Effect of corticosteroid and vitamin D3 as combined therapy on 25 (OH) vitamin D serum level and regulatory T (TReg) cells population in children with idiopathic nephrotic syndrome

Krisni Subandiyah,* Harinda Khanifa, Astrid Kristina Kardani

ABSTRACT

Background: Nephrotic syndrome (NS) is a chronic disease in children that correlates with T lymphocyte dysregulation (imbalance of regulatory T cells (TReg)/T-helper 17 (Th17) cells ratio). Vitamin D3 serum level was known decreased in children with nephrotic syndrome. Low vitamin D3 serum level can affect the outcome of nephrotic syndrome management with corticosteroid therapy. This study was aimed to investigate the effect of vitamin D as adjuvant therapy to 25 (OH) Vitamin D serum level and TReg population in children with idiopathic nephrotic syndrome.

Method: This study was designed as a randomized clinical trial, double-blind, pre and post-test control group which involved 30 subjects that newly diagnosed as NS. Subjects were divided into two groups, prednisone and vitamin D treated group and prednisone only treated group. TReg population in peripheral blood mononuclear cells (PBMC) was analyzed using flow cytometry. Vitamin D serum level was measured by ELISA method.

Results: Results showed that there was a significant elevation of TReg (independent t-test, $p = 0.001$) and 25 (OH) vitamin D serum level (independent t-test, $p = 0.001$) in prednisone and vitamin D treated group as compared to prednisone only treated group. Pearson testing first group showed that vitamin D level was positively correlated with TReg ($p = 0.332, r = 0.183$). The number of early remissions was higher in group treated with steroid and vitamin D3 as a combined therapy compared to group treated with steroid only (50% vs 20%). Whereas the number of late remission was higher in steroid only group (23% vs 8%).

Conclusions: We concluded that corticosteroid and vitamin D3 as a combined therapy increase both of TReg and 25 (OH) vitamin D level which affect glucocorticoid therapy response in subjects. The number of early remission was higher in group treated with corticosteroid and vitamin D3 as a combined therapy.

Keywords: 25 (OH) vitamin D, regulatory T (Treg) cells, corticosteroid, vitamin D3, nephrotic syndrome


INTRODUCTION

Idiopathic nephrotic syndrome is the most glomerular disease which characterized by several clinical manifestations such as severe proteinuria, hypoalbuminemia, hyperlipidemia, and edema.1,2 Prevalence of nephrotic syndrome was 12-16 cases per 100,000 children, and the peak was occurred at 2-5 years old. In Indonesia, the incidence of this disease was 6 cases per 100,000 children per year in children aged less than 14 years old and dominated by male (male : female ratio 2 : 1).1,3

Pathogenesis of nephrotic syndrome still based on immunological aberration which characterized by abundant circulating factor and immuno-regulatory imbalance.4-5 Regulatory T-cells (TReg) is an important tolerogenic T-cells which possessed protective effect on podocyte destruction.4,5

Elevated vascular permeability and angiogenesis are pathologic manifestations of nephrotic syndrome which caused by IL-6-induced vascular endothelial growth factor (VEGF) production. IL-6 and transforming growth factor-β (TGF-β) would lead to differentiation of IL-17 secreting cells (Th17) and inhibit TGF-β-induced TReg differentiation, suggesting that IL-16 played an important role in TReg/Th17 equilibrium.6 Vitamin D had been known as immune-modulator and possessed pleiotropic effects such as induction of TReg differentiation,8,10 inhibition of Th1 and Th17 effector cells,9,11 induction of immature and tolerogenic dendritic cells,12 inhibition of memory B cell formation and plasma cell differentiation.9,13

This study was aimed to investigate the effect of vitamin D as adjuvant therapy to 25 (OH) Vitamin D level and TReg cells population in pediatric nephrotic syndrome.
METHODS

Study Design
This study was designed as a randomized clinical trial (RCT) double blind, pre and post-test control group. There were two groups namely G1 (prednisone and vitamin D3) and G2 (prednisone only). TReg, and vitamin D level were measured before and after treatment. Treatment for G1 was prednisone 2 mg/kg body weight/day (maximal dose 80 mg/day) and vitamin D3 oral preparation 2000 IU (D-Vit, PT Gracia PharmindoTM) 2000 IU/day for four weeks. Treatment for G2 was prednisone 2 mg/kg body weight/day (maximal dose 80 mg/day) for four weeks according to ISKDC protocol. All the procedure and treatment of this study had been approved by Ethical Committee Faculty of Medicine, Brawijaya University, Malang.

