Assessing of oxidative stress related parameters in diabetes mellitus type 2: Cause excessive damaging to DNA and enhanced homocysteine in diabetic patients

Shazia Anwer Bukhari[1], Syed Ali Raza Naqvi[1]*, Saeed Ahmad Nagra[2], Fauzia Anjum[1], Sadia Javed[1] and Muhammad Farooq[1]
[1]Department of Chemistry, Government College University, Allama Iqbal Road, Faisalabad, Pakistan
[2]Institute of Chemistry, University of the Punjab, Lahore, Pakistan

Abstract: Oxidative stress and reactive oxygen species (ROS) have been documented subsist to the pathogenesis of many diseases including diabetes mellitus. The strength of both parameters could be estimated by measuring oxidative stress marker thiobarbituric acid reactive substances (TBARS), its related parameters and the antioxidants glutathione peroxidase and superoxide dismutase (SOD) in plasma of DM patients. Lipid peroxidation was measured as TBARS and presented as malondialdehyde, total cholesterol (TC), low-density lipoprotein (LDL), triglyceride (Tg), the antioxidants (vitamin A (β-carotene), vitamin E, vitamin C, glutathione peroxidase (GPx) and superoxide dismutase) levels. The results showed that these parameters, commonly, were declined appreciably in diabetic individuals as compared to the healthy individuals. In most cases, age and gender were appeared to involve in having greater values of diabetes marker. Further, increased level of lipid peroxidation and random behaviour of antioxidant potential also associated with Diabetes. For that reason these biomarkers might be of great important to diagnosis DNA damages of diabetic patients

Keywords: oxidative stress, malondialdehyde, superoxide dismutase, uric acid.

INTRODUCTION

It is widely reported that diabetes is a big threat to the world as an epidemic disease. According to a statistical analysis there are approximately 194 million citizens affected with diabetes which is >3% (5.1% for those aged 20 to 79) of the world's population. This percentage might be reached to 6.3% (333 million) by the year 2025 (Maskarinec *et al.,* 2009).

Oxidative stress plays major role in increasing diabetes in addition to cardiac, nervous stress and many other diseases. The metabolic aberrations of diabetes grounds overproduction of mitochondrial superoxide in endothelial cells of both large and small vessels, as well as in the myocardium. Superoxide and ROS are formed as a normal product of aerobic metabolism but can be elevated under diabetic conditions. In diabetes hexosamine pathway may participate in the development of β-cell dysfunction by provoking oxidative stress. Once, hyperglycemia becomes apparent, β-cell function deteriorates (Giacco and Brownlee, 2010). In diabetics, protein-bound hexose and hexosamine are higher in the presence of atherosclerosis (Sulaiman *et al.*, 2012).

Antioxidant defense involves both enzymatic and non enzymatic strategies including vitamin A, E, C SOD, catalase and GPx. Hyperglycemia is a well-known pathogenic factor of long-standing diabetic problems in diabetes mellitus (Kaplan *et al.*, 2012). Studies on animals and humans have demonstrated that insulin dependent diabetes mellitus (IDDM) is associated with elevated blood levels of lipid peroxidation products. Oxidative stress may be increased in IDDM because of disclosure to long-drawn-out periods of hyperglycemia which cause nonenzymatic glycation of plasma protein and binding of glucose to protein molecule (Suksomboon *et al.*, 2011). It is well known that diabetes provokes oxidative stress and that the follow-on oxidative damage is a key element in development of diabetic problems (Maeda *et al.,* 2010). Accumulation of free radical due to an increase oxidative stress in diabetic patients has gained deep interest in the last two decades (Madhur and Suresh, 2006).

Antioxidants secure aligned with the harmful effect of increase oxidative stress due to non-enzymatic autoxidative glycosylation and metabolic stress in diabetic individuals. Subjects with IDDM are among the groups at risk of having low vitamin concentrations (Adrian *et al.*, 2012). Diabetic individuals may also have higher antioxidant necessities due to higher amount of free radicals formation with hyperglycemia. Vitamin C and α-tocopherol are nutrients theorize to avert retinopathy by distressing supposed pathogenic factors like, retinal blood flow, protein glycosylation, insulin sensitivity, and oxidative stress. Pharmacological dosages of vitamin E develop insulin-mediated glucose dumping and elevated nutritional intake of vitamin C is related with a reduce rate of Type 2 diabetes (Williams *et al.*, 2012).

