Effects of vitamin D on serum levels and gene expression of enzymes aldose reductase, O-linked n-acetyl glucosamine transferase and glutamine fructose-6-phosphate aminotransferase in patients with type 2 diabetes: a randomized, double blind, placebo controlled clinical trial

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ABSTRACT

Although vitamin D deficiency has been associated with diabetes complications, the underlying mechanisms have not been clarified in human studies yet. This clinical trial was designed to evaluate the effect of vitamin D supplementation on serum levels and gene expression of some polyols and hexamine pathway enzymes, which play pivotal roles in the incidence of diabetes complications. Seventy-four patients with type 2 diabetes were randomly divided into two groups as receiving vitamin D (100 \( \mu \text{g/d} \) equal to 4000 IU/d) or placebo for a 3-month period. Moreover, serum levels of insulin, fasting plasma glucose (FPG), vitamin D, Hba1c, aldose reductase (AR), O-linked N-acetyl glucosamine transferase (OGT), and glutamine fructose-6-phosphate aminotransferase (GFPT), as well as the gene expression of mentioned enzymes in PBMCs were measured before and after the intervention. After 3-months intervention, 25 (OH) vitamin D level significantly increased in the vitamin D group. The expression of AR and GFPT genes significantly decreased and some significant differences were observed regarding the serum level of AR enzyme. Additionally, insulin showed significant increase following vitamin D intake. Our result show that, receiving 100 \( \mu \text{g/d} \) vitamin D in type 2 diabetes patients, for a 3-month period might be helpful for ameliorating diabetes complications not only by improving insulin level, but also by suppressing AR and GFPT gene expressions in PBMC.

Introduction

The global prevalence of diabetes mellitus amongst the adult population (aged between 20 and 79 years old) has been reported to be higher than 6.6% in 2010, which is estimated to reach 7.8% by 2030. To be more accurate, between 2010 and 2030, the prevalence rate of this disease will experience an increase by about 20 and 69% in the developed and developing countries, respectively. 1 According to WHO, 4 million people annually die due to diabetes, which in total accounts for 8% of global mortality. 2 Moreover, in diabetic individuals, long-standing hyperglycemia leads to nephropathy, neuropathy, and cardiovascular diseases. Despite applying different methods and consumption of various drugs to control blood sugar, diabetes complications such as cardiac, renal, neurological, and ocular injury, incur major medical costs on governments. 3,4 So far, various mechanisms have been proposed as the
causes of diabetes complications and the metabolic pathways involved. In this regard, the most important pathways seem to be the followings: I. Production of polyols by aldose reductase pathway, II. Hexosamines production pathway, III. Protein kinase C pathway, and IV. The Mitochondrial pathway of free radical production. Therefore, some important enzymes of these pathways involved in diabetic complications were chosen for this study, which were as follows: aldolase reductase (AR) that is the main enzyme in the polyols pathway, O-linked N-acetylgalactosamine transferase (OGT) that is an enzyme hexosamine pathway, and Glutamine: fructose-6-phosphate aminotransferase (GFPT) as the main regulatory enzyme of hexosamine pathway. Notably, excess glucose can be converted to sorbitol by the aldose reductase enzyme. Accordingly, alcohols inferred from this pathway are under-active; however, their accumulation in cells leads to osmotic injury. Additional glucose in cells is possibly converted to glucosamine by GFPT enzyme. Glucosamine produced in the hexosamine biosynthesis pathway (HBP) attaches to proteins by the OGT enzyme, in which the subsequent structural changes in proteins harm their performance. Several policies have been explored to reduce the complications of diabetes up to now. Although all these ways do not lead to disease treatment, they can increase the life expectancy and quality of life of the diabetic patients. In this regard, Lifestyle modification, especially a proper nutrition, is considered as one of the main treatments, which plays a significant role in controlling diabetes. Various studies have shown that, type II and I diabetes are associated with vitamin D deficiency. Additionally, several studies have shown that receiving VD supplementation can result in the reduction of some complications such as diabetic nephropathy and retinopathy; however, the VD mechanism of this effect on the reduction of diabetes complications is still unknown. Moreover, it was shown that, treatment with VD has improved the blood glucose and sensitivity to insulin in the patients with (type I, II.) diabetes as well as normal people. Also, the low level of VD in elderly men is associated with insulinemia and glucose intolerance, and besides, a low level of VD is generally accompanied with insulin resistance. Altogether, Vitamin D, as a supplement, can modulate the pathways involved in the pathogenesis of diabetes complications. The results of the above-mentioned studies showed that, polyols and hexosamine pathways play important roles in the incidence of diabetes complications. Accordingly, the effect of VD on the main enzymes of these pathways has not been studied in human studies so far. So, the present study was designed to investigate the effect of vitamin D on the concentration and gene expression of AR, OGT and GFPT, in the patients with type 2 diabetes to clarify the mechanism of action of the vitamin D in diabetes.

