ORIGINAL ARTICLE ASIAN JOURNAL OF MEDICAL SCIENCES

Patients with type 2 diabetes have reduced levels of plasma vitamin D_3 and are well correlated with the oxidative stress parameters

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Submitted: 23-11-2015 Revised: 27-12-2015 Published: 10-01-2016

ABSTRACT

Background: Diabetes has emerged as an epidemic in this country as well as worldwide. Increased oxidative stress and decreased antioxidant defense are established etiological factors of this multifactorial disease. Some of the recent studies reported deficiency of vitamin-D_3 in type-2 diabetic patients. **Aims and Objectives:** This study was aimed to estimate the total oxidative stress (TOS), the total antioxidant defense (TAD) and the plasma 25-hydroxy vitamin D_3 levels in patients of type-2 diabetes patients with an objective to elucidate if there is any significant correlation between the TOS, TAD and vitamin D_3 levels. **Materials and Methods:** The study was conducted in forty patients recently diagnosed type-2 diabetic and the findings were compared with age/sex matched healthy controls. Total oxidative stress and total antioxidant defense values was estimated by two simple colorimetric tests developed and standardized in our laboratory, plasma 25-hydroxy vitamin D_3 by standardized ELISA method. **Results and Discussion:** The levels of vitamin D_3 in patients was found to be 50.17 ± 15.85ng/ml which was significantly decreased ($P<0.001$) when compared to healthy control group (75.42 ± 9.59 ng/ml). The plasma vitamin D_3 levels show a significant positive correlation ($r=0.564$, $P<0.001$) with the TAD values and a significant negative correlation ($r=-0.561$, $P<0.001$) with the TOS values in the study subjects. There is significant positive correlation of 25-OH vitamin-D_3 with antioxidant defense and significant negative correlation with oxidative stress observed in the current study, and the levels of vitamin D_3 were significantly decreased in type-2 diabetes when compared to the healthy controls. **Conclusion:** The type-2 diabetes patients are usually associated with vitamin D_3 deficiency which is significantly correlated with the oxidative stress conditions in this group of patients.

Key words: Vitamin D_3, FBG, TOS, TAD, Diabetes

Access this article online

Website:
http://nepjol.info/index.php/AJMS

DOI: 10.3126/ajms.v7i3.13922
E-ISSN: 2091-0576
P-ISSN: 2467-9100

BACKGROUND

Incidence of type-2 diabetes is increasing in India and worldwide and it has taken the shape of an epidemic.[1] In 2010, the prevalence of type-2 diabetes mellitus in India was 51 million.[1] In spite of contemporary treatment is available with insulin and several hypoglycemic drugs, along with dietary and lifestyle management, the disease seems to be spreading heavily in the community. It is now well established that diabetes mellitus is well associated with oxidative stress. Type-2 diabetes is characterized by the combination of insulin resistance and β-cell dysfunction. The imbalance between total oxidative stress and antioxidant defense attributes largely to the pathogenesis of type-2 diabetes mellitus and its complications. Recently a few studies have reported the association of vitamin D_3 with diabetes.

Vitamin D can be obtained either through dietary intake or produced endogenously. It is found in foods such as oily

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fish (salmon, sardines, mackerel), egg yolks and fortified milk; however dietary intake only accounts for about 30% of the vitamin D obtained. The primary route via which people obtain vitamin D is through exposure to ultraviolet B (UVB) sunlight at wavelengths between 290-315 nm. UVB sunlight activates 7-dehydrocholesterol (7-DHC), a precursor synthesized from cholesterol and found within the skin.[2] The activation of 7-DHC and synthesis to previtamin D_3 within the skin and its subsequent isomerization to the inactive form, vitamin D_3 (cholecalciferol),[3] produce the endogenous stores of vitamin D that, once hydroxylated twice, perform many integral physiological functions.

