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Original article

Effects of Vitamin D supplementation on 8-hydroxydeoxy guanosine and 3-nitrotyrosine in patients with type 2 diabetes: a randomized clinical trial

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Summary. *Objective:* This study was done to assess the effects of vitamin D supplementation on oxidative stress biomarker in p patients with Type 2 Diabetes Mellitus (T2DM). *Methods:* This randomized double-blind clinical trial was done with 35 type 2 diabetic participants. The vitamin D group (n= 17) and the placebo group (n= 18) were stratified for BMI and age at the baseline before random allocation. The vitamin D group received 4000 IU (100µg) vitamin D3/day whereas the placebo group received matching placebo. The effect of vitamin D3 on 8-OHdG and 3- nitrotyrosine was measured by ELIZA kit at the beginning and at the end of the study. *Results:* After two months supplementation, the mean concentration of serum 8-OHDG had significantly decreased (P=0.015) in vitamin D group. The mean concentration of 3-NT decreased in both groups, whereas this reduction was significant only in vitamin D group (p=0.027). *Conclusions:* Vitamin D supplementation at the dose of 100µg for 2 months had beneficial effects on oxidative stress T2DM management. Results from this trial showed that there were beneficial effects associated with the use of vitamin D supplementation,specifically reducing 8-OHDG and 3-NT concentrations.

Key words: Vitamin D, 8-hydroxydeoxy guanosine, 3-nitrotyrosine, diabetes

Introduction

Diabetes is widely prevalent globally (1). At present, about 285 million people have diabetes and this number is expected to reach 438 million by the year 2030 (2). Hyperglycemia occurs in Type 2 Diabetes Mellitus (T2DM) which linked to progression of complication by production of free radicals such as reactive oxygen speices (ROS) and reactive nitrogen speices(RNS) (3). The production of advanced glycation end (AGE) contribute to diabetic progression by increasing oxidative damage of pancreas beta cell (4). Production of high free radicals in continues hyperglycemia causes autoxidation of glucose, activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases ,non enzamytic glycation of proteins and nitric oxide synthase that all of these events create free radical and stress oxidative (5).

There are low activity of antioxidant enzymes in pancreas, so the pancreas is sensitive to damage (6). Recent evidences suggest that the effects of glucotoxicity, endoplasmic stress and oxidative stress cause beta-cell loss in T2DM (7).

Several oxidative damage markers have been diagnosed. In recent years, 8 hydroxy deoxy guanosine (8-ohdg) has known as a DNA oxidation marker. The study of oxidative DNA marker is clinically important (8). ROS can damage cells by strand breaks in DNA and cause oxidation of guanine residues to 8-hydroxydeoxyguanosine (8-OHdG). Therefore,

8-OHDG can be a reliable marker of intracellular oxidative stress (9).

Peroxy nitrite is made by combination of ($O_2^{-\bullet}$) with NO• or reaction of OH• with NO_2 or H_2O_2 with NO• (10). High serum peroxy nitrite levels have been shown in diabetic patients by Al-Nimer et al (11). Another study have been reported that production of peroxy nitrite causes nitration of tyrosine along with activation of poly (ADP-ribose) polymerase (PARP) contribute to the endothelial dysfunction. PARP is an abundant nuclear enzyme that triggers oxidative DNA damage and leads to cellular damage (12). According to another study, inducible nitric oxide synthase (iNOS) in skeletal muscle of type 2 diabetic patients increased. The increased level of NO products, NO2 and NO3, and nitrotyrosine indicates reaction of nitric oxide with oxygen to produce peroxy nitrite that nitrated tyrosine. The superoxide that is necessary to react with NO is made in relation to AGE formation (13).

Peroxy nitrite causes nitration free tyrosine or in protein bound. It shows reactivity of protein nitration, DNA strand breakage and base modification that may have a mutagenic effect. It founds in many pathophysiological condition like inflammatory disease, diabetes, myocardial infarction, cancer, autoimmune disease and so on (14). The presence of nitrotyrosine in protein is considered as a biomarker of activity of peroxynitrite (15).

