

Level of 25(OH)D Serum, Expression of Interleukin 4 and Glucocorticoid Receptor of Mononuclear Cell in Steroid Resistance Nephrotic Syndrome Children

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ABSTRACT

Nephrotic syndrome (NS) is an autoimmune disease and its steroid resistance status supposed correlate with 25(OH)D level and IL-4 expression. The aimed of this study was investigated 25(OH)D plasma level, IL-4 and GR expression of PBMC in steroid sensitive and resistant pediatric NS patients and the association of those parameters. 27 subjects were divided into three groups (control group, steroid resistant NS group, and steroid-sensitive NS group). Peripheral blood mononuclear cells (PBMCs) isolated using the Ficoll-Hypaque method. Plasma 25(OH)D level was measured using ELISA method. IL-4 and GR expression were measured using flow cytometry of PBMCs. This study showed that 25(OH)D level and GR expression were significantly different in the control group compared to steroid resistant NS group ($p < 0.05$). Plasma 25(OH)D level, IL-4, and GR expression were not correlated each other in NS patients ($p > 0.05$). Plasma 25(OH)D level, IL-4 and GR expression were not contributed to steroid resistance in NS patients. However, GR expression has the highest contribution to steroid resistance of NS patient (Wald score 1.198). Plasma 25(OH)D level and GR expression were lower in steroid resistant NS group. GR expression has the highest contribution in steroid resistance of NS patients.

Keywords: 25(OH)D level, GR expression, IL-4 expression

INTRODUCTION

Nephrotic syndrome (NS) defined as a glomerular disease that characterized by proteinuria, edema, and hypoalbuminemia. This disease could affect all ages, especially 2-6 years old child with male: female ratio [1]. Nephrotic syndrome occurred 2-7 new case per 100.000 children [2]. Previous data had been reported that there was 101 case (34%) idiopathic NS from total 297 nephrology disease at Pediatric Department of Saiful Anwar Hospital [3].

Idiopathic NS is an autoimmune mediated disease that both steroid sensitive and steroid resistant possessed T cell dysfunction, decreased of 25(OH)D level, and glucocorticoid receptor (GR) expression. Nephrotic syndrome caused by an imbalance of Th1 and Th2 cytokines that could trigger podocyte dysfunction and massive proteinuria [4]. IL-4 and IL-13 could induce proteinuria in idiopathic NS patient [5].

A previous study showed that glucocorticoid receptor (GR) expression increase in the remission stage of NS and decreased GR expression in steroid resistant NS compared with steroid sensitive NS [6]. Furthermore, GR expression negatively correlated with histopathological damage of glomerulus [7].

25(OH)D level in NS patients also decrease caused by renal function impairment. Takeda has been described that an active form of vitamin D3 could modulate the immune response [8]. Vitamin D3 could reverse GR downregulation induced by ligand through its interaction with GC signaling pathway [8]. The role of vitamin D3 in immune response had been studied in steroid resistant asthmatic patients. A study showed that administration active form of vitamin D3 could increase IL-4, IL-5, IL-13 expression by Th2 and IL-10 and TGF by Treg [9].

Previous study showed enhancement of various cytokines production such as IL-1 IL-2, IL-4 and TNF- α , and also decreased of IL-10 production in minimal changes NS patient's peripheral blood mononuclear cells (PBMC) [10].

Its immunomodulatory effects of vitamin D could

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suppress Th1: IFN- γ ; IL-2; Th17: IL-17 IL-1; IL-6; TNF- α ; IL-12; IL-23 secretion, suppression of Treg: IL-10; TGF- β and also enhancement of Th2: IL-4; IL-5; IL-13 secretion [11]. In other hands IL-4 and IL-2 decrease GR α without affect GR β , which further concluded that steroid resistance induced by IL-4 and IL-2 and marked by decreased expression of GR α [12].

This study was conducted to investigate 25(OH)D plasma level, IL-4 and GR expression of PBMC in steroid sensitive and resistant pediatric NS patients and their association.

MATERIALS AND METHODS

Study Design

This study was cross-sectionally designed to measure vitamin D (25(OH)D) plasma level, GR and IL-4 expression of PBMC in steroid sensitive NS, steroid resistant NS, and control group. This study was conducted in nephrology polyclinic and ward of Pediatric Department, Dr. Saiful Anwar Hospital Malang, and Biomedical Laboratory, Faculty of Medicine, Brawijaya University Malang. This study had been approved by Ethical Committee of Medical Faculty of Medicine, Brawijaya University.

