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The Effects of Vitamin D Supplementation on Serum Levels and Genes Expression of Some Inflammatory and Endothelial Biomarkers in Patients with Type-2 Diabetes Mellitus: A Study Protocol for a Randomized Double-Blind Controlled Trial

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Abstract

Background:Diabetes mellitus has adverse effectson small and large vessels and causes micro- and macro-angiopathy.Vascular complications of diabetes are among the leading cause of mortality and morbidity in diabetic patients. Several studies have suggested the possible health benefit of vitamin D on the development of diabetic vascularcomplications, but little is known regarding theinvolvedmolecular mechanisms.Endothelial dysfunction is an importantearly event in the pathogenesis of vascular complications which is defined by apro-inflammatory state, and prothromboticproperties. The objective of our study was to determine the effect of vitamin D supplementation on bloodglucose indices, lipids, inflammatory profiles, endothelial dysfunction biomarkers, gene expression of enzyme glyoxalas-1, chitinase-3-like-1 (YKL40), and receptor for advanced glycation end products (RAGE) in peripheral blood mononuclear cells (PBMC) in type-2diabetes mellitus(T2DM)participants.

***Methods*:** For 3 months, 46 type-2 diabetic patients randomly divided into two groups (n=23 per group), receiving 100 µg/d (4000 IU) vitamin D or placebo.

General characteristics, dietary intakes (at the beginning, middle, and end), and physical activity (at the beginning and end) will be assessed using a general questionnaire, 24-h food recall, and short-form International Physical Activity Questionnaires (IPAQ), respectively. At the beginning and the endof trial, anthropometrics(weight, height, and waist circumference), blood pressure, and blood biomarkers, including serum glucose indices (fasting bloodsugar (FBS)),fasting blood insulin (FBI), homeostasis model assessment-insulin resistance (HOMA-IR), Quantitative Insulin SensitivityCheck Index (QUICKI), lipids (triglyceride (TG), low-density lipoprotein-cholesterol (LDL-c), high-densitylipoprotein-cholesterol (HDL-c), total cholesterol (TC)), inflammatory markers (tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6)), endothelial dysfunction factors (advanced glycation end products (AGEs)),YKL40, and plasminogen activator inhibitor -1 (PAI-1) will be determined. Gene expression of enzyme glyoxalas-1, YKL-40 factor, and RAGE in PBMC will be measured at the beginning and end ofthe study by real time PCR.

***Conclusion*:** This trial would be the first study to examine the effect ofvitamin D on certain genes and serumfactors amongT2DM patients.Further study is suggested to assess the potential of vitamin D in improving vascular complications and itsmolecular mechanisms.

Trial registration number:NCT03008057

Keywords: Diabetes, Vitamin D,Vascular complications

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I. BACKGROUND

The diabetes mellitus has reached a worldwide epidemic. The global prevalence of diabetes mellitus among the adult population (20 to 79 years) in 2010 was reported 6.6% and it is estimated to reach 7.8% by 2030[1]. Diabetic patients are at great risk for microvascular and macrovascular complications [2, 3]. Advanced

glycation end-products (AGEs) produced by uncontrolled hyperglycemia without mediating enzyme, has been consideredcausative initiator of diabetic vascular complications [4].AGEs have shown to increase the glomerular permeability, tubular inflammation which subsequently results innephropathy[5].

AGEs, throughbindingwith theirreceptors,RAGE, activatethe gene expression of pro-inflammatorycytokinessuch as TNF-α and IL-6. These inflammatory cytokines induce endothelial expression of plasminogen activator inhibitor-1 (PAI-1).PAI-1 as a major fibrinolytic inhibitor is associated with an increased risk of renal and cardiovascular disease and vascular thrombosis[6, 7]. In addition, IL-6 induces inflammatory glycoprotein, YKL-40, whichhigh level impair the endothelial function, cause inflammation and atherosclerosis[8].

The glyoxalase-1 enzyme is involved in degradation and elimination of AGEs from the body.Increased expression of glyoxalase-1 enzyme reduces the oxidative stress and carbonylation stress caused by AGEs production, suggesting that glucose-enzymes activity modulation may improve thediabetes complications[9].

Previous evidence has indicated that vitamin D reduces the development of T2DM as well as complications such as nephropathy and diabetic retinopathy, but the underlying mechanisms are still not well known[10]. In a study of PBMC cell culture and endothelial cells, it was demonstrated that vitamin D enhances the expression of the gene of glyoxalase-1 enzyme [11].

