

## Effect of Vitamin D3 Supplementation to 25(OH)D, IL-17, and HbA1c Level in Pediatric Type 1 Diabetes Mellitus

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### ABSTRACT

Type 1 Diabetes Mellitus (T1DM) is the consequence of autoimmune destruction process of  $\beta$  cells which associated with Th17 activity and low 25(OH)D level. This study was aimed to investigate the effect of vitamin D3 supplementation toward 25(OH)D level, Th17 activity (IL-17) and glycemic control (HbA1c) in pediatric T1DM. This study was designed as randomized clinical trials (RCT), double-blind, pre and post-test controlled study. Subject was children with T1DM who were divided into two groups: K1: subjects were treated with insulin 0.5–2 IU/day + vitamin D3 2000 IU/day for 3 months, K2: subjects were treated with insulin 0.5–2 IU/day + placebo for 3 months. Levels of 25(OH)D, IL-17 and HbA1c were evaluated after 3 months treatment using ELISA. After 3 months treatment, results showed that 25(OH)D level was significantly higher in K1 compared with K2 ( $p = 0.00$ ), IL-17 level was significantly lower K1 compared with K2 ( $p = 0.022$ ). Surprisingly, HbA1c level in K1 was not significantly different with K2 ( $p = 0.93$ ). Furthermore, in vitamin D-treated group, 25(OH)D level was elevated significantly after 3 months treatment with vitamin D ( $p = 0.00$ ), IL-17 level was reduced significantly after 3 months treatment with vitamin D ( $p = 0.001$ ) and HbA1c level was reduced insignificantly after 3 months treatment with vitamin D ( $p = 0.76$ ). Correlation study showed that there was no correlation between 25(OH)D level with IL-17 level ( $p = 0.160$ ,  $r = -0.284$ ) and 25(OH)D with HbA1c ( $p = 0.62$ ,  $r = -0.10$ ). This study can be conclude that vitamin D3 supplementation may elevate the 25(OH)D and reduce IL-17 level but did not change HbA1c level in pediatric T1DM.

**Keywords:** 25(OH)D, HbA1c, IL-17, type 1 diabetes mellitus, vitamin D

### INTRODUCTION

Type 1 Diabetes Mellitus (T1DM) is one of global health problem whereas its prevalence increase approximately 3% per year. In 2010, as many as 480.000 children were suffered from T1DM and its incidence was 75.800 case annually [1, 2, 3]. Management of T1DM should be comprehensively performed by pediatric endocrinologist together with dietician, psychiatrist, psychologist, and educator [4, 5].

In T1DM, destruction of pancreatic  $\beta$  cells caused by autoimmune process lead to decreased insulin secretion. The mechanisms underlying pancreatic  $\beta$  cells destruction are still unknown, some studies suggest it is caused by interaction of multiple gene and environmental exposure [5, 6]. However, there was an involvement of macrophage and T cells in the pathogenesis of T1DM which lead to inflammation and oxidative stress

[3, 7, 8]. T helper cells 17 (Th17) was involved in the autoimmunity which lead to destruction of pancreatic  $\beta$  cells [4, 9, 10]. Some chronic diseases such as T1DM, multiple sclerosis, rheumatoid arthritis, stroke, cardiovascular disease, and colorectal cancer were associated with low level of vitamin D [6, 10]. Vitamin D has a role as immunomodulatory substances in some autoimmune disease including T1DM [4, 10]. Interestingly, vitamin D supplementation decrease the risk of T1DM in dose-dependent manner. Instead of positive results in some clinical trials, there were some limitations in that cohort designed studies such as sample size, short follow-up period, and limited control group [11, 12, 13]. This study was aimed to investigate the effect of vitamin D3 supplementation toward 25(OH)D level, IL-17 level, and HbA1c in children with T1DM.

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## MATERIALS AND METHODS

### *Study design*

This experimental study was designed as randomized clinical trial (RCT) double blind, pre and post-test control group. Vitamin D3 supplementation was administered for 3 months. Before treatment, subjects with T1DM was measured for 25(OH)D, IL-17 and HbA1c level. Then, all subjects were randomly divided into 2 groups (K1 and K2) as follow: K1: subjects were treated with insulin 0.5 - 2 IU/day + vitamin D3 2000 IU/day (D-Vit, PT. Gracia Pharmindo) for 3 months, K2: subjects were treated with insulin 0.5 - 2 IU/day + placebo (flour containing capsules, PT. IMFARMIND Pharmaceuticals Industries) for 3 months. All procedures in this study had been approved by Ethical Committee of Research, dr. Saiful Anwar Public Hospital Malang, Indonesia

### *Subjects*

As many as 26 subjects were included in this study (13 subjects K1 and 13 subjects K2). Subjects were taken from Pediatric Endocrinology Outpatient Care, dr. Saiful Anwar General Hospital, Malang during November 2015-January 2016. Inclusion criteria for subjects are as follows: diagnosed as T1DM, age 1-18 years old, and allowed by his/her parents (informed consent). Exclusion criteria for subjects are T1DM patients with other autoimmune disease, severe infection, hepatic dysfunction, renal dysfunction, and anemia.