There have been evidence that vit D has an antioxidant activity (16). Treatment with intravenous calcitriol in hemodialysis patients could reduce oxidized albumin and increase serum thiol antioxidants (17).

Pancreas B cells express VDR gene, which also expressed in peripheral determinant of insulin sensitivity. These tissues may express the 1-hydroxylase gene in male Wistar rats (18). It has been reported that the number of VDR decreases in the kidney of db/db mice (19). In VDR knockout and hetrogenous mice, VDR loss is along with increasing expression of 8-OHdG and NF-κB (20).

Therefore, we hypothesized that vitamin D supplementation may have decreasing effect on 8-OHdG and 3-nitrotyrosine (3-NT), because it may decrease oxidative stress in T2DM. For this reason, we designed this study to examine the effects of vitamin D supplementation on 8-OHdG and 3-NT in type 2 diabetic patients.

Subjects and Methods

Participants

The participants consisted of 35 diabetic type 2 patients (15 women and 20 men who were 30 to 65 years old) who agreed to take part in our study. Based on the formula for parallel-design randomized controlled trials, the study was powered to detect 227 picogram/ml difference in 8-OHdG between the groups. The estimated sample size of 13 subjects in each group would give a power of 80% with 95% confidence interval (21). Nonsmoker and non menopause individuals aged 30-65 years old with type 2 diabetes (fasting blood glucose ≥ 126 mg/dl (≥6.9 mmol/l) or 2h post-glucose load≥200 mg/dl (≥11.1 mmol/l) or both) were included in this study. Patients with a history of renal failure, liver, nauseal or thyroid disease or any other inflammatory diseases were not included in the study. Individuals took corticosteroids, thiazolidondione, orlistat, sibotramin or anti-obesity drugs, and food and herbal supplement about 3 months before beginning of intervention or injecting insulin were not included as well. We did not also include those who took any kind of vitamin D or calcium supplements, those who were pregnant or lactating and those who had weight loss regime during the last year. All patients agreed to maintain their usual dietary and physical activity habits during the study. The criteria for eligibility were Iranian nationality, Body mass index (BMI) between 20 and 30. Overall, 36 patients who met all the inclusion criteria and during the analysis, one person were excluded from the study (Figure 1). All of the participants were obtained an informed consent.

This study was approved by the Tehran University of Medical Sciences (TUMS) ethical committee (ID: 17112) and registered on www.clinicaltrial .org as NCT 01876563.

Study design

This was a double-blind, parallel, randomized placebo-controlled clinical trial that was conducted in Tehran, Iran between September 2012 and February 2013 at Iranian Diabetes Association. Totally, 35 diabetic patients who met the inclusion criteria

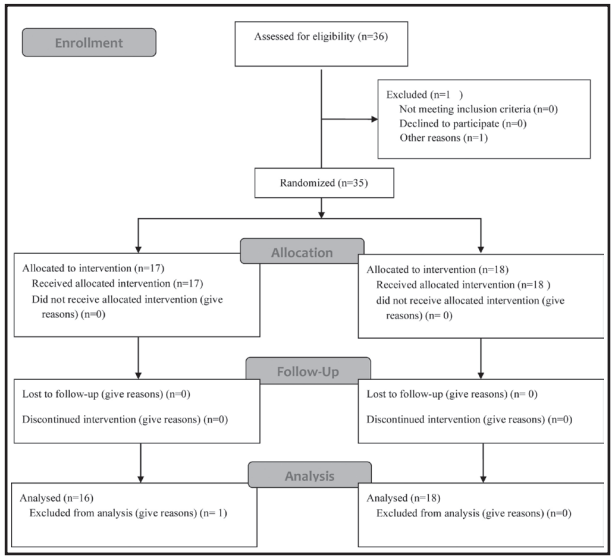

Figure 1. Follow diagram of enrolled participant in the study

completed the trial. Before randomization, we stratified participants based on age and BMI. All investigators and participants as well as laboratory technicians were blinded to the random assignment. Then patients were allocated by random permuted blocks within the strata (age and BMI) method in to the vitamin D group and placebo group. Individuals in the vitamin D group received 4000 IU vitamin D3 daily for 2 months and in the placebo group one placebo tablet per day consisting of only recipients without vitamin D for 2 months. Placebo and vitamin D supplement were manufactured by minoo pharmaceutical Co. The use of vitamin D supplements and placebos throughout the study was checked through asking participants to bring the medication containers. Compliance to the vitamin D supplementation was assessed through the quantification of serum vitamin D levels.

