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TYPE 2 DIABETES MELLITUS;

HOMOCYSTEINE, TOTAL ANTI-OXIDANT LEVELS AND VITAMINS IN PATIENTS OF DIFFERENT DURATION

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ABSTRACT… Objectives: To access the Homocystein and Antioxidant Status in Patient with Variation in Duration of Type 2 Diabetes Mellitus. **Data source:** 90 selected patients suffering from Diabetes Mellitus Type 2 (DMT2) and 30 subjects as control group. **Design of study:** Case Control Study. **Setting:** Rawal Institute of Health Sciences, Islamabad. **Period:** July 2013 – March 2014. **Materials & methods:** Out of 120 selected subjects, 30 were assigned as control, (group 1) and 90 of DMT2. Based on duration, patients of DMT2 were divided into; group 2 (DMT2 <5 years), 3 (DMT2 = 5-10 years) and group 4 (DMT2 >10 years). Smokers, renal failure, coronary artery disease, thyroid disease and patients on antioxidant treatment were excluded from the study. DMT2 was diagnosed according to American Diabetes Association standards. The fasting plasma glucose levels were measured by glucose oxidase method; HbA1c by automated kit, TAC by calorimetric TAC Assay Kit (BioVision), Vitamin C and E by ELISA Kit (HUMAN) while homocysteine measured by AXSYM HCY assay kit (ABBOTT). Cut off values for HbA1c was taken as ≤6%; FBS ≤110 mg/dl; TAC ≥1.16 mmol/L; Vitamin C ≥2 mg/dl; Vitamin E ≥ 9.5nmol/ml and homocysteine was < 6.3 μmol/L. **Results:** As the duration of DMT2 increases, levels of vitamin C and TAC fall significantly ($p < 0.05$) in all groups except between groups 1 & 2; however, vitamin E, decreased significantly in all the groups with increased DMT2 duration. A significantly increased level of HbA1c was noticed in groups 2, 3 and 4 compared to group 1 with increased DMT2 duration. The fasting blood sugar increased significantly in all the groups except between group 3 and 4. ANOVA showed significant differences ($p < 0.05$) between each group and within the groups when Hb1Ac, vitamin E, vitamin C, & TAC were compared. A positive significant correlation was observed when HbA1c was correlated with FBS; TAC correlated with vitamin C and E and between vitamin C and vitamin E. **Conclusions:** The levels of TAC, vitamin C and E gradually decrease with increased DMT2 duration; so should be supplemented in diabetics. TAC status can be taken as early marker to detect complications while homocysteine levels to prevent diabetic complications.

Key words: Homocysteine, Total Anti-Oxidant, Vitamins, Diabetes Mellitus Diabetes Mellitus type-2, Homocystein, Anti-oxidants

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INTRODUCTION

Diabetes mellitus is characterized by chronic elevated serum glucose levels either due to insufficient insulin production or resistance of body tissues to insulin.[1]

Diabetes is a documented biggest threat to the world as an epidemic disease. In 2013, according to International Diabetes Federation, an estimated 381 million people had diabetes. Its prevalence is increasing rapidly, and by 2030, this number is estimated to almost double.[2] In Pakistan, according to WHO statement in 2014 a total prevalence of 12.9 million people with diabetes (10% of total population) was estimated and that Pakistan is become 7th largest country in terms of Diabetes population and will be 4th largest by the year 2030.[3]

In DM there is increased production of reactive oxygen species. The oxidative stress in diabetes pathogenesis is by alteration in enzymatic systems, lipid peroxidation, and impaired Glutathione metabolism and decreased Vitamin

C levels. Lipids, proteins, DNA damage, catalase and superoxide dismutase are various biomarkers of oxidative stress in diabetes mellitus. The oxidative stress, through ROS, has been proposed as the root cause of insulin resistance, β-cell dysfunction, impaired glucose tolerance, type 2 diabetes mellitus and diabetic complications. Acute complications of diabetes, when left untreated, include diabetic ketoacidosis and hyperosmolar non-ketotic coma; while long-term chronic complications include stroke, cardiovascular disease, chronic kidney failure, foot ulcer and damage to the eyes.[4]

