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Research Article

# 1, 25$(OH_2)D_3$ INDUCE CATHELICIDIN BUT REDUCED *ESCHERICHIA COLI* KILLING IN NEUTROPHILS OF SYSTEMIC LUPUS ERYTHEMATOSUS PATIENT

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## ABSTRACT

1,25 $(OH)_2D_3$ as an active form of vitamin $D_3$ can induce the human antimicrobial peptide cathelicidin in the keratinocytes, respiratory epithelium, monocytes, and bladder. The main objective of this study was to examine the effect of 1,25 $(OH)_2D_3$ in increasing *Escherichia coli* (*E. coli*) killing via cathelicidin induction in neutrophils of systemic lupus erythematosus (SLE) patient. We used six groups of neutrophil culture of hypovitamin D SLE patient that treated with 1,25$(OH)_2D_3$ dose 0, $10^{-10}$M, $10^{-9}$M, $10^{-8}$M, $10^{-7}$M, and $10^{-6}$M, respectively, then induced with phorbol 12-myristate 13-acetate (PMA) to form neutrophil extracellular traps (NETs) as the last defense of neutrophil toward bacteria. Intracellular flow cytometry showed that 1, 25 $(OH)_2$ $D_3$ increasing cathelicidin expression, although statistically was not significant. And after infected with *E. coli*, the colony forming unit (CFU) counting was showed significant reducing of *E. coli* killing at the dose of $10^{-9}$M, $10^{-7}$M, and $10^{-6}$M ($p<0.05$). This reduced killing was linked with interaction of cathelicidin and DNA inside NETs ($r=-0.541$, $p<0.05$) so we can conclude that 1, 25 $(OH)_2D_3$ induced cathelicidin quantitatively but reduced cathelicidin qualitatively in neutrophils of SLE patient. This result may reveal the possibility of side effect of administering 1,25$(OH)_2D_3$ in SLE patient.

## KEY WORDS

*1, 25 $(OH)_2$ $D_3$, cathelicidin, Escherichia coli, neutrophil, SLE, patient.*

## INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic systemic autoimmune disease that is more often encountered [1]. Immune dysregulation and therapeutic factors in SLE can cause immunocompromised conditions so SLE patients are more susceptible to infection [2, 3]. Infection can induce flare and it is the highest cause of death in patients with SLE [4]. About 80% of SLE infection caused by bacteria with the highest incidence was by *Escherichia coli* (*E. coli*) [2, 5, 6]. *E. coli* is a gram negative bacteria that lives profitable in the human gut, but sometimes can be pathogenic and lethal [7]. The level of antibiotic resistance to *E. coli* in the

community is rapidly increasing, especially with regard to fluoroquinolones, third-generation of cephalosporins, and fourth-generation of cephalosporins [8].

Neutrophil are sometimes called "soldier of the body" because it is the first cell that is deployed to the site of infection [9]. Neutrophil kill bacteria intracellularly by phagocytosis and extracellularly by form neutrophil extracellular traps (NETS) [10]. The neutrophil function of SLE patients was disrupted because the phagocytosis function related to cathelicidin was decrease and the number of NETs was increase excessively so killing bacteria become inefficient [2, 4, 11, 12].

To date, there are over a hundred studies on SLE and vitamin D [13]. The serum levels of vitamin D {1,25(OH)$_2$D$_3$} of SLE patients in Indonesia are significantly lower when compared with healthy controls [1]. Although Indonesia is located on the equator that exposed to the sun throughout the year, SLE patients should not be exposed to sunlight and thus indirectly the synthesis of vitamin D in the body is reduced. Low levels of vitamin D in the SLE patients was associated with increased disease activity [14-17].

Vitamin D which has the classic function of regulating the balance of calcium and bone formation has a similar structure of molecule with steroids so it used by researchers to decrease the activity of SLE. Several studies have shown that administration of 1,25(OH)$_2$D$_3$ in SLE play role as an immunosuppressive agent of lymphocytes, decrease proinflammatory cytokines, inhibits the production of immunoglobulin on B cells, retain dendritic cells in immature condition, improve tolerogenesis, suppress excessive neutrophil response, and reduce NETs forming by neutrophil [10, 15, 18-22]. In another study also noted that the administration of vitamin D in humans can induce cathelisidin expression on keratinocytes, respiratory epithelium, monocytes, and bladder as a broad-spectrum antimicrobial peptides [23]. Cathelicidin expression in mice can mediate neutrophil defense against *Staphylococcus aureus* by increasing intracellular antimicrobial activity of the oxygen-independent pathway [24].

