doi: 10.11594/jtls.08.01.08
THE JOURNAL OF TROPICAL LIFE SCIENCE
OPEN ACCESS Freely available online
VOL. 8, NO. 1, pp. 43 – 47, January 2018
Submitted April 2017; Revised May 2017; Accepted December 2017

The Correlation of Regulatory T (TReg) and Vitamin D3 in Pediatric Nephrotic Syndrome

Yunika Nurtyas

Medical Faculty, Brawijaya University, Malang, Indonesia

ABSTRACT

Nephrotic syndrome (NS) is an autoimmune disease that correlates to the imbalance of regulatory T cells (T_{Reg}). This study was aimed to investigate the effect of vitamin D as adjuvant therapy of T_{Reg} population in pediatric nephrotic syndrome. This study was designed randomized clinical trial, double blind, with pre- and post-test control groups involving 15 subjects newly diagnosed with NS. Subjects were divided into 2 groups, namely K1 for group treated with prednisone+vitamin D and K2 group for prednisone treatment only. The population of T_{Reg} in peripheral blood mononuclear cells (PBMC) was analyzed using flowcytometry. Vitamin D serum level was measured through ELISA method. Results showed that there was a significant elevation of T_{Reg} (independent t-test, p = 0.010) in K1 group, which was higher than in K2 group. The Pearson test in the K1 group showed that vitamin D level was positively correlated with T_{Reg} (p = 0.039, r = 0.779).

Keywords: *Nephrotic syndrome, vitamin D, TReg*

INTRODUCTION

Idiopathic nephrotic syndrome is a glomerular disease characterized by several clinical manifestations such as severe proteinuria, hypoalbuminemia, hyperlipidemia, and edema [1, 2]. The prevalence of nephrotic syndrome stands at 12 – 16 cases per 100,000 children, which is mostly found at the age of 2 – 5 years old. In Indonesia, there were 6 cases found in every 100,000 children below 14 years old per year in children, which is dominated by male (male: female ratio = 2 : 1) [3].

Pathogenesis of nephrotic syndrome is based on the immunological aberration, characterized by abundant circulating factor [4] and immuno-regulatory imbalance [5]. Conversely, regulatory T-cells (T_{Reg}) is an important tolerogenic T-cell possessing protective effect on podocyte destruction [6, 7]. Vitamin D has been known as immunomodulator that able to induce T_{Reg} differentiation as it has pleiotropic effects [8, 9]. Therefore, this study was aimed to investigate the effect of vitamin D as adjuvant therapy to the T_{Reg} population in pediatric nephrotic syndrome.

MATERIALS AND METHODS

This study was conducted at Biomedical Laboratory, Faculty of Medicine, Brawijaya University. The duration of the study ranged between February until July 2015.

Study Design

This study was designed as a randomized clinical trial (RCT) double blind, with pre-and post-test control group. There were 2 groups namely K1 (prednisone and vitamin D3) and K2 (prednisone only). T_{Reg} population and vitamin D levels were measured before and after treatment. Treatment for K1 were prednisone 2 mg/kg body weight/day (maximal dose 80 mg/day) and vitamin D3 oral preparation (D-Vit, PT. Gracia Pharmindo™) 2000 IU/day for 4 weeks. Treatment for K2 was prednisone 2 mg/kg body weight/day (maximal dose 80 mg/day) for 4 weeks according to ISKDC protocol. All the procedures and treatments of this study had been approved by the Ethical Committee Faculty of Medicine, University of Brawijaya, Malang No. 314/EC/KEPK-S2/05/2015.

Subjects

There were 15 subjects included in this study (7 subjects in K1; 8 subjects in K2). Subjects were taken from the Pediatric Nephrology Outpatient Care and Pediatric

*Corresponding author:
Yunika Nurtyas
Medical Faculty, Brawijaya University
Jalan Veteran, Malang, Indonesia 65145
E mail: yunikanurtyas@gmail.com

How to cite:
Nurtyas Y (2018) The Correlation of Regulatory T (TReg) and Vitamin D3 in Pediatric Nephrotic Syndrome. J. Trop. Life. Science 8 (1): 43 – 47.

Ward, Dr. Saiful Anwar General Hospital, Malang during February – July 2015. The inclusion criteria for this study were newly diagnosed nephrotic syndrome patients aged 1 – 14 years old whose parents allowed them to participate in this study (informed consent). Meanwhile, the exclusion criteria for this study were secondary nephrotic syndrome, congenital nephrotic syndrome, relapse nephrotic syndrome, and steroid dependent nephrotic syndrome.

Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Blood samples in EDTA vacutainer were homogenized and added with PBS at a ratio of 1 : 1. The blood sample-PBS mixture was transferred slowly into falcon tube walls filled with Ficoll-Hipaque d = 1.077 g/dL (1 : 1). This mixture was centrifuged at 1,500 rpm at room temperature for 30 minutes which resulted in the formation of 4 layers namely plasma, PBMC, Ficoll-Hipaque, and erythrocyte. The PBMC ring was slowly transferred into 15mL centrifuge bottle. It was washed with 10mL PBS and centrifuged at 1200 rpm, at room temperature for 10 minutes. The supernatant was removed, washed using PBS, and centrifuged again at 1,200 rpm at room temperature for 10 minutes. After the second process of washing and centrifugation, PBMC will be formed as pellet at the bottom of centrifuge bottle.

Measurement of regulatory T-cell population

The population of T_{Reg} was measured trough flowcytometry method. Antibodies that were used phycoerythrin (PE) anti-human FOXP3, FITC anti-human CD4, and PE/Cy5 anti-human CD25 (eBioscience, San Diego, CA). PBMC was suspended at certain density (2 × 10^6 cells/mL) in culture medium (RPMI equipped with penicillin 100 U/mL, streptomy-cin 100 µg/mL, glutamine 2 mM, 10% calf fetal serum). The cell suspension was transferred into 24 wells then was stimulated with phorbol myristate acetate (PMA) 50 ng/mL and ionomycin 1 µM for 4 hours in monensin 500 ng/mL (Alexis Biochemical, San Diego, CA). The incubator was set at temperature of 37°C and air pressure of 5% CO_2. After 4 hours, the cell culture was transferred into sterile tubes and centrifuged at 1,500 rpm for 15 minutes.

T-cell lymphocyte was transferred into new tubes, washed with phosphate-buffered saline (PBS), and then incubated with fluorescein isothiocyanate (FITC) anti-human CD4 and PE CD25 at 4°C for 30 minutes. After incubation, speciments were stained with PE anti-human Foxp3. Specimens were transferred into cuvette ready for flowcytometry analysis. The population of T_{Reg} was analyzed with BD Cell Quest Pro.

Measurements of vitamin D level

Vitamin D level was measured through ELISA method as previously described. Briefly 200 µL pre-diluted serum samples were added into each well then it incubated for 2 hours at 25°C. After being washed, 100 µL enzyme conjugate was added into the tubes and incubated for 30 minutes at room temperature. Following this, 100 µL chromogen/substrates solution was added and incubated for 15 minutes at room temperature in dark room. Finally, 100 µL stop solution was added to each well. After 30 minutes, specimens were ready for analysis using ELISA reader at 650 nm.

Statistical analysis

Data distribution and homogeneity were statistically

Table 1. Subject Characteristics

Characteristics		*Combination Prednison+vitamin D (n = 7)*	*Prednisone only (n = 8)*
Age (years)	1 – ≤ 5	6	0
	> 5 - < 10	1	7
	≥ 10 – 14	0	1
Sex	Male	5	7
	Female	2	1
Vitamin D Status	Normal	0	6
	Insufficiency	6	2
	Deficiency	1	0
Nutritional Status	Good	5	6
	Undernutrition	2	2

Note: SSNS (steroid sensitive nephrotic syndrome), SRNS (steroid resistant nephrotic syndrome)

analyzed. Moreover, statistical differences of T_{Reg} and vitamin D levels between groups were analyzed by independent t-test. The differences of T_{Reg}, Th17, and vitamin D before and after treatment were analyzed by paired t-test. The correlation of T_{Reg} and vitamin D level was analyzed with the Pearson correlation test. Data was analyzed at 95% confidence interval (α = 0.05) using *SPSS version 17.0.* for *Windows*.

RESULTS AND DISCUSSION

Subject and baseline characteristics

Subject characteristics such as age, sex, vitamin D status, outcomes (steroid sensitive or resistant), and nutritional status were shown in Table 1. Morover the clinical outcome of subjects were shown in Table 2. Remission before 4 weeks and remission after 4 weeks were found in both groups that earlier was also found in K1. Most of the subjects were diagnosed with steroid sensitive nephrotic syndrome (SSNS), there was only one patient did not get remission and is classified as steroid resistant nephrotic syndrome (SRNS).