The vitamin D_3 which is obtained in the diet or through endogenous production is not biologically active.[4] In order for vitamin D_3 to become biologically active, it must receive two successive hydroxylations from the liver by 25-hydroxylase [25(OH)ase] to form 25(OH)D_3 (also known as calcidiol) and the kidneys by 25(OH)D_3-1α-hydroxylase [1α(OH)ase] to form 1,25 dihydroxyvitamin-D_3 (1,25$(OH)_2D_3$) (also known as calcitriol).[4] Once formed, 1,25(OH)2D_3 must bind to its vitamin D receptor (VDR) which forms a complex with the retinoid X receptor (RXR).[4]

Vitamin D is most well known for its function to maintain calcium and phosphorus homeostasis. However, recent evidence suggests that vitamin D and calcium homeostasis may also be important for a variety of non-skeletal outcomes including neuromuscular function, psoriasis, multiple sclerosis and colorectal and prostate cancer.[5] More recently, there is accumulating evidence to suggest that altered vitamin D may play a role in the development of type-2 diabetes mellitus.[6] Vitamin D deficiency appears to be related to the development of diabetes mellitus type 2.[6-11] The pathogenesis of type-2 diabetes mellitus is yet to be completely understood since a simultaneous array of malfunctioning mechanisms take part in an interrelated fashion to give the final outcome of type-2 diabetes. Vitamin D_3 plays a role in controlling free radicals in the liver of Streptozotocin-induced diabetic rats.[12] Though it has been already established by previous researchers that total oxidative stress is an important etiological factor for the causation of type-2 diabetes and simultaneously deficiency of vitamin D has also been observed in type-2 diabetes, but it is yet to be elucidated whether there is any significant links among them.

Aims and objectives

To estimate the total oxidative stress (TOS), the total antioxidant defense (TAD), and plasma vitamin D_3 levels in type-2 recently diagnosed diabetic patients and to find out whether there is any association between plasma vitamin D_3 levels and TOS and TAD levels in type-2 diabetes.

MATERIALS AND METHODS

Forty recently diagnosed type-2 diabetic patients (18 males and 22 females) were enrolled in the current study, with their ages ranging from 20 to 50 years, and age/sex matched healthy controls (21 males and 19 females) of the same age group were recruited. The study was preapproved by the Institutional Ethics Committee of Nilratan Sircar Medical College, Kolkata, India. Patients having other endocrine disorders like type-1 diabetes, thyroid disorders, pregnant mothers, patients suffering from polycystic ovarian disease, renal failure, any malignant disease and patients receiving antioxidant agents are excluded from the study.

Sample collection

Five ml of whole blood (after 8 to 10 hours calorie deprivation) was drawn aseptically from superficial vein, in heparin containing vials, centrifuged for 5 minutes at 3000 r.p.m., plasma was used immediately for estimation of different biochemical parameters and stored in aliquots at -40°C for further use.

Assay of total oxidative stress (TOS) in plasma:[13,14,15]

Principle

This test is based on iron catalysed breakdown of hydroperoxides into alkoxyl (RO•) and peroxyl (ROO•) radicals which reacts with the chromogen (N,N-dimethyl- p- phenylenediamine sulphate) towards formation of a colored compound the absorbance of which is photometrically detectable at 505 nm. The method has been modified from the original FORT (Free Radical Oxygen Test), due to the unstability of nonlyophilized chromogen in solution procured and used in our laboratory. The intensity of the color correlates directly with the quantity of radical compounds, according to the Lambert-Beer's law, and it can be related to the oxidative status of the sample.

Assay procedure

One hundred microliters of plasma diluted to 20 times in PBS, was dissolved in one ml of acetate buffer. Twenty-five microliters of working chromogen solution (N,N-dimethyl- p- phenylenediamine sulphate) added, absorbance taken at 505nm by 6min time-scan in UV-VIS spectrophotometer. The absorbance values obtained at 4 to 6 minutes for each sample against blank, were compared to the curve obtained using H_2O_2.

Standardization

Standard curve of TOS by modified FORT was prepared using different dilutions of hydrogen peroxide (H_2O_2) in milimolar concentrations per litre and difference in absorbance values taken at 505nm in a six-minute time-scan between 6th and 4th minute (increase in absorbance noted maximum in this period). Each data is the mean of three

different values by the same procedure performed in four different occasions. The assay was completed in 6 minutes.

Intra-assay and Inter-assay coefficients of variation

The maximum intra-assay variation at 4 min was 4.762 and that at 6 min was 4.145 and inter-assay variations at 4 and 6 minutes were 2.717 and 2.105 respectively for TOS. The maximum sensitivity of the assay was 1.22 milimol/l H_2O_2 and the linearity was up to 120 milimol/l.

