Carboxylated Osteocalcin and Adiponectin Correlate with Glycated Hemoglobin and 25(OH) Vitamin D Levels in Saudi Females with Type 2 Diabetes Mellitus Following Ingestion of Vitamin D Supplements

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Authors’ contributions

This work was carried out in collaboration between all authors. Author SMB designed the study, wrote the protocol, and wrote the first draft of the manuscript. Author SQ choose analytical methods and supervised all practical aspects of the study, author FK collected samples, performed all biochemical estimations, statistical analysis and presentation of results, author KAS provided clinical insight in research design, and was responsible for recruitment of correctly diagnosed subjects providing needed medical history, author HJ managed the literature searches and organizing references, author EH reviewed the manuscript critically, submitted it and helped in formulating final version. All authors read and approved the final manuscript.

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ABSTRACT

**Background:** Type 2 diabetes (T2D) is one of the fastest growing public health problems worldwide. Evidence linking vitamin D (VTD) status and T2D was reported. However, its role, and whether it acts directly or through stimulating synthesis of osteocalcin is still unclear. Vitamin D deficiency is common amongst Saudi adults. However, vitamin D supplement is not part of usual management regimen for diabetic patients.

**Objectives:** To study: 1-The effect of self-administered minimum dose of vitamin D supplements on glycemic control, insulin sensitivity, and lipids profile in T2DM females. 2-The relationship between serum levels of VTD and different forms of osteocalcin (OC), and the active circulating form of adiponectin.

**Subjects and Methods:** Sixty T2DM females were recruited to form two groups matched in age and body mass index (BMI). Group A (GA) was the vitamin supplemented group, with a minimum of 3 months intake of cholecalciferol (400IU/day), while Group B (GB) was the control group receiving no supplements. Fasting blood samples were drawn for the measurements of glucose, lipids profile, insulin, glycated hemoglobin (HbA1c), 25(OH) vitamin D, total OC, carboxylated OC (cOC), uncarboxylated OC (ucOC), and High molecular weight adiponectin (HMWApn).

**Results:** Means of 25(OH) vitamin D, triglycerides, total OC, cOC and HMWApn were all significantly higher in Group A compared to corresponding means in Group B, while mean HbA1c was significantly lower (P< 0.05 in all cases). VTD correlated positively with total and cOC in both groups. However, it correlated positively with HMWApn, and negatively with HbA1c in Group A only. No correlation between VTD and ucOC was found. cOC correlated highly positively with HMWApn, and negatively with HbA1c in both groups.

**Conclusion:** VTD supplemented subjects showed better glycemic control, possibly indirectly by stimulating the synthesis of osteocalcin, and hence adiponectin. Caboxylated OC is most likely the active form. Therefore, vitamin K status must be monitored.

**Keywords:** Vitamin D; glycemic control; osteocalcin; carboxylated osteocalcin; adiponectin.

1. INTRODUCTION

Type 2 diabetes (T2D) is one of the fastest growing public health problems in both developed and developing countries imposing a high financial burden on health care costs [1,2]. Reports indicate that Saudi Arabia has one of the highest prevalence rates of T2D in the world [2-4]. The hyperglycaemia in T2D has long been known to be associated with reductions in both insulin sensitivity and beta cell function [5], with genetic, environmental, dietary and lifestyle factors playing major roles in its development [6-8]. Once established, T2D is difficult to treat, and despite pharmacologic treatment blood glucose levels tend to increase over time leading to serious health complications [9,10]. This rapidly increasing incidence and prevalence emphasizes the importance of innovative approaches for the management and prevention of the disease. Indeed, various efforts were made to study its pathogenesis and etiology, in order to prevent its development, or; at least; to control associated complications.

More than fifty years ago, animal studies indicated that calcium is needed for insulin secretion [11]. These studies showed that an adequate vitamin D (VTD) status is necessary for pancreatic islets function and glucose tolerance [12,13]. Furthermore, evidence linking VTD status and T2D was reported [14], and an association between VTD deficiency, beta cell dysfunction and insulin resistance was observed [15]. Hence, an association between the onset of T2DM and VTD deficiency was suggested [16,17]. Further evidence came from studies reporting low VTD levels in patients with T2DM [14,18].