Materials and methods

Study design and participants

Patients with type 2 diabetes attending the Iranian Diabetes Association in Tehran were entered into this double-blind clinical trial. The main inclusion criteria were patients with aged 30–60 years old, having 20 to 30 kg/m² body mass index (BMI) receiving no dietary supplements at least 2 months before and also during the trial and patients on diet and/or hypoglycemic agents other than insulin. Exclusion criteria were consuming vitamin D supplements within 3 months, pregnancy, lactation and having a clinical disease.

Randomization and ethical consideration

74 cases of patients with type II diabetes, who voluntarily referred to Iran’s diabetes center were enrolled in this study. Participants divided into two groups (vitamin D or placebo) by the permuted-block randomization method. Patients were matched by using stratified randomization based on sex (male/female) and BMI. Vitamin D and placebo tablets were purchased from the Pars Minoo Pharmaceutical, Cosmetic, and Hygienic Company (Iran). Each placebo tablet contained gelatin starch, lactose powder, magnesium stearate, and citric acid. The lactose powder percentage has
a reduction in vitamin D supplements, and 100 μg or 4000 IU of vitamin D was added instead of it. Placebo and vitamin D tablets had the same shape, size, and color. Patients were advised to continue their usual anti-diabetic drugs, usual diets and physical activity, but to receive no herbal products or dietary supplements at least 3 months before and also in the meantime of the trial, willing to maintain.

**Ethics approval and consent to participate**

The present study was approved by the Ethics Committee of biology, Science and Research Branch, Islamic Azad University Tehran, Iran. The research was recorded in the Iranian Web site for registration of clinical trials (http://www.irct.ir IRCT20170813035665N2). All participants read and signed a written informed consent prior to enrollment.

**Sample blood collection**

At baseline and the end of the study (after 3 months), 15 ml fasting blood samples were taken from patients, 10 ml of blood were transferred to tubes with EDTA k3 anticoagulant for isolating peripheral blood mononuclear cells (PBMCs) and 5 ml was transferred into tubes without anticoagulant for serum separation. Serum samples were separated by centrifugation at 3000 RPM for 10 min and then were stored at −80°C until assayed.

**Assessment of biochemical variables**

The FPG levels were measured by enzymatic methods (Pars Azmoon kit, Iran). Insulin and vitamin D were measured using Electrochemiluminescence (ECL) method, HbA1c was assessed with the HPLC method by the Tosoh G8 instrument (South San Francisco, CA). Aldose reductase, glucosamine transferase, glutamine-fructose aminotransferase concentration was quantified by Crystal day Elisa kits (Shanghai China).

**PBMCs isolation**

PBMCs were isolated after blood collection by using the ficoll (Lymphodex, Inno-Train) and standard density gradient centrifugation, for real-time PCR assay PBMCs were stored.

