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VITAMIN D STATUS AND ITS ASSOCIATION WITH OXIDATIVE STRESS IN DIABETIC PATIENTS

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ABSTRACT

The aim of study was to reveal the association between Vitamin D deficiency and oxidative stress biomarkers in diabetes mellitus. Diabetes mellitus is a progressively prevalent disease correlated with increased incidence of morbidity and mortality. Oxidative stress has been considered as a major hallmark for the pathogenesis of diabetes mellitus. Vitamin D deficiency was also observed in diabetes mellitus.

This is a case-control study conducted on 100 diabetic patients recruited from Baqai Institute of Diabetology and Endocrinology, Karachi and 50 normal healthy control categorized as; Group I: Normal healthy control, Group II: Diabetes patients less than 10 years of diagnosis of disease and Group III: Diabetes patients more than 10 years of diagnosis of disease. Exclusion criteria included the use of drugs which affect calcium and bone metabolism, chronic disorders of liver and kidney, cancer and other complications. Anthropometric indices, Fasting blood glucose (FBG), Glycated hemoglobin (HbA1c), Urea, Creatinine, serum levels of Vitamin D, Calcium, Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline phosphatate (ALP) were measured in all groups. Catalase, Superoxide dismutase and Malondialdehyde (MDA) levels were estimated as markers for oxidative stress.

The results revealed the significant increased Body mass index (BMI), FBG, HbA1c, Urea, Creatinine, ALT, AST, ALP, total cholesterol, triglyceride, Low density lipoprotein (LDL)-cholesterol, Calcium and MDA in diabetes. Vitamin D, Catalase and Superoxide dismutase and HDL-cholesterol were markedly decreased in both diabetic groups. Pearson correlation analysis showed a marked association between oxidative stress biomarkers and Vitamin D deficiency in both diabetic groups. Present study concluded a strong association among Vitamin D deficiency and oxidative stress biomarkers in diabetes. Hence it is suggested that Vitamin D supplementation may reduce deleterious effects of diabetes mellitus.

KEY WORDS: Diabetes mellitus; Vitamin D; Oxidative stress; Reactive oxygen species; Impaired fasting glucose.

INTRODUCTION

Vitamin D 25(OH) D levels and oxidative stress biomarkers in diabetic patients compared with non diabetic healthy Diabetes is an enormous and emergent clinical and public health problem (Zafar *et al*., 2011; Grover *et al*., 2005). The International Diabetes Federation (IDF) estimated that 415 million adults had diabetes in 2015 and estimated number will increase to approx 642 million by 2040 (Herman, 2017). Diabetes is considered as an epidemic disease; attributed to biological as well as socio-economic impact. Pathogenic basis of Diabetes mellitus include hyperglycemia, gradual impairment of insulin action by pancreatic β-cells, resistance to insulin, or both and many other metabolic disturbance. (Akter *et al*., 2011; Alharbi *et al*., 2014)

The growing evidences on the pathophysiology of diabetes mellitus has displayed that reactive oxygen species and oxidative stress are among the main causal factors that are responsible for the pathogenesis of insulin resistance, impaired insulin secretion and glucose utilization and abnormal hepatic glucose production (Folli *et al*., 2011; Akash *et al*., 2013; Rehman and Akash, 2016). In addition, this mechanism has been implicated as the underlying cause of both the microvascular (involving small vessels, such as capillaries) and macrovascular (involving large vessels, such as arteries and veins) complications associated with diabetes mellitus (Brownlee, 2001). Previous study supports the evidence that in diabetic patients hyperglycemia play a crucial role in the generation of oxidative stress leading to endothelial dysfunction in blood vessels (Ceriello, 2006).

Previous studies showed the functional relationship between vitamin D and insulin sensitivity (Gulseth *et al*., 2017). Vitamin D also plays a vital role in β-cell function, insulin sensitivity, and secretion by both direct and indirect actions (Harinarayan, 2014).Several epidemiological studies showed inverse associations between serum 25-hydroxyvitamin and fasting blood glucose, insulin resistance and prevalence of Type 2 diabetes mellitus (T2DM) (Chiu *et al*., 2004; Forouhi *et al*., 2008; Ford *et al*., 2005; Gradinaru *et al*., 2012; Schottker *et al*., 2013). Vitamin D status, also inversely associated with oxidative stress biomarkers (Shea *et al*., 2007; Tarcin *et al*., 2009).Studies suggested that impaired glucose metabolism and hypovitaminosis D is inversely associated with circulating oxidative stress biomarkers (Gradinaru *et al*., 2012).