Assay of total antioxidant defense (TAD) in plasma:[13,15]

Principle

In an acidic medium (pH = 5.2) and a suitable oxidant ($FeCl_3$), the chromogen (N, N-dimethyl- p- phenylenediamine sulphate) develops a stable and colored radical cation that is photometrically detectable at 505 nm at 37° C. Antioxidant compounds in the sample reduce the radical cation of the chromogen, quenching the color and producing a discoloration of the solution, which is proportional to their concentration. The absorbance values obtained for the samples are compared with a standard curve obtained using Trolox.

Assay procedure

One ml of acetate buffer (pH = 5.2) taken in a test tube. Twenty-five microliter chromogen reagent that contains N, N-dimethyl- p- phenylenediamine sulphate and 10 microliter $FeCl_3$ solutions were added. Ten microliter of 20 times diluted plasma was added to the mixture, the antioxidant compounds in the sample reduce the chromogen, quenching the color and producing a discoloration of the solution, which is proportional to their concentration.

Standardization

Standard curve of assay of TAD was prepared by using different dilutions of trolox, a potent antioxidant and it quenches the color of the chromogen maximally between 4th and 6th minute. With different concentrations of trolox, (6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid), a water soluble form of vitamin E, the difference in absorbance between the absorbance values at 505nm at 4th and 6th minute of the 6 minutes time scan was plot and a calibration curve was constructed.

Intra-assay and inter-assay coefficients of variation

The maximum intra-assay variations at 4 and 6 minutes were 7.914 and 9.009 respectively and inter-assay variation at 4minutes and 6minutes were 2.173 and 4.717 respectively, whereas the linearity ranged from 0.25 to 10.0 mmol/l equivalent of trolox.

Estimation of fasting blood glucose

Estimation of fasting blood glucose (FBG) was done by glucose oxidase-peroxidase method colorimetrically in semi-autoanalyser using standardized reagent kit.

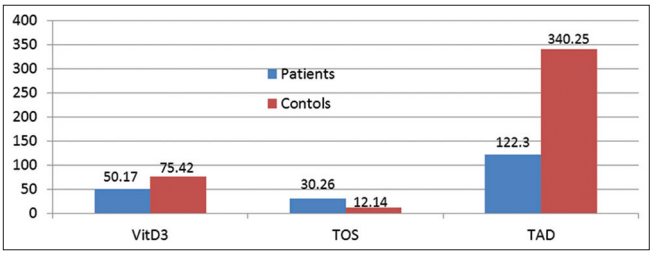

Figure 1: Comparison of mean Vitamin D_3, TOS & TAD in type-2 diabetic patients and healthy controls

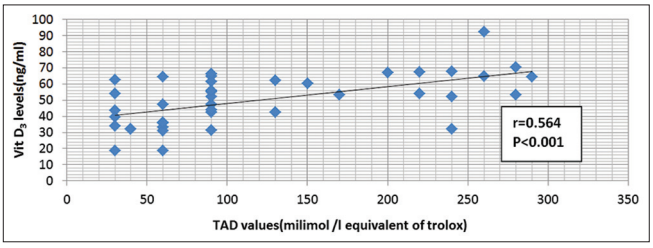

Figure 2 : Scatter plot showing correlation between Vitamin D_3 and TAD values in patients

Assay of plasma Vitamin D_3 level

Estimation of plasma 25- OH-vitamin D_3 level is done using standardized competitive binding Enzyme Linked Immunosorbant Assay (ELISA) kit (calibrator range 2.5-150ng/ml) and absorbance taken in an ELISA microplate reader at 450nm. All samples were assayed in triplicate with calibrators before every run. The mean of the three readings was considered as final value.

Storage

All the above reagents in our method should be stored for future use in 6 to 8 degree for two weeks except the chromogen reagent (N, N-dimethyl- p- phenylenediamine sulphate) and $FeCl_3$ which were required to be prepared freshly before the tests. Plasma samples were stored in -40°C deep freezer. Tests were performed within a week after sample collection.

Statistical analysis

Data were expressed as mean ± standard deviation (SD), comparison of data was done using unpaired two-tailed Students' t-test and Pearson's correlation, $P<0.05$ was considered as significant. Statistical analysis was done using Microsoft Office Excel-2007 and Statistical Package for the Social Sciences version 2020.

RESULTS AND DISCUSSION

The clinical and biochemical parameters of the study subjects as well as healthy controls have been depicted in Table 1.

