7} M and 10^{-8} M (Verway et al., 2013), then added using 100 μ L of autologous serum. After that, it was re-incubated in a CO₂ incubator for 3 days.

Phagocytosis test

Macrophages that have been cultured in 24 well culture plates are washed with PBS 1 time. Latex suspension was added as much as 100 μ L, then incubated for 60 minutes. Each well was fixed with ethanol for 30 seconds. Ethanol is then discarded and dried at room

temperature. Each well was stained with 20% giemsa for 30 minutes and washed with aquadest. Coverslip is removed from the pit and dried at room temperature, then examined under a microscope at 400x magnification.

Data Analysis: The obtained data were then tabulated, then ANOVA statistical analysis was carried out and continued with the Post Hoc Test Multiple Comparisons, Bonferroni type with a significant value if $p < 0.05$.

Percentage of macrophages that phagocyte latex in children infected with tuberculosis

The results of the average percentage of macrophages that phagocyte latex according to the study group in children infected with tuberculosis can be seen in the table below:

Table 1. Average number of macrophages phagocytting latex between study groups

Groups	Mean±Std Dev	P
K	53,00±3,20	
P1	58,11±4,59	0,01
P2	64,78±6,72	

Where:

K=Control, P1=Giving a concentrated 10^{-8} M and P2= Giving a concentrated 10^{-7} M

Based on table 1, the ANOVA test results, we obtained a value of $p < 0.05$, which means there is a significant difference in the mean percentage of macrophages that phagocyte latex between

the control group and the treatment group.

To see a significant difference in the mean percentage of macrophages that phagocyte latex in each group, proceed with the Bonferroni Post Hoc Test. The test results can be seen in the following table 2:

Table 2. Post Hoc Bonferroni Test Results on the Average Percentage of Macrophages that Phagocyte Latex Between Study Groups

Groups	P		
	K	P1	P2
K	-	0,13	0,01
P1	0,13	-	0,03
P2	0,01	0,03	-

Where:

K=Control, P1=Giving a concentrated 10^{-8} M and P2= Giving a concentrated 10^{-7} M

Based on the Post Hoc Bonferroni in Table 2, the mean percentage of macrophages that phagocyted latex was found to be significant in the group without giving 1,25 (OH) 2D with Treatment group 2 using a concentration of 10^{-7} M 1,25 (OH) 2D ($p = 0.01$) and Treatment group 1 using a concentration of 10^{-8} M with Treatment group 2 using a concentration of 10^{-7} M 1,25 (OH) 2D ($p = 0.03$), while the mean percentage of macrophages that phagocyted latex was not significant in the group without administration 1, 25 (OH) 2D with Treatment group 1 using a concentration of 10^{-8} M ($p = 0.13$).

The Average Number of Latex Particles Phagocytosed by Each Macrophage in Children Infected with Tuberculosis

The results of the average examination of the number of latex particles phagocytosed by each macrophage based on the study group in children infected with tuberculosis can be seen in the table below:

Table 3. Average Number of Latex Particles Phagocytosed by Each Macrophage by Research Group

Group	Mean±Std Dev	<i>p</i>
K	4,58±1,22	
P1	9,26±1,16	0,01
P2	8,64±1,31	

Where:

K=Control, P1=Giving a concentrated 10^{-8} M and P2= Giving a concentrated 10^{-7} M

Based on table 3, the ANOVA test results obtained *p*-value <0.05 , which means there is a significant difference in the average number of latex particles phagocytosed by each macrophage between the control group and the treatment group. To see a substantial difference in the mean percentage of macrophages that phagocyte latex in each group, proceed with the Bonferroni Post Hoc Test. The test results can be seen in the following table 4:

Table 4. Post Hoc Bonferroni Test Results on the Average Number of Latex Particles Phagocytosed by Each Macrophage Between Research Groups

Groups	<i>P</i>		
	K	P1	P2
K	-	0,01	0,01
P1	0,01	-	0,91
P2	0,01	0,91	-

Where:

K=Control, P1=Giving a concentrated 10^{-8} M and P2= Giving a concentrated 10^{-7} M

Based on the Post Hoc Test Multiple Comparisons in table 4, the mean number of latex particles phagocytosed by each macrophage is significant in the group without 1,25 (OH) 2D treatment group 1 using a concentration of 10^{-8} M 1,25 (OH) 2D ($p = 0.01$) and the group without giving 1,25 (OH) 2D with Treatment group 2 using a concentration of 10^{-7} M 1,25 (OH) 2D ($p = 0.01$). In contrast, the average number of latex particles phagocytized by each macrophage were not significant in the Treatment group 1 using a concentration of 10^{-8} M 1,25 (OH) 2D with Treatment group 2 using a concentration of 10^{-7} M 1,25 (OH) 2D ($p = 0.91$).

DISCUSSION

The phagocytic activity of macrophages can be assessed from the percentage of macrophages that phagocyte latex particles (counted from 100 activated macrophage cells) and from the phagocytosis index (the average number of latex particles phagocytated by each macrophage). In this study the phagocytosis index was determined based on the number of latex phagocytes by 50 macrophage

cells.⁽¹³⁻¹⁴⁾

Percentage of Macrophages that Phagocyte Latex Particles in Children Infected with Tuberculosis

Based on research, it is known that the administration of a concentration of 10^{-7} M 1,25 (OH) 2D can increase the percentage of macrophages that phagocyte latex particles are statistically significant. In contrast, the effect of giving a concentration of 10^{-8} M 1,25 (OH) 2D on increasing the percentage of macrophages that phagocyte particles latex when compared with without the administration of 1,25 (OH) 2D was not significantly different, meaning that the administration of 1,25 (OH) 2D at a concentration of 10^{-8} M has not been able to increase the percentage of macrophages that phagocyte statistically significant latex particles.