More recently, osteocalcin; a non-collageneous protein produced by osteoblasts under the stimulation of the active form of VTD [19]; was reported to increase insulin secretion and β-cell proliferation, in addition to regulating body fat mass and adiponectin gene expression in a genetically modified mouse model [20]. This was confirmed by other studies in wild-type mice [21,22]. Human studies demonstrated further the positive association between serum osteocalcin levels, insulin secretion and insulin sensitivity [23-25]. In addition, serum osteocalcin levels were reported to be inversely associated with dysmetabolic phenotypes such as atherogenic...
dyslipidemia, abdominal obesity, and metabolic syndrome [26, 27]. Since circulating osteocalcin is found in carboxylated and un- (or under) carboxylated forms, there is considerable debate regarding which form is the active one with respect to these effects. Earlier animal studies and in vitro data [21, 28], as well as some human studies [24, 29] suggested that only the uncarboxylated form of osteocalcin (ucOC) functions hormonally in the regulation of glucose homeostasis and energy metabolism, leading to the suggestion that VTD promotes insulin production and sensitivity by stimulating ucOC secretion by osteoblasts [29]. However, most human studies related effects to level of total osteocalcin [23, 25-27], in addition to the carboxylated form [30]. Debate about how osteocalcin regulates insulin sensitivity is still ongoing, with some suggesting that osteocalcin regulates insulin sensitivity through an effect on adiponectin rather than through a direct effect on insulin [28], and others disputing this [25].

Vitamin D deficiency and insufficiency are common amongst Saudi adults, and in particular females [31, 32], with even lower levels among diabetic patients [33]. However, vitamin D supplement is not part of usual management regimen for diabetic patients.

Therefore, we aimed to study the effect of self-administered minimum available dose of vitamin D supplements on glycemic control; measured by glycated hemoglobin (HbA1c); and insulin resistance measured by the homeostasis model assessment of insulin resistance (HOMA-IR) in T2DM female patients. In addition, the relationship between vitamin D level, different forms of osteocalcin, the active form of adiponectin (high molecular weight adiponectin; HMWApn) [34], insulin resistance, and glycemic control was also investigated, in the hope of clarifying the overall picture further.

2. SUBJECTS AND METHODS

2.1 Study Design

A comparative study between two groups of type 2 diabetic females was implemented. A total of 60 patients; treated by oral hypoglycemic drugs; were recruited from attendants of “outpatients clinics” at King Abdulaziz University Hospital between mid December 2010 and mid February 2011. Pregnant subjects, and subjects on insulin, or those suffering from serious diabetic complications (such as cardiovascular disease, fatty liver or chronic renal failure) were excluded. Subjects suffering from other endocrine and/or metabolic disorders; including metabolic bone disease were also excluded. The exclusion included as well subjects taking cortisone or other steroids, diuretics, under phenobarbital and phenytoin medications, or taking mineral oil products, using antacids regularly, or having gastrointestinal disorders. Subjects reporting daily intake of vitamin D supplements at minimum available dose (400 IU as cholecalciferol) for at least three months were allocated to the experimental group (Group A). Selected subjects were interviewed for demographic data, and their heights and weights were measured and recorded to calculate their body mass index (BMI) by dividing weight (Kg) by height squared (m²). The patients’ medical records were reviewed for the type and dosage of used therapy. The control group (Group B) was selected from patients not taking any supplements, to match Group A subjects in age, BMI, smoking status and treatment regimen. Fasting blood samples were obtained from all selected subjects, and separated serum and plasma were frozen at - 80°C for later analysis in one batch to avoid inter-batch variability. All participants signed a written informed consent. The study was approved by the Committee on "the Ethics of Human Research" at the Faculty of Medicine- King Abdulaziz University.

2.2 Biochemical Estimations

Glucose, HbA1c, total cholesterol, HDL-cholesterol, and triglycerides (TG) were estimated using automated enzymatic methods (Dimension Vista 1500T Intelligent Lab System from SIEMENS Company). Low density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald equation [35]. Fasting insulin and 25(OH) vitamin D were measured using a direct electrochem-luminescence immunoassay on modular E170 (Elecsys module) immunoassay analyzer, all supplied by Roche Diagnostics GmbH in the biochemistry laboratory at the university hospital.