Based on the age factor, subjects were mostly from kids aged under 10 years old. The subjects were dominated by male or 12 boys from 15 subjects. This finding was also in accordance with previous studies and has been considered to be correlated with abnormal T cell clones in male thymus gland [10]. Based on nutritional status, it is revealed that most subjects had good nutritional status. However, it is important to evaluate nutritional status of children with nephrotic from syndrome because they are at high risk of suffering from malnourishment.

Vitamin D level status in nephrotic syndrome patients (9 of 15) was low. This result was in accordance with previous study conducted in the General Hospital Dr. Cipto Mangunkusumo that 22 of 26 nephrotic patients had low vitamin D levels (10 insufficiency, 16 deficiency) [11]. Loss of vitamin D-bounded protein through urine had been considered as etiologic factor for low plasma concentration in nephrotic patients [12]. Low vitamin D levels cause hyper-reactivity o dendritic cell, T cell, B cell, T_{Reg} suppression, and pro-inflammatory cytokines elevation [13] that would lead to nephrotic syndrome. Several factors affect 25(OH)D level such as age, race, season, and milk consumption [14].

Regulatory T cell population

The results demonstrated that there was no significant difference in the population of T_{Reg} in K1 and K2 (independent t-test, p = 0.97 pretest) before treatment.

Table 2. Clinical outcome

Outcome	*Combination Prednisone+ vitamin D (n=7)*	*Prednisone only (n=8)*
Classification		
SNSS	6	8
SNRS	1	0
Remission		
Early responder	5	4
Late responder	1	4
Resistance	1	0

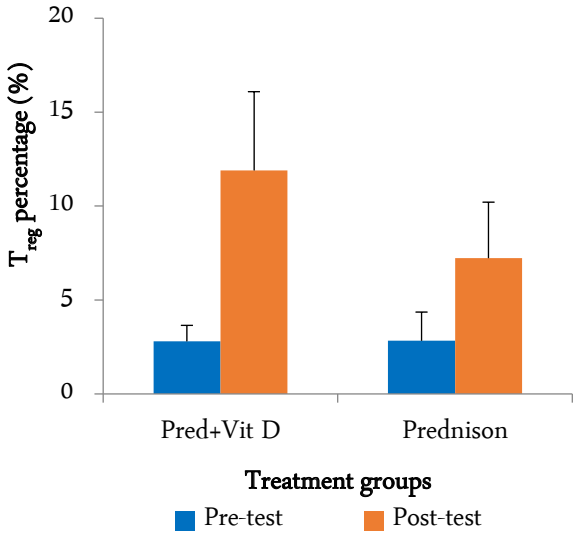

Figure 1. T_{reg} percentage before treatment (pre-test) and after treatment (post-test)

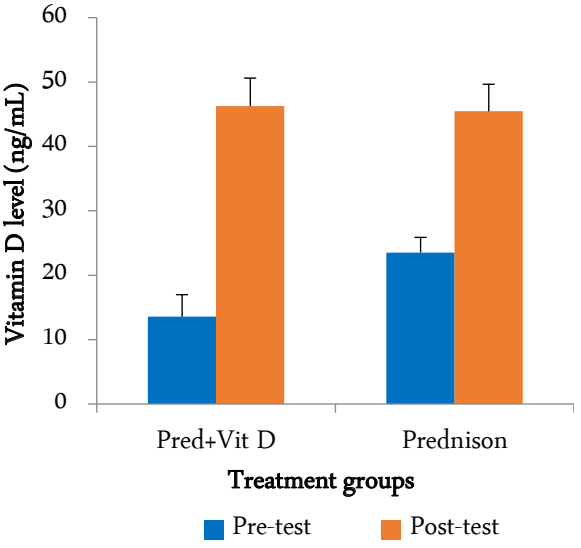

Figure 2. Vitamin D Level before treatment (pre-test) and after treatment (post-test)

However, the differences were found significantly after treatment (independent t-test, p = 0.03 post test). Furthermore, the elevation of the T_{Reg} population in K1 and K2 (before and after treatment, independent t-test, p = 0.01) were significantly different. Figure 1 shows the T_{Reg} percentage before treatment, after treatment, and its enhancement after treatment. Furthermore, the enhancement of T_{Reg} percentage was significantly different in both groups (paired t-test, K1 p = 0.00, K2 p = 0.00).