Participants were requested to consume their usual diets throughout the study. They were also asked not to change their routine physical activity levels. To make sure of these recommendation during intervention, 3 days of dietary records (one weekend day and two weekdays) were obtained. Blood sampling was done at study baseline and at the end of intervention to quantify 8-OHdG and 3-NTand 25-hydroxy vitamin D3 (25(OH) D).

Measurements

In the beginning of the study and after 2 months, blood samples were taken after 12-14 hours overnight fasting. After centrifuging, serums were separated and stored at -80 C for measuring the concentration of serum 8-OHdG and 3-NT. Serum 8-OHdG was measured by human 8-OHdG ElIZA kit (cat no:E1436Hu). Serum 3-NT was measured by Human 3-NT ELIZA kit (cat no: E1425Hu).

Statistical analysis

Normal distribution of all variables was assessed by the Kolmogorov-Smirnov test. Log transformation was applied for non-normally distributed variables. All variables were reported as mean ± Standard Deviation (SD). Within group comparisons were made by paired-sample t test. The Student t test was used to detect differences between the two groups independently. P <0.05 was considered as statistically significant. All statistical analyses were done using the Statistical Package for Social Science version 17 (SPSS Inc., Chicago, Illinois, USA).

Result

Thirty-six participants were randomly assigned into two groups of the vitamin D group (n=18) and the placebo group (n=18); there was one drop out at the analysis report. At the end of the trial, 35 subjects allocated, vitamin D group (n=17) and placebo group (n=18) were analyzed.

The baseline characteristics of the participants are presented in Table 1. There was no significant difference in weight, Waist circumference (cm), Hip circumference (cm), duration of diabetes, Sun exposure of diabetes between groups.

The oral anti-diabetic drugs included glibenclamida and metformin.All participants were traditional attire-when outdoor, which limits surface area exposure to direct sunlight. Vitamin D groups included of 17 patients (9 men, 8 women) and placebo groups included of 18 patients (11 men,7 women) that there were not any significant correlation (P=0.418).

Table 1. Anthropometric parameters of two study

	Vitamin D group (n=17)	Placebo group (n=18)	
Age(yr)	50±6.13	50.27±6.63	0.898
Weight(kg)	80.05±18.39	83.75±13.04	0.496
BMI(kg/c)	28.94±5.52	29.2±5.10	0.879
Waist circumference(cm)	94.97±14.03	97.26±10.43	0.587
Hip circumference(cm)	107.38±10.89	108.32±6.97	0.762
WHR	0.88±0.08	0.89±0.06	0.579
Duration(years)	6.76±4.68	6.47±3.87	0.841
Sun exposure	2.12±0.928	2.11±0.832	0.983

a: Data are expressed as mean±SD
b: t-test was used to observe differences between the groups

The Pearson correlation coefficient (r) at the baseline (n=35) showed an inverse relationship between serum 25(OH) D and 8–OHDG (r= -394, P =0.023) and serum 25(OH) D and 3-NT (r=-0.415, p=0.015). The pearson correlation is shown in table 2.

The effect of vitamin D3 supplementation is shown in table 3. As shown in table 3, serum 25(OH) D increased (P<0.001) by the end of intervention, whereas serum 8-OHDG log and 3-NT statistically decreased, (P =0.015 and P =0.027, respectively) at the end of the intervention, compared with baseline.

The result of this study showed that the mean serum 8-OHdG significantly decreased in vitamin D group compared to the placebo group (P =0.003) at the end of the intervention. The 8-OHDG percent change in vitamin D group compared with placebo group significantly reduced (P =0.025).