The aim of present study was to evaluate the oxidative stress biomarkers in diabetes mellitus with respect to time of diagnosis of the disease. The study also focuses on the association of 25-hydroxy controls.

MATERIALS AND METHODS

The study was carried out in the Department of Biochemistry, University of Karachi, Pakistan. Study protocol was approved by institutional review board of University of Karachi. Written inform consents were obtained from patients

and control. Inclusion criteria included diabetic patients with diabetes (<10 years of diagnose) and diabetes (>10 years of diagnose) along with normal healthy control with known history and no other complication or disease were selected for this study. Exclusion criteria included use of drugs which affect calcium and bone metabolism, chronic disorders of liver and kidney, cancer, endocrinology disorders, insulin injection, use of anticonvulsant drugs, and other supplementation.

Experimental design: Total 100 blood sample of diabetic patients of both sexes were collected from Baqai institute of Diabetology and Endocrinology (BIDE), Karachi, Pakistan, with written informed consent.
Total 150 subjects were included in the study both patients and control categorized as;
Group1: (n=50) Control (Normal healthy individual without Diabetes)
Group 2: (n=50) Diabetes patients (patients with known history<10 years of diagnosis)
Group 3: (n=50) Diabetes patients (patients with known history>10 years of diagnosis)

Sample collection: Under aseptic condition using, sterile syringes and vaccutainers 3 cc venous bloods was drawn in a fasting state from control and patients. All tubes were kept at 4^{o}C in cold box. Tubes were centrifuge at 3000 rpm for 10 minutes at 4^{o}C to separate plasma. Plasma were collected in eppendroff and stored at -80^{o}C until analysis.

Clinical examination: Body mass index (BMI) and Blood pressure and fasting blood glucose of each individual control and patient were measured and recorded.

Estimation of serum lipid profile: Plasma samples were analyzed for total cholesterol (TC) (Meiattini *et al*., 1978), triglyceride (TG) (Bucolo and David, 1973), high density lipoprotein (HDL) (Grove, 1979), by autoanalyzer and low density lipoprotein (LDL) calculated by using Friedwald formula (Warnick *et al*., 1990).

Estimation of serum urea and creatinine: Serum urea level was estimated by diacetyl monoxime method (Mather and Roland, 1969). Serum creatinine was measured by modified Jaff's method (Spierto *et al*., 1979).

Estimation of glycated haemoglobin (HbA1c): HbA1c level were estimated by using auto analyzers. (Shima *et al*., 1988).

Estimation of serum calcium ions using ion selective electrode (ISE) (Ion Meter 3345 Jenway): By using ion selective electrode (ISE) serum calcium level was estimated using ion meter 3346 (Jenway). Jenway's available manual operating procedure was followed. The value was recorded as mmol/l.

Estimation of serum vitamin D: Vitamin D was estimated by the Enzyme Linked Immuno Sorbent Assay (ELISA). The assay utilized a competitive ELISA technique with a selective monoclonal antibody recognizing 25 (OH) vitamin D.

Oxidative Stress Biomarkers

Estimation of Malondialdehyde (MDA): Lipid peroxidation in plasma was determined by following the method of Okhawa *et al*. (1979). Malondialdehyde (MDA) is one of the end products of lipid peroxidation. The plasma Malondialdehyde (MDA) was allowed to react with thiobarbituric acid (TBA) to form pink color complex. The absorbance was measured at 532 nm.

Estimation of catalase: Plasma Catalase (CAT) activity was estimated by the Sinha, (1972). The method is based on the fact that dichromate in acetic acid is reduced to dichromic acetate when heated in the presence of H_2O_2 and measured spectrophotometrically at 570nm.

Estimation of superoxide dismutase (SOD): Superoxide dismutase (SOD) activity was estimated by the method of Kono *et al*., 1978. The method is based on the principle of the inhibitory effect of superoxide dismutase (SOD) on the reduction of nitro-blue tetrazolium (NBT) dye by superoxide radicals, which are generated by the autoxidation of hydroxylamine hydrochloride. The reduction of nitro-blue tetrazolium (NBT) is measured at 560 nm and the results were expressed as U/mL.