Vitamin D level and T_{reg} population

The vitamin D level was found higher in K1 than K2 (before and after treatment, independent t-test, p = 0.00). Figure 2 shows vitamin D levels before treatment, after treatment, and its elevation after treatment. Furthermore, the elevation of vitamin D level was significantly different in both groups (paired t-test, K1 p = 0.00, K2 p = 0.00).

The elevation of T_{Reg} population in prednisone and vitamin D treated group was higher than in prednisone only treated group. Reduction of T_{Reg} population and its dysfunction in nephrotic syndrome would lead to disability to suppress effector T cells [15] that is associated to proteinuria [16, 17]. T_{Reg} also acts as anti-inflammatory T cells through secretion of several anti-inflammatory cytokines such as IL-10 and TGF-β [18].

Vitamin D administration could induce and stimulate T_{Reg} directly through antigen presenting cells or dendritic cells also indirectly through endocrine or intracrinal conversion of 25(OH)D becoming 1,25(OH)2 D3 [18]. Furthermore, vitamin D administration was correlated with elevation of T_{Reg} $Foxp3^+$ population [9, 19]. Several mechanisms focused on how vitamin D affects T_{Reg} have been studied. Administration of 1,25(O H)2D3 could enhance STAT5 phosphorylation in $Foxp3^+$ cells via TGF-β and IL-2 that lead to T_{Reg} differentiation [20, 21]. Conversely, low vitamin D levels would lead to IL-6 upregulation instead of TGF-β downregulation causing Th17 differentiation [21].

Glucocorticoid had been known as one of anti-inflamation drugs that induces T cell apoptosis, T cell energy, and suppress T cell function [22]. Furthermore, glucocorticoid could induce IL-10 upregulation resulted in immature dendritic cells or macrophage thus inducing differentiation of T_{Reg}/suppressor T cells [22]. Adjuvant therapy with vitamin D3 could induce immunosupressive effects of T_{Reg} through upregulation of Foxp3 and IL-10 [23, 24, 25].

CONCLUSION

In conclusion, there was significant elevation of T_{Reg} population in the prednisone and vitamin D treated group than in prednisone only treated group. However, the vitamin D level was positively correlated with T_{Reg} population.

ACKNOWLEDGMENT

Authors thank to Brawijaya University for facilitating this research.

REFERENCES

1. Bagga A (2008) Management of steroid sensitive nephrotic: revised guidelines. Indian Journal of Nephrology 18 (1): 31 – 39. doi: 10.4103/0971-4065.41289.
2. Zhang S, Audard V, Fan Q et al. (2011) Immunopathogenesis of idiopathic nephrotic syndrome. In: Hererra GA (ed) Experimental models for renal diseases: Pathogenesis and diagnosis. Basel, Karger. pp 94 – 106. doi: 10.1159/000313 947.
3. UKK Nefrologi Ikatan Dokter Anak Indonesia (2008) Tatalaksana sindrom nefrotik idiopatik pada anak. Jakarta, Badan Penerbit Ikatan Dokter Anak Indonesia.
4. Hafez MA, Shimada M, Lee PY et al. (2009) Idiopathic nephrotic syndrome and atopy: Is there a common link?. American Journal of Kidney Disease 54 (5): 945 – 953. doi: 10.1053/j.ajkd.2009.03.019.
5. Wang L, Li Q, Wang L et al. (2013) The role of Th17/IL-17 in the pathogenesis of primary nephrotic syndrome in children. Kidney and Blood Pressure Research 37 (1): 332 – 345. doi: 10.1159/000350161.
6. Wang (2008) Regulatory T cells in renal disease. International Journal of Clinical and Experimental Medicine 1 (4): 294 – 304.
7. Pereira WF, Brito-Melo GEA, Guimaraes FTL et al. (2014) The role of the immune system in idiopathic nephrotic syndrome: A review of clinical and experimental studies. Inflammation Research 63 (1): 1 – 12. doi: 10.1007/s00011-013-0672-6.
8. Terrier B, Derian N, Schoindre Y et al. (2012) Restoration of regulatory and effector T cell balance and B cell homeostasis in systemic lupus erythematosus patients through vitamin D supplementation. Arthritis Research and Therapy 14 (1): 1 – 10. doi: 10.1186/ar4060.
9. Urry Z, Chambers ES, Xystrakis E et al. (2012) The role of 1α,25 dihydroxyvitamin D3 and cytokines in the promotion of distinct Foxp3+and IL-10+ CD4+ T cells. European Journal of Immunology 42 (10): 2697 – 2708. doi: 10.1002/eji.2012 42370.