Serum HMWApn, total OC and ucOC levels were measured manually using commercially available ELISA kits (ALPCO Company, Germany). Carboxylated OC was separated from ucOC by adsorption on hydroxyapatite (Calbiochem, USA) according to published methods [36]. The cOC concentration was calculated as the total OC concentration minus the ucOC concentration. HOMA-IR was calculated using the equation: fasting glucose x fasting insulin < 22.5 [37].
2.3 Statistical Analysis

Analysis was performed using SPSS statistical package version 16. Descriptive statistics, such as mean ± SEM, were calculated for all estimated parameters. Unpaired Student t-test, and the Mann Whitney-U test were employed for comparison of normally distributed and non-normally distributed parameters, respectively. Partial correlation analysis was performed to study association between 25(OH) vitamin D, different forms of osteocalcin, HMW adiponectin and HbA1c. Significance was assigned at \( P < 0.05 \).

3. RESULTS

Age of recruited subjects ranged between 40-62 years, with no significant difference between the means of the experimental and control groups (\( P = 0.221 \)), and mean ± SEM being 53.20 ± 1.22 and 50.20 ± 1.15 for GA and GB respectively. The BMI ranged between 22.1- 40.6, with most subjects being overweight or obese, no significant difference between the means of the experimental and control groups (\( P = 0.306 \)), and mean ± SEM being 32.16 ± 1.03 and 30.75 ± 0.896 for GA and GB respectively. Daily dose of vitamin D in GA varied from 400 - 800 IU as cholecalciferol.

3.1 Biochemical Characteristics of Study Groups

Biochemical characteristics of both study groups are presented in Table 1.

The mean 25(OH) vitamin D concentration was significantly higher in the experimental group (GA) than in the control group (GB). Using the latest suggested classification of vitamin D status [38], one subject in each group had 25(OH)

| Table 1. Biochemical characteristics of subjects in experimental (Group A) and control (Group B) groups |
|-----------------------------------|-----------------|-----------------|-----------|
| **Group A** | **Group B** | **P-Value** |
| **25 (OH) D\(_3\)** (ng/ml) | 10.00 – 70.71 (30.27 ± 3.36) | 10.00 – 43.00 (18.57 ± 1.74) | 0.003 |
| Fasting Glucose (mmol/L) | 3.5 – 20.7 (7.8 ± 0.6) | 5.2 – 21.8 (9.0 ± 0.6) | 0.191 |
| HbA1c (%) | 6.1 – 12.7 (7.97 ± 0.27) | 5.6 – 15.1 (9.05 ± 0.43) | 0.036 |
| Fasting Insulin (mU/L) | 5.10 – 35.63 (14.50 ± 1.34) | 4.91 – 30.70 (13.68 ± 1.28) | 0.659 |
| HOMA-IR | 1.54 – 13.74 (4.94 ± 0.56) | 1.40 – 17.19 (5.47 ± 0.66) | 0.542 |
| Total Cholesterol (mmol/L) | 3.28 – 5.96 (4.50 ± 0.14) | 1.88 – 6.97 (4.80 ± 0.22) | 0.252 |
| HDL (mmol/L) | 0.92 – 1.94 (1.35 ± 0.06) | 0.81 – 1.83 (1.32 ± 0.06) | 0.787 |
| LDL (mmol/L) | 1.90 – 4.66 (2.79 ± 0.12) | 1.13 – 4.76 (3.17 ± 0.18) | 0.082 |
| TG (mmol/L) | 0.66 – 3.35 (1.67 ± 0.13) | 0.57 – 2.52 (1.34 ± 0.08) | 0.033 |
| Total Osteocalcin (ng/ml) | 2.0 – 16.1 (8.02 ± 0.77) | 1.9 – 11.2 (5.84 ± 0.43) | 0.016 |
| Uncarboxylated Osteocalcin (ng/ml) | 1.0 – 4.4 (2.21 ± 0.20) | 0.9 – 6.3 (2.46 ± 0.22) | 0.406 |
| Carboxylated Osteocalcin (ng/ml) | 0.7 – 14.6 (5.81 ± 0.76) | 0.9 – 8.3 (3.38 ± 0.33) | 0.005 |
| HMW Adiponectin (ng/ml) | 1.1 – 6.3 (4.18 ± 0.24) | 0.8 – 6.5 (3.07 ± 0.29) | 0.005 |
vitamin D below the recognized severe deficiency cut-off level of ≤ 10 ng/ml, while most subjects (22 or 73.3%) in the control group, and 3 in the experimental group were in the vitamin D insufficiency range (11-20 ng /ml). Only one subject in the control group, compared to 16 in the experimental group had serum concentrations ≥ 30 ng/mL, suggested by some as the cutoff value for defining optimal vitamin D status [39,40].