There has been no previous study that describes the effects of vitamin D on neutrophil of patients with SLE, especially those associated with bacterial killing effect via induction of cathelicidin. So in this study we want to examine the effect of 1,25(OH)$_2$D$_3$ in increasing *E. coli* killing via cathelicidin induction in neutrophils of SLE patient.

## MATERIALS AND METHODS

### *Ethical Clearance*

This study protocols was approved by Health Research Ethics Committee Faculty of Medicine Brawijaya University (Ethical Clearance No. 016 / EC / KEPK / 01 / 2014) and informed consent had been obtained.

***Neutrophils isolation***

Neutrophils was isolated from peripheral venous blood of female hypovitamin D {25(OH)$_2$D$_3$ serum level < 30 ng/ml} SLE patient in active disease state (SLEDAI score > 3). 7 ml fresh blood was drawn from median cubital vein and directly put into vacutainer tubes containing EDTA anticoagulant, then brought to room temperature. We made a layer of 3.5 ml blood in the top of 3.5 ml polymorphprep within 15 ml centrifuge tube, then centrifuge of 1400 rpm for 33 minutes. After that, the plasma and mononuclear ring at the top were discarded and polymorphonuclear (PMN) ring at the bottom were harvested. PMN put in a new 15 ml centrifuge tube and washed twice with 5 ml of PBS. Then centrifuge 1200 rpm for 10 minutes and resuspension with culture medium (RPMI 1640 + FBS 10%). The purity of neutrophils was tested by flow cytometry and detected in CD14$^{lo}$ and CD10.

Actually we isolated neutrophils from 4 female SLE patients with hypovitamin D in active disease state, but we failed to get their PMN ring, so in this study we only use the neutrophils isolation from 1 patient and divided it into 4 replication. This patient characteristics are 25(OH)$_2$D$_3$ serum level = 15.5 ng/ml, SLEDAI score = 17, age = 20 years old, duration of illness = 4 years, current medication = methylprednisolone, calcium, folic acid, and chloroquine.

***Neutrophils treatment***

Isolated neutrophils was counted with haemocytometer and @5x10$^5$ cells/ml was seeded in 24-well plate that contain @500 ul culture medium. We divided our 24 wells into six groups and treated with dose of 1,25(OH)$_2$D$_3$: M1 (0), M2 (10$^{-10}$M), M3 (10$^{-9}$M), M4 (10$^{-8}$M), M5 (10$^{-7}$M), and M6 (10$^{-6}$M). The confluence of culture tested under an inverted microscope then incubated in 5% $CO_2$ incubator at 37°C overnight (18-24 h).

***NETs induction***

To make this *in vitro* condition like *in vivo*, we put 20 nM phorbol 12-myristate 13-acetate (PMA) into each well to stimulate NETs forming after incubation overnight then incubated again for 2 h.

***Intracellular flow cytometry of cathelicidin***

After 2 h incubation, 4x10$^5$ cell of neutrophil was harvested and put into eppendorf. Into each eppendorf we added 0.5 ul LL-37 primary antibody (sc-166770 Santa Cruz) and 0.5 ul scundair flow antimouse FITC antibody (KPL, 02-18-06) then measured with flow cytometer and detected in LL-37.

***Escherichia coli infection***

On the first day of the study, *E. coli* strain O157 was grown in Luria-Bertani broth overnight. The next day, an aliquots of microorganisms cultured dissolved in Luria-Bertani broth, grown for 2-3 hours, and measured with spectrophotometry λ = 625 nm to determine the 10$^3$/ml of *E. coli*. 1x10$^1$ neutrophils in the well-24 plate was harvested and put into eppendorf that contain 10$^3$ *E. coli*

in 1 ml of 0.9% NaCl. Infected neutrophils were further incubated for 20 minutes in waterbath with medium speed at 37°C and plated into EMB medium to determine colony forming unit (CFU). The *E. coli* killing data was obtained from subtraction of CFU of infected neutrophil (*E. coli* survival) and CFU of *E. coli* without neutrophil and 1,25(OH)$_2$D$_3$.

***Statistics***

Data of cathelicidin expression from flow cytometry and *E. coli* lysis were analyzed using SPSS 16.0 for Windows. The effect differences among each treatment groups of 1,25(OH)$_2$D$_3$ analyzed with One-Way ANOVA. And further, correlation and regression between cathelicidin expression and *E. coli* lysis analyzed with Pearson and linear regresion. Statistical significance was taken as $p < 0.05$.