SUBJECTS
As many as 30 subjects were included in this study (15 subjects G1, 15 subjects G2). Subjects were taken from Pediatric Nephrology Outpatient Care and Pediatric Ward, dr. Saiful Anwar Public Hospital, Malang during January - December 2016. Inclusion criteria for this study were the newly diagnosed nephrotic syndrome, age 1-14 years old, and allowed by his/her parents (informed consent). Exclusion criteria for this study were the secondary nephrotic syndrome, congenital nephrotic syndrome, relapse nephrotic syndrome, and steroid dependent nephrotic syndrome.

Isolation of Peripheral Blood Mononuclear Cells (PBMC)
Blood sample in EDTA vacutainer was mixed until homogeneous before added PBS 1:1. Blood sample-PBS mixture was taken by using micropipette and transferred slowly into falcon tube wall that already filled with Ficoll-Hipaque d=1.077 g/dl (blood sample-PBS mixture volume: Ficoll-Hipaque volume was 1:1). This mixture was centrifuged at 1500 rpm, room temperature, for 30 minutes and would be resulted in the formation of 4 layers that was plasma, PBMC, Ficoll-Hipaque and erythrocyte. PBMC ring was taken slowly using micropipette and transferred into 15 mL centrifuge bottle. Washed with 10 mL PBS and centrifuged at 1200 rpm, room temperature for 10 minutes. The supernatant was removed, washed using PBS, and centrifuged again at 1200 rpm, room temperature, for 10 minutes. After two times washing and centrifugation process, PBMC formed as a pellet in the bottom of centrifuge bottle.

Measurement of regulatory T-cell population
Measurement of TReg was based on flow cytometry. In this experiment, we used phycoerythrin (PE) anti-human FOXP3, FITC anti-human CD4 and PE/Cy5 anti-human CD25 (eBioscience, San Diego, CA). PBMC was suspended at a certain density (2 × 106 cells/ml) in culture medium (RPMI which equipped with penicillin 100 U/ml, streptomycin 100µg/ml, glutamine 2mM, 10% calf fetal serum). The cell suspension was transferred into 24 wells. Cell culture then stimulated with phorbol myristate acetate (PMA) 50 ng/ml and ionomycin 1µM for 4 hours in monensin 500ng/ml (Alexis Biochemical, San Diego, CA). Incubator temperature set at 37ºC and 5% CO2. After 4 hours culture, cell culture was transferred to a sterile tube and centrifuged at 1500 rpm for 15 minutes.

T-cell lymphocyte was transferred into a new tube, washed with phosphate-buffered saline (PBS), and then incubated with fluorescein isothiocyanate (FITC) anti-human CD4 and PE CD25 at temperature 4ºC for 30 minutes. After incubation, specimens were stained with PE anti-human Foxp3. Specimens were transferred into cuvette ready for flow cytometry analysis. TReg population was analyzed with BD Cell Quest Pro.

Measurements of Vitamin D Level
Measurement of vitamin D level was based on ELISA method as previously described. Briefly, 200µl prediluted serum samples were added to each well and then incubated for 2 hours at 25ºC. After washing process, 100 µl enzyme conjugate was added and incubated for 30 minutes at room temperature. After washing process, 100µl chromogen/ substrates solution was added and incubated for 15 minutes at room temperature and dark room. Finally, 100µl stop solution was added to each well. After 30 minutes, specimens were ready for analysis using ELISA reader at 650 nm.

Statistical analysis
The statistical test was based on data distribution and homogeneity. Statistical differences of TReg, and vitamin D level between groups were analyzed by independent t-test. Correlation of TReg and vitamin D level was analyzed by Pearson correlation test. Data were analyzed at 95% confidence interval (α=0,05) using SPSS for Windows version 19.0.

RESULTS

Subject Characteristics
The subject characteristics such as age, sex, vitamin D status, outcomes (steroid sensitive or resistant),
### Table 1  Baseline Characteristic

<table>
<thead>
<tr>
<th>Sample Characteristic</th>
<th>Prednisone + Vit D (n = 15)</th>
<th>Prednisone (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-≤ 5 years old</td>
<td>6 (6/30)</td>
<td>0</td>
</tr>
<tr>
<td>&gt;5 -&lt;10 years old</td>
<td>11 (11/30)</td>
<td>10 (10/30)</td>
</tr>
<tr>
<td>≥ 10-14 years</td>
<td>0</td>
<td>3 (3/30)</td>
</tr>
<tr>
<td>Sex:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boys</td>
<td>12 (12/30)</td>
<td>11 (11/30)</td>
</tr>
<tr>
<td>Girl</td>
<td>3 (3/30)</td>
<td>4 (4/30)</td>
</tr>
<tr>
<td>Vitamin D status :</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
<td>9 (9/30)</td>
</tr>
<tr>
<td>Insufficiency</td>
<td>11 (11/30)</td>
<td>8 (8/30)</td>
</tr>
<tr>
<td>Deficiency</td>
<td>1 (1/30)</td>
<td>1 (1/30)</td>
</tr>
<tr>
<td>Nutritional status:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well nourished</td>
<td>13 (10/30)</td>
<td>13 (11/30)</td>
</tr>
<tr>
<td>Undernourished</td>
<td>2 (2/30)</td>
<td>2 (2/30)</td>
</tr>
</tbody>
</table>