**Corresponding author:* e-mail: a_naqvi313@yahoo.com

Red blood cell SOD activities are often measured in humans as an index of defense aligned with superoxide in blood. The interest in SOD has increased markedly in relation to aging and pathogenesis, including diabetes mellitus. Superoxide dismutase catalyses the disproportionation of O_2^- that presumably rises in autoxidation of diuric acid (Uyanik *et al.*, 2012).

Homocysteine (tHcy) which is a sulfur-containing amino acid. Recent research on plasma tHcy in diabetic patients shows diverging results. High tHcy levels are a significant risk factor for macrovascular obstacles (Balogh *et al.*, 2012). Homocystein levels are contingent lying about varied everyday life. Coffee utilization and smoking are coupled with high tHcy levels. A relationship is found among blood pressure, diabetes, heart rate and increase level of tHcy (Harris *et al.*, 2012).

Sialic acids are acetylated derivatives of neuraminic acid. SAs are inflammatory markers and strong predictor of diabetes, coronary heart disease as well as stroke and cardiovascular mortality (Payam *et al.,* 2012). Increased SAs concentrations have been observed in tumors, myocardial infarction, diabetes, inflammatory disorders and alcoholism (Varki and Varki, 2007).

Testosterone is an essential circulating and obviously occurring androgen in both men and women. Increased testosterone concentration is linked with insulin level and diabetes mellitus. Low plasma testosterone is linked with increased level of glucose and insulin (Reynolds *et al.,* 2012), whereas hyperandrogenicity is linked with a greater risk for Type 2 diabetes in women (Tiano and Jarvis, 2012). The level of ROS-induced oxidative harm to lipids, proteins, polysaccharides cell membranes and DNA damage aggravated due to reduce effectiveness of antioxidant defense mechanism. It has been accepted that antioxidants in addition to scavenge, stop the production of free radicals, which is conscientious for reperfusion induced damage and lipid peroxidation. This may increase the chance of diabetes development.

In this study evaluation of oxidative stress level, activity of enzymatic antioxidant, non-enzymatic antioxidants and blood pressure in diabetes patients, both males and females, was carried out. Further, oxidative damage of DNA and elevated level of homocysteine link with other markers was studied.

METERIAL AND METHOD

The study population consisted of 160 subjects, 80 males and 80 females. All cases and controls were enrolled under well-versed approval. University ethical committee permitted the study and work was performed in harmony with the Declaration of Helsinki. The diabetic patients those who visited the Allied Hospital and Civil Hospital for checkup/treatment were picked up randomly. The all patients were belong to middle socio-economic class of Faisalabad and rural areas of Faisalabad. Age, gender, immunization status, fever or vomiting and any prior use of prescription as well as dietary status were recorded. No mineral supplements were taken by these individuals. The individual was discontinued from the treatment if she/he experienced any complications. A comprehensive physical examination of each young and old individual was performed by one of the experienced physician examining the patients. These subjects were separated into male and female groups and further divided into four groups (young male patients, young female patients, old male patients, old female patients and similar number of groups of healthy control subjects). A total of one hundred and sixty (160) blood samples were collected between 06-09 h in the morning in a cold test tube from normal and diabetic patients. Tubes were centrifuged at 769 g for 15 min. Serum was separated and stored into small aliquots at -20°C till further analysis.

The patients that have total cholesterol level more than 220 mg/dL or triglycerides concentration >200 mg/dL, or receiving lipid lowering drugs were considered as having hyperlipidemia. Diabetes mellitus was diagnosed if the fasting plasma glucose concentration was ≥126 mg/dL or if the patient was receiving insulin treatment or oral hypoglycemic agents. Body mass index (BMI; kg/m^2) was calculated by using the following formula: BMI = weight (kg)/height (m^2), body temperature (°F) was recorded by thermometer and blood pressure (mmHg) was measured by sphygmomanometer of every individual.