In the present study, we hypothesized that vitamin D has a beneficial effect on diabetic angiopathyvia inducing the glyoxalase-1 enzyme pathway, which in turn triggers the degradationof AGEs. Therefore, we aimed to conduct a clinical trial to examine the effect of vitamin D supplementation on blood glucose indices, lipids, inflammatory profiles, endothelial dysfunction biomarkers, gene expression of glyoxalase-1 enzyme, YKL-40 factor, and RAGE inPBMC of type-2 diabetes mellitus participants.

II. METHODS AND DESIGN

Study design

A double-blind randomized clinical trial design is to beused in this study. The Ethics Committee of Tehran University of Medical Sciences has approved study protocol (95-03-161-32615). The flow diagram of thestudy protocol is shown in Figure 1.

Sample size

The largest sample size was obtained based on serum YKL40 variable [12]. The sample size was 23 patients in each group with a CI of 95%, power of 80% and loss of 15%. A total of 46 patients will be invited and divided into two equal groups by using the block randomization method. According to this sample size, if the mean difference of YKL40 variable is13 ng/ml, the hypothesis of H0 with CI of 95% and power of 80% will be rejected.

Study subjects

Patients with type 2 diabetes attending Iranian Diabetes association (IDA) in Tehran are to be invited to the study. Diabetes type 2 has been diagnosed by an internist and within at least two years since the initial diagnosis.

Patients who meet the eligibility criteria and agree to enroll in study will be referred to the principal investigator. First, the goals, methods, and benefits of the intervention will be explained and informed Consent Forms approved by Ethics Committee of theTehran University of Medical Sciences (TUMS) will be signed.

Inclusion criteria

- Type 2 diabetes diagnosed by an internist and within at least two years since the initial diagnosis.
- Age 30–60 years
- (20 < BMI <30)
- Informed consent formsigned and dated by the subject and investigators.
- History of consuming stabilized dose of oral anti-diabetic drugs and statins.

Exclusion criteria

- Suffering from cognitive impairment or other psychotic illnesses diagnosed by the psychiatrist
- History of consuming vitamin D supplements within 3 months before the beginning of the study
- Having complication of diabetes, thyroid disorders, liver damage, inflammatory diseases
- Using insulin or thiazolidinedione or anti-obesity drugs
- Any diagnosed malignancy
- Lactation, pregnancy
- Any drastic change in regular diet and lifestyle
- Any change in type and dosage of regular medication(s)

• Alcohol consumption and smoking (at least 5 cigarettes per day during the last 6 months)
• Intake of drugs that interact with vitamin D including anticonvulsants drugs (Phenytoin, and Phenobarbital)
• History of consuming vitamin B6 supplements
• Patients who consume less than 90% of their intervention.

Randomization and intervention

Participants of this study are divided into two randomly allocated groups (vitamin D and placebo) by random permuted blocks within the strata (BMI) method. In this study, theratio of vitamin D and placebo supplementation groups is 1:1.

The block randomization is performed by an assistant and the intervention allocation is blinded for both the investigator and subjects. The participants are randomly placed into two groups receiving vitamin D supplements or placebo supplements. Vitamin D and placebo tablets are prepared by the Pars Minoo Pharmaceutical, Cosmetic and Hygienic Company (Iran).

Each tablet of vitamin D contains 100 µg or 4000 IU [13, 14] of vitamin D. Each tablet of placebo contains gelatin starch, lactose powder, magnesium stearate, and citric acid. In vitamin D supplement the percentage of lactose powder has decreased and vitamin D is added instead.

Placebo and vitamin D tablets are similar in shape, size, and color. The type of supplements is blinded as A and B packages for investigators and patients.

Objectives

• Compare the mean of serum lipid (TG, TC, LDL-c,and HDL-c) and glucose indices including FBS, FBI, HOMA-IR, and QUICKI between the two groups and within each group, before and after the intervention.
• Compare the mean of serum inflammatory factorsincluding TNF-α, IL-6 between the two groupsand within each group, before and after the intervention.
• Compare the mean of serum endothelial dysfunction factors includingAGEs, YKL40, and PAI-1 between the two groups and within each group, before and after the intervention.
• Compare the mean of gene expression of YKL-40, glyoxalase-1, and RAGE between the two groups and within each group, before and after the intervention.

Measurements and assessments

At the start of the study, a setof questionnaires including a general informationquestionnaire, IPAQ, and 24-hour food recallquestionnaire will be completed by interviews. General characteristics will be defined by filling out the general information questionnaire asparticipants will be asked about age, education, job,smoking, andalcohol consumption,medical history and so on as well as the history of drug use, and taking any dietary supplements and vitamin/minerals.

For nutritional assessment three 24- hour dietary recall questionnaire at the beginning,after1.5 months, and end of the trialconsist of two typical days and a holiday areto be taken.The patients will be asked to remember all consumedfoods and drinks during the past 24 h when completingthe 24-h food recall questionnaire.This questionnairehas previously been validated in Iran [15]. The intakevalves will be changed to g/dayand the dietary intakes are to be assessed by using the DFP (Dorosty Food Processor) software that contains Iranian food composition tables [15, 16]. The intake of macronutrients and micronutrients includingdietary vitamin D will bedefined.