**RNA extraction and cDNA synthesis**

Messenger RNA (mRNA) was extracted using the Hybrid-R RNA isolation kit (Gene All, Seoul, South Korea). The amount and purity of RNA were evaluated by Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, Del., USA) and the ratio of 260/280 and 260/230 ~ 2.0 were considered as acceptable values. RNA was reverse-transcribed into cDNA with Revert Aid RT Reverse Transcription Kit (Thermo, Fisher, and USA). Then, cDNA was made, and the expression levels of AR, OGT, and GFPT genes were assessed by quantitative real-time PCR using SYBR Premix Ex-Taq II (Takara Bio Inc., Shiga, Japan). β-actin gene was utilized as housekeeping. The fold changes were calculated by the use of the $2^{-\Delta\Delta CT}$ method. Sequences of primers were designed by using OligoCalc, Primer blast and GeneRunner softwares that their specifications are shown in Table 1. The primers sequence is presented in Table 1.

**Sample size calculation and statistical analyses**

Sample size calculation was according to changes in the mean value of the plasma GFPT as a primary outcome. To detect a 25% change in the primary outcome at a two-sided 0.05 significance level with a power of 0.8, 19 participants were required in each group with respect to 25% dropped out...
estimation a final sample size of 37 patients per group was planned for requirement in this study. The findings of this study were analyzed using SPSS software version 25. To evaluate normality of data distribution, Kolmogorov–Smirnov test was done for data sets. For RT-PCR results, the statistical analyses were exerted to the normalized ΔCt values (Ct target – Ct β actin). Qualitative data are evinced as proportions. Quantitative data are expressed as the mean value ± SD or as the median. Baseline data were analyzed to determine the possible significant starting intergroup differences. A paired t-test and independent t-test were used for numerical normally distributed data. The Wilcoxon signed-rank test and the Mann–Whitney U test were used for nonparametric distributions. Additionally, to remove the effect of confounding variables, the analysis of covariance (ANCOVA) was used. In all analysis, P < .05 was considered statistically significant.

Results

Total 116 patients volunteered to complete the study. At the baseline, 74 participants were randomly assigned to vitamin D group (n = 37) and placebo group (n = 37). Of 74 participants, two from the placebo and vitamin D groups dropped out. The flow diagram of the trial is shown in Figure 1.

Baseline characteristics

The baseline characteristics of the participants are detailed in Table 2. The mean age of subjects in the group that received vitamin D supplementation and the placebo group was 50.57 ± 5.60 and 51.04 ± 6.31 years, respectively, and no statistically significant difference was observed between two groups. At the beginning of the study, there was no significant difference between the group that received VD supplementation and the group that received placebo in none of the investigated variables (fasting plasma glucose, insulin, HbA1C, vitamin D, in addition to AR, OGT, GFPT concentration, and gene expression).

Table 3 shows the comparison of the change in laboratory parameters at the baseline and after 3 months of the vitamin or placebo intake. There was a significant difference in the mean change of 25 (OH) D serum level after 3 months (P < .001). Although a slight decline was detected in the level of FPG and HbA1c in vitamin D group compared to the baseline value, there was no statistically significant difference between the two groups. Vitamin D group had a larger increase in serum insulin levels compared with the placebo group (P < .013). A significant difference was observed between the two groups in the serum AR level (P = .012). Melt curve and amplification plot of the three genes are depicted in Figure 2. Vitamin D decreased AR and GFPT gene expression (in the vitamin D vs in the placebo) in isolated PBMCs (Figure 3).

Discussion

Several studies have shown that, vitamin D deficiency plays a role in the incidence and progression of diabetes, in which the incidence risk of type 2 diabetes decreases along with increasing the vitamin D serum
Figure 1. Schematic chart of the clinical trial process.

Table 2. Baseline characteristics of the patients in placebo and vitamin D groups.