The difference between the serum calcidiol of vitamin D and placebo groups before intervention was not statistically significant (P=0.669), but this difference after intervention was statistically significant (P =0.005).

There was not any significant change in the intake of vitamin D through food in vitamin D group compared to placebo group before and after intervention (P = 0.08and P =0.33, respectively).

The mean nitrotyrosine percent change had been decreased in vitamin D groups, but this reduction were not significant (P =0.124). Also, the mean nitrotyrosine percent change had been increased in placebo group.

Discussion

In this randomized placebo-controled clinical trial of vitamin D supplementation in patients with T2DM results showed a significant reduction in the biomarker of stress oxidative. It is the first time to assess 8-OHdG and 3-NT in the T2DM disease.

We found that vitamin D supplementation led to reduction of serum 8-OHdG and 3-NT.It is found that stress oxidative is involved in the pathophysiology of T2DM (22, 23). In a study on diabetic rats, maxacalcitol (0.2μg/kgbw) decreases the progression of diabetic nephropathy by inhibition of oxidative stress and modifying the Nrf2–Keap1 pathway

Table 2. Fasting serum calcidiol, 8-OHDG and 3-NT at before and after

variables	p-valu	r
8-OHDG after and calcidiol after	0.23	-0.394
3-NT after and calcidiol after	0.15	-0.415

A: p-value for pearson correlation
**P<0.05 is statistically significant*

Table 3. Fasting serum calcidiol, 8-OHDG and 3-NT of two groups at before and after

		Vitamin D group (n=17)	Placebo group (n=18)	*p-value*[b]	*p-value*[b]
calcidiol (ng/ml)	Baseline	14.51±11.51	12.83±11.65	0.669	
	Post-intervention	25.74±11.89	13.74±11.30	0.005*	<0.001
	difference	0.11±0.01	0.02±0.11	<0.001	
	p-value[c]	<0.001	0.087		
8-OHDG	Baseline	32.57±26.97	53.49±43.88	0.10	
	Post-intervention	22.25±6.72	55.83±41.26	0.003*	<0.001
	Percent change difference	-17.45±24.32	24.16±68.42	0.025*	
	p-value[c]	0.015*	0.418		
3-NT	Baseline	631.02±744.79	1222.76±1202.88	0.089	
	Post-intervention	381.16±175.70	1037.20±861.04	0.005*	<0.001
	3-NT percent change	-16.70±44.95	27.67±108.57	0.124	
	p-value[c]	0.027*	0.762		

a: Data are expressed as mean±SD
b: t-test was used to observe differences between the groups
c:paired t-test used to observe differences in the group before and after intervention.
d:p values for ANOCOVA after adjustment for variables baseline value
**P<0.05 is statistically significant.*

in T2DM without a significant effect in blood pressure and glomerular filtration rate. Maxacalcitol was administered 3 times per week. Expressions of Nrf2 and heme oxygenase-1(HO-1), glutamate-cysteine ligase catalytic subunit (GCLC) and a modifier subunit (GCLM) that are downstream genes significantly increased and Keap1 expression decreased in the vitamin D group compared with the DM group. Nrf2 is negatively regulated by Keap1 and activate antioxidant and detoxifying enzyme. Expression of VDR decreased in the DM group compared with insulin and vitamin D groups. Expression of NADPH oxidase and urinary excertion of 8-OHDG as a marker of oxidation DNA devastation significantly reduced in the vitamin D group. Oxidative stress decreased in the kidney of vitamin D group. This suggests that vitamin D can prevent progression of diabetic nephropathy by decreasing of oxidative stress. Vitamin D can modify suppression of Nrf2-Keap1 pathway and NF-KB and NADPH oxidase activity (24).

In a study, VDRA(vitamin D receptor activator) reduced oxidative stress in the hemodialysis patients, so that 1.5 mg per week calcitriol injected into a vein of 11 hemodialysis patients for 4 weeks. Results showed that calcitriol therapy can increase serum thiol antioxidants in hemodialysis patients. Vitamin D can reduce oxidative stress through increasing free thiol content. A description of possible systemic effects of VDRA on oxidative stress, is its inhibitory effect on renin-angiotensin system. Since inflammation is closely related to oxidative stress and antioxidant effect of VDRA can be attributed to its anti-inflammatory effect.