For the evaluation of the physical activity level theIPAQwill be used. The shortversion of theIPAQ is suitable for use in nationaland regional surveillance systems and provides the informationrequired for research work or evaluation purposes. Threelevels (categories) of physical activity are proposed: low, moderate, and high [17]. This questionnaire has been validatedin previous studies [18–20] including in Iran [21, 22].

Anthropometric parameters including weight, height and waist circumference will be measured. The weight of participants will be measured with minimal clothing and without shoes with an accuracy of 100 gusing a digital scale. Height will bemeasured using a wall stadiometerin standing position without shoes with an accuracy of 0.5cm. Waist circumference will be measured in standing position in the middle of the last rib and the iliac crestbynonelastic,tape with an accuracy of 0.5 cm.

Blood samples (10-15 cc) will be collected from patients in asterile tube with EDTA as an anticoagulant. Two ml of whole blood are poured in CBC steriletube for counting blood indexes and HbA1c. For serum separation, samples are centrifuged at 3000 RPM for 10 minutes and then serum samples will be collected in a sterile micro tube to be stored at -80 c until analyzed. PBMCs were isolated using the Ficoll-Histoprep gradient (BAG Health Care Gmbh, Germany) centrifugation protocol[23].

Serum calcidiol will be measured using chemiluminecense method withELECSYS system with Roche kit (codenumber: 05894973). Blood glucose profiles, including FBS, FBI, HOMA-IR, and QUICKI willbe

determined by a glucose specific kit (glucose oxidase method), electrochemiluminescence ((ECL) by thecobas e 411® analyzer device) and the following formulae, respectively:

QUICKI = 1 / **(**log(fasting insulin μU /ml) + log (fasting glucose mg /dl))

HOMA_IR =**(**FBI(mU/ l) × FBS (mmol/ l)**) /** 22.5

Serum level of inflammatory and endothelial dysfunction markers will be measured in separated serum obtained from patients. For this purpose the serum level of IL-6, TNF-α,AGEs,YKL40, and PAI-1 will be measured by ELISA kit according protocol of company(eBioscience USA) in two groups of study before and after of intervention.
Peripheral blood mononuclear cells are isolated using standard protocols then RNA will be isolated from PBMC cells by RNAase Mini Kit (Qiagene –USA). After that, cDNA will be synthesized from RNA by a QuantiTect Rev (Qiagene –USA) and the gene expression of YKL40, glyoxalase-1 enzyme and RAGE will be quantified by RT-PCR in two groups before and after the intervention.

Data analysis

The Kolmogorov-Smirnov test will be used for determining normality of the parameters. Wilcoxon test and Mann-Whitney test will be used to analysis of non-normal distribution variables within and between groups. Clinical and biochemical variables before and after study (pre-and post-intervention variables) will be expressed as means± SDs. To compare the differences in clinical and biochemical variables between pre-and post-intervention periods, pair *t*-test will be used. Comparisons the values between vitamin D and placebo groups will be performed by using *t*-test. Pearson's correlation coefficient statistical tests will be also applied. In all analysis, *P* value <0.05 will be considered statistically significant.

Ethical considerations

1. Explainingthe trial methods and goals to patients
2. Obtaining written informed consent from all patients
3. Ensuring that this dose of vitamin D supplementation is not harmfulto patients according to previous studies.
4. No change in the patients' treatment protocols are to be considered
5. Confidentiality of patientsinformation is also to be considered
6. Any patient receiving placebo after completing the study will receive vitamin D supplement in the cases of being deficient.
7. Patients will be able to withdraw from the trialif they desire

III. CONCLUSION

T2DM is a common chronic diseasewhich has adverse effects on small and large vessels. Vascular complications are the main etiology for mortality and morbidity among the patient with diabetes[2, 3].

Poor vitamin D status is suggested to berelated to endothelial dysfunction in diabetic patients [24]. Several studies have shown the possible health benefit of vitamin D on the development of diabetic vascular complications, but little is known regarding the involved molecular mechanisms [10]. Endothelial dysfunction is an important early event in the pathogenesis of vascular complications [25]. It is of highrelevance due to the various health benefit of vitamin D and vascular complications and lack of any studiesrelated to molecular mechanisms [11]. Due to remarkable changes of some blood biomarkers and some gene expressions in diabetic patients, and the lack of human studies on the effects of vitamin D on molecular mechanisms and signaling pathways, this trial is designed.

The strengths of this study are its randomized double-blindeddesign, protocol publication, determining dietary and physical activity statuses and registering any patient-reported complications.