Assessment of liver function: Plasma levels of alanine aminotransferases (ALT) and Aspartate aminotransferase (AST) were estimated by using Enzymatic Kit (Randox, UK) followed by the method of Bergmeyer and Horder (1980).The method of Tietz *et al*. (1983) were used for the estimation of serum alkaline phosphatase via enzymatic kit (Erba Diagnostics, Germany).

Statistical analysis: Results are expressed as mean ± SD. Statistical significance and difference from control and test values were analyzed by ANOVA one way analysis of variance using SPSS version 19. Tukey test was used for multiple comparisons. $p<0.001$, $p<0.01$ and $p<0.05$ were considered significant. Pearson's correlation analysis was performed to find the association between Vitamin D and oxidative stress biomarkers.

RESULTS

Clinical examinations: The results showed that BMI, systolic blood pressure and fasting blood glucose levels were significantly increased in both diabetic groups (patients with <10 years and >10 years of diagnosis) in comparison with control ($p<0.001$). Further no significant results were observed (Table 1).

Estimation of serum lipid profile: Results of lipid profile showed that the total cholesterol was markedly increased ($p<0.05$) in both diabetic groups (patients with <10 years and >10 years of diagnosis) as compared with control. Whereas, no significant changes in Serum triglyceride was observed in both diabetic groups (patients with <10 years and >10 years of diagnosis) as compared with control. However, HDL-Cholesterol was significantly decreased in diabetic group 1(patients with <10 years of diagnosis) ($p<0.01$) as well as diabetic group 2 (patients with >10 years of diagnose) ($p<0.05$) as compared with controls (Fig. 1).

Estimation of serum urea and creatinine: Plasma urea and creatinine were markedly elevated in Diabetes patients (with <10 years of diagnosis) as well as Diabetes patients (with >10 years of diagnosis) as compared with control ($p<0.001$) (Table 1). However, creatinine levels were more significantly raised in Diabetes patients (with >10 years of diagnosis) as compared with Diabetes patient (with <10 years of diagnosis) ($p<0.05$). No significant change was observed in urea level between both diabetic groups (with <10 years and >10 years of diagnosis).

Estimation of glycated haemoglobin (HbA1c): Plasma HbA1c was significantly elevated in both diabetic groups (patients with <10 years and >10 years of diagnosis) as compared with control ($p<0.001$). However no significant result was observed when both diabetic groups were compared (Table 1).

Estimation of serum calcium ions using ion selective electrode (ISE) (Ion Meter 3345 Jenway): Marked elevated calcium levels were observed in both Diabetes patients (with <10 years of diagnosis) as well as Diabetes patients (with >10 years of diagnosis) in comparison with control ($p<0.001$). Plasma calcium levels in Diabetes patients (with >10 years of diagnosis) was significantly high as compared with Diabetes patients (with <10 years of diagnosis) ($p<0.001$) (Table 1).

Estimation of serum vitamin D: Vitamin D levels in normal healthy control and both diabetic groups were depicted in Table 1. Results showed marked reduction in Vitamin D levels in both diabetic groups (with <10 years and >10 years of diagnosis) as compared with control ($p<0.001$). However, no significant results were observed between both diabetic groups.

Oxidative Stress Biomarkers

Estimation of malondialdehyde (MDA): MDA levels were markedly elevated in Diabetic group 1 (patients with <10 years of diagnosis) and Diabetic group 2 (patients with >10 years of diagnosis) in comparison with control ($p<0.001$). No significant result was observed among both diabetic groups (Table 2, Fig. 2).

Estimation of catalase: Marked decreased Catalase activity was observed in both Diabetic group 1 (patients with <10 years of diagnosis) and Diabetic group 2 (patients with >10 years of diagnosis) as compared with normal healthy control ($p<0.001$) as depicted in Table 2. Diabetic group 2 (patients with >10 years of diagnosis) showed significant decreased Catalase activity in comparison with Diabetic group 1 (patients with >10 years of diagnosis) ($p<0.001$) (Fig. 3).