### Table 2  Clinical Outcome

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Prednisone + Vit D (n = 15)</th>
<th>Prednisone (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSNS</td>
<td>15 (15/30)</td>
<td>13 (13/30)</td>
</tr>
<tr>
<td>RSNS</td>
<td>0</td>
<td>2 (2/30)</td>
</tr>
<tr>
<td>Remission</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early</td>
<td>13 (13/30)</td>
<td>6 (6/30)</td>
</tr>
<tr>
<td>Late</td>
<td>2 (2/30)</td>
<td>7 (7/30)</td>
</tr>
<tr>
<td>No remission</td>
<td></td>
<td>2 (2/30)</td>
</tr>
</tbody>
</table>

### Table 3  TReg Percentage Before Treatment, After Treatment, and Its Enhancement After Treatment

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th>Prednisone + Vit D</th>
<th>Prednisone</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treg Percentage (%)</td>
<td>Group</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td></td>
</tr>
<tr>
<td>Pretest</td>
<td>3.44 ± 2.55</td>
<td>3.66 ± 1.80</td>
<td>0.791</td>
<td></td>
</tr>
<tr>
<td>Posttest</td>
<td>17.27 ± 6.7</td>
<td>7.85 ± 7.52</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Deviation</td>
<td>13.82 ± 6.04</td>
<td>4.19 ± 7.40</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

### Table 4  Vitamin D level before treatment, after treatment, and its elevation after treatment.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th>Prednisone + Vit D</th>
<th>Prednisone</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vit D level ng/mL</td>
<td>Group</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td></td>
</tr>
<tr>
<td>Pretest</td>
<td>14.87 ± 7.47</td>
<td>27.25 ± 14.99</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>Posttest</td>
<td>46.34 ± 14.19</td>
<td>40.66 ± 13.96</td>
<td>0.281</td>
<td></td>
</tr>
<tr>
<td>Deviation</td>
<td>31.46 ± 9.69</td>
<td>13.43 ± 17.25</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>
Clinical outcomes of subjects were shown in Table 2. Remission before four weeks and remission after four weeks were found in both of group but earlier in G1. Most of subjects were steroid sensitive nephrotic syndrome (SSNS), only two patients did not get remission and classified as steroid resistant nephrotic syndrome (SRNS).

and nutritional status were shown in Table 1. Clinical outcomes of subjects were shown in Table 2. Remission before four weeks and remission after four weeks were found in both of group but earlier in G1. Most of subjects were steroid sensitive nephrotic syndrome (SSNS), only two patients did not get remission and classified as steroid resistant nephrotic syndrome (SRNS).

### Table 5 Correlation of TRegand Vitamin D Level

<table>
<thead>
<tr>
<th>Variable correlation</th>
<th>n</th>
<th>Correlation Coefficient (r)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treg elevation associated with elevation of Vit D level</td>
<td>30</td>
<td>0.183</td>
<td>0.332 &gt;</td>
</tr>
</tbody>
</table>

Figure 1 TReg percentage before (pretest) and after (post test) treatment. Treg mean percentage in G1 (3.44 ± 2.55%) and G2 (3.66 ± 1.80%) on pretest evaluation and Treg mean percentage in G1 (17.27 ± 6.7%) and G2 (7.85 ±7.52%) on post test evaluation.

Figure 2 TReg percntation on new nephrotic syndrome before and after prednisone treatment. Above: R2 are PE stained CD4 cell before treatment. The upper right figure is CD4CD25+Foxp3+ percentation which stained using FITC before treatment. Below: R2 is PE stained CD4 cell after treatment. The lower right figure is CD4CD25+Foxp3+ percentation which stained using FITC after treatment.

Figure 3 TReg percentage before (pretest) and after prednisone and vitamin D treatment. Above: R2 are PE stained CD4 cell before treatment. The upper right figure is CD4CD25+Foxp3+ percentation which stained using FITC before treatment. Below: R2 is PE stained CD4 cell after treatment. The lower right figure is CD4CD25+Foxp3+ percentation which stained using FITC after treatment.