In hyperglycemia, overload cells with energy substrate, augment the flux of electron donors (NADH and $FADH_2$) to mitochondrial electron transport chain result in excessive production of superoxide anion radical ($^{\bullet}O_2^-$).[5] This results in metabolic events like increased polyol pathway activity, increased formation of advanced glycation end products, protein kinase C and nuclear transcription factor κb activation and increased hexosamine pathway flux.[6] In oxidative stress glucose-mediated intracellular pathway activates RUNX2 DNA-binding transcription factor which plays an important role in endothelial cell function and angiogenesis, cause micro-vascular complications (diabetic retinopathy, diabetic neuropathy, diabetic nephropathy) and macro vascular complications (coronary artery disease, stroke, peripheral vascular disease) and insulin resistance due to impaired signaling.[7]

There is a mark reduction in the risk of the diabetic complications when antioxidant micronutrients are provided to the diabetic patients in different studies. Endogenous compounds (glutathione, ubiquinol, urate, bilirubin), enzymes (superoxide dismutase, catalase, glutathione peroxidase) and some dietary components such as vitamin C, vitamin E, carotenoids, and polyphenols are responsible for detoxification of reactive oxygen species.[8]

Vitamin E is the most effective antioxidant; after Vit E, Vit C has also strong antioxidant properties but it is also suggested that the therapeutic effects caused by the antioxidant action of these two vitamins appear in those persons who have poor baseline status. The antioxidants taken in low amount increase cardiovascular risks many folds. The association of water soluble Vit C with diabetic complications is not as marked as with Vit E. A strong association exists between oxidative stress and atherosclerosis and this association has been proved in many experimental studies and animal experiments.[9,10]

Elevated homocysteine (Hcy) blood levels are responsible for endothelium damage causing blood vessel inflammation which in turn may lead to atherogenesis and resulting ischemic injury. Hyperhomocysteinemia is therefore a possible risk factor for coronary artery disease. The superoxide anion formed by homocysteine auto-oxidation can cause vascular injury by inducing the oxidation of LDL. Besides, Hcy affects nitric oxide binding or production, which hinders vasodilatation. Hcy induces a procoagulative state due to increased thromboxane formation and platelet aggregation, factor XII activation, inhibition of protein C activator, and the facilitation of lipoprotein binding to fibrin. The toxic influence of Hcy to the endothelium can be blocked by antioxidant enzyme supplementation. Hcy levels can be reduced by folate administration while the production and effects of free radicals can be controlled by antioxidants.[11,12]

In this study evaluation of oxidative stress level, antioxidant, homocysteine, and vitamin levels were measured in patients of diabetes mellitus type 2 at different time intervals.

MATERIAL AND METHODS

The study was conducted at Rawal Institutte of Health Sciences Islamabad from July 2013 to March 2014. Out of 120 selected subjects (mean age = 49.11±5.98 years**)**, 90 were of DM type 2 and 30 were assigned as control group (group 1). Based on duration, patients of DM type 2 were divided into 3 groups; group 2, 3 and 4; group 2 – patients with DM duration less than 5 years, group 3 with duration between 5-10 years and group 4 with duration of DM more than 10 years.

Smokers, patients on previous antioxidant treatment, of renal failure, coronary artery disease and thyroid disease were excluded from the study. Diabetes mellitus type 2 was diagnosed according to the standards set by American Diabetes Association. Included diabetics were not getting aspirin, statins, or antihypertensive medications. Informed consent was obtained from all participants before participation.

The overnight fasting blood samples were collected from anti-cubital vein. A soft rubber tourniquet was applied above the elbow. The punctured site was cleaned with spirit swab and was air dried. Five ml of blood was collected using aseptic techniques, tourniquet was removed and the punctured site was sealed. Blood for glucose was collected in grey top (acetoacetate) tube, for HbA1c in purple (EDTA) tube and for vitamin C, vitamin E, homocysteine and total antioxidant capacity in red top tube.

The fasting plasma glucose levels were measured by glucose oxidase method; HbA1c by automated kit on Cobas Integra of Roche; Ascorbic acid measured by calorimetric Assay Kit Biovision (FRASC). The Vitamin C and E were measured by using ELISA Kit (HUMAN) while homocysteine by AXSYM HCY assay (ABBOTT) is based on the FPIA (Fluorescence Polarization Immunoassay) technology.