No significant difference in the means of fasting serum glucose, insulin or calculated HOMA-IR was found between the experimental group when compared to control group. However, the majority of subjects in the control group (21 or 70%) had poorly controlled glucose level reflected on their HbA1c values. The opposite was noted in the experimental group with almost half the subjects (14 subjects or 46.7%) showing good glycemic control, reflected on HbA1c values ≤ 7%.

The means of total, HDL- and LDL- cholesterol did not differ significantly between the two groups. However, the mean serum triglycerides concentration was significantly higher in the experimental group (P= 0.033).

Most (> 70%) of the circulating osteocalcin was in the carboxylated form, with significantly higher means of total and carboxylated osteocalcin being found in the experimental group (Table 1). Similarly higher mean HMW adiponectin was found in the experimental group.

The results of partial correlation analysis to study the association between the concentrations of 25(OH) vitamin D, different forms of osteocalcin, HMW adiponectin and HbA1c showed that there is a significant negative correlation between 25(OH) vitamin D and HbA1c in GA (Fig. 1a), but not in GB (Fig. 1b). There was also significant positive correlation between 25(OH) vitamin D in both study groups with total OC (r=0.421, P= 0.01 for GA and r=0.511, P= 0.009 for GB) and cOC (Figs. 2a & b), as well as with HMW adiponectin (r= 0.505, P=0.004) in GA, but not in GB (r= 0.295, P= 0.112). However, no significant correlation between 25 (OH) vitamin D and undercarboxylated osteocalcin was found in either group.

Significant negative correlation was also found between concentrations of total and cOC osteocalcin and HbA1c in GA (Correlation coefficient, r = -0.385, P= 0.040 for total, and r= -0.404, P= 0.026 for cOC), and in GB (r= -0.493, P=0.01 for total, and r= -0.549, P= 0.002 for cOC) (Figs. 3a & b).

Moreover, Significant positive correlation between concentrations of total and cOC with HMW adiponectin in GA (Correlation coefficient, r=0.396, P=0.031 for total, and r=0.437, P=0.015 for cOC), and in GB (r=0.484, P=0.009 for total, and r=0.560, P=0.001 for cOC) (Figs. 4a & b). However, no significant correlation between undercarboxylated osteocalcin and HbA1c or HMW adiponectin was found in either group. Finally, significant negative correlation between the concentration of HMW adiponectin and HbA1c in GA (Correlation coefficient, r = -0.480, P= 0.007), and in GB (r= -0.378, P=0.039).

![Fig. 1. Correlation between 25(OH) vitamin D and HbA1c in Group A (a) and Group B (b)](image-url)
Fig. 2. Correlation between 25(OH) vitamin D and carboxylated osteocalcin (cOC) in Group A (a) and Group B (b)

Fig. 3. Correlation between carboxylated osteocalcin (cOC) and HbA1c in Group A (a) and Group B (b)

Fig. 4. Correlation between carboxylated osteocalcin (cOC) and high molecular weight adiponectin (HMWApn) in Group A (a) and Group B (b)

4. DISCUSSION

As reported by studies in our region [31,32] a high percentage of the studied groups had below optimal vitamin D status, reflected on low 25(OH) vitamin D serum concentration. Ingestion of 400 IU/ day of the vitamin for at least three month increased the mean concentration of circulating 25 (OH) vitamin D significantly (P= 0.003, Table 1). However, half of the subjects still had levels below suggested optimal vitamin D status value of ≥ 30 ng/mL [39,40]. This could be due to
variation in original VTD status between selected subjects, as well as duration of using supplement. In addition, there is well documented wide variation between individuals with regard to the 25(OH) vitamin D level achieved with the same oral dose of vitamin D [41,42].

Different effects of supplementation with VTD on glucose homeostasis and insulin secretion were reported by different research groups, with some reporting no effect on fasting blood sugar, insulin and HbA1c in VTD supplemented group of subjects compared with placebo group [43-46], and others reporting a significant improvement in insulin secretion and/or glucose tolerance in supplemented subjects [47-49]. The reasons for discrepancy seen in different studies might be related to differences in duration of the study, sample size, ethnicity and health status of study population, dose and formulation of used supplements, adherence and non-study supplement use, as well as difference in lifestyle, body composition and original VTD status of subjects prior to supplement [50,51].

