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The Relationship Between Serum Vitamin D ($25(OH)D_3$), C-Peptide, and Interleukin-2 Level in Children with Type 1 Diabetes Mellitus

Tip 1 Diabetes Mellitus'lu Çocuklarda Serum D Vitamini ($25(OH)D_3$), C-Peptid ve İnterlökin-2 Seviyeleri Arasındaki İlişki

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Abstract

Introduction: Vitamin D is very important for Type 1 diabetes mellitus (T1 DM) pathogenesis, and plays major role in the regulation of pancreatic beta cells. However, serum vitamin D ($25(OH)D_3$) level related to inflammatory condition in T1 DM has not been sufficiently investigated. Therefore, we aimed to investigate the relationship between serum vitamin D ($25(OH)D_3$), C-Peptide, and Interleukin-2 levels in children with T1 DM.

Material and Methods: Blood samples from 20 subjects of T1 DM patients and healthy patients were collected, and analyzed. Serum level of vitamin D ($25(OH)D_3$), C-Peptide, and Interleukin-2 between two groups were measured by using Indirect ELISA.

Results: Serum vitamin D ($25(OH)D_3$), C-Peptide, and Interleukin-2 level between both groups were significantly different ($p<0.001$). It was shown that serum vitamin D ($25(OH)D_3$) and C-Peptide level had significant positive correlation, while serum C-Peptide and Interleukin-2 level had significant negative correlation ($p=0.002$; $r=-0.658$). Serum vitamin D ($25(OH)D_3$) and Interleukin-2 had also significant negative correlation ($p<0.001$; $r=-0.753$).

Discussion: Serum vitamin D ($25(OH)D_3$) level is related to inflammatory condition in T1 DM. Low level of serum vitamin D ($25(OH)D_3$) in T1 DM patient tends to increase the level of Inteleukin-2, and it is highly correlated with pancreatic beta cells' impairment, which is marked by low C-Peptide level and high Inteleukin-2 level.

Keywords: C-Peptide, interleukin-2, T1 DM, vitamin D ($25(OH)D_3$)

Öz

Giriş: D vitamini, tip 1 diabetes mellitus (T1 DM) oluşumunda pankeasta beta hücre düzenlenmesinde çok önemlidir ve pankreastaki beta hücrelerini düzenler. Ancak, serumdaki D vitamini ($25(OH)D_3$) seviyesinin T1 DM hastalarında, inflamatuvar durum ile ilişkisi yeterli düzeyde araştırılmamıştır. Bu nedenle, çalışmamızda T1 DM tanısı alan çocuklarda serum D vitamini, C-peptid ve İnterlökin-2 düzeyleri arasındaki ilişkinin saptanmasıı amaçlanmıştır.

Gereçler ve Yöntemler: T1 DM olan 20 hastanın ve sağlıklı bireylerin kan örneklerini alarak irdeledik. Serum örneklerinde D vitamini ($25(OH)D_3$), C-Peptid ve İnterlökin-2 düzeyleri inderekt ELISA tetsi ile ölçüldü.

Bulgular: Her iki gruptaki serum D vitamini ($25(OH)D_3$), C-peptid ve İnterlökin-2 düzeyleri anlamlı düzeyde birbirinden farklılık gösterdi ($p<0.001$). Serum D vitamini ($25(OH)D_3$) ve C-peptid seviyeleri arasında doğrusal bir ilişki saptanmasına karşılık, C-peptid ve İnterlökin-2 düzeyleri arasında ters bir bağlantı saptanmıştır ($p=0.002$; $r=-0.658$). Ayrıca Serum D vitamini ($25(OH)D_3$) ve İnterlökin-2 düzeyleri arasında da ters bir bağlantı saptanmıştır ($p<0.001$; $r=-0.753$).

Sonuç: Serumdaki D vitamini ($25(OH)D_3$) düzeyi, T1 DM'deki inflamatuvar durum ile ilişkilidir. D vitamininin düşük olması T1 DM'lu hastalarda İnterlökin-2 düzeyinin artışı ile bağıntılı gibi gözükmektedir ve pankreastaki beta hücrelerinin yetersizliğini gösteren düşük C-peptid ve yüksek Interleukin-2 düzeyi ile önemli ölçüde ilişkilidir.

