Serum IL-18 and hsCRP Correlate with Insulin Resistance without Effect of Calcitriol Treatment on Type 2 Diabetes

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ABSTRACT

Background: Chronic low-grade systemic inflammation presented in Type 2 diabetes mellitus plays a major role in disease progression as well as development of micro- and macro-vascular complications of diabetes. Therefore, reducing inflammation can be beneficial in prevention of diabetes complications. **Objectives:** To investigate the association between insulin resistance and inflammatory markers, and assessing the effects of oral Calcitriol on inflammatory cytokines in type 2 diabetic patients. **Methods:** In this double-blind randomized placebo-controlled trial, 70 participants with type-2 diabetes were randomly divided to two groups. One group received two capsules of Calcitriol (0.25 μg 1,25-dihydroxy cholecalciferol per each capsule) per day. The second group received placebo tablets. At the beginning of the study, we assessed insulin resistance and its relation to inflammatory profile. Serum high sensitive C-reactive protein (hs CRP), interleukin-6 and interleukin-18 were also measured at the beginning and the end of the 12-week supplementation trial. **Results:** Mean calcium, phosphorus and vitamin D concentrations in the study participants were 8.98 ± 0.79 mg/dl, 3.86 ± 0.50 mg/dl and 40.91 ± 30.9 ng/ml, respectively. IL-18 and hsCRP had significant positive associations with insulin resistance markers and negative associations with insulin sensitivity markers. At the end of the 12-week supplementation trial, no significant difference was seen in serum levels of hsCRP, IL-6 and IL-18 between the two groups, while these values were adjusted for baseline values. **Conclusion:** Inflammation was associated with insulin resistance in diabetic patients. No anti-inflammatory effect of Calcitriol in terms of decreasing hsCRP, IL-6 and IL-18 detected.

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INTRODUCTION

Type 2 diabetes (T2DM) is a major public health problem and its prevalence is rapidly growing worldwide (1). This chronic disease has significant impacts on patients' quality and length of life (2). Major features of T2DM include insulin resistance, glucose intolerance, hyperinsulinemia, hyperglycemia, and dislipidemia (3).

Chronic low-grade systemic inflammation presented in T2DM plays a major role in disease progression and development of micro- and macro-vascular complications of DM (3,4). Low grade systemic inflammation acts as a key pathophysiological factor in insulin resistance, type 2 diabetes mellitus, steatosis and cardiovascular diseases (5). Inflammation and insulin resistance are also common in chronic diseases such as metabolic syndrome (6). Many studies have shown the relationship between insulin resistance and inflammation, but the mechanisms involved are not completely understood (7).

Compared to healthy individuals, diabetic patients are at greater risk of coronary heart disease (8) and atherosclerosis is the leading cause of morbidity and mortality in type 2 diabetic patients. Accordingly, more than 70 percent of mortality in diabetic patients is due to atherosclerosis (9).

There are several potential pathways for endothelial injury and vascular complications that are related to higher prevalence of CHD in diabetic patients, including chronic inflammation, increased oxidative stress and activation of the immune responses (9). Systemic inflammation is a marker of cardiovascular risk in adult population (10). Therefore, any action aimed to reduce inflammation in diabetic patients, can be beneficial in the prevention of diabetes complications. A newly discovered anti-inflammatory agent is vitamin D (11). The biologically active form of vitamin D, 1,25 (OH)2D binds to its nuclear receptors in the target tissues and exerts its biologic effects (11,13).

Classic roles of vitamin D are the maintenance of calcium homeostasis and bone health (11,12). However, in recent years, new functions such as modulation of the immune system and inflammation are proposed for this vitamin (12,14-16). Several studies have indicated anti-inflammatory effects of vitamin D (11). These data show the relationship between vitamin D deficiency and autoimmune and inflammatory diseases (11,12). It is also shown that macrophage dysfunction and cytokine overproduction occur in vitamin D insufficiency (12). Although in vitro and animal studies confirmed anti-inflammatory effects of active vitamin D or Calcitriol (11,17-19), human studies have reached inconsistent results (11).