### *Measurement of vitamin D level*

Vitamin D level measurement was conducted at Clinical Pathology Laboratory, Saiful Anwar General Hospital, Malang. Blood plasma was stored at -20°C until all sample's subject collected. Measurement of vitamin D level was performed according to manufacturer's instruction (Alegria human vitamin D kit, ORG 270). Biotin solution 25-D (1 mL) was added into all tubes, and then mixed using vortex for 10 seconds. As many as 200 µL of dilution callibrator was added into each well of antibody coated plate and then incubated for 2 hours at 18–25°C. After 3 times washing process, 200 µL conjugate was added into each well. Microplate was covered with plastic and then incubated for 30 minutes at 18–25°C. After addition of 200 µL TMB substrate into each well, microplate was covered and then incubated for 30 minutes at 18–25°C. Finally, 100 µL stop solution was added into each well. After 30 minutes, specimens were ready for

analysis using ELISA reader at 450 nm.

### *Measurement of IL-17 level*

The method of malondialdehyde Measurement of IL-17 level was conducted at Physiology Laboratory, Faculty of Medicine, Universitas Brawijaya, Malang. Blood plasma was stored at -20°C until all sample's subject collected. Measurement of IL-17 level was based on ELISA method according to manufacturer's instruction (BioLegend LEGEND MAX™ Human IL-17A ELISA Kit, catalogue number 433917). First of all, human IL-17A standard should be prepared in different concentration (assay buffer A as diluent; 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.3 pg/mL, 15.6 pg/mL, 7.8 pg/mL, and 3.9 pg/mL) for making standard curve of IL-17. Microplate washed 4 times using 300 µL wash buffer. As many as 50 µL assay buffer A was added into each well which containing standard dilution or sample, add 50 µL standard dilution into each well. After that process, microplate was sealed using plate sealer and then incubated for 2 hours while squeezed at 200 rpm. After mixing process, microplate was washed using wash buffer for 4 times. 100 µL human IL-17A detection antibody solution was added into each well then incubated for 1 hours at room temperature. After incubation process, microplate was washed using wash buffer for 4 times. 100 µL Avidin-HRP D solution was added into each well and then incubated for 30 minutes at room temperature. Microplate was washed again using wash buffer for 5 times. 100 µL substrate solution F was added into each well and then incubated for 30 minutes in dark room. 100 µL stop solution was added into each well. After 30 minutes, specimens were ready for analysis using ELISA reader at 450 nm.

### *Measurement of HbA1c level*

HbA1c level measurement was conducted at Clinical Pathology Laboratory, Saiful Anwar General Hospital, Malang. As many as 1 mL blood sample (whole blood) was collected into EDTA-coated tube from subjects. 5 µL of sample was mixed with 1.5 ml wash/ diluent solution before analysis. Measurement of HbA1c was performed using Bio-Rad D-10TM.

### *Statistical analysis*

Statistical test was based on data distribution and homogeneity. Statistical differences of 25(OH)D, IL-17, and HbA1c level between groups were analyzed by independent t-test. 25(OH)D, IL-17, and HbA1c differences before and after treatment were analyzed by paired t-test. Correlation of 25(OH)D, IL-17, and

HbA1c level was analyzed by Pearson correlation test.

Table 1. Subject Characteristics

Characteristics	K2 (n=13)	K1 (n=13)	p-value
Sex			0.691 <sup>a</sup>
- Male	5	6	
- Female	8	7	
Age (years)	12.15±3.26	12.46±2.99	0.804 <sup>b</sup>
Duration of T1DM			0.695 <sup>a</sup>
- ≤ 5 y.o	7	6	
- > 5 y.o	6	7	
Insulin dose	1.19±0.29	1.14±0.16	0.672 <sup>c</sup>
Nutritional status			0.185 <sup>a</sup>
- Good nutrition	11	8	
- Undernutrition	2	5	

Note: - p-value<sup>a</sup> no significant differences using Chi-Square test  
 - p-value<sup>b</sup> no significant differences using t-test  
 - p-value<sup>c</sup> no significant differences using Mann-Whitney test

Data was analyzed at 95% confidence interval ( $\alpha=0.05$ ) using SPSS for Windows version.