Population and Subject

Research subject included in this study was nine subjects in each group. Inclusion criteria of topics are classified as steroid sensitive and steroid resistant NS aged 1-14 years old and allowed by his/her parents to join in this study (informed consent). Exclusion criteria of subjects is NS which classified as secondary NS (caused by extra-renal factor such as chemical/drugs, systemic disease such as SLE or Henoch Schonlein, infectious disease such as malaria, hepatitis), congenital/infantile NS, patient with autoimmune disease (SLE, DM), patient with allergy (asthma, rhinitis, atopic dermatitis), patients with severe infection, liver dysfunction, and patient with vitamin D supplementation.

PBMC Isolation

Blood sample in Vacutainer EDTA/heparin mixed properly, and then mixed with PBS (1:1). Blood sample-PBS take using micropipette and then overlaid to falcon tube which already contains Ficoll-Hipaque d=1.077 g/mL through its wall (volume sample: Ficoll-Hipaque 1:1). This mixture then centrifuged at room temperature 1000 rpm for 30 min. After centrifuged, this mixture will form 4 layer (plasma, PBMC, Ficoll-Hipaque, and granulocyte or erythrocyte). PBMC ring

has taken slowly using micropipette and then placed in new 15 mL centrifuge bottle. PBMC solution and then washed with PBS 10 mL and centrifuged at room temperature, 1200 rpm for 10 min. Pellet washed again with PBS and then centrifuged at room temperature, 1200 rpm for 10 min (this process is repeated two times). PBMC formed as a pellet in the bottom of the bottle.

Measurement of GR Expression in CD3

PBMC's pellet was added with PE-conjugated anti-human CD357 (GITR) antibody (catalog number 311604) and then incubated for 15 min in the dark room. After that, the pellet was centrifuged at 2500 rpm for 3 min. Then, the pellet was added to one mL fixation buffer solution in each tube, centrifuged at 2500 rpm, 4°C for 3 min. The supernatant was removed. Cell pellet that has been fixated then resuspended by added 1x permeabilization washed buffer and centrifuged at 2500 rpm, 4°C for 3 min. After this process, the cell pellet was resuspended in added 0,5 ml cell staining buffer and then analyzed using flow cytometry. Expression of GR analyzed using software BD Cell Quest Pro.

Measurement of IL-4 Expression

PBMC's pellet was added with PerCP/Cy5.5 anti-human IL-4 (catalog number MP 4-25D2) and then incubated in the dark room for 15 min. After that, the pellet was added to 1 mL cell staining buffer and then centrifuged for 3 min at Mystral-1000 rt and hatch centrifuge at 2500 rpm, room temperature continuously. Supernatant was removed, then added with 1 ml fixation buffer, incubated for 15 min in dark room, room temperature, and centrifuged for 3 min in Mystral-1000 rt and hettch centrifuge at 2500 rpm at room temperature continuously.

The supernatant was removed, the pellet was added with permeable wash buffer and then incubated for 15 min in a dark room at room temperature. After this process, centrifuged again for 3 min in Mystral-1000 rt and hatch centrifuge at 2500 rpm at room temperature continuously. The supernatant was removed, and the pellet was added with cell staining buffer 500 μ L, and then placed in a sample tube for flow cytometry machine, BD FACS Calibur. IL-4 expression analyzed using software BD Cell Quest pro.

Measurement of Plasma 25(OH)D Level

Three polypropylene tube that used for control, sample, and calibrator were prepared. 1 ml 25-D biotin

solution was added to all tube, and then vortex for 10 seconds. Each of calibrator dilution added 200 μ l, control or sample in each appropriate well according to an antibody-coated plate/microplate. The plate was covered with plastic and then incubated in 18-25°C for two hours. All well then washed three times using wash solution. 200 μ l conjugate were added to each well using multichannel pipette. Plate covered again with plastic, incubated in 18-25°C for 30 min (this process repeat 2 times). 200 μ l TMB substrate was added into each well, and then covered with plastic, incubated in 18-25°C for 30 minutes. 100 μ l stop solution was added to each well. Absorbance was measured in each well at 450 nm using microplate reader in 30 min after added stop solution.