Laboratory analysis

Serum was acidified with 10% trichloracetic acid (1:1 v/v) and stored at -40°C for determination of vitamin C using dinitrophenylhydrazine by the method of Omaye *et al.* (1979). For simultaneous measurement of vitamin E and A, reversed phase HPLC (Shimadzu Model-7125) was used as reported by Bieri *et al.,* (1979) and modified by Islam *et al.* (2005). SOD activity was measured using the protocol reported by Sun *et al.,* (1988). GP_X activity was measured using a coupled assay system. The oxidation of reduced glutathione was coupled to NADPH oxidation in a reaction catalyzed by glutathione reductase (Avissar *et al.,* 1991). Total homocystine was measured by a homocysteinemicrotiter plate assay for the determination of tHcy in serum of normal and diabetic patients. Plasma samples were prepared in polyethylene culture tubes with a reducing agent tris(2-carboxyethyl) phosphine hydrochloride. To reduce the protein bound Hcy to free Hcy that was subsequently converted to S-adenosyl-L-homocysteine (SAH), SAH-hydrolase was used and quantitated by horseradish peroxidase (HRP-SAH) competitive assay (Diazyme, Cat. No. DZ012A).

Sialic acid was measured with a slightly modified protocol described by Crook (1993). Hexosamine (Hydrogen peroxide assay; mg/dL) levels were determined using a Peroxi Detect^{TM} kit (Sigma). Hydrogen peroxide levels were expressed relative to sample treated with 10 μM H_2O_2, with the value arbitrarily was set at 100 (Hideaki *et al.*, 2001). Radio Immunoassay (RIA, REF IM1119) kit was used for determination of testosterone level. Plasma triiodothyronine (T_3; ng/dL) was measured using ELISA kit biocheck enzyme immunoassay (EIA test kit Lot/RN15685 Cat. No./BC-1005 Bio Check Inc., USA).

For the quantitative determination of the total thyroxine (T_4) concentration Enzyme Immunoassay (EIA) kit was used (Lot/RN-21055 Cat. No./BC-1007 Bio Check Inc. USA).

Comet assay

DNA damage was measured by performing comet assay as reported by Martin *et al.* (2000). Briefly, 0.05 gm of normal agarose gel was dissolved in 5 mL PBS, heated 2-3 min at ambient temperature and kept in hot water. About 100 μL of normal gel was dropped on to the glass slide ((Eric Scientific; Cat No. Xes 370) and cover it with cover slip (24 x 50nm). One mL of the whole blood was drawn into hepranized tube from normal and diabetes victim individuals. In these samples 1 mL histopaque was added slowly and centrifuge at 2100 rpm (548×g) for 30 min. Likewise, 0.04 of low melting gel was added in 4 mL PBS, heated 2-3 min and kept at 37 °C. After eluting the middle layer having white blood cell into a new tube, added 1 mL PBS (1: 10) and centrifuged at 1600 rpm for 10 min. The supernatant of the centrifuged sample was discarded.

A small volume (10μL) of residual material was taken in ependorf tube and mix it with 80 μL of low melting agarose gel. Poured this mixture on slide already prepared with normal gel after removing the cover slip. After proper solidification fixed these slides in a tray having lyzed solution for 50 min. Washed these slides with deioninzed water for 3-4 times. Fixed these slides in electrophoresis apparatus having alkaline buffer for 30 min and operated the electrophoresis at 17 V and 300 mA for 25 min in the dark. Washed these slides with neutralizing buffer 2-3 times. Each slide was viewed by fluorescence microscopy at 200x magnification using an excitation filter of 546 nm and a barrier filter of 590 nm. The degree of damage of DNA was estimated by visual image analysis as described by Collins and Dusinska (2002).

STATISTICAL ANALYSIS

Analysis of variance was applied to determine the difference between groups (Steel and Torri, 1997) and Duncan Multiple Range test was applied to test the difference between means (Duncan, 1955).

RESULTS

General and anthropometric characteristics such as Weight (kg), Body Mass Index (kg/m^2), Systolic Blood Pressure (mm/Hg) and Diastolic Blood Pressure (mm/Hg) of the young and old male and female subjects were measured and data was shown in table 1. Quantitative bio-analysis for important biochemical parameters of serum was carried out for normal and diabetic patients of different age and gender and results obtained was reported in table 2. The concentrations of serum vitamin E, vitamin C, vitamin A, and the activities of SOD, GPx, concentrations of sialic acid, heosamine, and hormones are presented in table 3. Correlation coefficients of biochemical characteristics, vitamins and hormonal profile with homocysteine of normal and diabetic individuals was also constructed and the results was shown in Table 4. All these findings was carried out using standard protocols and precautions.