Previous studies support the need for further research on anti-inflammatory effects of vitamin D, so the objective of the present study was to investigate the associations between insulin resistance and inflammatory markers and the effects of oral Calcitriol treatment of inflammatory cytokines in type 2 diabetic patients. To our knowledge, this is the first study on anti-inflammatory effects of Calcitriol in diabetic patients.

MATERIALS AND METHODS

The present study was a double-blind randomized placebo-controlled trial. Seventy participants (35 male and 35 female) with type-2 diabetes, aged 30-75 years old, on treatment with oral hypoglycemic drugs were recruited from the outpatient Motahari

Clinic at Shiraz University of Medical Sciences. Criteria for case inclusion were well-controlled fasting plasma glucose, serum calcium <10.5 mg/dl, normal liver and kidney function (GFR between 90 - 120 ml/min/1.73 m^2), and no history of kidney stone and hypercalcemia, chronic infectious and inflammatory disorders. Exclusion criteria included taking insulin for diabetes control, taking calcium and vitamin D supplements, history of diseases affecting vitamin D status and intestinal malabsorption disease.

The participants were asked to read and sign an informed consent document. The study protocol was reviewed and approved by the Human Ethics Committee of Research Council of the vice chancellor for Research Affairs in Shiraz University of Medical Sciences.

Demographic data were collected by interviews and anthropometric indices including measurement of weight and height were determined for each subject. Body weight was measured to the nearest 0.1 kg using the Seca 713 scale, while subjects were minimally clothed. Height was determined using measuring tape without shoes and subsequently body mass index was calculated by dividing weight (kg) by squared height (m^2). Waist circumference was obtained by measuring the distance around the smallest area below the rib cage and above the umbilicus and the hip circumference was measured at its widest part of the buttocks or hip. Waist to hip ratio was calculated by dividing these two measures (20).

The patients were randomly allocated into one of the two study groups using balanced block randomization method. Treatment group received two capsules of Calcitriol (0.25 µg 1,25-dihydroxy cholecalciferol per each capsule) per day (Zahravi Pharmacy Co., Tehran, Iran). The control group received identical-looking placebo capsules.

At the beginning and the end of the 12-week supplementation trial, fasting venous blood samples were drawn from the arm. Blood was collected for measurement of hsCRP, interleukin-6 and interleukin-18. Circulating levels of hsCRP (ELISA, Monobind, USA), IL-6 (ELISA, eBioscience, USA) and IL-18 (ELISA, eBioscience, USA) were measured in the laboratory of Endocrinology and Metabolism Research Center of Shiraz University of Medical Sciences. All measurements were performed using a sandwich ELISA according to manufacturer's protocol. Serum levels of fasting insulin, glucose and lipid profile were also determined. Insulin resistance and sensitivity were calculated as shown in Table 1 (21).

Table 1. Insulin resistance and sensitivity assessment.

	Definitions
Quicki Index	1/ log (glucose mg/dl) + log (insulin µU/ml)
HOMA-IR	(Fasting insulin [micro units per milliliter] × fasting glucose [millimoles per liter]) / 22.5
McAuley	Exp [2.63−0.28ln (insulin) − 0.31ln (triglyceride)]
FIRI	Insulin (µU/ml) × glucose (mmol/l) /25
Bennetts Index	1/ log [glucose(mmol/l)] × log[insulin(µU/ml)]

HOMA-IR: Homeostasis Model Assessment-Insulin Resistance; FIRI: Fasting Insulin Resistance Index.

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Statistical Analysis. Data processing and analysis were done using SPSS version 17 for windows (SPSS Inc, Chicago, USA). Normally distributed data were expressed as mean (± Standard Deviation). We investigated the association between insulin resistance and sensitivity indexes, inflammatory markers and serum levels of 25 (OH) D using bivariate correlation analysis.

To analyze the intervention outcomes, basal values of hsCRP, IL-6 and IL-18 were compared between treatment and control groups using independent student's *t-test*. Also, to determine the effects of Calcirtiol on each one of outcome variables (hsCRP, IL-6 and IL-18) at the end of the 12-week intervention adjusted for basal values, we used covariance analysis. This analysis adjusts basal values of each variable. Significance level was set at p<0.05.

RESULTS

70 diabetic patients (35 males and 35 females) participated in our study. Baseline and biochemical characteristics of the participants are displayed in Table 2.