RESULTS AND DISCUSSION

Characteristics of Subjects

This study involved 27 subjects divided into three groups: control group, steroid resistant NS group, and steroid-sensitive NS group. The subject characteristic such as sex, age, nutrition status described in Table 1.

Level of Plasma 25(OH)D

Figure 1 showed 25(OH)D level was 23.86 ng/mL for the control group, 15.50 ng/mL for steroid sensitive NS group, and 10.88 ng/mL for steroid-resistant NS group. One way ANOVA showed significant differences between groups ($p < 0.05$). Post Hoc Tukey test showed significant differences between the control group and steroid resistant group ($p = 0.003$).

IL-4 Expression

Figure 2 showed IL-4 expression was 2.89% for the control group, 0.86% for steroid-resistant NS group, and 1.09% for steroid-sensitive NS group. Non-parametric test Kruskal-Wallis showed no significant differences among groups ($p = 0.095$).

GR Expression

Figure 3 showed GR expression was 3.86% for the control group, 1.74% for steroid-resistant NS group, and 2.66% for steroid-sensitive NS group. One way ANOVA showed significant differences between groups ($p < 0.05$). Post Hoc Tukey test showed significant differences between the control group and steroid-resistant group ($p = 0.008$).

Correlation of Plasma 25(OH)D Level, IL-4 and GR Expression

Pearson correlation test showed no significant cor-

relation between 25(OH)D level and IL-4 expression ($p > 0.05$, $r = 0.382$), 25(OH)D level and GR expression ($p > 0.05$, $r = 0.417$), IL-4 expression and GR expression ($p > 0.05$, $r = 0.271$). Logistic regression test showed 25(OH)D level, IL-4 expression, and GR expression has no significant contribution for steroid resistance. However, from 3 parameters, GR expression has highest Wald score (1.198) which mean GR expression is the most significant predictor for steroid resistance in NS patients.

Characteristics of Subjects

Data showed a range of patient age was 5-10 years old for steroid sensitive NS, 10-14 years old for steroid-resistant NS. This result consistent with the previous study reported that 75% NS aged less than 10 (ten) years old [2].

This study showed in NS groups dominated by the female. Epidemiological data showed in children aged younger than 8 (eight) years old at the onset of disease, male: female ratio varied from 2:1 until 3:2. However, in older children, male: female ratio almost same. Other data indicated that 66% patients with MCNS (minimal changes nephrotic syndrome) or FSGS (focal segmental glomerulosclerosis) was male, but 65% patients with MPGN was female [16]. Berg and Wening [10] speculated that a higher prevalence of NS in the male might be correlated with abnormal T cell clone that frequently occurred in thymus gland [10].

Nutritional status showed in all three groups has good nutrition status. NS patients possessed higher risk factor for developmental impairment caused by NS itself or its complication [13].

Plasma 25(OH)D Level, IL-4 and GR Expression

Data showed that plasma 25(OH)D level in the control group significantly higher than NS groups.

Table 1. Characteristic of subject

Characteristics	Control (n=9)	SSNS (n=9)	S R N S (n=9)
a. Age (year)			
1 - \leq 5 years old	0 (0/9)	2 (2/9)	1 (1/9)
5 - \leq 10 years old	5 (7/9)	5 (5/9)	2 (2/9)
10 - 14 years old	4 (4/9)	2 (2/9)	6 (6/9)
b. Sex			
- Male	1 (1/9)	4 (4/9)	3 (3/9)
- Female	8 (8/9)	5 (5/9)	6 (6/9)
c. Nutritional Stat			
- Over-nutrition	2 (2/9)	1 (1/9)	0 (0/9)
- Good-nutrition	9 (7/9)	7 (7/9)	8 (8/9)
- Under-nutrition	0 (0/9)	1 (1/9)	1 (1/9)
- Malnutrition	0 (0/9)	0 (0/9)	0 (0/9)

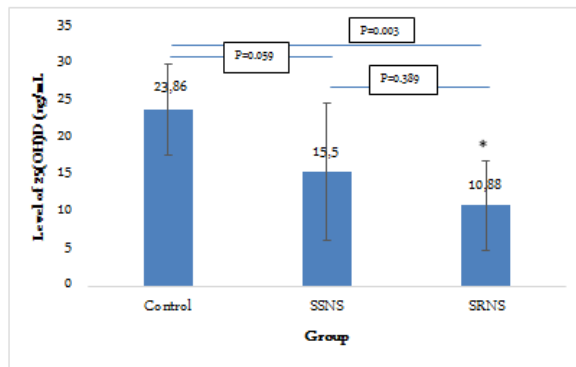


Figure 1. Mean of Plasma 25(OH)D level (ng/mL) in control, steroid resistant NS, and steroid-sensitive NS group.
* Post Hoc Tukey showed significant differences with the control group.