Figure 4 Vitamin D level before treatment, after treatment, and its elevation after treatment. Vitamin D level in G1 (14.87 ±7.47) and G2 (27.25 ± 14.99) on pretest evaluation and Vitamin D level in G1 (46.34 ± 14.19) and G2 (40.66 ± 13.96) on post test evaluation. Vitamin D level before treatment, after treatment, and its elevation after treatment. Vitamin D level in G1 (14.87 ±7.47) and G2 (27.25 ± 14.99) on pretest evaluation and Vitamin D level in G1 (46.34 ± 14.19) and G2 (40.66 ± 13.96) on post test evaluation. Four weeks were found in both of group but earlier in G1. Most of subjects were steroid sensitive nephrotic syndrome (SSNS), only two patients did not get remission and classified as steroid resistant nephrotic syndrome (SRNS).
Regulatory T cell population
The result showed that the degree of elevation Treg was higher in G1 compared to G2 (before and after treatment, independent t-test, \( p = 0.001 \)). Table 3 and Figure 1-3 showed TReg percentage before treatment, after treatment, and its enhancement after treatment.

Vitamin D level
The result showed that the degree of elevation vitamin D was higher in G1 compared to G2 (before and after treatment, independent t-test, \( p = 0.001 \)). Table 4 and Figure 4 showed vitamin D level before treatment, after treatment, and its elevation after treatment.

Correlation of TReg and vitamin D level
The result showed that TReg percentage was positively correlated with vitamin D level (\( p = 0.332, \tau = 0.183 \)).

DISCUSSION
Baseline Characteristics
Based on the age factor, data showed that the most age distribution of subjects was less than 10 years old. 30 subjects included in this study, 23 was a male. This finding was also in accordance with the previous study and had been considered to be correlated with abnormal T cells clone in male thymus gland. Nutritional status revealed that most subjects were in good nutrition. However, it is important to evaluate the nutritional status of children with nephrotic syndrome because they were at high risk to be suffered from malnourished.

Vitamin D status in nephrotic syndrome patients (21 of 30) was at a low level. This result was accordance with the previous study conducted in General Hospital dr. Cipto Mangunkusumo which had been reported that 22 of 26 nephrotic patients had low vitamin D level (10 insufficiency, 16 deficiency). Loss of vitamin D-bounded protein through urine had been considered as an etiologic factor of its low plasma concentration in nephrotic patient. Low vitamin D level caused hyper-reactivity dendritic cell, Tcell and Bcell; TReg suppression and elevated pro-inflammatory cytokines which would lead to nephrotic syndrome. Several factors which affect 25(OH)D level such as age, race, season, and milk consumption.

Vitamin D and TReg Population
There was significant elevation TReg in prednisone and vitamin D treated group as compared to prednisone only treated group. Reduction of TReg count and its dysfunction in nephrotic syndrome would lead to inability to suppress effector T cells and associated with degree of proteinuria. TReg was also act as anti-inflammatory T cells through secretion of several anti-inflammatory cytokines such as IL-10 and TGF-β. Vitamin D administration could induce and stimulate TReg directly through antigen presenting cells or dendritic cells and indirectly through endocrine or intracrine conversion 25(OH)D became 1,25(OH)2D. Furthermore, vitamin D administration was correlated with elevation of TReg Foxp3+ count. Several mechanisms which focused on how vitamin D affects TReg had been studied. Administration of 1,25(OH)2D3 could enhance STAT5 phosphorylation in Foxp3+ cells via TGF-β and IL-2 stimulation which in turn would lead to TReg differentiation. Conversely, low vitamin D level would lead to IL-6 upregulation instead of TGF-β downregulation which in turn could cause Th17 differentiation.

Glucocorticoid had been known as anti-inflammation drugs which induce T cell apoptosis, induce T cell anergy, and suppress T cell function. Furthermore, glucocorticoid could induce IL-10 upregulation in immature dendritic cells or macrophage thus induce differentiation of TReg/ suppressor T cells. Adjuvant therapy vitamin D3 could potentiate immunosuppressive effects of TReg through upregulation of Foxp3 and IL-10.

Correlation of TReg and Vitamin D Level
This study had been proved that vitamin D have positively correlated with TReg. Zhou and colleagues (2008) had been reported that Th17 cytokines level such as IL-17 and IL-23 were elevated in nephrotic patients and this condition was related to glomerular destruction, proteinuria, and hypoalbuminemia. Furthermore, imbalance of Th17/TReg population which represents pro-inflammatory condition occurred in patients with minimal change nephrotic syndrome. Our data are suggesting that vitamin D played an important role in the regulation of TReg cells.

Limitation of Study
Several factors as follow could affect the result of study such as less subject number, other factors which could affect vitamin D level (nutrition intake such as milk, sun exposure), other biomolecular factors that didn't studied such as IL-23, vitamin D receptor, and CD80.

CONCLUSION
We concluded that there was a significant elevation of Vit D serum level and TReg in prednisone and vitamin D treated group as compared to prednisone only treated group. The number of early remission
was higher in group treated with corticosteroid and vitamin D3 as a combined therapy.

CONFLICT OF INTEREST

All authors declare there is no conflict of interest regarding publication of this manuscript.

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REFERENCES