Estimation of superoxide dismutase (SOD): Activity of SOD was significantly reduced in both diabetic groups (patients with <10 years and >10 years of diagnosis) as compared with control ($p<0.001$). Marked reduction in SOD activity was observed in Diabetic group 2 (patients with >10 years of diagnosis) when compared with Diabetic group 1 patients with >10 years of diagnosis) ($p<0.001$) (Table 2, Fig. 4).

Assessment of liver function: Plasma AST and ALT levels were significantly raised in both diabetic groups (with <10 years and >10 years of diagnosis) as compared with control ($p<0.001$). However, ALT levels were markedly raised in Diabetic group 2 (patients with >10 years of diagnosis) as compared with Group 1 (patients with <10 years of diagnosis) ($p<0. 01$). ALP levels were markedly increased in Diabetic group 1 (with <10 years of diagnosis) as compared with control ($p<0.001$) and no significant result was observed when ALP levels of both diabetic groups were compared (Table 3).

Pearson correlation analysis: Pearson correlation analysis showed a strong negative association among MDA and Vitamin D ($r = -0.714$, $p<0.01$) (Fig. 5), MDA and Catalase ($r = -0.309$, $p<0.05$) (Fig. 6) of Group 1 (patients with <10 years of diagnosis). However, Catalase and vitamin D showed a significant positive correlation in Group 1 (patients with <10 years of diagnosis) (Table 4, Fig. 7).

Table 1. Anthropometric indices, FBG, HbA1c, urea, creatinine, calcium and Vitamin D levels in normal healthy individual and diabetic patients.

Parameters	Control	Diabetic group-1	Diabetic group-2
BMI	20.82 ± 3.69	29.27 ± 7.63***	28.26 ± 4.63 ***
Systolic blood pressure	118.80 ±7.18	136.20 ± 26.64 ***	136.20 ± 20.98 ***
Diastolic blood pressure	83.40 ± 6.26	87.60 ± 14.22	88.20 ±9.24
Fasting blood glucose l (FBG) (mg/dL)	114.80 ±10.12	226.18 ± 75.69 ***	228.94 ± 69.64 ***
HbA1c	6.84 ± 0.39	10.60 ±2.58 ***	9.98 ± 1.99 ***
Urea (mg/dL)	26.31 ± 4.9	37.93 ± 14.30 ***	37.21 ± 4.73 ***
Creatinine (mg/dL)	0.96 ± 0.13	1.35 ± 0.36 ***	1.63 ± 0.86 ***, #
Calcium (mmol/L)	2.63 ± 0.49	4.26 ±1.55 ***	5.90 ± 1.53 ***, ###
Vitamin D (ng/mL)	44.40 ±13.86	13.85 ± 3.44***	13.46 ± 3.76 ***

Values represent the mean ±SD

***P< 0.001, with Control, ###P<0.001, #P<0.05 with Diabetic group 1(patients with <10 years of diagnosis), Diabetic group 2 (patients with >10 years of diagnosis) (One way ANOVA)

Table 2. Oxidative stress biomarkers in normal healthy individual and diabetic patients.

Parameter	Control	Diabetic Group1	Diabetic Group 2
Malondialdehyde (mM/ml)	5.01 ± 1.00	6.94 ± 2.59 ***	7.61 ± 2.77 ***
Catalase (µM/ml)	346.41 ± 60.67	271.009 ± 25.55***	121.93 ± 9.22 ***, ###
Superoxide dismutase (U/ml)	319.27 ± 35.34	279.89 ± 44.32 ***	202.43 ± 40.48 ***, ###

Values represent the mean ±SD

***p< 0.001, with Control, ###P<0.001 with Diabetic group 1 (patients with <10 years of diagnosis), Diabetic group 2 (patients with >10 years of diagnosis) (One way ANOVA)

Table 3. ALP, AST, and ALT levels in normal healthy individual and diabetic patients.

Parameter	Control	Diabetic Group1	Diabetic Group 2
ALP (U/L)	140. 27 ± 18.54	185.41 ± 69.60 ***	161.26 ± 54.60
AST (U/L)	42.36 ± 14.82	87.60 ± 20.29 ***	84.79 ± 31.83 ***
ALT (U/L)	24.24 ± 11.98	59.87 ± 45.81 ***	85.18 ± 51.15 ***, ##

Values represent the mean ±SD

***p< 0.001, with Control, ##P<0.01 with Diabetic group 1 (patients with <10 years of diagnosis), Diabetic group 2 (patients with >10 years of diagnosis) (One way ANOVA)

ALP (Alanine aminotransferases); AST (Aspartate aminotransferase); ALT (Alkaline phosphatase)

Table 4. Pearson correlation analysis between Oxidative stress biomarkers and vitamin D of normal healthy *individual* and diabetic patients.