## RESULTS AND DISCUSSION

### Subject characteristics

Subject characteristics such as sex, age, duration of disease (T1DM), insulin dose, and nutritional status (age and insulin dose was listed as mean  $\pm$  standard deviation) was shown in Table 1.

This study included 26 subjects with age ranged from 7-17 years old and its average in each group was 12 years old. This data was in accord with peak incidence of T1DM which ranged from 5 to 7 years old and at puberty [8, 14, 15]. This study also showed male: female ratio was 1.4 : 1 and this data similar with previous report about the proportion of T1DM patient based on sex (male : female 1 : 1) [9]. Nutritional status of subjects was mostly good nutrition. This data indicated that nutritional status didn't directly associated with T1DM because basic mechanism of T1DM was autoimmune process.

### Level of 25(OH)D, IL-17 and HbA1c

Level of 25(OH)D, IL-17, and HbA1c before treatment with vitamin D3 or placebo was shown in Table 2. As shown in Table 2, there was no significant differences between K1 and K2 based on 25(OH)D (independent t-test,  $p = 0.36$ ), IL-17 (independent t-test,  $p = 0.16$ ), and HbA1c level (independent t-test,  $p = 0.07$ ).

Level of 25(OH)D, IL-17, and HbA1c after treatment with vitamin D3 or placebo was shown in Table

3. As shown in Table 3, 25(OH)D level was significantly higher in vitamin D-treated group compared with placebo-treated group (independent t-test,  $p = 0.00$ ). Furthermore, IL-17 level was significantly lower in vitamin D-treated group as compared to placebo-treated group (independent t-test,  $p = 0.02$ ). Surprisingly, HbA1c level in vitamin D-treated group wasn't significantly different with placebo-treated group (independent t-test,  $p = 0.93$ ).

Level of 25(OH)D, IL-17, and HbA1c in vitamin D-treated group was shown in Table 4. As shown in Table 4, 25(OH)D level was elevated significantly after 3 months treatment with vitamin D3 (paired t-test,  $p = 0.00$ ). IL-17 level was also reduced significantly after 3 months treatment with vitamin D3 (paired t-test,  $p = 0.01$ ). However, HbA1c was reduced insignificantly after 3 months treatment with vitamin D3 (paired t-test,  $p = 0.77$ ).

Our data showed that 10 subjects were suffered from vitamin D deficiency and 6 subjects were suffered from vitamin D insufficiency. Several observational studies showed that T1DM subjects has low level of vitamin D [2, 16, 17]. Children with vitamin D deficiency has higher risk to have autoimmune disease. Furthermore, the risk of T1DM in children with rachitis at their first year of life was tripled. Supplementation of 2000 IU/day vitamin D had been reported to decrease the relative risk of T1DM (RR 0,22 with CI 0,05-0,89) [11, 13]. This study showed that after treatment, 25(OH)D level was significantly higher in vitamin D-treated group as compared to placebo-treated group. Furthermore, vitamin D supplementation 2000 IU/day for 3 months was also increase 25(OH)D significantly at the end of study.

This study showed that IL-17 level in vitamin D-treated group was significantly lower as compared to placebo-treated group at the end of study. Interestingly, statistical analysis in vitamin D-treated group, vitamin D supplementation didn't significantly reduced IL-17 level. Our data was similar with previous observational study which showed that IL-17 secretion and expression was higher in T1DM subjects [18]. Furthermore, another study also reported that IL-17-secreted CD4(+) and CD8(+)-T-cells were increased in new onset T1DM subjects [19]. Interestingly, administration of beta cell autoantigens (proinsulin, insulinoma-associated protein, and GAD65 peptide) was increase IL-17 reactivity in 54% subjects with new onset of T1DM as compared to control (10%) [20]. Currently, IL-17 had been investigated for its role in pathogenicity of

T1DM. Previous study showed that IL-17 increase the

Table 2. Level of 25(OH)D, IL-17, and HbA1c before treatment

Variable	K2	K1	p-value
	Mean ± SD	Mean ± SD	
25(OH)D level (ng/mL)	26.78±15.54	21.97±10.27	0.361
IL-17 level (pg/mL)	832.15±272.02	1002.15±325.57	0.161
HbA1c level (%)	11.67±3.21	9.38±2.92	0.070

Note: - K1: insulin + vitamin D-treated group

- K2 (insulin-only-treated group)