The value of fasting plasma glucose in the patients included in the current study was 119.6 ± 28.02 mg/dl which was

significantly higher (P<0.05) than those with healthy controls (78.49 ± 12.56 mg/dl). The levels of vitamin D_3 in patients was 50.17 ± 15.85ng/ml which was also significantly lower (P<0.001) when compared to healthy control group (75.42 ± 9.59 ng/ml) as depicted in Figure 1. The TOS value in patients is 30.26 ± 13.68 milimol/l equivalent of H_2O_2 which is significantly higher (P<0.001) than TOS value in controls (12.14 ± 4.73 milimol/l equivalent of H_2O_2). The TAD values in the study subjects is 122.3 ± 85.92 milimol/l equivalent of trolox, which is significantly lower (P<0.001) than the TAD values of healthy controls (340.25 ± 155.04 milimol/l equivalent of trolox).

The plasma vitamin D_3 levels show a significant positive correlation (r=0.564, P<0.001) with the TAD values (Figure 2) and a significant negative correlation (r=-0.561, P<0.001) with the TOS values (Figure 3) in the study subjects.

Considering the mean value of TOS of patients and subtracting the standard deviation a round value of 20 milimol of H_2O_2/l was taken as cut off for higher and lower values for total oxidative stress. Further, considering the mean total antioxidant status of patients, we selected a round-off value of 100milimol/l equivalent of trolox, and categorized patients with plasma values of TAD<100 milimol/l equivalent of trolox to have less anti-oxidant defense and those with values>100 milimol of trolox/l to have more antioxidant defense. With this cut-off TAD as 100, the patients were subcategorized as per the following Table 2 and Figure 4.

The fact that type-2 diabetes mellitus is associated with increased oxidative stress and decreased antioxidant defense has already been established. Similarly low levels of circulating plasma Vit-D_3 is also associated with type-2 diabetes patients. An increased prevalence of diabetes has been observed in vitamin D-deficient individuals.

The Vit-D receptor (VDR) has been identified in many cells, like intestinal mucosal cells, immune cells (T and B cells), kidney cells and pancreatic b-cells.[4,16] The binding of 1,25$(OH)_2D_3$ to the VDR/RXR (retinoid X receptor) complex and subsequent binding to its specific DNA sequence known as the vitamin D response element (VDRE) leads to an increased expression of proteins, such as calbindin-D9K found in the intestine and calbindin-D28K found in pancreatic β-cells, thus facilitating calcium influx into these tissues.

Although serum 25$(OH)D_3$ concentrations of 80 nmol/L have shown to be efficacious in preventing many diseases associated with vitamin D deficiency, it is important to note

Table 1: The clinicobiochemical parameters of type-2 diabetic patients and healthy controls

Variables	Patient (N=40)	Control (N=40)	P value
Age (years)	43.7±5.6	39.45±9.16	NS
Sex (M/F)	18/22	21/19	NS
Body mass index (BMI)	25.0±4.63	24.15±2.51	NS
Fasting blood glucose (mg/dl)	119.6±28.02	78.49±12.56	<0.001*
TOS (mM/l equivalent of H_2O_2)	30.26±13.68	12.14±4.73	<0.001*
TAD (mM/l equivalent of trolox)	122.3±85.92	340.25±155.04	<0.001*
Vitamin D_3 (ng/ml)	50.17±15.85	75.42±9.59	P<0.001

Student's t-test was done, *Indicates significant (P<0.05), with 95% confidence level, NS: Not significant

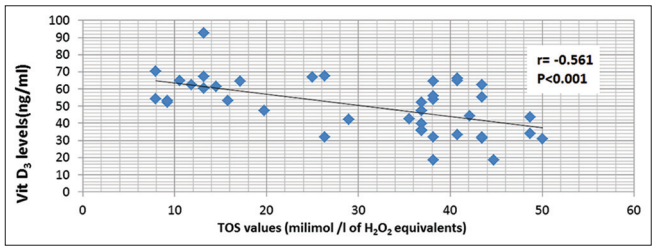

Figure 3: Scatter plot showing correlation between Vitamin D_3 and TOS values in patients