## RESULTS

*1,25(OH)$_2$D$_3$ induce cathelicidin expression in neutrophils of SLE patient* Vitamin D has been shown to activate the antimicrobial peptide cathelicidin in a range of human cell types. Therefore, we investigated if this process also occur in the neutrophils of SLE patients. After 6 doses of 1,25(OH)$_2$D$_3$ administration to neutrophils of SLE patient, we found higher expression of LL-37 of treatment group than control in a dose of $10^{-10}$M (72.48±2.58% vs 67.66±1.52%, p = 0.85), $10^{-9}$M (73.00 ±2.54% vs 67.66±1.52%, p = 0.79), $10^{-7}$M (70.98±7.56% vs 67.66±1.52%, p = 0.965), and $10^{-6}$M (75.30±5.69% vs 67.66±1.52%, p = 0.467) (**Figure 1**). There were no significant difference between treatment groups and control statistically, but we can see that in the dose of $10^{-8}$M have a significant different with dose of $10^{-6}$M (60.83±9.99% vs 75.30±5.69%, p=0.026).

***1,25(OH)$_2$D$_3$ reduced E. coli killing in neutrophils of SLE patient***

Subtracted the number of *E. coli* survival from the number of *E. coli* were plated without neutrophils and 1,25(OH)$_2$D$_3$, we obtained *E. coli* killing data. Unexpected, the administration of 1,25(OH)$_2$D$_3$ in neutrophils of SLE patient even reduced *E. coli* killing and we found lower presentation of bacteria lysis of treatment group than control in a dose of $10^{-9}$M (54.17±6.00% vs 35.83±13.25%, p = 0.08), $10^{-7}$M (55.92±12.49% vs 35.83±13.25%, p = 0.012), and $10^{-6}$M (35.83±13.25% vs 35.83±13.25%, p = 0.00) (**Figure 2**). In that diagram, we can see that the pattern of *E. coli* killing inversely proportional with the pattern of cathelicidin induction after treated with six dose of 1,25(OH)$_2$D$_3$.

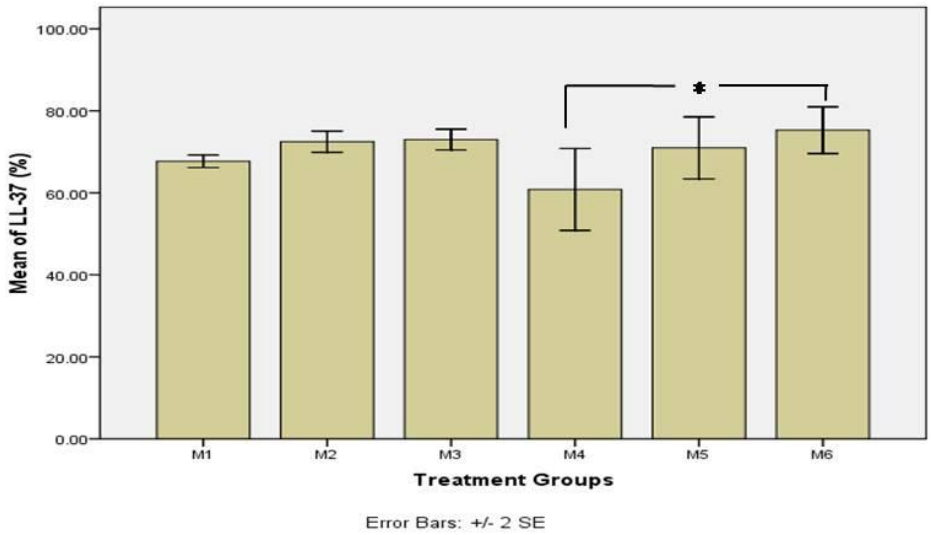


**Figure 1** Expression of LL-37 in neutrophils of SLE patient after administration of six dose of 1,25(OH)$_2$D$_3$ (M1 = 0, M2 = $10^{-10}$M, M3 = $10^{-9}$M, M4 = $10^{-8}$M, M5 = $10^{-7}$M, M6 = $10^{-8}$M). There were higher mean of LL-37 expression among M2 ($10^{-10}$M), M3 ($10^{-9}$M), M5 ($10^{-7}$M), and M6 ($10^{-6}$M) compared to M1 (0). M6 was the highest trend compared to control (M1). There were significant difference between M6 and M3. *significant ($p < 0.05$).