Comet assay was performed to measure the DNA damaging of DM patients according to the method reported by Martin and coworkers. The visual results obtained are shown in fig. 1.

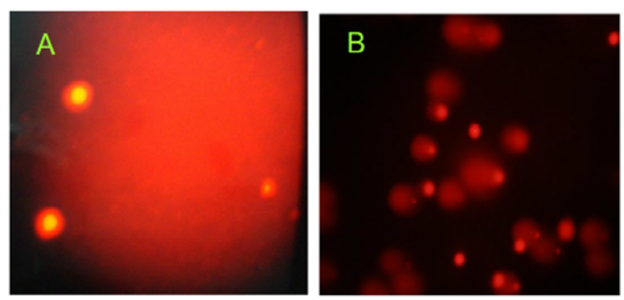

Fig. 1: comet images showing different levels of DNA damaging: A) zero level comet assay (undamaged DNA); B) various degrees of damage from minor (grade 0) to severe (grade 4).

DISCUSSION

Anthropometric characteristics of male and female such as body weight and body mass index were appreciably greater (*P*≤0.01) in diabetic individuals as matched up with that of non-diabetic complements (16.75-22.98 kg/m^2) and was a self-sufficient inconsistent in both young and old males and females. Overall mean systolic blood pressure (SBP) was considerably (*P*≤0.001) higher in female diabetic patients, followed by old diabetic males and diabetic young males and females respectively. Similarly diastolic blood pressure (DBP) was significantly (*P*≤0.001) higher in diabetic patient followed by their respective normal subjects.

This parameter was highest in old male diabetic patients, followed by female diabetic patients.

Serum biochemical parameters showed mean serum glucose concentration (10.4±0.15mmol/L) which was considerably higher in old females diabetic cases ($P \leq 0.01$) as compared to old and young females and male control counterparts respectively. Mean serum cholesterol (7.5±0.07 mmol/L) and triglyceride (3.4±0.14mmol/L) were significantly higher ($P \leq 0.01$) in old female and male diabetic cases than those in young male and female diabetic cases as compared to their normal individuals (Table 2). HDL-cholesterol (4.4±0.11moml/L) was significantly higher in both old normal subject (old group). They were followed by normal male and female (young group). Young diabetic group (both male and female) had appreciably ($P \leq 0.01$) lower HDL-C with respect to this variable. However, LDL-cholesterol (4.6 ±0.14mml/L) was significantly higher ($P \leq 0.01$) in old diabetic female and males, diabetic young females followed by normal individuals. Total tHcy (21.7±0.68µmol/L) content was significantly higher in old diabetic male cases and old female diabetic patients (20.2±1.0µmol/L). Among other participants, young female diabetic patients had significantly higher values followed by young male diabetic patients. Young normal males and normal old females had the same level of tHcy compound. DNA damaging in old female diabetic patients (20.8±1.5) was noted significantly higher than that of old male diabetic patients (14.9±0.6µm) but both young female and male diabetic patients had statistically same level of DNA damage (14.2 and 13.8 µm). The activities of erythrocyte antioxidants such as SOD and GPx significantly (P=0.02 and 0.001 respectively) decreased in diabetic patients (Table 3).

GPx catalyzes peroxide decrease and utilizes GSH as the substrate and converts to GSSG. A decrease in the GPx activities in the erythrocyte of diabetic patients have been observed and is a normal phenomenon. GSH acts as a substrate in the scavenging reaction catalyzed by GPx and as a scavenger of vitamins C and E radicals. In our study, the plasma and erythrocyte GSH concentrations significantly decreased in diabetic patients. It may be due to an increased utilization of GSH.

In diabetic patients, we found significantly lower levels of vitamins C compared with that in controls (table 3).