Table 2. Baseline and biochemical characteristics of treatment and control group.

Variable	Mean±SD		P Value **
	Treatment	**Control**	
Age (year)	53.8 ± 8.9	52.4 ± 7.8	0.462
Weight (kg)	72.9 ± 12.7	70.9 ± 12.5	0.514
Height (cm)	160.3 ± 8.9	161.1 ± 10.4	0.718
WHR	0.99 ± 0.07	0.95 ± 0.13	0.080
BMI (kg/m^2)	28.3 ± 4.4	27.0 ± 3.4	0.164
Diabetes duration (year)	6.3 ± 5.3	6.6 ± 4.8	0.819
Glybenclamide (number of 5 mg tablets/day)	3.2 ± 1.3	2.9 ± 1.7	0.437
Metformin (number of 500 mg tablets/day)	2.9 ± 1.4	2.4 ± 1.2	0.110
FBS (mg/dl)	145.6 ± 52.1	141.8 ± 48.5	0.753
Hb.A1C (%)	7.08 ± 1.6	7.03 ± 1.7	0.676

** Independent Samples *t-test*
WHR: waist to hip ratio

Serum 25(OH)D, calcium and phosphorus levels are shown in Table 3.

Vitamin D Status, Inflammation and Insulin Resistance. Association analysis between insulin resistance and sensitivity indices and inflammatory profile are

presented in Table 4. As shown, IL-18 and hsCRP showed significant positive associations with insulin resistance markers (HOMA-IR and FIRI) and negative associations with insulin sensitivity markers (QUICKI, McAuley, and Bennetts Index) at the beginning of the study.

Table 3. Biochemical variables at baseline and end of the intervention.

Variable	Control			Treatment		
	Baseline	3 months	P Value	Baseline	3 months	P Value
25(OH) vitamin D (ng/ml)	38.54 ± 29.9	30.92 ± 22.2	0.013$^{£}$	43.28 ± 32.1	34.10 ± 31.5	0.017
Calcium (mg/dl)	9.01 ± 0.6	9.14 ± 0.3	0.331	8.94 ± 0.9	9.26 ± 0.5	0.059
Phosphorus (mg/dl)	3.87 ± 0.5	4.07 ± 0.5	0. 057	3.87 ± 0.5	4.13 ± 0.4	0.013

* Mean ± SD, £: Paired *t-test*

Mean calcium, phosphorus and vitamin D concentrations in the study participants were 8.98 ± 0.79 mg/dl, 3.86 ± 0.50 mg/dl and 40.91 ± 30.9 ng/ml, respectively. Sixteen subjects out of our 70 participants were vitamin D deficient (serum 25(OH)D under 20 ng/ml).

Table 4. Association between Insulin resistance and sensitivity indexes and inflammatory markers.

Variables	HOMA-IR	QUICKI	McAuley	FIRI	Bennetts Index
IL-6 (pg/ml)	N.S.	N.S.	N.S.	N.S.	N.S.
IL-18 (pg/ml)	0.301* (0.011)**	-0.285 (0.017)	-0.240 (0.046)	0.301 (0.001)	-0.285 (0.017)
hsCRP (μg/ml)	0.468 (0.00004)	-0.355 (0.003)	-0.261 (0.029)	0.468 (0.00004)	-0.285 (0.017)

N.S: not significant, * Correlation coefficient (r), ** P-value

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At the beginning of the study, There was no significant relationship between serum 25(OH)D level and IL-6 and hsCRP. A near to statistically significant negative relationship between 25(OH)D level and IL-18 was seen (r=-0.233, p=0.052).

Impact of Calcitriol Treatment of Inflammatory Markers. Comparison of the mean baseline and outcome values between the treatment and control groups is shown in Table 5. There were no significant differences between the serum inflammatory markers of the two groups at the beginning of the study. Also at the end of the study, comparing level of serum inflammatory markers adjusted for baseline values, no significant difference was seen in hsCRP, IL-6 and IL-18 between the two groups.

Table 5. Comparison of the mean baseline and outcome values between the treatment and control groups.