Parameters	MDA	Catalase	SOD	Vitamin D
Control				
MDA	1	0.091	0.021	-0.045
Catalase	-	1	0.126	0.261
SOD	-	-	1	0.229
Vitamin D	-	-	-	1
Diabetes group 1				
MDA	1	-0.309 *	-0.206	-0.714**
Catalase	-	1	0.275	0.285*
SOD	-	-	1	0.254
Vitamin D	-	-	-	1
Diabetes group 2				
MDA	1	0.162	-0.094	-0.262
Catalase	-	1	-0.161	0.275
SOD	-	-	1	0.101
Vitamin D	-	-	-	1

**Correlation is significant at the 0.01 levels (2-tailed)

*Correlation is significant at the 0.05 levels (2-tailed)

Control: Normal healthy individual

Diabetic group 1: (patients with <10 years of diagnosis)

Diabetic group 2: (patients with >10 years of diagnosis)

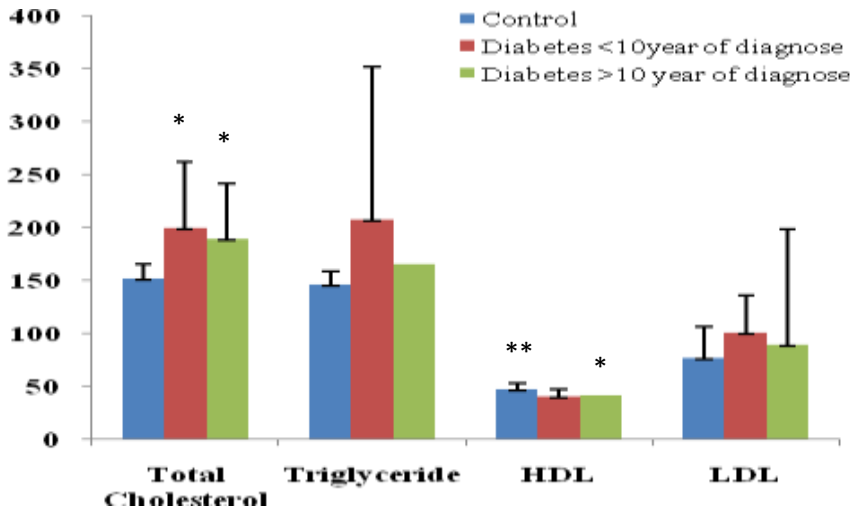

Fig. 1. Comparison of lipid profile in control, Diabetic group 1 (patients with <10 years of diagnosis) and diabetic group 2 (patients with >10 years of diagnosis)
**p<0. 01, *p<0.05 with control

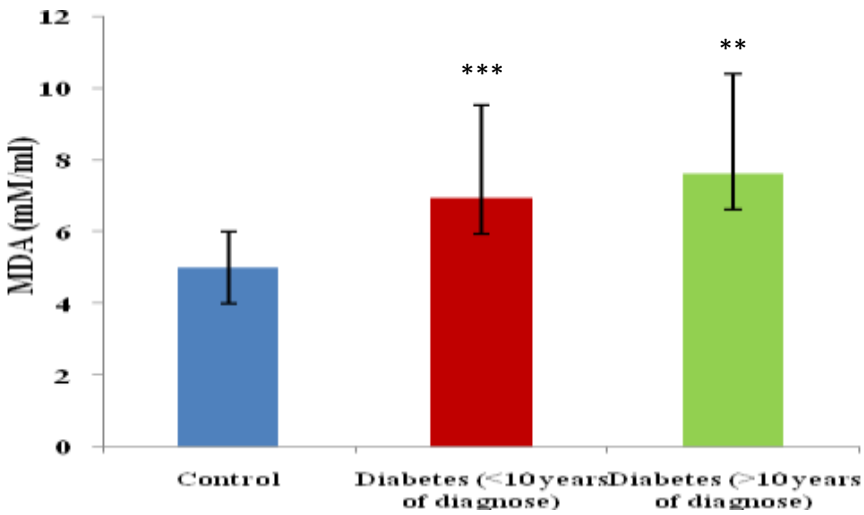

Fig. 2. Lipidperoxidation in control, Diabetic group 1 (patients with <10 years of diagnosis) and Diabetic group 2 (patients with >10 years of diagnosis)
**p<0. 01, *p<0.05 with control