This indicates that there was a severe damage to antioxidant system, which is unable to combat oxidative stress and result in increased inflammation by increasing sialic acid in diabetic patients (Table 3) and the serum concentrations of vitamin C, vitamin E and vitamin A considerably decreased in diabetic patients as compared to their values in normal subjects. Normally, the concentration of vitamin C in blood and tissue is relatively elevated (Williams *et al.*, 2012) and plays a key role in the production and defense of our connective tissues and elaborate matrix that hold the body together. Vitamin C also supports our cardiovascular system, protects tissue from free radicals damage and helps nervous system to convert amino acids into neurotransmitters. Therefore, vitamin C is important for our many vital features of biochemical functioning.

Serum vitamin E and A decreased significantly in diabetic patients irrespective to their age and gender. With age vitamin C and A declined in young and old individuals. In comparison with gender, vitamin C and E were significantly lower in females while vitamin E was the highest in females as compared to that in males. Patients with DM have decreased vitamin C as compared to normal individuals. This deficit could be one factor to contribute to amplified threat of infections, harm to connective tissues and might have caused oxidative tissue damage (Giacco and Brownlee, 2010). Several reasons for reduced vitamin C concentration in diabetic individuals included renal re-absorption of vitamin C can be abridged due to hyperglycemia, blood glucose may compete with vitamin C for uptake into definite cells and tissues, cellular regulation of vitamin C may be impaired and finally, increased oxidative stress may deplete antioxidant reserve (Pfister *et al.,* 2011).

Vitamin E as an antioxidant is proposed to protect, prevent and be used as a treatment agent for cardiovascular diseases, cancer, in normal cellular aging and in infertility (Williams *et al.,* 2012). Low levels of vitamin are associated with increased incidence of diabetes (Hope and Hope, 2005). Individuals with diabetes have low level of antioxidant; on the other hand, people with diabetes need more antioxidant requirements due to increase in free radical's production with increase in serum glucose concentration (Suleiman *et al.*, 2005).

When vitamin E therapy was given to diabetic rats has remarkable effect in reduction of lipid peroxidase and elevation of catalase, GPx and glutathione-s-transferase and these finding along with its reduction of malonaldehyde level in liver, brain and myocardial homogenate confirm known effect of vitamin E (Halim and Mukhopadhyay, 2006). Likewise, high nutritional intake of vitamin C was related with a lower incidence of type 2 diabetes (Song *et al.*, 2009). Vitamin A has numerous biological activities including antioxidant capacity, blue light filtering, and modulation of immune function and regulation of cellular differentiation as well as proliferation. Serum levels of vitamin A are linked with a minor risk of increasing chronic and degenerative diseases (Mullin, 2011).

Table 1: Anthropometric parameters of normal and diabetic patients with gender and age

Parameters	Young				Old			
	Male (N=40)		Female (N=40)		Male (N=40)		Female (N=40)	
	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic
Weight (kg)	70.17 ±1.14^{d}	72.00 ±0.73^{c}	47.67 ±1.05^{g}	57.50 ±1.26^{f}	78.50 ±1.09^{b}	82.50 ±1.09^{a}	56.67 ±0.96^{f}	59.83 ±1.56^{e}
Body Mass Index (BMI; kg/m^{2})	22.33 ±0.92^{f}	27.75 ±0.51^{a}	21.83 ±0.53^{f}	24.15 ±0.65^{e}	24.83 ±0.53^{d}	26.62 ±0.18^{b}	22.50 ±0.62^{f}	25.53 ±0.45^{c}
Systolic Blood Pressure (SBP; mm/Hg)	127.83 ±2.67^{d}	134.33 ±2.01^{c}	127.67 ±1.47^{d}	134.17 ±2.57^{c}	136.83 ±3.97^{b}	138.17 ±3.13^{b}	128.50 ±2.11^{d}	145.17 ±2.30^{a}
Diastolic Blood Pressure (DBP; mm/Hg)	82.83 ±0.70^{d}	88.83 ±0.30^{c}	79.33 ±1.40^{e}	89.17 ±0.83^{c}	87.17 ±0.60^{c}	97.67 ±0.71^{a}	83.00 ±0.81^{d}	94.00 ±0.85^{b}

Data are shown as means ± SD. *P*-values were 0.01 to 0.0001. Similar alphabets in a row do not differ significantly at $P \geq 0.05$

Table 2: Comparison of serum biochemical parameters changes in control and diabetic subjects with age and gender