Table 3. Level of 25(OH)D, IL-17, and HbA1c after treatment

Variable	K2	K1	p-value
	Mean ± SD	Mean ± SD	
25(OH)D level (ng/mL)	24.99±12.37	143.52± 29.81	0.000
IL-17 level (pg/mL)	910.34±374.41	582.08± 305.67	0.022
HbA1c level (%)	10.31± 2.36	10.20± 3.89	0.933

Note: - K1: insulin + vitamin D-treated group

- K2 (insulin-only-treated group)

Table 4. Level of 25(OH)D, IL-17, and HbA1c in treatment group

Variable	Before	After	p-value
	Mean ± SD	Mean ± SD	
25(OH)D level (ng/mL)	24.37±13.13	84.25 ± 64.45	0.000
IL-17 level (pg/mL)	917.15±306.44	746.21 ± 374.37	0.001
HbA1c level (%)	10.52±3.22	10.25 ± 3.15	0.765

Table 5. Correlation of 25(OH)D level with IL-17 and HbA1c level

Correlation	N	Correlation coefficients (r)	p-value
25(OH)D level and IL-17 level	26	-0.284	0.160
25(OH)D level and HbA1c level	26	-0.101	0.624

production of local chemokines which lead to insulinitis and pancreatic beta cells apoptosis [21].

This study indicated that vitamin D supplementation didn't change glycemic control of T1DM subjects based on HbA1c level. Some experimental studies which investigate the effect of vitamin D supplementation on glycemic control has controversy results. Bizzari and colleagues had been reported that vitamin D3 supplementation at dose 0,25mcg/day didn't decrease HbA1c level as compared to control at several points of follow-up period (6, 12, and 24 months) [22]. Conversely, Aljabri and colleagues had been demonstrated that HbA1c level was significantly lower after treatment with vitamin D3 4000 IU/day [23]. Later study only involved vitamin D deficiency subjects (25(OH)D level < 50 nmol/L) and designed as non blinded and

non randomized controlled trial [23]. This contradictory results might be caused by different dosage and duration of vitamin D3 supplementation.

#### Correlation level of 25(OH)D level with IL-17 and HbA1c

Table 5 showed correlation of 25(OH)D level with IL-17 and HbA1c level. As shown in Table 5, 25(OH)D level was insignificantly correlated with IL-17 level (Pearson correlation test,  $p= 0.160$ ,  $r= -0.284$ ). Consistent with previous result, there was no significant correlation between 25(OH)D level with HbA1c level (Pearson correlation test,  $p= 0.624$ ,  $r= -0.101$ ).

This study showed that 25(OH) level was insignificantly correlated with IL-17 level. Previous in vitro study showed that administration of 1,25(OH)2D3 to human T cell CD4(+) CD25(-) decrease its proinflammatory cytokines production (IFN $\gamma$ , IL-17 and IL-21) and induce adaptive regulatory T cell activator (CTLA-4 and FoxP3) [24]. This contradictory result might be caused by different method in assessing IL-17 level. Ferraro and colleagues reported that there was elevated regulation of Th17 and functional defect of TReg CD4+CD25bright cells in PLN tissue and target organ, but not in peripheral blood [25].

Consistently, 25(OH)D level was also insignificantly correlated with HbA1c level. Regarding to the effect of vitamin D supplementation on HbA1c level, there were controversial result in the previous studies. Nwosu and colleagues reported that vitamin supplementation for 3 months didn't correlated with HbA1c level in T1DM patient [26]. Branco and colleagues also reported that there was no correlation between 25(OH)D level and HbA1c level [27]. Conversely, observational studies showed that 25(OH)D was negatively correlated with HbA1c level and insulin dosage requirement in T1DM patient [28, 29].

Active form of vitamin D, 1,25(OH)2D<sub>3</sub> possesses immunomodulator potency that can be used for prevention and treatment of autoimmune and infection disease. Active form of vitamin D could inhibit lymphocyte proliferation and its cytokine production, induce beta cell tolerance and decrease its sensitivity to apoptosis process [7, 14, 30, 31]. Activation of VDR signaling inhibit DC maturation which represented by decrease DC marker, class II MHC, costimulator molecules (CD40, CD80, and CD86) and other maturation marker (such as CD83) [7, 32]. DC which had been modulated by 1,25(OH)2D3 will shift Th1 and Th17-dominant into Th2-dominant response and increase IL-10 secretion which lead to proliferation of



suppressive Treg [7, 32, 33, 34].

## CONCLUSION

We concluded that vitamin D3 supplementation may elevate the 25(OH)D and reduce IL-17 level but didn't change HbA1c level in pediatric T1DM.

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