| Variable       | Placebo (n = 37) | Vitamin D (n = 37) | P-value |
|----------------|------------------|--------------------|---------|
| Age (years)    | 51.04 ± 6.31     | 50.57 ± 5.60       | 0.799   |
| Gender (female/male) | 10/11            | 11/10              | 0.75    |
| FPG (mg/dl)    | 180.28 ± 46.36   | 173.28 ± 35.65     | 0.58    |
| HbA1c (%)      | 7.92 ± 1.20      | 7.50 ± 0.79        | 0.19    |
| Insulin (μU/ml)| 13.20 (9.20,14.65)| 8.20 (6.45,12.30)  | 0.092   |
| HOMA-IR        | 5.40 ± 2.41      | 4 ± 2.88           | 0.19    |
| Vitamin D (ng/ml) | 14.70 ± 13.70  | 14.6 ± 10.39       | 0.99    |
| AR (ng/ml)     | 1.90 (1.075,5.56)| 2.1 (0.923,7.8)    | 0.74    |
| OGT (ng/ml)    | 4.1 (2.94,21)    | 4 (3.16,9.8)       | 0.93    |
| GFPT (ng/ml)   | 0.36 (0.3,2.58)  | 0.45 (0.33,1.39)   | 0.52    |

Values are mean ± SD for data with normal distribution and median (interquartile ranges) for data not normally distributed. Abbreviations: FPG: fasting plasma glucose; HbA1c: Hemoglobin A1c; AR: Aldose reductase; OGT: O-linked N-acetyl glucosamine transferase; GFPT: Glutamine fructose-6-phosphate aminotransferase.

The result of this study show that, aldose reductase enzyme level and gene expression decreased significantly in the vitamin D group. The AR enzyme catalyzes the attachment of the N-acetyl glucosamine unit to the serine and threonine residues of proteins. A previous study has evinced that; the expression of this gene among diabetic people can increase diabetes complications, while suppressing the gene may ameliorate the patient’s condition. Additionally, vitamin D significantly reduced the GFPT gene expression in the vitamin D group patients. The expression of the GFPT gene, as an important enzyme in the hexosamine pathway, was investigated in a study conducted by Fricovsky et al., which was performed on the effect of hexosamine pathway in the experimental model of type 2 diabetes. One month following diabetes induction, the weight, glucose, and insulin levels have increased in mice. After two months, the hexosamine pathway products amount has increased. Additionally, the amount of hexosamine pathway regulatory enzyme i.e., GFPT, significantly increased and the vascular tissue proteins glycosylation had also rose. Overall, the findings of this study show that, the hexosamine pathway play...
a significant role in the incidence of diabetic cardiomyopathy by increasing the proteins glycosylation. Some previous studies showed that, a high glucose concentration causes an increase in cell apoptosis. Accordingly, this increase in apoptosis was concurrent and correlated with the increased activity of the
hexosamine pathway and the production of oxygen-free radicals. On the other hand, the use of antioxidant and hexosamine pathway inhibitors caused a reduction in cardio myoblast apoptosis.\textsuperscript{[17]} Therefore, by decreasing the expression of the GFPT cellular receptor gene, it is expected that vitamin D may delay the complications of diabetes. In the present study, the level of OGT enzyme and its gene expression showed no significant difference between groups after performing the intervention. It seems that, a follow up period and number of the samples of this study were inadequate to measure changes in this gene. In an \textit{in silico} study, Inbathamizh et al. investigated the inhibitory effect of calcitriol and 5, 5'-Dithiobis-2-nitrobenzoic acid on OGT enzyme with the aim to find a treatment for prostate cancer. In such studies, researchers predict the 3D structure of proteins and then find some suitable inhibitors for them using
several methods such as homology modeling and molecular docking. In this regard, the analysis results indicated that, calcitriol probably plays an inhibitory role for OGT enzyme in terms of 3D structure and active sites.\textsuperscript{[11]} So, the repressing character of vitamin D on OGT might be due to its structural inhibitory effect, not a change in the enzyme’s gene expression. There are other studies in agreement with the current study, indicating that insulin show a significant increase in the diabetic patients after receiving vitamin D supplementation. Grineva et al. conducted a statistical analysis on the findings showing that, the lower levels of vitamin D were related to obesity, the increased level of blood glucose, and the reduced insulin sensitivity. Therefore, the authors concluded that, vitamin D deficiency is regarded as one of the risk factors for the incidence of obesity and insulin resistance.\textsuperscript{[18]} Moreover, Yokoyama et al. investigated the relationship between serum vitamin D level and vitamin D receptor (VDRs). They showed that, the higher serum vitamin D levels are associated with the improvement of kidney status and function in the patients with diabetes.\textsuperscript{[19]} Taken together, our results demonstrated that, vitamin D supplementation is effective on decreasing the AR serum levels, as well as the AR and GFPT gene expressions in type 2 diabetic patients with vitamin D deficiency or insufficiency. Our study strengths were as follows: it was performed on humans for the first time, it had a double-blind randomized placebo-controlled design, and only two patients missed through trial. Also, our limitation was a short follow up period. Despite the obvious usefulness and advantages of vitamin D supplementation, more studies are needed to evaluate the levels of other enzyme activity, and cell receptors could definitely develop the reliability of this study.