Parameters	Young				Old			
	Male (N=40)		Female (N=40)		Male (N=40)		Female (N=40)	
	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic
Glucose (mmol/L)	7.1 ±0.08^{d}	7.80 ±0.27^{c}	6.7 ±0.13^{e}	8.3 ±0.16^{b}	7.4 ±0.11c	10.4 ±0.15^{a}	7.5 ±0.09^{c}	8.33 ±1.41^{b}
Cholesterol (mmol/L)	5.3 ±0.12^{f}	6.4 ±0.09^{d}	5.4 ±0.11	6.8 ±0.09^{b}	5.6 ±0.08^{d}	7.50 ±0.08^{a}	6.7 ±0.08 ^{c}	7.5 ±0.07^{a}
Triglyceride (mmol/L)	1.1 ±0.08^{g}	2.1 ±0.08^{d}	1.3 ±0.08^{f}	2.4 ±0.08^{c}	1.8 ±0.04^{e}	3.2 ±0.11^{b}	2.0 ±0.04^{d}	3.4 ±0.14^{a}
HDL-Cholesterol (mmol/L)	4.0 ±0.05^{b}	3.5 ±0.04^{d}	3.7 ±0.03^{c}	3.1 ±0.07^{e}	4.4 ±0.11^{a}	3.5 ±0.19^{d}	3.9 ±0.07^{b}	3.6 ±0.13^{d}
LDL-Cholesterol (mmol/L)	3.00 ±0.05^{f}	4.0 ±0.04^{d}	3.0 ±0.03^{f}	4.1 ±0.07^{c}	3.21 ±0.12^{e}	4.5 ±0.20^{b}	3.3 0±0.07^{e}	4.6 ±0.14^{a}
Homocysteine (μmol/l)	3.7 ±0.29^{e}	8.9 ±0.67^{d}	2.8 ±0.14^{g}	11.0 ±0.51^{c}	5.6 ±0.51^{f}	21.70 ±0.68^{a}	3.60.13^{e}	20.2 ±1.0^{a}
DNA damage (μm)	5.4 ±0.5^{g}	13.8 ±1.1^{c}	7.0 ±0.5^{d}	14. 2±0.5^{c}	6.6 ±1.2^{f}	14.9 ±0.6^{b}	6.9 ±0.4^{e}	20. 8±1.5^{a}

Data are shown as means ± SD. *P*-values were 0.01 to 0.0001. Means followed by similar alphabets in a row do not differ significantly at $P \geq 0.05$

Results showed (table 3) T_3 and T_4 decreased with age and their concentration was the lowest in old as compared to that in young individuals, In diabetic patients, T_3 and T_4 decreased significantly, however decrease in T_3 was evident in young and old individuals. In numerous pathophysiological conditions such as malnutrition, cirrhosis of liver, protein caloric malnourishment and in carbohydrate scarcity, thyroid hormone metabolism is reported to be distorted with decline in serum triiodothyronine (T_3) and a reciprocal increase in TR_3.

Investigations have suggested that change in concentration of T4 is an indication of a change in the factors that determine the decreased T4 secretion during the initial period of in sulinopemia. Likewise, a significant decrease in serum T3 was observed after 15 days of DM in rats (Isidro *et al*., 2007) and decreased hepatic and renal type I iodothyronine-deiodinare activity in experimental DM has also been reported (Maia *et al*., 2005; Wintergerst *et al*., 2011).

Testosterone was significantly decreased in both diabetic old and young male patients (table 3). Similar trend was also found in female subjects. The consequence of testosterone on insulin action and glucose metabolism is deficient; however, numerous unusual models and methods have been used to study androgen effect. A number of studies indicated that high testosterone levels are related with increased insulin resistance. High level of testosterone is frequently found in polycystic ovary syndrome such affected patients have a high risk of diabetes. Further as compare to hypergonadotropic and hypogonadotropic men, hypoandrogenism have increased insulin resistance level (Lee *et al.*, 2005).

Decline in testosterone may be the lack of ability of the pituitary gland to counter suitably, under the effects of elevated serum glucose and relations between nervous and endocrine system. Rise in body lipids decrease testosterone level due to increased activities of aromatase present in fat tissue thereby converting testosterone and

Table 3: Changes in serum vitamins, health biomarkers and hormone profiles of normal and diabetic patients with gender and age.