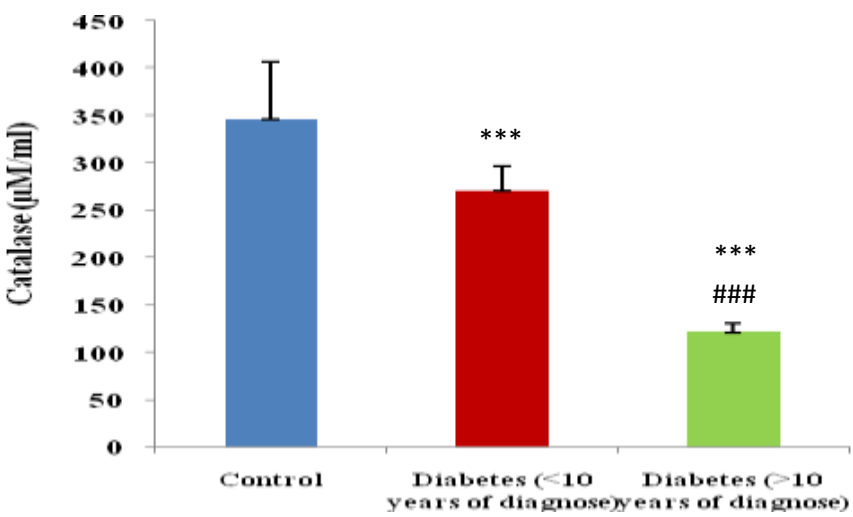

Fig. 3. Catalase activity in control, Diabetic group 1 (patients with <10 years of diagnosis) and Diabetic group 2 (patients with >10 years of diagnosis)
**p<0. 01, *p<0.05 with control

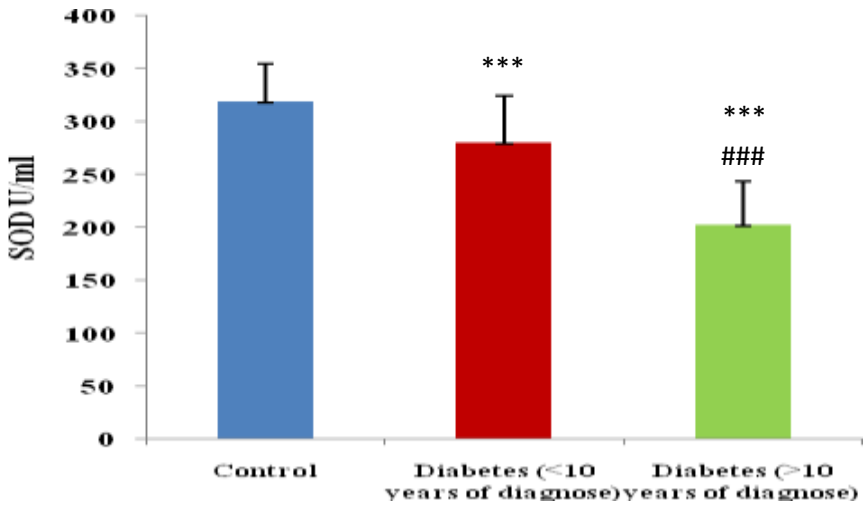

Fig. 4. Superoxide dismutase activity in control, Diabetic group 1 (patients with <10 years of diagnosis) and Diabetic group 2 (patients with >10 years of diagnosis)
**p<0. 01, *p<0.05 with control