LIMITATIONS

The limitations of this trial are slow patient recruitments due to the multiple eligibility criteria which lead to increase of the study period, self-reporting of the drugs and supplement consumptions, dietary intakes, and physical activities.

ABBREVIATION:

RAGE: Receptor for advanced glycation end products, PBMC: Peripheral blood mononuclear cells, T2DM: Type-2 diabetes mellitus, IPAQ: International Physical Activity Questionnaires, FBS: Fasting blood sugar, FBI: Fasting blood insulin, HOMA-IR: Homeostasis model assessment-insulin resistance, QUICKI: Quantitative

Insulin Sensitivity Check Index lipids, TG: triglyceride, LDL-c: low-density lipoprotein-cholesterol, HDL-c: high-density lipoprotein-cholesterol, TC: total cholesterol, TNF-α: tumor necrosis factor-alpha, IL-6: interleukin-6, AGE: Advanced glycation end products, PAI-1: Plasminogen activator inhibitor -1, AGE:Advanced glycation end-products, IDA: Iranian Diabetes association, DFP: Dorosty Food Processor

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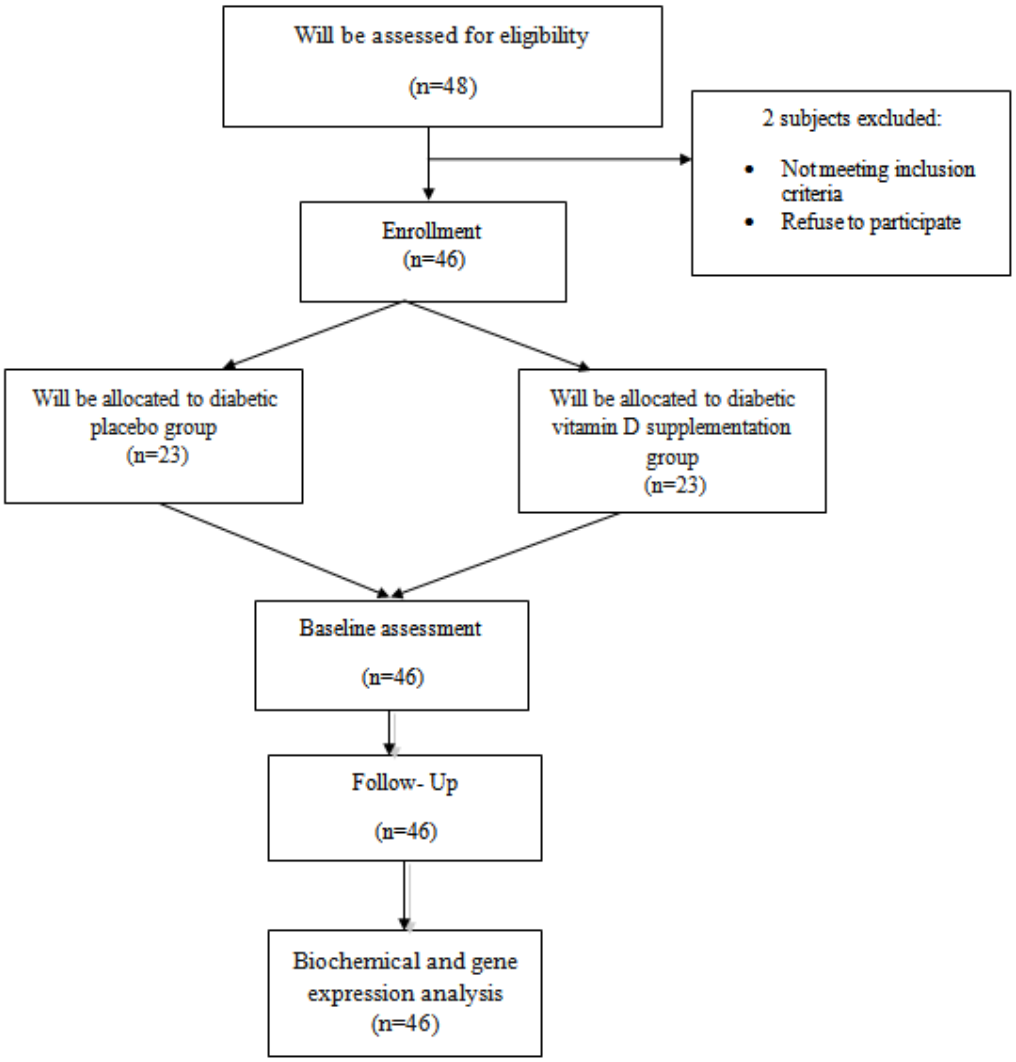
Fig. 1 Participants flow diagram



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