10. van den Berg JG, Weening JJ (2004) Role of the immune system in pathogenesis of idiopathic nephrotic syndrome. Clinical Science 107 (2): 125 – 136. doi: 10.1042/CS20040095.
11. Septarini AD, Tambunan T, Amalia P (2012) Calcium and vitamin D supplementation in children with frequently relapsing and steroid-dependent nephrotic syndrome. Paediatrica Indonesiana 52 (1): 16 – 21. doi: 10.14238/pi52.1.2012.16-21.
12. Esmaeeili M, Azarfar A, Hoseinalizadeh S (2015) Calcium and vitamin D metabolism in pediatric nephrotic syndrome: An update on the existing literature. International Journal of Pediatrics 15 (3): 103 – 109. doi: 10.22038/IJP.2015.3932.
13. Ginanjar E, Sumariyono, Setiati S, Setiyohadi B (2007) Vitamin D and autoimmune disease. Acta Medica Indonesiana 39 (3): 133 – 141.
14. Weng FL, Schults J, Heskovitz RM et al. (2005) Vitamin D insufficiency in steroid-sensitive nephrotic syndrome in remission. Pediatric Nephrology 20 (1): 56 – 63. doi: 10.1007/s00467-004-1694-7.
15. Araya C, Diaz L, Wasserfall C et al. (2009) T regulatory cell function in idiopathic minimal lesion nephrotic syndrome. Pediatric Nephrology 24 (9): 1691 – 1698. doi: 10.1007/s00467-009-1214-x.
16. Liu LL, Qin Y, Cai JF et al. (2011) Th17/ Treg imbalance in adult patients with minimal change nephrotic syndrome. Clinical Immunology 139 (3): 314 – 320. doi: 10.1016/j.clim.2011.02.018.
17. Shao XS, Yang XQ, Zhao XD et al. (2009) The prevalence of Th17 cells and FOXP3 regulatory T cells (Treg) in children with primary nephrotic syndrome. Pediatric Nephrology 24 (1): 1683 – 1690. doi: 10.1007/s00467-009-1194-x.
18. Lang CL, Wang MH, Chiang CK, Lu KC (2014) Vitamin D and the Immune system from the nephrologist's viewpoint. ISRN Endocrinology 14 (1): 1 – 11. doi: 10.1155/2014/105456.
19. Smolders J, Thewissen M, Peelen E et al. (2009) Vitamin D status is positively correlated with regulatory T cell function in patients with multiple sclerosis. PLoS ONE 4 (8): 63 – 45. doi: 10.1371/journal.pone.0006635.
20. Chambers ES, Suwannasaen D, Mann EH et al. (2014) 1,25-dihydroxyvitamin D3 in combination with transforming growth factor-β increases the frequency of Foxp3+ regulatory T cells through preferential expansion and usage of interleukin-2. Immunology 143 (1): 52 – 60. doi: 10.1371/journal.pone.0006635.
21. Gordillo R, Spitzer A (2009) The nephrotic syndrome. Pediatric in Review 30 (3): 94 – 104. doi: 10.1371/journal.pone.0006635.
22. Franchimont D (2004) Overview of the actions of glucocorticoids on the immune response: a good model to characterize new pathways of immunosuppression for new treatment strategies. Annals of the New York Academy of Sciences 1024 (1): 124 – 137. doi: 10.1196/annals.1321.009.
23. Heine G, Niesner U, Chang HD et al. (2008) 1,25-dihydroxyvitamin D3 promotes IL-10 production in human B cells. European Journal of Immunology 38 (8): 2210 – 2218. doi: 10.1002/eji.200838216.
24. Barrat FJ, Cua DJ, Boonstra A et al. (2002) In vitro generation of interleukin-10-producing regulatory $CD4^+$ cells is induced by immunosuppressive drugs and inhibited by T helper type 1 (Th1)- and Th2-inducing cytokines. Journal of Experimental Medicine 195 (5): 603 – 616. doi: 10.1084/jem.20011629.
25. Zhou L, Lopes JE, Chong MMW et al. (2008) TGF-β-induced Foxp3 inhibits TH17 cell differentiation by antagonizing RORγt function. Nature 453 (1): 236 – 240. doi: 10.1038/nature06878.