Anahtar Kelimeler: C-Peptid, İnterlökin-2, Tip 1 Diabetes Mellitus, D Vitamini ($25(OH)D_3$)

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Introduction

Type 1 diabetes mellitus (T1 DM) is one of the most common autoimmune diseases in children (5–10% cases), and it is characterized by the destruction of pancreatic beta cells, resulting in absolute insulin deficiency. Loss of tolerance to self antigens (central and peripheral tolerance defect) is related to the destruction of pancreatic beta cells in T1 DM.[1] Active form of vitamin D is responsible to prevent T1 DM occurrence, by modifying T cell differentiation, modulating dendritic cell activity, inducing cytokine secretion and promoting T regulator cell.[2]

Interleukin-2 (IL-2) is one of the most dominant pro-inflammatory cytokine that is related to T1 DM. It activates natural killer cells and also cytotoxic T cells to destroy target cells.[3] Low activity of vitamin D might cause pancreatic beta cells' functional defect, and cytokine imbalance (pro-inflammatory cytokines are dominant). C-Peptide level is highly related to pancreatic beta cell function. Therefore, low level of C-Peptide in a patient with T1 DM might indicate that most of pancreatic beta cells (70–90%) are impaired.[4]

Vitamin D is very important for pathogenesis of T1 DM and plays a major role in the regulation of pancreatic beta cells. Low activity of vitamin D might be influenced by a genetic factor such as synthesis defect and vitamin D receptor (VDR) polymorphism, and also by environmental factors.[5] Many studies have consistently shown that vitamin D profile is highly correlated with autoimmune diseases. However, circulating vitamin D ($25(OH)D_3$) levels related to inflammatory condition in T1 DM has not been sufficiently investigated. Therefore, it is important to investigate the relationship between serum vitamin D ($25(OH)D_3$), C-Peptide, and Interleukin-2 levels in children with T1 DM. We aimed to assess the impact of vitamin D level on immunological status, and metabolic control of T1DM patients.

Material and Methods

Samples Collection

Patients were recruited from outpatient clinic of the Department of Pediatrics (Endocrinology Division), Saiful Anwar Public Hospital, Malang, Indonesia in the period from January to March 2017. They were asked to participate in this study during their initial visit to the outpatient clinic, by allowance of their parents. Informed consents were received from parents. All new patients that are diagnosed with T1 DM according to American Diabetes Association criteria were eligible for participating in this study. The mean age of 20 T1 DM patients was 12.1±3.1 years old (ranging from 1 y.o. to 18 y.o.). All subjects with autoimmune diseases (Systemic Lupus Erythematosus, Noonan Syndrome), infectious diseases (tuberculosis, sepsis, HIV), abnormal liver and renal function, and history of previous vitamin D therapy for at least 1 month were excluded. The mean age of 20 healthy individuals (control group) was 10.55±2.64 years old (ranging from 1 y.o. to 18 y.o.). All subjects with abnormal liver and renal function, previous history of diabetes, previous vitamin D therapy for at least 1 month, and autoimmune diseases were excluded.

All blood samples were collected during the initial visit, and the serum level of vitamin D ($25(OH)D_3$), C-Peptide, and Interleukin-2 were measured in both groups. Each patient underwent blood sampling after 8 hours of fasting, during the initial visit in the morning. Three mL of venous blood were taken from each patient by sterile heparinized tubes. Blood plasma was stored at -20°C until all samples were collected. The study protocol was approved by the medical ethical committee of the Saiful Anwar Public Hospital. The ethical clearance was approved by Regional Medical Sciences Research Ethics Committee of Faculty of Medicine, Brawijaya University. All patients were on insulin treatment, based on standard therapy for T1 DM.