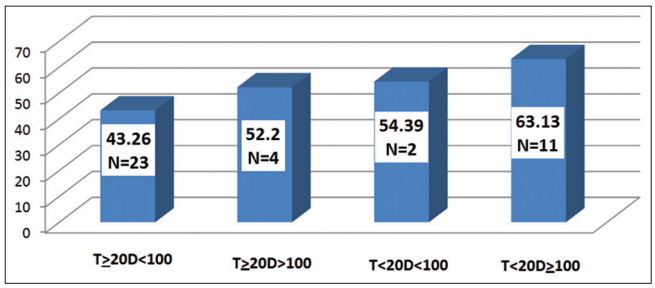

Figure 4: Vitamin D_3 levels in different stress and antioxidant groups

that this is an arbitrary cut-off point, i.e., it may be sufficient for some individuals while grossly inadequate for others.[17]

The most effective way of measuring vitamin D status is to measure serum concentration of 25$(OH)D_3$, not 1,25$(OH)_2D_3$; this is due to the rapid clearance rate of the latter.

In this study we have measured the total oxidative stress and total antioxidant defense in both type-2 diabetic patients as well as healthy controls by two simple colorimetric tests developed and standardized in our laboratory and observed that the TOS values are significantly higher and TAD values significantly lower in study subjects than the control group. Simultaneously the 25-hydroxy vitamin D_3 levels measured are significantly lower in the patients than the control group. For further analysis, we have categorized the patients in subgroups according to TOS and TAD values and observed that vitamin D_3 levels are significantly lower

Table 2: Plasma vitamin D_3 levels in subgroups of patients with TOS (milimol/l of H_2O_2) and TAD (milimol/l equivalent of trolox) parameters

No	Sub groups	N (%)	Description	Vit D_3 (ng/ml) Mean±SD	P value
A	T>20, D<100		Patients with increased oxidative stress and decreased antioxidant defense	43.26	<0.001*
B	T>20, D>100		Patients with increased oxidative stress and increased antioxidant defense	52.20	<0.001*
C	T<20, D<100		Patients with decreased oxidative stress and decreased antioxidant defense	54.39	<0.001*
D	T<20, D>100		Patients with decreased oxidative stress and increased antioxidant defense	63.13	<0.001*

*One way ANOVA test done, P value considered significant if P<0.05. (TOS=T) and (TAD=D)

in groups with more oxidative stress and lesser antioxidant defense. Researchers have already investigated the effects of vitamin D_3 in oxidative stress in animal models like streptozotocin induced diabetic rats.[12] In a study conducted by Dalgard et al on 158 type 2 diabetics more than 50% of the study population was vitamin D deficient.[18] In another study administration of vitamin D in diabetic subjects produced negative effects on control of glycemic status and insulin resistance.[19] In a study conducted in India there is hypovitaminosis D among type 2 diabetic cases than healthy controls. A significant negative association was obtained between Vitamin D level and DM.[20] In a study conducted in Iranian population, results indicate an inverse relationship between serum levels of 25-OH-D and activities of GSH-PX(glutathione peroxidase) and GR (glutathione reductase) in diabetic patients.[21] Vitamin D in one of the nutrients that was investigated as an antioxidant recently and antioxidant effects of vitamin D were shown in previous experimental and in vitro studies.[22]

The significant positive correlation of 25-OH vitamin-D_3 with antioxidant defence and significant negative correlation with oxidative stress observed in our study may lead researchers in future to investigate whether vitamin D_3 should be included in the treatment protocol of type-2 diabetes patients.

CONCLUSION

The current study elucidated that plasma level of vitamin D_3 is significantly reduced in type-2 diabetic patients. The level of plasma vitamin D_3 is significantly positively correlated with total antioxidant defense and significantly negatively correlated with the total oxidative stress. Further studies in larger number of subjects may open up a new horizon regarding the exact role of vitamin D_3 in patients suffering from type-2 diabetes mellitus with oxidative stress as an etiological factor.

Limitations of the study

The sample size for the current study was small as it was a pilot study. Further studies are required to validate the results of the current study with large sample size.

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Authors Contribution:
PS - Designed the study, Data Acquisition, Data Analysis and Drafting of Manuscript; **UKB -** Data Analysis, Drafting of Manuscript, Review of Manuscript; **AK -** Manuscript Preparation, Data Analysis, Review of Manuscript, Final Approval.

Source of Support: Nil. **Conflict of Interest:** None.