Cut off values for HbA1c was taken as <6%; FBS <110 mg/dl; TAC >1.16 mmol/L; Vitamin C >2 mg/dl; Vitamin E > 9.5nmol/ml, and of homocysteine < 6.3 µmol/L.

Ethical Consideration

The study protocol was approved by ethical committee of the institution. Previous permission regarding enrolment in the study protocol was taken and participant identify was kept secret by the use of a unique ID number.

Statistical Analysis

Data was entered and analyzed in SPSS (Statistical package for social sciences) version 20.

Mean ± SD was used to present quantitative variables i.e. HbA1c, fasting blood sugar (FBS), Vitamin E, Vitamin C and Homocysteine. The sensitivity and specificity of individual assay and combinations of various assays was determined at 95% confidence interval (CI).

To determine the association between quantitative variables of four groups, ANOVA was applied. Pearson Correlation was computed with level of significance <0.05. The data was represented as tables. The p -value <0.05 was considered statistically significant.

RESULTS

Table-I shows demographic and different variables in patients with diabetes mellitus type 2.

The levels of TAC decrease significantly (p <0.05) in all groups except between groups 1 & 2 (P = 0.234) as the duration of diabetes increases. A similar trend of significant difference(p <0.05) in the levels of vitamin C was seen in all groups of patients with type 2 diabetes mellitus as the duration of diabetes increases; while the difference was found to be non-significant (p = 0.750) when the levels were compared between group 1 & 2. On the other hand anti-oxidant vitamin E, was found to be significantly decreased (p <0.05) in all the groups as the duration of type 2 diabetes increases.

A significantly increased (p <0.05) levels of HbA1c were noticed in groups 2, 3 and 4 as compared to group 1 as the duration of diabetes increases; however, the levels were found to be non-significant when group 2 was compared with that of group 3 (p = 0.493) and group 4 (p = 0.407). A similar non-significant trend was noticed between group 3 and group 4 (p=0.108).

When fasting blood sugar was compared between the diabetic groups a significant increased (p <0.05) levels were noticed in all the groups with the exception between group 2 and 3 (p =0.674). The levels of homocysteine showed a statistically significant difference (p <0.05) in all groups except between control and group 1 (p=0,215)

Variable	Group I	Group II	P Value 1-2	Group III	P Value 1-3	Group IV	P value 1-4	P value 2-3	P value 2-4	P value 3-4
Age	53.56 ±10.00	53.63 ± 1.69	.979	56.26 ± 9.79	.295	54.80 ± 9.55	.627	.290	.634	.559
weight	71.23 ± 5.98	64.73 ± 7.36	.000	68.60 ± 7.78	.147	69.26 ± 8.68	.311	.053	.033	.755
Systolic Pr	151.00 ± 8.38	147.60 ± 10.23	.165	149.40 ± 8.16	.457	151.63± 6.61	.746	.455	.075	.249
Diastolic Pr	87.33 ± 3.67	88.63 ± 3.81	.184	88.76 ± 3.89	.148	88.56 ± 3.70	.200	.894	.946	.839
FBS	87.10 ± 13.88	188.00 ± 49.80	.000	182.83 ± 44.75	.000	160.47 ± 28.00	.000	.674	.011	.024
HbA1c	4.91 ± 13.88	8.41 ± 1.87	.000	8.08 ± 1.79	.000	8.77 ± 1.44	.000	.493	.407	.108
Hsy	11.92 ± 4.60	13.29 ± 3.83	.215	17.89 ± 6.57	.000	27.75 ± 6.74	.000	.002	.000	.000
Tac	1.00 ± 0.13	1.04 ± 0.10	.234	0.79 ± 0.091	.000	0.47 ± .14	.000	.000	.000	.000
Vit.C	1.31 ± 0.37	1.28 ± 0.35	.750	0.80 ± 0.13	.000	0.24 ± 0.10	.000	.000	.000	.000
Vit.E	6.42 ± 1.21	3.25 ± 0.39	.000	1.94 ± 0.40	.000	1.31 ± 0.34	.000	.000	.000	.000

Table-I. Demographic variables in patients with diabetes mellitus type 2.
FBS= Fasing blood sugar Hsy = Homocysteine TAC = Total antioxidant capacity
Vit C = Vitamin C Vit E = Vitamin E