Vitamin D ($25(OH)D_3$) Serum Level Measurement

Serum level of circulating vitamin D ($25(OH)D_3$) was measured in ng/mL at 450 nm wavelength by means of Alegria Human Vitamin D ELISA Kit (ORG 270), made in Orgentec. Indirect ELISA (Enzyme Link Immuno Sorbent Assay) was performed to measure the level of vitamin D ($25(OH)D_3$) in all samples. Biotin solution 25-D (1 mL) was added into all sample's tube and then mixed using vortex for 10 seconds. A hundred µL of Dilution Callibrator was added to each well. All samples (100 µL of each samples) were also added to each well and covered using adhesive strip. After antigen had been coated to micro plate, it was incubated for 2 hours at room temperature. Then, the coating solution was removed and the plate was washed using Wash Buffer (3 times). Human Conjugate (200 mL monoclonal antibody) was added to each well and incubated for 2 hours at room temperature. New adhesive strip was used to cover it. The plate was

washed two times using PBS-Tween. Substrate solution (200 µL) was added to each well, incubated for 20 minutes at room temperature. At last, 50 µL of stopping solution (NAOH 3M) was added. The result was read by observing the optical density at 450 nm using plate reader (normal circulating vitamin D level = >30 ng/mL, vitamin D insufficiency = 20–30 ng/mL, vitamin D deficiency = <20 ng/mL).

C-Peptide Serum Level Measurement

Serum level of C-Peptide was measured in ng/mL at a 450 nm wavelength by means of Immulite 2000 C-Peptide Assay (L2KPEP2), made in Sevron, USA. Indirect ELISA (Enzyme Link Immuno Sorbent Assay) was performed to measure the level of C-Peptide in all samples. As many as 100 µL of Assay Diluent was added to each well. All samples (100 µL of each sample) were also added to each well and covered using adhesive strip. After antigen had been coated to micro plate, it was incubated for 2 hours at room temperature. Then, the coating solution was removed and the plate was washed using Wash Buffer (3 times). Human Conjugate (200 ml monoclonal antibody) was added to each well and incubated for 2 hours at room temperature. New adhesive strip was used to cover it. The plate was washed two times using PBS-Tween. Substrate solution (200 µL) was added to each well, incubated for 20 minutes at room temperature. At last, 50 µL of stopping solution (NAOH 3M) was added. The result was read by observing the optical density at 450 nm using plate reader (normal fasting C-Peptide level=0.9–7.1 ng/mL).

Interleukin-2 Serum Level Measurement

Serum level of Interleukin-2 (IL-2) was measured in pg/mL at 450 nm wavelength by means of Human IL-2 Quantikine ELISA Kit, made in R&D Systems. Indirect ELISA (Enzyme Link Immuno Sorbent Assay) was performed to measure the level of IL-2 in all samples. A 100 µL of Assay Diluent RD1W was added to each well. All samples (100 µL of each sample) were also added to each well and covered using adhesive strip. After antigen had been coated to micro plate, it was incubated for 2 hours at room temperature. Then, the coating solution was removed and the plate was washed using Wash Buffer (3 times). Human IL-2 Conjugate (200 ml monoclonal anti IL-2, antibodies against recombinant human IL-2) was added to each well and incubated for 2 hours at room temperature. New adhesive strip was used to cover it. The plate was washed 2 times using PBS-Tween. Substrate solution (200 µL) was added to each well, incubated for 20 minutes at room temperature. At last, 50 µL of stopping solution (NAOH 3M) was added. The result was read by observing the optical density at 450 nm using plate reader.

Statistical Analysis

Statistical analysis was conducted by SPSS software (17.0). Serum vitamin D, C-Peptide, and Interleukin-2 levels between groups were analyzed by using Independent T-Test; to find out whether the level of serum vitamin D, C-Peptide, and Interleukin-2 between two groups were significantly different. The correlation between serum vitamin D and C-Peptide level, vitamin D and Interleukin-2 level, C-Peptide and Interleukin-2 level were analyzed by using Pearson correlation test. Data was analyzed at 95% confidence interval (α=0.05).

Results

Subject Characteristics

Subject characteristics such as sex, age, duration of disease (T1 DM), insulin dose, complete blood count result (hemoglobin, leukocyte, thrombocyte), renal function test, liver function test, glutamic acid decarboxylase autoantibodies test (GAD test), blood glucose self-monitoring frequency, and nutritional status (age and insulin dose was listed as mean±standard deviation) was shown in Table 1. The study included 40 subjects with age ranged from 5–15 years old, that were divided into two groups (20 subjects in T1 DM group and 20 subjects in control group). The average age in T1 DM group was 12.1±3.1, and the average age in control group was 10.55±2.64. Subjects from this study were 35% male and 65% female. Nutritional status of subjects in T1 DM group (65% subjects) were mostly in a good nutrition. But, there was equal proportion of nutritional status between good and under nutrition, in control group. While all T1 DM patients had GAD+ (autoimmune marker for T1 DM), none of healthy persons had it. Most T1 DM patients (18 (90%) subjects) had normal body mass index (BMI) between 18.5–25. All subjects (T1 DM patients and healthy persons) had normal complete blood count, liver function test and renal function test.