Conclusion

The results obtained in this study show that, daily vitamin D supplementation and its increased serum level lead to a significant reduction in AR level in serum, as well as a decrease in the AR and GFPT genes expression. Vitamin D might ameliorate diabetes complications through polylol and hexosamine pathways.

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References

[1] International, D. F.; Five Questions on the IDF Diabetes Atlas. Diabetes Res. Clin. Pract. 2013, 102(2), 147.
[2] Belen’s, J. W.; Grobbee, D. E.; Nealb, B. The Global Burden of Diabetes and Its Complications: An Emerging Pandemic. European Journal of Cardiovascular Prevention & Rehabilitation. 2010, 17(1 suppl), s3–s8. DOI: 10.1097/01.hjr.0000368191.86614.5a.
[3] Zhang, P.; Zhang, X.; Brown, J.; Vistisen, D.; Sicree, R.; Shaw, J.; Nichols G. Global Healthcare Expenditure on Diabetes for 2010 and 2030. Diabetes Res. Clin. Pract.. 2010, 87(3), 293–301. DOI: 10.1016/j.diabres.2010.01.026
[4] Clarke, P.; Gray, A.; Legood, R.; Briggs, A.; Holman, R. The Impact of Diabetes-related Complications on Healthcare Costs: Results from the United Kingdom Prospective Diabetes Study (UKPDS Study No 65). Diabetic Med. 2003, 20(6), 442–450. DOI: 10.1046/j.1464-5491.2003.00972.x.
[5] Madonna, R.; De Catarina, R. Cellular and Molecular Mechanisms of Vascular Injury in Diabetes—part I: Pathways of Vascular Disease in Diabetes. Vasc. Pharmacol. 2011, 54(3–6), 68–74. DOI: 10.1016/j.vph.2011.03.005.
[6] Hosseinzadeh, P.; Javanbakht, M. H.; Mostafavi, S. A.; Djalali, M.; Derakhshanian, H.; Hajianfar, H.; Bahonar, A.; Dijazayery, A. Brewer’s Yeast Improves Glycemic Indices in Type 2 Diabetes Mellitus. Int. J. Prev. Med. 2013, 4 (10), 1131–1138.

[7] Brock, K. E.; Huang, W.-Y.; Fraser, D. R.; Ke, L.; Tseng, M.; Mason, R. S.; Stolzenberg-Solomon, R. Z.; Michal Freedman, D.; Ahn, J.; Peters, U., et al. Diabetes Prevalence Is Associated with Serum 25-hydroxyvitamin D and 1,25-(OH)₂D in US Middle-aged Caucasian Men and Women: A Cross-sectional Analysis within the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial. British Journal of Nutrition. 2011, 106(3), 339–344.

[8] Baz-Hecht, M.; Goldfine, A. B. The Impact of Vitamin D Deficiency on Diabetes and Cardiovascular Risk. Current Opinion in Endocrinology, Diabetes and Obesity. 2010, 17(2), 113–119. DOI: 10.1097/MED.0b013e3283372859.

[9] Patrick, P. A.; Visintainer, P. F.; Shi, Q.; Weiss, I. A.; Brand, D. A. Vitamin D and Retinopathy in Adults with Diabetes Mellitus. Archives of Ophthalmology. 2012, 130(6), 756–760. DOI: 10.1001/archophthalmol.2011.2749.