ANOVA						
		Sum of Squares	df	Mean Square	F	Sig.
hbaic	Between Groups	511.269	75	6.817	5.301	.000
	Within Groups	56.586	44	1.286		
hys	Between Groups	5353.162	75	71.375	1.091	.383
	Within Groups	2878.139	44	65.412		
tac	Between Groups	5.381	75	.072	1.267	.200
	Within Groups	2.493	44	.057		
vit.c	Between Groups	21.399	75	.285	1.272	.195
	Within Groups	9.870	44	.224		
vit.e	Between Groups	479.021	75	6.387	6.627	.000
	Within Groups	42.407	44	.964		
weight	Between Groups	4300.575	75	57.341	.863	.717
	Within Groups	2923.217	44	66.437		

Table-II. Association between and within the groups using ANOVA
FBS= Fasing blood sugar Hsy = Homocysteine TAC = Total antioxidant capacity
Vit C = Vitamin C Vit E = Vitamin E

		FBS	HbA1c	Hsy	Tac	Vit.C	Vit.E
FBS	PC	1	.822**	.155	-.212*	-.213*	-.584**
	Sig. (2-tailed)		.000	.092	.020	.019	.000
HbA1C	PC	.822**	1	.315**	-.386**	-.366**	-.627**
	Sig. (2-tailed)	.000		.000	.000	.000	.000
Hsy	PC	.155	.315**	1	-.628**	-.605**	-.538**
	Sig. (2-tailed)	.092	.000		.000	.000	.000
Tac	PC	-.212*	-.386**	-.628**	1	.774**	.607**
	Sig. (2-tailed)	.020	.000	.000		.000	.000
Vit.C	PC	-.213*	-.366**	-.605**	.774**	1	.682**
	Sig. (2-tailed)	.019	.000	.000	.000		.000
Vit.E	PC	-.584**	-.627**	-.538**	.607**	.682**	1
	Sig. (2-tailed)	.000	.000	.000	.000	.000	

Table-III. Correlation between different variables.
FBS= Fasing blood sugar Hsy = Homocysteine TAC = Total antioxidant capacity
Vit C = Vitamin C Vit E = Vitamin E

Table-II shows association between and within the groups using ANOVA.

A significant differences ($p < 0.05$) between each group and within the groups when Hb1Ac, vitamin E, were compared using ANOVA. In contrast a non-significant difference was noticed when vitamin C ($p=0.195$), Homocyctein ($p=0.383$) & TAC ($p=0.200$) were compared between the groups and within the groups.

Table-III shows correlation between different variables.
A statistical significant correlation was seen when HbA1c was correlated with FBS ($r=0.822$; $p=0.000$) and homocyctein ($r=0.315$; $p=0.000$), however, it showed an inverse relationship with TAC ($r= -0.386$, $p = 0.000$), vitamin C ($r= -0.366$, $p = 0.000$) and vitamin E ($r=-0.627$, $p = 0.000$). A significant inverse correlation of FBS was noticed with TAC ($r=-0.212$, $p = 0.000$), vitamin C ($r= -0.213$, $p = 0.000$), and vitamin E ($r= -0.584$, $p=0.000$) while a positive non-significant correlation was seen with homocystien ($r=0.155$; $p=0.092$). A significant positive correlation was seen when TAC was correlated with vitamin C ($r=0.774$, $p=0.000$) and vitamin E ($r= 0.607$, $p=0.000$) while a negative correlation noticed with homocysteine ($r=0.628$; $p=0.000$), HbA1c ($r= -0.386$, $p=0.000$), and FBS ($r= -0.212$, $p=0.000$). When vitamin C was correlated with vitamin E ($r=0.682$, $p=0.000$) and homocysteine ($r=0.605$; $p=0.000$), a significant positive correlation was noticed.

DISCUSSION

Our study showed an overall reduction in the levels of TAC, vitamin C and vitamin E in type 2 diabetics with time duration as compared to control group. These results are in consistent with Odum et al[13] and Peerapatdit et al[14], who showed a significant reduction in the levels of vitamin C, vitamin E and TAC in type 2 diabetic patients compared to control group. However, Maxwell et al[15] showed that TAC of plasma was increased in patients with uncomplicated type 2 diabetes despite high levels of oxidative stress depending upon mitochondrial functions.