Vitamin D (25(OH)D_3), C-Peptide, and Interleukin-2 Serum Level

Independent T-test was performed based on normality test, the collected data was normally distributed. It was also found that serum vitamin D (25(OH)D_3)

Table 1. Subject Characteristics

Characteristics		Type 1 DM (n=20)	Control (n=20)
Age (years)		12.1±3.10	10.6±2.64
Age Distribution (years old)	<5 y.o	0	0
	5-10 y.o	6/20	11/20
	11-15 y.o	11/20	9/20
	>15 y.o	3/20	0
Sex	Male	7/20	7/20
	Female	13/20	13/20
Nutritional Status	Over Nutrition	2/20	0
	Good Nutrition	13/20	10/20
	Under Nutrition	5/20	10/20
Body Mass Index	<18.5	0	0
	18.5-25	18/20	20/20
	>25	2/20	0
Duration of T1 DM (years)		3.85±1.30	-
Insulin Dose IU/kg/d)		1.18±0.25	-
Blood Glucose Self Monitoring Frequency	1-2 times/day	20/20	-
	>2 times day	0	
Hemoglobin (g/dL)		12.19±0.55	12.48±0.55
Leukocyte (number/mm^3)		8925±528	7871±1151
Thrombocyte (count/mm^3)		347250±27082	312650±29805
Urea (mg/dL)		24.24±3.87	24.32±3.26
Creatinine (mg/dL)		0.60±0.10	0.59±0.10
SGOT (U/L)		30.35±3.15	29.45±2.90
SGPT (U/L)		30.65±2.11	31.85±3.06
GAD + subject		20/20	0/20

Over Nutrition: >110% of Ideal Body Weight ; Good Nutrition: 90%-110% of Ideal Body Weight; Under Nutrition: <70% of Ideal Body Weight

(20.82±5.53 vs 33.14±2.17) (Figure 1), C-Peptide (0.27±0.12 vs 3.26±1.08) (Figure 2), and Interleukin-2 levels (1566.00±432.39 vs 187.50±39.59) (Figure 3) between T1 DM and control group were significantly different (p<0.001). The level of serum vitamin D (25(OH)D_3) and C-Peptide in T1 DM patients was lower than that in control group. On the other hand, the level of Interleukin-2 in T1 DM patients was higher than that in control group.

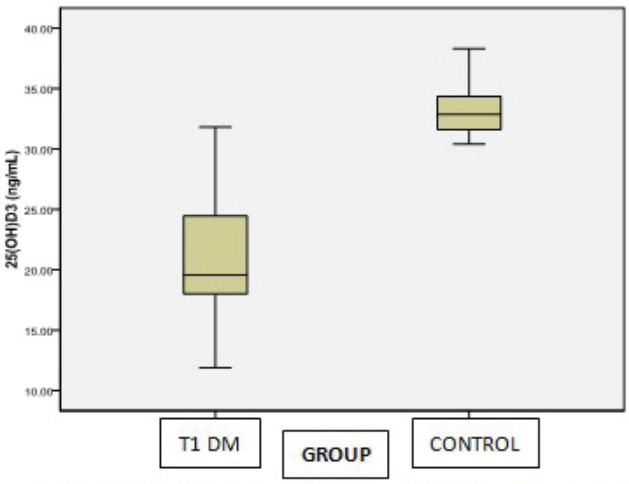

Figure 1. Mean Vitamin D (25(OH)D3) levels in patients with type 1 diabetes mellitus patients and Control Group p<0.001.