[10] Grammatiki, M.; Rapti, E.; Karras, S.; Ajjan, R. A.; Kotsa, K. Vitamin D and Diabetes Mellitus: Causal or Casual Association. Reviews in Endocrine and Metabolic Disorders. 2017, 18, 227–241.

[11] Inbathamizh, L.; Padmini, E. In Silico Studies on the Inhibitory Effects of Calcitriol and 5, 7-dihydroxy-2-nitrobenzoic Acid on Human Glucosaminyl N-acetyl Transferase 1 Activity. Asian J. Exp. Biol. Sci.. 2012, 3, 14–21.

[12] Buijssse, B.; Boeing, H.; Hirche, F.; Weikert, C.; Schulze, M. B.; Gottschald, M.; Ku‘hn, T.; Katzke, V. A.; Teucher, B.; Dierkes, J., et al. Plasma 25-hydroxyvitamin D and Its Genetic Determinants in Relation to Incident Type 2 Diabetes: A Prospective Case-cohort Study. Eur. J. Epidemiol. 2013, 28, 743–752.

[13] Kneckt, P.; Laaksonen, M.; Mattila, C.; Härkänen, T.; Marniemi, J.; Heliovaaara, M.; Rissanen, H.; Montonen, J.; Reunanen, A. Serum Vitamin D and Subsequent Occurrence of Type 2 Diabetes. Epidemiology. 2008, 19(5), 666–671.

[14] Kawai, T.; Takei, I.; Tokui, M.; Funae, O.; Miyamoto, K.; Tabata, M.; Hirata, T.; Saruta, T.; Shimada, A.; Itoh, H. Effects of Epalrestat, an Aldose Reductase Inhibitor, on Diabetic Peripheral Neuropathy in Patients with Type 2 diabetes, in Relation to Suppression of N-E-carboxymethyllysine. J. Diabetes Complications. 2010, 24, 424–432. DOI: 10.1016/j.jdiacomp.2008.10.005.

[15] Hashimoto, Y.; Yamagishi, S. I.; Mizukami, H.; Yabe-Nishimura, C.; Lim, S. W.; Kwon, H. M.; Yagihashi, S. Polyl Pathway and Diabetic nephropathy Revisited: Early Tubular Cell Changes and Glomerulopathy in Diabetic Nephropathy. J. Diabetes Investig. 2011, 2, 111–122. DOI: 10.1111/j.2040-1124.2010.00071.x.

[16] Fricovsky, E. S.; Suarez, J.; Ihn, S.-H.; Scott, B. T.; Suarez-Ramirez, J. A.; Banerjee, I.; Torres-Gonzalez, M.; Wang, H.; Ellrott, I.; Maya-Ramos, L. Excess Protein O-GlcNAcylation and the Progression of Diabetic Cardiomyopathy. American Journal of Physiology - Regulatory, Integrative and Comparative Physiology. 2012, 303(7), R689–R699. DOI: 10.1152/ajpregu.00548.2011.

[17] Rajamani, U.; Essop, M. F. Hyperglycemia-mediated Activation of the Hexosamine Biosynthetic Pathway Results in Myocardial Apoptosis. American Journal of Physiology-Cell Physiology. 2010, 299(1), 139–147. DOI: 10.1152/ajpcell.00020.2010.

[18] Grineva, E.; Karonova, T.; Micheeva, E.; Beljava, O.; Vitamin, N. I. D Deficiency Is a Risk Factor for Obesity and Diabetes Type 2 in Women at Late Reproductive Age. Aging (Albany NY). 2013, 5(7), 575–581. DOI: 10.18632/aging.100582.

[19] okoyama, K.; Nakashima, A.; Urashima, M.; Suga, H.; Mimura, T.; Kimura, Y.; Kanazawa, Y.; Yokota, T.; Sakamoto, M.; Ishizawa, S., et al. Interactions between Serum Vitamin D Levels and Vitamin D Receptor Gene FokI Polymorphisms for Renal Function in Patients with Type 2 Diabetes. PloS One. 2012, 7(12), e51171.