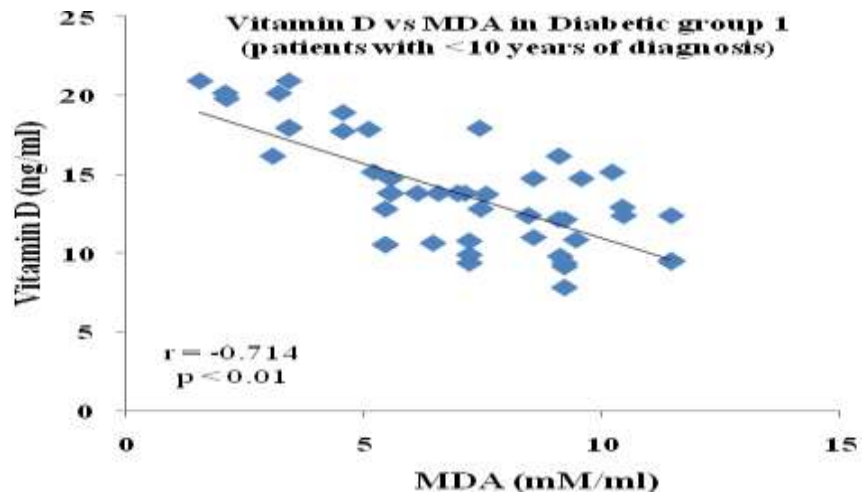

Fig. 5. Pearson correlation analysis among vitamin D and MDA of Diabetic group 1 (patients with <10 years of diagnosis).

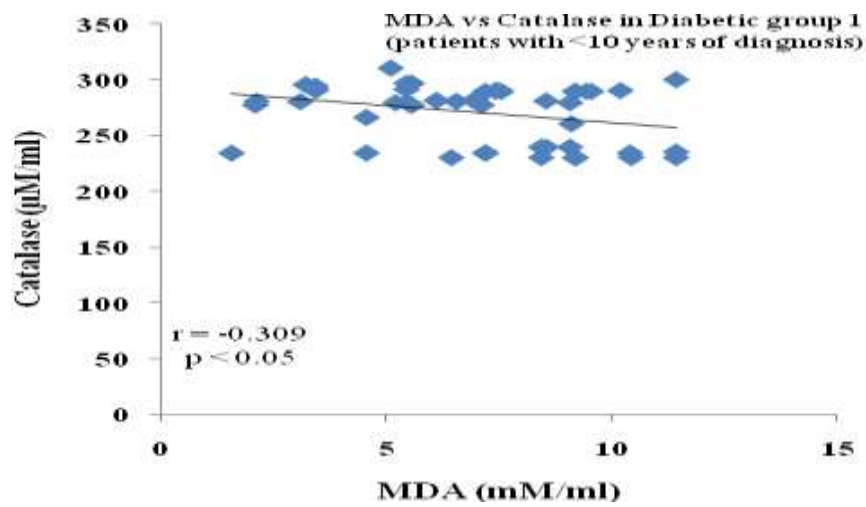

Fig. 6. Pearson correlation analysis among Catalase and MDA of Diabetic group 1 (patients with <10 years of diagnosis).

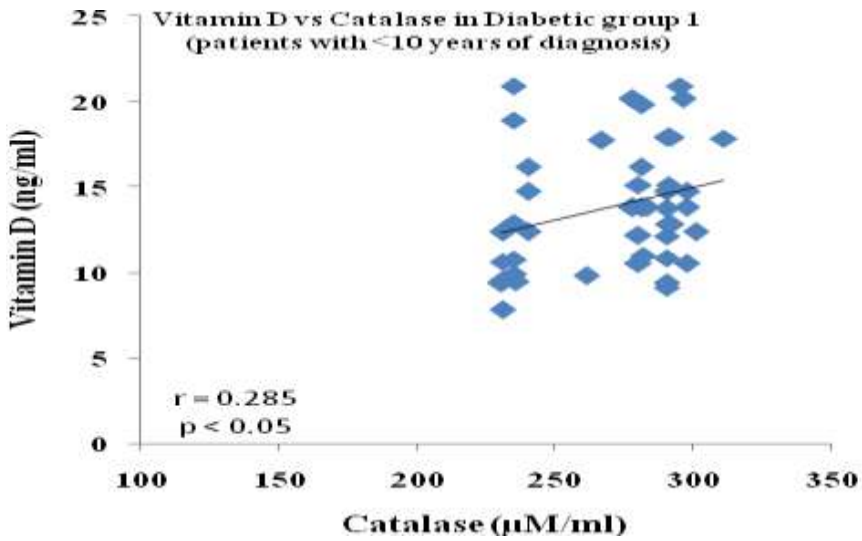

Fig. 7. Pearson correlation analysis among vitamin D and Catalase of Diabetic group 1 (patients with <10 years of diagnosis).