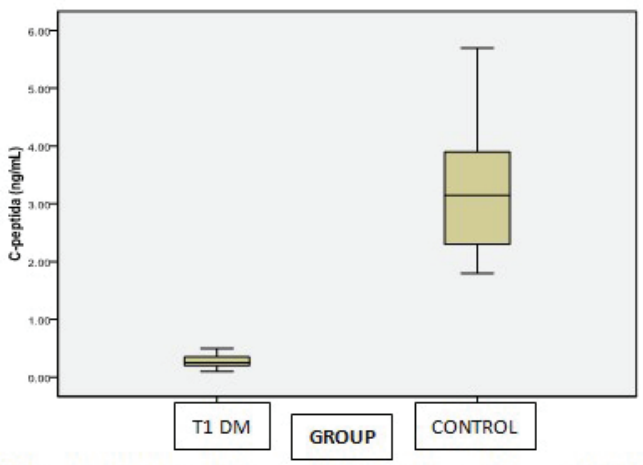

Figure 2. C-Peptide levels in Type 1 Diabetes Patients and Control Group (p<0.001).

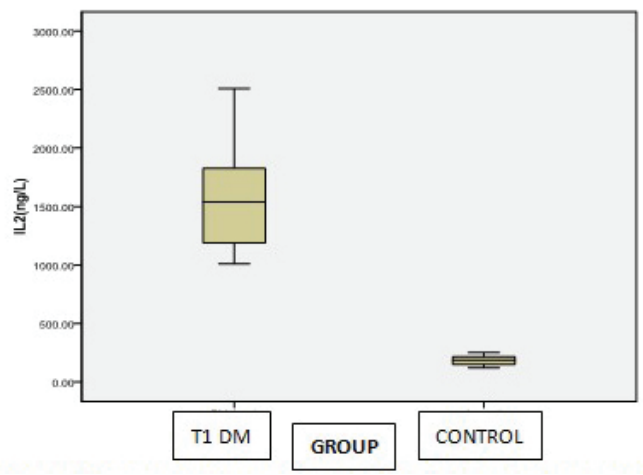

Figure 3. IL-2 Levels in Type 1 Diabetes Mellitus patients and Control Group (p<0.001).

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Correlation Between Vitamin D (25(OH)D_3), C-Peptide, and Interleukin-2 Serum Level in Children with Type 1 Diabetes Mellitus

Figure 4 showed that serum vitamin D (25(OH)D_3) and C-Peptide level had significantly positive correlation in children with T1 DM (p<0.001; r=+0.961). On the other hand, serum C-Peptide and IL-2 level had significant negative correlation in children with type I diabetes mellitus (p=0.002; r=-0.658) (Figure 5). Serum levels of vitamin D (25(OH)D_3) and IL-2 had also significant negative correlation in children with T1 DM (p<0.001; r=-0.753) (Figure 6).

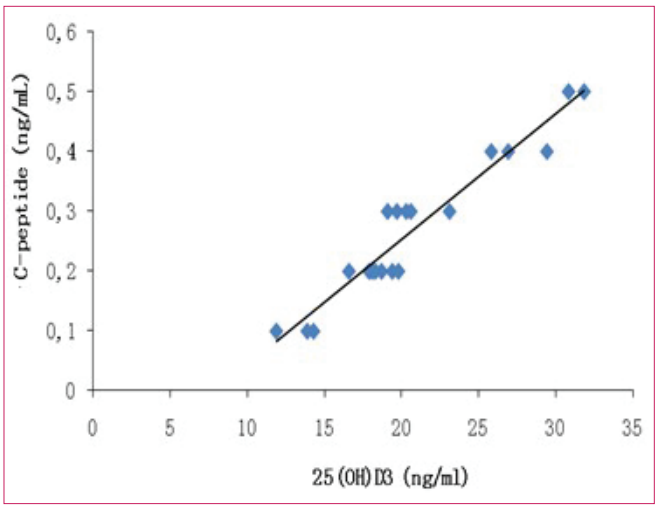

Figure 4. Correlation Between Vitamin D (25(OH)D3) and C-Peptide Levels in Children with Type 1 Diabetes Mellitus (p<0.001; r=+0,961).