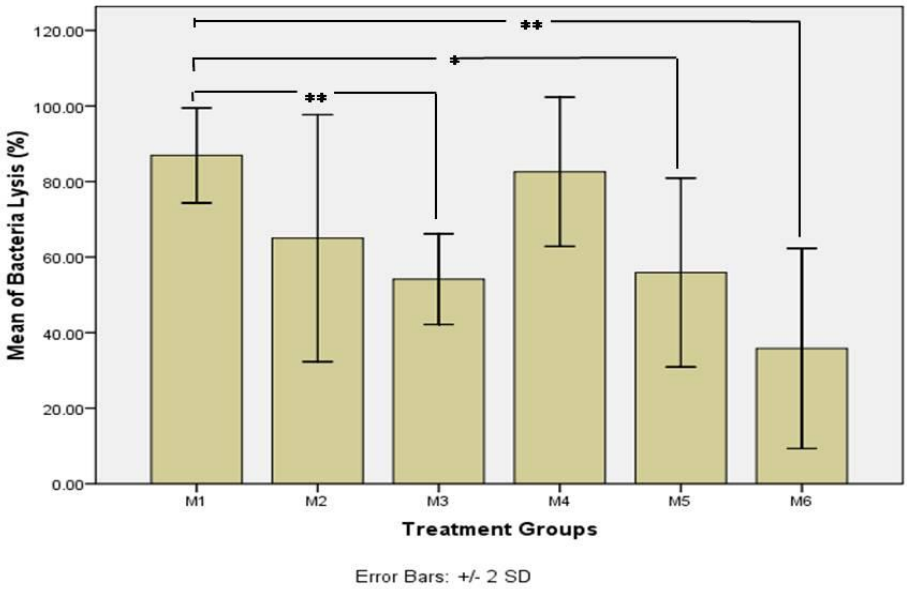


**Figure 2** Colony Forming Unit (CFU) of bacteria (*E. coli*) after administration of six dose of 1,25(OH)$_2$D$_3$ (M1 = 0, M2 = $10^{-10}$M, M3 = $10^{-9}$M, M4 = $10^{-8}$M, M5 = $10^{-7}$M, M6 = $10^{-8}$M). There were significant lower mean of bacteria lysis among M3 ($10^{-9}$M), M5 ($10^{-7}$M), and M6 ($10^{-6}$M) compared to M1 (0). M6 was the lowest trend compared to control (M1). *significant ($p < 0.05$) **significant ($p < 0.01$)

***Reduced killing of E. coli had negative moderate correlation with increasing of cathelicidin expression***

Linked *E. coli* killing with increased cathelicidin expression on neutrophils of SLE patient, we found a negative moderate correlation among them (r=-0.541, p<0.05). It means that if the cathelicidin expression were increased, the *E. coli* killing will be reduced, conversely.

***Cathelicidin induction predicted 29.3% cause E. coli lysis in neutrophils of SLE patient***

The linear regression results showed that the lysis of *E. coli* in neutrophils of SLE patient predicted 29.3% caused by cathelicidin induction. It was mean that 70.7% of *E. coli* killing by neutrophils of SLE patient was predicted to be caused by other factors.

## DISCUSSION

There are two forms of vitamin D (calciferol), vitamin $D_2$ (ergocalsiferol) and vitamin $D_3$ (cholecalsiferol). Both can be found in foods or supplements, but only vitamin $D_3$ is produced in the skin. Vitamin $D_2$ and vitamin $D_3$ have different structures, but these differences did not affect the metabolism (activation) in the body and they both function as a prohormone [25].

Vitamin D from food are absorbed along with fat absorption in the intestine, joined with chylomicrons, and transported through the lymphatic system into the venous circulation. Vitamin D in the skin or derived from food can be stored in fat cells and are released from the cell. Vitamin D in the circulation is bound to vitamin D binding protein (VDBP), which is then taken to the liver, where vitamin D is converted by vitamin D-25-hydroxylase to 25-hydroxyvitamin D {25(OH)D}. This form of 25(OH)D in the circulation is what is used to determine the vitamin D status [25].

Experts agree that the levels of 25(OH)D in the body if less than 20 ng/ml is said deficiency and if the levels are 21-29 ng/ml is said insufficiency. The level of vitamin D in children and adults should be kept at levels above 30 ng/ml in order to obtain the maximum benefit of vitamin D on health [26]. In this study, the vitamin D level of SLE patient is less than 20 ng/ml, so it must be increase to obtain the maximum benefit of vitamin D on health, consider that low levels of vitamin D in SLE patients was associated with increased disease activity [14].