DISCUSSION

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia, altered insulin activity and other metabolic disorders (Matsushita, 2010). There is a growing scientific evidence suggested that excess generation of reactive free radicals species, largely due to elevated glucose levels, leads to oxidative stress, which further exacerbates with a variety of pathophysiological conditions including diabetes mellitus (DM) as well as other metabolic disorders (Johansen *et al*., 2005; Matough *et al*., 2012). Previous studies revealed that hyperglycemia generate ROS and results in disturbed antioxidant defense mechanism that eventually leads to oxidative stress (Seghrouchni *et al*., 2002). Oxidative stress is an important mediator of diabetic complications (Eiichi and Takeshi, 2010).

The results of the present study showed elevated BMI, FBG, HbA1c, plasma urea and creatinine in both diabetic groups (patients with <10 years and >10 years of diagnosis) (Table 1). These parameters were considered as the markers for diabetic complications as reported in previous studies (Anjaneyulu *et al*., 2004, Abdelsalam *et al*., 2011).

Disturbed lipid profile was also observed in diabetic groups as compared with the normal healthy control as shown in Figure 1. The result of the study was in accordance with previous study that demonstrated that hyperglycemia induced oxidative stress increased generation of lipidperoxides that disturbed lipid profile (Shodehinde and Oboh, 2013).

It has been proved from the previous researches that hyperglycemia elicits oxidative stress biomarkers (Cederberg *et al*., 2001). Prolonged hyperglycemia due to diabetes mellitus considered as a major contributor of increased lipid peroxidation (Suvarna and Sinha, 2010). Results of the present study also revealed that there was significant increased MDA in both diabetic groups (patients with <10 years and >10 years of diagnosis) as compared with normal healthy individuals (Table 2). Presently the Catalase levels were decreased in diabetes patients as compared with control as shown in Table 2. Diabetic group 2 (patients with >10 years of diagnosis) showed more significant decreased Catalase activity as compared with diabetic group 1 (patients with <10 years of diagnosis). This may be due to deleterious effects of prolonged hyperglycemia. The results are in accordance with previous research that showed low level of Catalase in diabetes patients (Adeneye *et al*., 2014). Prolonged hyperglycemia markedly reduced catalase as compared with non diabetic individuals**.** Similarly results also showed that SOD levels were significantly decreased in both diabetic groups as compared with control. SOD activity was markedly decreased in Diabetic group 2 (patients with >10 years of diagnosis) as compared with diabetic group 1 (patients with <10 years of diagnosis) showing the effects of hyperglycemia and its complications (Fig. 4).

Vitamin D is essential not only for maintenance of bone but also for other metabolic activities. Low vitamin D with an increase risk of diabetes mellitus (type 1 and 2) has been reported previously (Forouhi *et al*., 2008). The results of our study showed reduced vitamin D levels in both diabetics groups (patients with <10 years and >10 years of diagnosis) in comparison with normal healthy individuals (Table 1).

In diabetic patients, accumulation of glycogen in hepatocytes may lead to disturbed liver enzyme which causes mild to moderately elevated ALT, AST and ALP **(**Chatila *et al*., 1996). In the present study elevated ALT, AST and ALP levels were observed (Table 3).

Pearson correlation analysis showed a strong association among oxidative stress biomarkers and vitamin D deficiency (Table 4). Previous studies also showed significant correlation among oxidative biomarkers and Vitamin D. Considering these results, it is concluded from the study that hyperglycemia is one of the leading cause of oxidative stress in diabetes mellitus. Prolonged hyperglycemia also elicits the risk of microvascular and macrovascular complication in diabetes mellitus. A strong association was observed among oxidative stress biomarkers and vitamin D.

CONCLUSION

The findings of present research are of merit in revealing that elevated oxidative stress biomarkers and Vitamin D deficiency in diabetes mellitus could be a probable diagnostic biomarker for early diabetic complications. There is a significant association between elevated oxidative stress biomarkers and Vitamin D deficiency in diabetes mellitus.

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