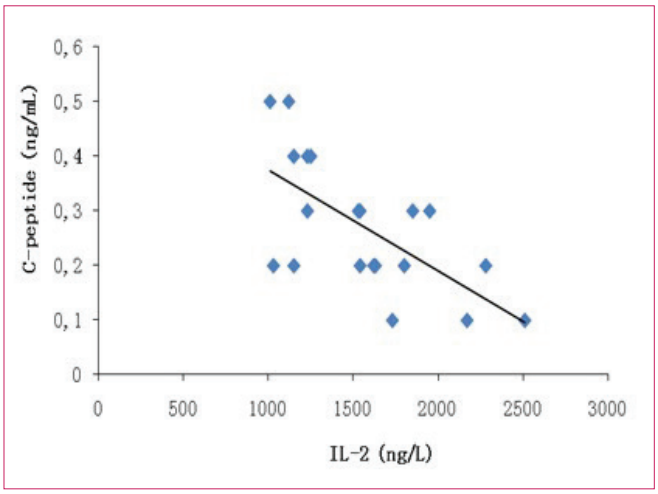

Figure 5. Correlation Between C-Peptide and IL-2 Level in Children with Type 1 Diabetes Mellitus (p=0.002; r=-0,658).

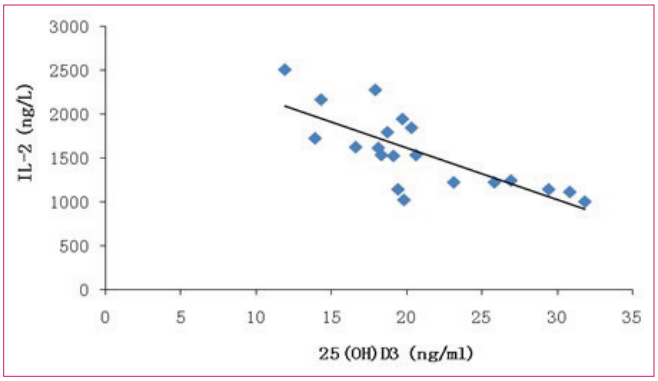

Figure 6. Correlation Between Serum Vitamin D (25(OH)D3) and IL-2 Levels in Children with Type 1 Diabetes Mellitus (p<0.001: r=-0,753).

Discussion

Vitamin D deficiency might play major role in the pathogenesis of T1 DM. It is influenced by genetic and also environmental factor.[2] The most responsible gene that associated with T1 DM is HLA gene class II, which is located in chromosome 6p21.3. HLA-DR and HLA-DQ locus in class II region have a very strong risk, in association with T1 DM pathogenesis. Non-HLA locus such as INS and CTLA-4 also influence genetic susceptibility in T1 DM patients.[5] Deterioration of vitamin D synthesis might be associated with age, liver disease, melanin pigment, skin lesion, and exposure intensity. Malabsorption and obesity can also cause the decrease of vitamin D bioavailability, in which vitamin D is easily sequestered in adipose tissue. Vitamin D catabolism might increase because of medications such as anti-convulsant, glucocorticoid, and certain types of anti-retroviral therapy.[6]

In Holland, about 60–84% children with T1 DM has vitamin D deficiency.[7] The number of T1 DM patients with vitamin D deficiency has also been increased to the prevalance of 90.6% in Qatar.[8] Vitamin D serum levels in Australian T1 DM patients were found to be lower than that of healthy patients.[9] Cross sectional study upon 128 patients with T1 DM in America, showed that 15% patient had vitamin D deficiency, 61% patient had vitamin D insufficiency, and only 24% patient had sufficient vitamin D level.[10] Consistent with the result of previous study, this research also showed that 60% patient with T1 DM had vitamin D deficiency, while 30% patient had vitamin D insufficiency, and only 10% of patients had sufficient vitamin D level.

Vitamin D ($25(OH)D_3$) level was used to assess the level of vitamin D in circulation, instead of the active form of vitamin D (1.25 $(OH)_2D$). This active form of vitamin D is not a good indicator for vitamin D deposition parameter because of its short half life (4 hours) and low concentration in circulation (100–1000 times lower than vitamin D ($25(OH)D_3$) level). Moreover, vitamin D deficiency might increase PTH (parathyroid hormone) which can induce the activity of 1-alpha hydroxylase. It will cause the level of vitamin D (1.25 $(OH)_2D$) increase and mimic normal assessment.[11] 1.25-Dihydroxyvitamin D3 (1.25 $(OH)_2D$) inhibits lymphocyte activation and affects other elements of the immune system, such as cytokines and immunoglobulin production, as well as Major Histocompatibility Complex (MHC) class II and the cluster differentiation 4 (CD4) expression.[12]