Parameters	Young				Old			
	Male (N=40)		Female (N=40)		Male (N=40)		Female (N=40)	
	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic	Normal	Diabetic
Vitamin C (µmol/L)	29.67 ±0.91^{B}	27.17 ±0.70^{C}	30.33 ±0.55^{A}	25.33 ±0.42^{D}	27.50 ±0.56^{C}	20.50 ±1.31^{E}	27.00 ±0.63^{C}	17.83 ±0.70^{F}
Vitamin E (µmol/L)	24.50 ±1.18$^{B\text{-}D}$	25.83 ±0.54^{B}	25.50 ±0.76^{BC}	22.67 ±0.95^{D}	28.50 ±0.95^{A}	23.00 ±0.97^{CD}	24.67 ±0.61$^{B\text{-}D}$	22.00 ±0.73^{D}
Vitamin A (µmol/L)	2.62 ±0.19^{C}	1.63 ±0.05^{E}	3.03 ±0.13^{A}	1.90 ±0.08^{D}	2.85 ±0.09^{B}	1.41 ±0.06^{G}	3.05 ±0.07^{A}	1.54 ±0.06^{F}
Superoxide Dimutase (SOD; U/L)	225.83 ±4.72^{G}	354.337 .69^{C}	210.83 ±6.01^{H}	317.83 ±4.89^{D}	270.00 ±4.52^{E}	443.67 ±13.56^{A}	245.67 ±1.94^{F}	393.33 ±5.95^{B}
Glutathione Peroxidase (GPX; U/L)	129.33 ±1.35^{B}	111.33 ±2.19^{E}	121.17 ±2.71^{D}	102.67 ±1.30^{F}	150.33 ±2.90^{A}	112.17 ±3.12^{E}	124.17 ±1.83^{C}	103.5 ±2.12^{F}
Sialic Acid (mg/dL)	31.50 ±0.80^{F}	43.83 ±1.22^{C}	30.67 ±0.84^{G}	43.83 ±0.60^{C}	42.17 ±1.10^{D}	54.67 ±1.33^{A}	41.50 ±1.08^{E}	50.00 ±0.58^{B}
Hexosamine (mg/dL)	60.67 ±3.08^{G}	82.00 ±3.51^{D}	53.17 ±1.56^{H}	90.33 ±1.22^{C}	75.00 ±1.63^{F}	99.00 ±4.09^{A}	77.50 ±2.55^{E}	96.50 ±1.45^{B}
Testosterone (ng/mL)	540.17 ±20.36^{B}	343.33 ±23.91^{D}	41.17 ±1.67^{E}	28.67 ±1.62^{F}	644.67 ±21.71^{A}	438.83 ±23.31^{C}	51.00 ±1.31^{E}	32.83 ±1.44^{F}
Triiodothyronine (T_3; ng/mL)	140.50 ±2.48^{B}	113.00 ±2.16^{D}	137.83 ±2.86^{C}	108.5 ±1.20^{E}	153.67 ±2.10^{A}	101.50 ±1.10^{F}	137.00 ±2.58^{C}	92.17 ±2.67^{G}
Thyroxine (T_4; µg/dL)	6.50 ±0.09^{A}	5.35 ±0.17^{D}	6.05 ±0.29^{B}	4.68 ±0.46^{E}	5.67 ±0.17^{C}	4.50 ±0.15^{F}	5.37 ±0.18^{D}	3.63 ±0.07^{G}

A-H, Different alphabets in a row differ significantly ($P\leq0.01$)

Table 4: Correlation coefficients of biochemical characteristics, vitamin and hormonal profile with homocysteine of normal and diabetic individuals

Parameters	Normal Homocysteine (µmol/L)	Diabetic Homocysteine (µmol/L)
Vitamin C (µmol/L)	-	-0.953(0.000)
Vitamin A (µmol/L)	-	-0.879(0.004)
Superoxide Dimutase (SOD; U/L)	-	0.949(0.000)
Glutathione Peroxidase (GPX; U/L)	0.985(0.015)	-0.774(0.024)
Sialic Acid (mg/dL)	-	0.882(0.004)
Hexosamine (mg/dL)	-	0.874(0.005)
Testosterone (ng/mL)	0.955(0.045)	-
Triiodothyronine (T_3; ng/mL)	-	-0.879(0.004)
Thyroxine (T_4; µg/dL)	-	-0.882(0.004)