Biologically, 25(OH)D is inactive form and must be converted in the kidney into the active form 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] by 25-hydroxyvitamin D-1α-hydroxylase (1α-OHase). The cells in the immune system may express the 1α-OHase enzyme so they can hydroxilate 25(OH)D from the circulation into 1,25$(OH)_2$D. 1,25$(OH)_2$D on immune cells will work autocrine or paracrine. Furthermore, 1,25$(OH)_2$D from the circulation into target cells will bind to the vitamin D receptor (VDR) in the cytoplasm and enter the nucleus then act as the transcription factors, and experienced

heterodimerization with the retinoid X receptor (RXR). Complex of 1,25(OH)$_2$D-VDR-RXR binds to vitamin D response elements (VDRE) located in the DNA [27].

When complex 1,25(OH)$_2$D-VDR-RXR binding to the VDRE in the CAMP (cathelicidin gene expression in humans) the synthesis of cathelicidin will increase. Cathelicidin are small cationic peptides that measuring 12-100 amino acids and has a broad-spectrum antimicrobial activity. Cathelicidin is referred to as the body's natural antibiotic and mature form in human known as LL-37 [23].

In this study, the effect of administering 1,25(OH)$_2$D$_3$ on neutrophils of SLE patient can induce the cathelicidin expression as the body's natural antibiotic, although statistically was not significant between treatment groups and control. The pattern of cathelicidin expression in this research were increase, decrease in the dose of $10^{-8}$ M, then increase again along with increasing dose of 1,25(OH)$_2$D$_3$. Dose of $10^{-6}$M was the highest trend compared to control and there was a significant difference between dose $10^{-8}$M and $10^{-6}$M. This probably caused by something matter in the dose of $10^{-8}$M and further study is needed.

After infected with *E. coli*, the bacterial killing were reduced and showed the opposite pattern with the cathelicidin induction. This was possible caused by the formation of NETs as the last defence of neutrophils. The cathelicidin activity was reduced when associated with NETs. Indeed, the presence of DNA reduced the antimicrobial activity of cathelicidin [24].

The administration of 1,25(OH)$_2$D$_3$ is able to cause inhibition of genes transcription of encoding proteins gp91 phox (Cybb) which is the component of the PHOX. PHOX itself is a key enzyme in the formation of the NETs so that the PHOX inhibition can reduce the spending of NETs [10]. In addition, 1,25(OH)$_2$D$_3$ is also capable to inhibit the mammalian target of rapamycin of (mTOR) through its interaction with VDR, increasing the gene transcription of phosphatase and tensin homolog (PTEN), and increasing the gene transcription of DNA-damage-inducible transcript 4 (DDIT4) so the formation of NETs can be derived. This is cause bactericidal effect of the NETs also be decreased [20, 21].

The optimal vitamin D level in serum was > 30 ng/ml (> 75 nM). It means that dose 1 nM equals with 0.4 ng/ml [28]. Previous studies described that 1,25(OH)$_2$D$_3$ at physiological concentration (0.1 nM) is inactive and has less potential effect on the immune response regulation of SLE patients. 1,25(OH)$_2$D$_3$ at 10 nM (4 ng/ml) is effective at physiological concentration and at 100 nM (40 ng/ml) give a toxic effect on the immune response regulation of SLE patients because the nutrient response threshold has been reached [29].

Current study showed that at a dose of 10 nM, which should provide an optimal effect on the immune response regulation of SLE patients, did not cause a significant decrease in

bacterial lysis. This decreasing lysis of bacteria was significant at physiological dose and toxic doses. This means that the use of optimal dose (10 nM) of vitamin D on SLE patient will not cause adverse effects in reducing bacterial lysis or in other words safe to use.

This study showed that the lysis of *E. coli* in neutrophils of SLE patient predicted 29.3% caused by cathelicidin induction. It was mean that 70.7% of *E. coli* killing by neutrophils of SLE patient was predicted to be caused by other factors, like ROS from NADPH oxidase, α-defencin, β-defencin, lyzozim, lactoferin, ROI, proteolytic enzyme, cathepcin G, cationic protein, etc. Cathelicidin localization in response to bacteria was independent of the NADPH oxidase, whereas killing was partially dependent on a functional NADPH oxidase [9, 24].

## CONCLUSION

Administration of 1,25(OH)$_2$D$_3$ in neutrophils of SLE patient induce cathelicidin quantitatively, but reduced cathelicidin qualitatively (reduced *E. coli* killing). This result may reveal the possibility of side effect of administering 1,25(OH)$_2$D$_3$ in SLE patient, especially on physiologic dose and lethal dose of 1,25(OH)$_2$D$_3$.

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