	Variables	Treatment	Control	P Value
	hsCRP (µg/ml)	1.72 ± 2.4	1.93 ± 1.8	0.695
Baseline*	IL-6 (pg/ml)	5.05 ± 3.8	4.75 ± 3.8	0.749
	IL-18 (pg/ml)	199.58 ± 190.2	247.87 ± 178.1	0.277
	hsCRP (µg/ml)	1.98 ± 2.0	2.11 ± 1.8	0.830
Outcome**	IL-6 (pg/ml)	2.96 ± 1.2	3.24 ± 2.1	0.433
	IL-18 (pg/ml)	213.04 ± 200.3	266.08 ± 210.5	0.755

* Independent student *t-test*, ** Co-variance Analysis

DISCUSSION

Inflammation and insulin resistance are both common in several chronic diseases such as type-2 diabetes mellitus (5). Chronic low-grade systemic inflammation is a key factor in progression of type-2 diabetes mellitus and development of its micro- and macrovascular complications (3). Low grade systemic inflammation is also considered as a main pathophysiological factor in insulin resistance, type-2 diabetes mellitus, steatosis and cardiovascular diseases (5).

In our study, IL-18 and hsCRP levels at the beginning of the study had significant positive associations with insulin resistance markers (HOMA-IR and FIRI) and negative associations with insulin sensitivity markers (QUICKI, McAuley and Bennetts Index).

In vitro studies revealed an association between cytokines and insulin resistance, but clinical studies reached conflicting results (22-24). A large epidemiological study by Marques-Vidal et al. (23) on subjects with diabetes and insulin resistance showed elevated levels of pro-inflammatory cytokines, such as IL-6, TNF-α and hs-CRP;

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however, in a study by Abbatecola et al. no significant associations were observed between the cytokines and impaired glucose tolerance (22).

Based on the evidence showing the presence of VDR in immunocompetent cells (25), and the influence of 1,25(OH)2D on inflammatory cytokines in vitro (11,26), one can propose a relationship between vitamin D status and pro-inflammatory cytokines (12). 1,25 (OH)2D modulates Ca^{2+} signaling and reactive oxygen species (ROS) production and each of these can modulate inflammatory cytokines production and release. Therefore, it is anticipated that Calcitriol can modulate cytokine production in adipocytes and macrophages (27). In fact, Calcitriol can modulate cytokine production in adipocytes and macrophages through Ca^{2+}-dependent and mithochondrial uncoupling-dependent mechanisms (27). Human studies regarding the relationship between vitamin D status and cytokines in vivo demonstrate inconsistent results (12).

Peterson et al., assessed the relationship between vitamin D status and inflammatory markers in 69 healthy women. The results showed no significant relationship between serum 25(OH)D concentration with serum IL-6, IL-10 and CRP levels. However, linear regression models revealed a significant inverse relationship between serum concentrations of 25(OH)D and TNF-α levels (12). In a cross-sectional study on 1381 Framingham offspring study participants, there was no significant relationship between serum 25(OH)D concentration and systemic inflammatory markers (17). In our study, there was no significant relationship between serum 25(OH)D level and IL-6 and hsCRP. A nearly statistically significant negative relationship between 25(OH)D level and IL-18 was seen. Moreover, after Calcitriol treatment, there was no significant difference between the treatment and control groups in IL-6, IL-18 and hsCRP levels.

Regarding anti-inflammatory properties of vitamin D, human studies have yielded different results. In a study by Schelithoff et al., daily intake of 50 µg vitamin D for 9 months in CHF patients increased the serum concentrations of anti-inflammatory cytokine IL-10, and prevented any increase in the serum concentrations of pro-inflammatory cytokine TNF-α (28). In a study by Beilfuss et al., one-year supplementation with high-dose cholecalciferol in overweight and obese subjects decreased serum IL-6 and increased serum hsCRP level (11). Gannage-Yared et al., reported that calcium plus vitamin D supplementation for 12 weeks has no effect on circulatory cytokines in healthy post-menopausal women (17,29).