Furthermore, a post hoc test showed that plasma 25(OH)D level significantly different between a control group and steroid-resistant group, but no significant differences between steroid-resistant and steroid-sensitive groups. This results indicated that vitamin D has a role in the development of NS. However, because of limitation of study design (observational), we cannot conclude whether vitamin D caused NS or NS caused vitamin D dysfunction. This result also indicated that vitamin D might be a role for steroid resistance in NS patient.

Cheng et al. [14] reported that dexamethasone combined with vitamin D3 protect podocyte from experimental injury. Colin et al. [15] showed that 1,25(OH)2D3 could inhibit tumor necrosis factor (TNF), interleukin-17 (IL-17) and interferons (IFN) production, and also triggered IL-4, IL -5, and IL-10 expression in PBMC or CD4T-cell.

Calcitriol administration in T cell culture could inhibit proinflammatory cytokine production in Th1, Th9, and Th22 and enhance production of anti-inflammatory cytokines in Th2. Clinically, 1,25(OH)2D3 has the beneficial effect of reducing proteinuria and altered renin-angiotensin-aldosterone (RAAS) system. Histopathologically, 1,25(OH)2D3 could preserve the structural and functional integrity of podocytes and also suppress renin expression directly in transcriptional stage [16].

This study also showed that IL-4 expression lower in the control group compared with NS group and significantly different with steroid resistant NS group. This data indicated that NS group showed activation of Th2-cells. Kanai described that in NS occurred imbalance of Th1 and Th2 ratio whereas Th2 more dominant and cause increased expression of IL-4 and IL-13

[17]. In relapse NS, activated T cell induce IL-4 and IL-13 secretion, which in turn triggered IgE isotype switching [17].

Higher IgE group showed increased of IL-4 and IL-5 expression significantly [25]. [25] further explains that increased of IL-4 and IL-5 production in idiopathic NS could indicate several cytokines involved in IgE production. Allergic treatment with single agent could not induce NS remission, suggested that increased of IgE did not cause NS, but reflected allergic response toward their humoral immunity perturbation [10,25,26].

Pan et al. and [18] consistently agree with Hafez [4] about imbalance of Th1/Th2 and domination of Th2 response in NS [4,18,27]. In MCNS, there was an increased of IL-4, IL-8 and GRO- γ (growth regulated protein) production. IL-8 caused altered permeability of glomerular basement membrane (GBM) through a production of HSPGs (Heparan Sulfate Proteoglycans) which in turn could induce proteinuria in a rat model. The IL-4 and IL-13 receptor also found in podocytes and enhanced expression of both receptors found in MCNS. Overexpression of IL-13 in the animal model caused podocyte damage through transduction signal and activation of the transcriptional pathway. However, the role of IL-4 in podocyte damage still can not be understood [4].

In this study, lower expression of IL-4 in NS groups might be caused by administration of glucocorticoid and cytostatic drugs. The subject included in this study has been a routine visit to pediatric nephrology polyclinic and hospitalized for cytostatic drugs therapy. Because of this, IL-4 expression mean still did not reflect steroid resistant NS, which newly diagnosed. Cytostatic drugs that act in T lymphocyte and usually used in NS is cyclophosphamide, chlorambucil or cyclosporine. Cyclophosphamide and chlorambucil are alkylating agents and cross-linked DNA during all cell

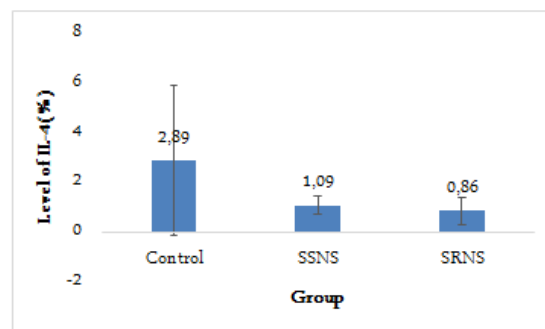


Figure 2. Mean of PBMC's IL-4 expression (%) in control, steroid-resistant NS, and steroid-sensitive NS group.