The 1,25-dihydroxyvitamin D is a pleiotropic hormone, which contributes to regulating calcium homeostasis, induces differentiation, and inhibits the proliferation of various normal cells and cancer cells.¹⁵ 1,25-dihydroxyvitamin D can also act as a powerful modulator of immune function, which can activate monocytes or macrophages, suppress lymphocyte proliferation, play a role in the production of immunoglobulins and cytokine synthesis, thus playing a role in human innate immunity for certain

infectious agents.¹⁶

Some previous studies using 1,25 (OH) 2D including, Chandra et al. (2005), found that administration of 1,25 (OH) 2D at a concentration of 10^{-7} M significantly increased the phagocytosis of macrophages against *M. tuberculosis* in normal subjects even with potential low. Still, they found 1,25 (OH) 2D at concentrations of 10^{-7} M, and 10^{-8} M significantly decreases spontaneous lymphoproliferative response and increases apoptosis of peripheral blood mononuclear cells in PTBPs (pulmonary tuberculosis patients) .¹⁷ The study of Kobayashi et al. (2005) proves administration of 1,25 (OH) 2D in cell cultures with concentrations of more than 10^{-7} M can significantly reduce the ability of keratinocytes to produce MMP-2 induced by LPS stimulation, then also seen the effect of 1,25 (OH) 2D on TIMP-production 1 (tissue inhibitor of metalloproteinase 1) and TIMP-2 (tissue inhibitor of metalloproteinase 1) of keratinocytes. The minimum concentration of 1,25 (OH) 2D which shows significant emphasis is 10^{-8} M for TIMP-1 and 10^{-9} M for TIMP-2.¹⁸

The Average Number of Latex Particles Phagocytosed by Each Macrophage in Children Infected with Tuberculosis

The second indicator of phagocytic activity of macrophages can be seen from

the phagocytosis index, which is the average of latex particles phagocytized by each macrophage. Based on the research that has been done, the average of latex particles phagocytized by each macrophage in the treatment group, either by giving a concentration of 10^{-7} M 1,25 (OH) 2D or giving a concentration of 10^{-7} M 1,25 (OH) 2D twice as much compared to the group without giving 1,25 (OH) 2D, is \pm nine latex particles respectively, while the average of latex particles in the group without giving 1,25 (OH) 2D is a number of \pm five latex particles. In the group without 1,25 (OH) 2D administration, it is possible that some macrophages have not been activated or are activated by lower phagocytic activity.

Based on the results of research and observations it can be concluded that the higher number of activated macrophages and the increase in the number of latex particles that are phagocytized by macrophages in the administration of 1,25 (OH) 2D when compared to the group without 1,25 (OH) 2D may be related to the role of immunomodulators 1,25 (OH) 2D on macrophage function. 1,25 dihydroxyvitamin D is a functional modulator of macrophages. Monocytes and macrophages are able to express CYP27B1 and vitamin D receptors (VDR).¹⁹ Thus, 1,25 (OH) 2D may, directly and indirectly, affect the innate immune system and adaptive immune system.²⁰

The 1,25-dihydroxyvitamin D can mediate biological responses, including cell differentiation through genome pathways, which involve vitamin D receptors in the nucleus to modulate gene transcription further and through non-genomic pathways involving cell membrane receptors to produce fast effects.²¹ In the immune system body, 1,25 (OH) 2D promotes monocyte differentiation and inhibits lymphocyte proliferation and cytokine secretion, such as IL-2 and INF- γ .²² 1,25 dihydroxyvitamin D also induces gene expression of cathelicidin antimicrobial peptides and defensins in monocytes, increasing NO synthesis and iNOS expressions.⁽⁸⁻⁹⁾

The presence of CYP27B1 in macrophages is very important for the immunomodulatory role of 1,25 (OH) 2D. CYP27B1 is an enzyme that is responsible for the final hydroxylation of 1,25 (OH) 2D. In the kidney, CYP27B1 is induced by parathyroid hormone in response to calcium homeostasis, but in monocytes and macrophages, CYP27B1 expression is regulated by immune stimuli, such as IFN- γ and TLR signals. TLR signals can induce VDR and CYP27B1 expression in monocytes and macrophages.⁽²³⁻²⁴⁾

The introduction of antigens through TLRs will increase the expression of vitamin D receptors (VDR) and the 1α -hydroxylase (CYP27B1) gene in

macrophages. The biological action of 1,25 (OH) 2D is carried out by binding to the VDR and Retinoid X (RXR) receptors in the nucleus of various body cells. VDR forms a heterodimer complex with a retinoid X receptor (RXR) and functions as an activated transcriptional regulatory ligand. The RXR-VDR complex regulates gene expression through Vitamin D Responsive Elements (VDRE) in responsive gene promoters of 1,25 (OH) 2D. 25 Receptor complexes with 1,25 (OH) 2D interact with DNA in the nucleus of target cells and can stimulate selective gene expression or suppress specific gene transcription.²⁶

In this study, it appears that in vitro 1,25 (OH) 2D can increase the phagocytic activity of macrophages against latex particles when compared without giving 1,25 (OH) 2D, which might also increase phagocytic activity against *M. tuberculosis* in infected children TB.

This study has the limitation of not using alveolar macrophages for the examination of phagocytic activity because it is not possible to use child samples and the resulting macrophages have not been activated properly, because they do not use stimulation factors (for example, GM-CSF) as activating macrophages.

CONCLUSION

The conclusion of this study is that

1,25 (OH) 2D affects the number of activated macrophages and the average number of latex particles phagocytosed by each macrophage. However, there is no significant difference in the average number of latex particles phagocytosed by each macrophage between 10^{-8} M and 10^{-7} M concentration of 1,25(OH) 2D.

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