C-reactive protein is a non-specific marker of inflammation. It increases with chronic infection, increased age and tissue damage (12). CRP has long been used to assess high degree inflammation in infections and chronic inflammatory diseases. But highly sensitive assays can report even mild increases of CRP in the range of 2-6 mg/L (10). Highly sensitive CRP is a marker of endothelial dysfunction and is an adjunctive method to assess global cardiovascular risk (30). There are strong associations between hsCRP level and future cardiovascular risk in adults (10). Studies published on diabetic patients have revealed an inverse association between serum 25(OH)D and CRP level (12). But an intervention preformed on post-menopausal women with CHF, vitamin D supplementation did not have any effect on the CRP level (12).

IL-6 is an acute phase mediator with pro- and anti-inflammatory properties. Increased level of IL-6 contributes to endothelial dysfunction and predicts myocardial infarction, and is also related to cardiovascular death (11). In some studies, increased level of IL-6 was associated with insulin resistance or type-2 diabetes mellitus (31).

Several in vitro studies reported that 1,25(OH)2D can inhibit IL-6 production in different cell types (12). But in vivo studies did not establish the effects of vitamin D on

IL-6 level in human (12,17,28). In a study on patients with renal failure undergoing hemodialysis, six months of supplementation with oral and intravenous Calcitriol reduced the serum IL-6 level significantly (32). But we cannot extrapolate the results of vitamin D supplementation in uremic patients to diabetic population because uremic patients have difficulty activating vitamin D and they are most probably 1,25(OH)2D deficient. Therefore, calcitriol intake makes significant effect in reducing the serum IL-6 level.

Interleukin-18 is a pro-inflammatory cytokine, a member of IL-1 family cytokines. It is produced by vascular endothelial cells and activated machrophages (33,34). Therefore, IL-18 is a vascular injury induced inflammatory marker and is of prognostic value for future cardiovascular events (34). The serum concentration of IL-18 is reported to be elevated in type 2 diabetic patients (33).

IL-18 directly causes plaque destabilization and cardiac dysfunction. It also promotes vascular remodeling through upregulating macrophage infiltration and increasing the medial thickness of the aortal wall (33). IL-18 also contributes to microangiopathy and is, thus, related to diabetic nephropathy. Besides its pro-inflammatory effects, it directly affects renal function and, therefore, contributes to nephropathy progression (34). In all, IL-18 is a predictor of cardiovascular and renal complications of diabetes mellitus (35). We could not find any published study on the effects of vitamin D on IL-18, and it seems that our study is the first one assessing the effects of vitamin D on serum IL-18 level; however, we did not find any effect of Calcitriol on IL-18.

In several in vitro studies, active vitamin D or Calcitriol are reported to reduce the inflammatory cytokine production. But not all in vivo studies found similar results. Probably, it is because of tight body control exerted on 1,25(OH)2D production, and its narrow physiologic range in the serum (17).

Regarding the lack of any effect of Calcitriol on inflammatory markers in the present study, there are a number of plausible explanations. First, type 2 diabetes is a pro-inflammatory condition, but the role of immune system in this disease may be marginal relative to auto-immune diseases such as multiple sclerosis, IBD and chronic hepatitis (11) where the effects of vitamin D are more prominent. Therefore, vitamin D anti-inflammatory effects cannot be seen in low-grade systemic inflammation that occurs in type 2 diabetes mellitus. Second, our participants had no problem converting 25(OH)D to 1,25(OH)2D. Our data therefore make sense with respect to the fact that the effects of Calcitriol supplementation is more pronounced in subjects with low concentration of 1,25(OH)2D. Third, as it was mentioned before, some anti-inflammatory effects of vitamin D are exerted through Ca++ ion modulatory properties of vitamin D, therefore, probably co-supplementation of calcium and vitamin D could better show anti-inflammatory properties of vitamin D.

We found a nearly statistically significant negative relationship between 25(OH)D level and IL-18. The mean vitamin D concentration in our diabetic participant was in normal range therefore we assume that Calcitriol treatment may better show anti-inflammatory effects in diabetes patients with low vitamin D concentration.

Limitations of our study include short duration of the intervention, using formulas to assess insulin function instead of the standard method of Clamp test, and assessment of a few number of interleukins.

In conclusion, inflammation was associated with insulin resistance in diabetic patients. Calcitriol treatment had no effect on the level of inflammatory cytokines in type 2 diabetic patients in a 12-week supplementation trial.

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