Our results are in consistent with Kenzo et al[16] who showed reduced levels of vitamin C in type 2 diabetics. In individuals with type 2 diabetes, there were reduced levels of serum vitamin C indicating strong relationship with dysfunction of kidney and inflammation. Levels of erythrocyte vitamin E are more important than plasma levels of Vitamin E in the case of diabetic children. So levels of Vitamin E must be restored to original levels by considering the erythrocyte levels of Vitamin E. These levels can be restored to normal

if Vitamin C is given along with metformin. It has been shown that if plasma level of Vitamin C is high along with increase intake of fruits and vegetables, risk of diabetes decreases many folds.[17]

An inverse correlation between fasting plasma glucose and total anti-oxidant level shows that in poorly controlled Type 2 diabetics, there is a defect in antioxidant defense of the body against oxidative stresses suggesting that in an impaired glucose tolerance state when blood glucose levels are high there is an increase in oxidative stresses and some deficiency in the antioxidant defense. These factors cause increase oxidative DNA damage, leading to pancreatic beta-cell dysfunction, insulin resistance and more enhanced hyperglycemia. This vicious circle is responsible for making the diabetes more deleterious.[10]

In type 2 DM if vitamins are administered for three months, many beneficial effects appear like reduction in blood pressure, blood glucose levels and a rise in SOD and GSH enzyme activity that has ability to decrease insulin resistance by reduction of oxidative stress parameters. Vitamin C supplementation has an important role in reducing fasting and postprandial oxidative stress thus protecting the development of many diabetic complications.[10,18]

There also occurs an associated rise in lipid peroxidation mediated by free radicals. Vitamin E, a lipid soluble vitamin having antioxidant actions and shows a greater protection of membranes against damage, produced by cholesterol oxidation products. Vitamin E also decreases the risk of cardio metabolic events, and for the same reason, it must be given in diabetic patients on long term basis. The erythrocyte vitamin E levels are more important than plasma levels of Vitamin E in case of diabetic children which can be restored if vitamin C is given with metformin.[19]

Our results showed an inverse correlation of FBS with TAC. These results are in consistent with Akinosun et al[20] and Song et al[21] in poorly controlled Type 2 diabetics associated with a defect in antioxidant defense of the body against oxidative stress.

A good control of FBS could possibly help reduce free radical activity and probably minimize the chronic complications in diabetic patients. Increased blood glucose is associated with an increase in oxidative stresses and some deficiency in the antioxidant defense. These factors cause increase oxidative DNA damage, pancreatic beta-cell dysfunction, insulin resistance and more enhanced hyperglycemia. This vicious circle is responsible for making the diabetes more deleterious.

According to our and study done by Dominguez LJ[22] and Leung SBI[12] are in agreement with that the homocysteine levels rises with increase duration of diabetes in patients with type 2 DM. Earlier studies have suggested that in DM type 2 subjects with diabetic complications, there is elevated level of Hcy associated with oxidative stress regardless of resistance to insulin. It was found by other authors that type 2 diabetic patients having cardiovascular problems had higher Hcy levels than those without cardiovascular problems.

Hcy level are likely to rise in complications associated with diabetes mellitus. With extended time period, complications of DM increases and so does Hcy. Hcy might be related to extended period and micro-vascular problems of diabetes mellitus. It has been demonstrated that individuals with recognized macroangiopathy have elevated homocysteine levels. Homocysteine considerably hinders Ca2+ activated K+ channel (BKCa is major role player in mediating the contraction mechanism in the muscles of the vessels) current separately in humans and rats arteries. So the abnormalities encountered in vascular diseases might be the result of decreased and damaged BKCa by increased Hcy levels. There is also an indication that generation of NO by eNOS (epithelial nitric oxide synthase) is affected and current facts indicate that Hcy reduces the phosphorylation level of eNOS.[23,24]

CONCLUSION

The levels of Total anti-oxidant capacity, vitamin C, and vitamin E gradually decrease with duration of diabetes and are associated with oxidative stress and be added in diabetics to increase their quality of life. TAC status may be taken as early marker to detect complications in diabetic type 2 patients especially of longer duration. The levels of homocysteine should be kept at low levels to prevent from diabetic complications.

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