In another study, calcitriol endovascular treatment resulted in a significant change in the proportion of oxidized albumin. Albumin remove free radicals. This proportion show cys34 situation in human serum albumin (25).

In one study, rats were nephrectomy, follow-up for four months. The rats were divided in 5 categories. 1-uremic rats 2-uremic rats treated with enalapril 3-uremic rats treated with paricalcitol 4-uremic rats treated with paricalcitol and enalapril, and 5-control group. Results showed that enalapril, paricalcitol and their combined treatment could protect the heart from oxidative stress. The antioxidant effect of vitamin D may be related to inhibition of NADPH oxidase (26).

In a study, 29 diabetic individuals participated and were divided in to two groups. In the group (T2D-H), glycosylated hemoglobin were more than 7%,blood sugar higher than 140 and in the group (T2D-N), blood glucose were lower than 140 and glycated hemoglobin were lower than 7%. In the group H, DNA damage were more than group N (27).

In one study, bronchial cells extracted and cultured with air pollution particles. In this environment, vitamin D resulted in increased expression of G6PD and reducing interleukin-6. This enzyme is an enzyme necessary for the production of limiting substrates in an antioxidant pathway (28).

Other mechanisms of antioxidant effects of vitamin D, strengthening the gene expression of glucose-6-phosphate dehydrogenase, a key enzyme in estrogen and testosterone levels is required for regulation of insulin secretion and insulin receptor. Vitamin D reduces lipid peroxidation in kidney and liver function, and the amount of AST, ALT, PAL, total and direct bilirubin, creatinine and blood urea will be reduced (29).

In a study that was conducted on rats, the effect of vitamin D on oxidative stress and nitrosative in a group with particularly low vitamin D levels. 3-NT increased. These increases cause the disruption of NF-KB pathway. NF_KB mediates translation of nitric oxide synthase i (inducible) .3-NT transfer NF-KB to the nucleus and the increase in iNOS. iNOS can cause nitrosative stress. The result indicates that vitamin D deficiency in the brain is effective in increasing nitrosative stress. Cognitive decline may increase in middle-aged and older.

Several brain proteins in people who were deficient in vitamin D more than the control group . PPIA has several roles including protein folding, protein kinase and phosphatase regulation, immune regulation, cell signaling and redox status. Vitamin D may regulate glutathione levels by regulation of the activity of gamma-glutamyl trans-peptidase (30).

In a study, the effect of calcium and vitamin D on damage of DNA, 8- hydroxydeoxy guanosine, in normal colorectal mucosa was investigated. Ninety two patients with colorectal adenoma with 2 g of calcium and 800 units of vitamin D were treated for 6 months. Another group received placebo. The marker of oxidative stress of colorectal mucosa declined 22% and 25% respectively in vitamin D and calcium group in woman. This reduction effect was not observed in men and both calcium and vitamin D –treated group. Perhaps because the women were in postmenopausal stage, estrogen and progesterone intake may activate the VDR in colorectal mucosa in menopause. In total, calcium and vitamin D may reduce oxidative stress of nucleic acid in human colorectal mucosa. These effects were seen stronger among participants who vitamin D receptor expression was more than others in colon crypts. In colonocyte, vitamin D leads to increase of expression of antioxidant enzymes and inhibit lipid proxidation-induced iron in liposome. Vitamin D can reduce the levels of glutathione reductase, increase superoxide dismutase and glutathione peroxidase activity-dependent manganese and also glutathione. So the oxidative stress can be reduced in the colorectal epithelium (31).