In our study, no significant difference was noted in the mean of fasting glucose between the two groups. This could be explained by the noted dietary adjustment by patients before going in for laboratory testing to avoid reprimand by their physicians in the case of fasting glucose. However, since patients cannot manipulate their HbA1c, this could be a better measure of long term glucose homeostasis. In our study, the mean HbA1c was found to be significantly lower in the experimental group (P=0.036, Table 1), indicating better glycemic control among the subjects taking the supplement. A well designed and controlled clinical trial, with the supplement supplied to the patients might yield even more control. The noted effect on glycemic control could reflect either improved β-cell function, and/or improved tissue sensitivity to insulin. However, the lack of difference in the means of insulin and HOMA-IR between the two groups suggests that VTD supplement had no effect on insulin sensitivity.

Low VTD status was reported to be associated with increased cardiovascular risk which includes atherogenic lipid profile [52,53]. However, studies so far have shown conflicting results regarding the effect of VTD supplementation on blood lipids [54-61]. A recent large cross sectional combined with cohort study found that correcting VTD deficiency was associated with an increase in mean total and high-density lipoprotein cholesterol, but non-significant changes in low-density lipoprotein cholesterol and triglycerides, and concluded that supplementation to correct for VTD deficiency might not lead to clinically meaningful changes in lipid concentrations [62]. Thus, our finding of higher mean serum triglycerides might not be a consequence of VTD supplementation, but due to differences in dietary and lifestyle practices, as well as differences in used medications.

Vitamin D regulates the synthesis of osteocalcin [19]. Therefore, the noted higher mean concentration of total osteocalcin in the supplemented group (P=0.016, Table 1), as well as the significant positive correlation between the concentrations of VTD and total osteocalcin in both studied groups (r=0.421, P= 0.01 for GA and r=0.511, P= 0.009 for GB) was expected. Similar results were found for cOC (Table 1, Figs. 2a & b), but not for ucOC. This might be because the concentration of ucOC is reported to be dependent on vitamin K status rather than VTD [63-65].

The noted significant correlation between VTD concentration and glycemic control; reflected on HbA1c level; in the experimental group, but not in the control group (Figs. 1a & b) leads to the suggestion that VTD concentration must reach a certain level before any association is detected. However, since osteocalcin is reported to regulate adiponectin gene expression [21,28], and significant correlation between HMWApn and osteocalcin in both studied groups was found (Figs. 4a & b), but only in GA with VTD, a second suggestion is that VTD is associated indirectly to glycemic control through its effect on osteocalcin synthesis.

Furthermore, the noted significant negative correlation of HbA1c, and the positive correlation of HMWAdpn with total and cOC, but not ucOC, leads to the suggestion that cOC is the active form involved in regulating adiponectin synthesis, as well as glucose homeostasis.

Our study has its limitations. First of all, supplements were taken voluntarily and had to be bought, so adherence to daily intake might have been inadequate. Secondly, the duration of the supplement was not the same for all subjects, varying between three months and more than three years, which could explain the variation in estimated level within the supplemented group (GA). However, this should
not affect the interpretation of results since the mean 25 (OH) vitamin D in GA was significantly higher, and the conclusions were based on studied correlations between estimated parameters rather than actual values. Thirdly, the initial VTD status and measures of glycemic control and glucose homeostasis of studied subjects before taking the supplement was not known. However, it is hoped that our results will stimulate further studies in our region to investigate the role of VTD supplements in diabetes management.

5. CONCLUSION

In conclusion; and in spite of the limitations; it can be suggested that supplementing T2DM females in our region; even with the minimum available dose of VTD of 400 IU; has beneficial effects on their glycemic control. The effect is most likely due to stimulation of osteocalcin synthesis, with the carboxylated form appearing to be the active hormone. Therefore, care must be taken to ensure adequate vitamin K status, especially that many diabetics are on medications that could compromise that status.

CONSENT

All authors declare that written informed consent was obtained from all involved patients for publication of the obtained results anonymously, after the objectives of the study was thoroughly explained to them individually.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.
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