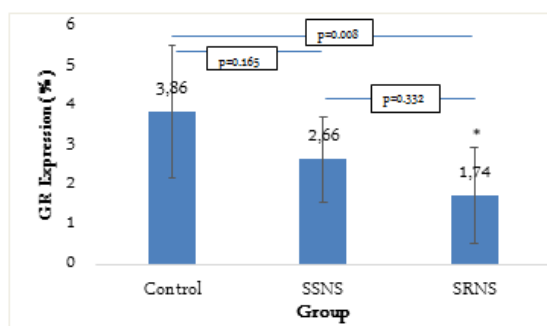


Figure 3. Mean of PBMC's GR expression (%) in control, steroid-resistant NS, and steroid-sensitive NS group.
* Post Hoc Tukey showed significant differences with the control group.

cycle phase. Cyclosporine is calcineurin inhibitor that blocked the production of several cytokines such as IL-2, IL-3, IL-4 and interfered IL-2 receptor expression (CD25) which in turn prevent T cell activation [20].

Other possible cause-related with the role of Th1/Th2 ratio in the pathogenesis of NS. Glucocorticoid treatment could change Th1/Th2 ratio through suppression of proinflammatory cytokines and induced of anti-inflammatory cytokines as described by Barnes (2010) [19]. Barnes stated that GC treatment could trans-repressed inflammatory cytokines (IL-2, IL-3, IL-4, IL-5, IL-6, IL-23, IL-15, TNF- α , GM-CSF, SCF, TSLP); chemokines (CCL1, CCL5, CCL11, CXCL8); inflammatory enzymes: iNOS (inducible nitric oxide synthase), COX-2 (inducible cyclo-oxygenase), cPLA2 (inducible phospholipase A2); inflammatory peptides (endothelin-1); mediator receptor: NK1 (neurokinin), bradykinin (B2)-receptor; adhesion molecules (iCAM-1, VCAM-1) and also trans-activated lipocortin-1; B2-Adrenoceptors; Secretory leukocyte inhibitory protein; I κ B- α (inhibitor NF- κ B); MKP1 (inhibitor MAP kinase); Glucocorticoid inducible leucine zipper (GILZ); anti-inflammatory cytokines or inhibitor: IL-10, IL-12, IL-1 receptor antagonist [19].

This study also showed lower GR expression in steroid-resistant NS group compared with control and steroid-sensitive NS group. Among three variables, GR has the highest contribution to steroid resistance in NS patients. This data are consistent with the previous study conducted by [21] which reported that GR density in peripheral T cell was important factor determined GC sensitivity. GR expression could be used as the predictor for steroid responsivity rate in adult MCNS [28]. Wasilewska et al. [6] stated that lower expression of GR caused a worse response to GC

treatment. Carlotti et al. [22] showed that GR might be mediated the occurrence of glucocorticoid resistance in idiopathic NS.

Other study showed that autoregulation of GR was not related with GC response in idiopathic NS patients [23]. GC sensitivity depends on functionality and expression of its receptor. GR expressed almost in all cells, include convoluted distal tubule cells, collecting duct cells, and all part of glomerular cells. GC cellular response directly correlated with the hormone-binding capacity [24].

This study was designed as observational (cross-sectional) that has a limitation in investigating the causal effects. Because of that, we suggested to do further research which developed experimentally both in vivo or in vitro to investigate the role of IL-4, vitamin D, and GR in NS (especially in newly diagnosed steroid resistant NS patients and focused on Th1/Th2 ratio). Further investigation in GR isomer (GR α and GR β) and their role in steroid resistance of NS should be performed. Development knowledge in the pathophysiology of steroid resistance especially in NS could be followed by the development of drugs targeting in molecules that act as the predictor in steroid resistance.

CONCLUSIONS

We conclude that plasma 25(OH)D level, IL-4 and GR expression was lower in steroid resistant NS group. GR expression has the highest contribution to steroid resistance of NS patients.

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