VDR expression in endothelial cells is influenced by vitamin D and VDR expression increases through the mechanisms of transcription and translation. VDR activation causes reduction of oxidative stress in endothelial cells. Superoxide is converted to O2 and H2O2 by superoxide dismutase. These important antioxidant defense mechanisms are in all cells that are exposed to oxygen. Superoxide dismutase copper-zinc are in all cytosol of all eukaryotic cells .Increased expression of superoxide dismutase increased expression of VDR shows that activation or expression of VDR can affect by oxidative stress. Activity of VDR is related to antioxidant activity.Therefore, VDR is sensitive to oxidative stress and adequate amounts of vitamin D protect VDR from oxidative stress (16). Kynuta et al. showed vitamin D plays a key role in the biosynthesis of estrogen in general and specifically the direct regulation of aromatase gene expression (32).

Studies have shown that diabetes reduces plasma estradiol (a hormone needed to regulate the secretion of insulin and its receptors) (33). Vitamin D prevents decreased estrogen levels, thus can reduce diabetes speed (34).

The effects of prophylactic and therapeutic vitamin D 3 in diabetes and its side effects on the liver, pancreas and kidneys were examined. The results showed that vitamin D 3 at a rate of 5,000 units per kilogram of body weight in diabetic rats increased plasma insu-

lin levels, reaching the optimal level of blood glucose and appropriate liver glycogen concentration . In addition, the amount of superoxide dismutase, catalase, glutathione peroxidase increased compared to diabetic rats. Oxidative stress in cells is due to dysfunctional internal antioxidant enzymes, mainly superoxide dismutase, glutathione peroxidase and catalase. Hyperglycemia decreases non-enzymatic antioxidant defense. Vitamin D in diabetic mice resulted in increased levels of magnesium, calcium in plasma, which are needed for insulin exocytosis and oxidation of glucose and stimulation of the mitochondria metabolism. In fact, magnesium deficiency can cause dysfunction in the insulin receptor tyrosine kinase activity that ultimately leads to insulin resistance. Also it increases the TNFa and causes the development of chronic inflammatory syndrome with low intensity. Impaired glucose metabolism, increases in free radicals and severity of complications of diabetes will happen. Glucose concentrations outside the beta cells trigger the opening channels dependent on the calcium. An increase in cytosolic calcium causes calcium entry into the mitochondria and improves the oxidation of glucose and production of ATP for secretion of insulin. Vitamin D also has hypocholesterolemic and hypolipidemic effects and converts cholesterol to bile acids (29).

In a study, the role of the VDR in response to stimulation of 1:25 dihydroxy vitamin D 3 of endothelial oxidative stress was assessed. It was found that 1.25-dihydroxy vitamin D3 not only in dose and time -dependent increase VDR expression but also upregulate VEGF (vascular endothelial growth factor) and its receptors and superoxide dismutase antioxidant. VDR inhibition blocks the 1.25-dihydroxyvitamin vitamin D 3 ,VEGF, proliferation and migration of endothelial. In this study, by the hypoxia simulation, it was found that the state of oxidative stress as a result of hypoxia not only reduced the level of superoxide dismutase, but also reduced VDR expression in endothelial cells and correct this by adding 1.25 dihydroxy vitamin D 3 in the environment. These findings suggest that VDR expression in endothelial was affected by oxidative stress and decreased in endothelial cells. Adequate amounts of vitamin D and appropriate VDR expression is essential in angiogenic and antioxidative defense of endothelial . In this study, vitamin D concentrations of 0,5, 20, 100 nM were added to the culture environment and VDR expression in environment treated with vitamin D was greater than the untreated (16).
We do not suggest the suitable dosages for vitamin D supplements for diabetic patients. Other studies with different dosages of vitamin D are required. The present study have some limitations that must be considered in interpretation of results. The number of participants was small and the duration of intervention was short. Some non-significant changes may have become statistically significant with longer follow up.

Conclusion

In conclusion, the present trial has indicated that there was a negative inverse relationship between serum calcidiol with 3-NT and 8-OHDG at the end of the intervention. According to our clinical trial, vitamin D supplement can decrease diabetic complication followed by decreasing oxidative stress. Also vitamin D supplementation at the dose of 100μg for 2 months had beneficial effects on oxidative stress and T2DM management. Further studies to determine the appropriate doses of vitamin D for these patients are warrented. According to our clinical trial, vitamin D supplement can decrease diabetic complication followed by decreasing oxidative stress.

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