The level of serum vitamin D ($25(OH)D_3$) in T1 DM patients was found to be significantly lower than that in control group. Significant positive correlation between serum vitamin D ($25(OH)D_3$) and C-Peptide level in children with T1 DM showed that low level of serum vitamin D ($25(OH)D_3$) in T1 DM patients was highly correlated with low C-Peptide levels.[13] It was also found that level of serum C-Peptide in T1 DM patients was significantly lower than that in control group. Low levels of C-Peptide in all T1 DM patients (<0.6 mg/dL) indicates that 70–90% pancreatic beta cells are impaired and malfunctioning.[14] Exogenous insulin therapy has no suppressive effect on the ability of beta-cells to secrete C-Peptide. However, the effect of insulin administration was not strong enough to enhance the secretion of C-Peptide endogenously.[15]

Vitamin D might have indirect immunomodulator effect on T-cell regulation, therefore it will inhibit pro-inflammatory cytokine and provide protection to target tissue such as pancreatic beta cells. Vitamin D can also produce inhibition cytokine and counter act the pro-inflammatory cytokine.[2] Pro-inflammatory cytokine is more dominant than anti-inflammatory cytokine in patients with T1 DM. Consistent with the study, level of serum IL-2 in T1 DM patients was significantly higher than that in control group. This inflammatory condition will affect the pancreatic beta cells and reduce its function.[16] In a study by Hussain MJ et al.,[17] TNF-α and soluble IL-2 receptor were found to be increased in patients with T1 DM, and also in their first generation. Serum vitamin D ($25(OH)D_3$) and IL-2 had also significant negative correlation in children with T1 DM. Therefore, low level of vitamin D tends to increase the level of IL-2. Increasing level of IL-2 and IL-2R were associated with low levels of vitamin D.[17]

Vitamin D receptor (VDR) polymorphism is often associated with the pathogenesis of T1 DM because vitamin D exerts its action via the nuclear VDR.[18] The VDR belongs to the steroid receptor super-family and is widely expressed in pancreatic cells. Although the exact role of VDR polymorphisms in TI DM pathogenesis has been unclear yet, several studies have suggested association between VDR single nucleotide polymorphism (SNP) and TI DM.[12] Between four well known SNPs, FokI polymorphism (rs2228570) is associated with increased risk of T1 DM.[18] This SNP results in an alternative transcription initiation site, leading to new protein variant.[19]

In this study, two T1 DM patients had normal vitamin D level, while the level of C-Peptide was low and the level of IL-2 was high. It might be possible that those patients had normal level of vitamin D, but could not work properly. This condition tends to increase the inflammatory state and influence the function of pancreatic beta cells. In fact, specific polymorphism of VDR will make the structure does not fit anymore to bind to active vitamin D. VDR with FokI polymorphism has totally different structure, in comparison with full length VDR. New structure of VDR will undergo conformational changes after both DNA-ligand interaction, and also provide different response to 1.25 $(OH)_2D$.[2]

Conclusion

Circulating vitamin D ($25(OH)D_3$) level seems to be related to inflammatory condition in T1 DM. Low level of serum vitamin D ($25(OH)D_3$) in patients with T1 DM tends to increase the level of IL-2. Moreover, low level of serum vitamin D ($25(OH)D_3$) in T1 DM patients was also highly correlated with pancreatic beta cells' impairment, which is marked by low C-Peptide level and high IL-2 levels.

Peer-review: Externally peer-reviewed.

Ethics Committee Approval: The use of blood samples in this research was approved by local ethical committees, and all patients were given written consent prior to their participation in this study. This research was conducted according to the Declaration of Helsinki Principles..

Conflict of Interest: No conflict of interest was declared by the authors.

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Author Contributions: Harjoedi Adji Tjahjono carried out design of the study, most of the experiment and drafted the manuscript. Vivi Ratnasari and Edi Widjajanto carried out technical execution and molecular analysis. Andreas Budi Wijaya participated in bioinformatical analysis, coordination and also revised the manuscript. All authors read and approved the final manuscript.

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