Values in parenthesis are *P*-value for significance

androstenedione to estrogen (Elbers *et al*., 2011). This increase alteration may account for the decrease in testosterone in the present study. Oxidative stress due to diabetes, also lead to tissue injury (Maneesh *et al*., 2005a) which also occurs in the testis (Maneesh *et al*., 2005b) thus damage testosterone producing leyding cells as well as sertoli cells. SOD acts as an antitoxic to superoxide anion. Therefore, over appearance of SOD might be an adaptive reaction and may result in increased dismutation of superoxide to hydrogen peroxide. Maneesh *et al*. (2006) concluded that declilne in testosterone involving control effect of high serum glucose and above all dietary insufficiency increased oxidative stress and increased aromatase activities due to excess fats.

In the present study, serum GSHPx concentration increased significantly with age, however, in diabetic patients, either young or old, its concentration decreased significantly as compared to their enzymes in diabetic patients might result into an increase in lipid peroxidation thus increase risk for a potential for formation of atherosclerosis. Pari *et al*. (2012) reported a significant decrease in GPx activities in aorta homogenates of rats made diabetic 4 to 8 months ago. In diabetic patients, hyperglycemia leads to glucose oxidation and protein glycation in order to increase free radicals thus increasing the oxidative stress. Increased ROS exposure to endothelial cell display a number of adverse biological effects such as expression of adhesion molecules (Hughes *et al*., 2005). These adhesion molecules with E-selectin, P-selectin, vascular cell adhesion molecules 1 (ICAM-1) were articulated on endothelial cells (Lawson and Wolf, 2009).

In distinction, Salil *et al* (2012) observed decrease in SOD activity in kidney, spleen, liver, brain, muscles, heart, pancreas apart from lungs of streptozotocin and alloxan treated rats. In diabetic, red blood cell SOD activity has been shown to be increased (Ahmed, 2005). Glycation was exposed to influence the c-terminal end of SOD, tumbling its heparin-binding affinity (Prasad and Sinha 2010) thus harm in diabetes parting the endothelium to be more vulnerable to damage by superoxide anion.

tHcy has been intensively investigated with regard to its possible role in the pathogenesis of cardiovascular disease. Individuals with nutritional deficiency of folates or cyanocabalamin have high levels of homocysteine. tHcy is not in foodstuff but is produced from methionic. Its transformation occurs from methionine to tHcy by means of a de-methylation pathway which gives a methyl group with glycocyamine in the formation of creatine (Song *et al*., 2009). tHcy is sulfydryl amino acid that has painstaking role in vascular injury resulting in the improvement of peripheral and coronary arterial syndrome.

Elevated levels of tHcy supposed to promote the creation of oxidation products like, thiolactone and tHcy disulfides which have the capability to damage endothelial cell by disproportionate sulfation of collagen leading to thrombosis and arteriosclerosis. tHcy is moreover converted to cysteine via B6-dependent process or is remethylated to methionine. Later response proceeds by two pathway. One of which is reliant on vitamin B-12 and folate and the other on betaine (Song *et al*., 2009). tHcy levels are directly related with creation of creatinine. Higher levels of tHcy in patients can be observed in weakened renal functions or in patients with ending stage renal diseases (Title *et al*., 2006). Correlation coefficient of tHcy with anthropometric, biochemicals, hormonal and mineral profiles have been given in table 4.

tHcy levels were low in streptozotocin-induced diabetic rats and increased when insulin treatment was given to these rats (Title *et al*., 2006). The association between plasma insulin and tHcy explains the inequality reported in diabetic patients.

The whole cell embedded in agarose was subjected to lysis and then to an electric field for pulling of the nucleus in the direction of the node in order to form a comet like structure (Galjart, 2010). In the present study however, significant damaging of DNA was noted in all types of DM patients i.e. young and old male and female diabetic patients.

Studies have shown that DNA in human is more resistant to oxidant challenge following antioxidant pre-treatment of cells. Homocysteine as well as glycocylated HbIC and proteins significantly higher in diabetic patients were indicative of fairly high oxidative stress in the present study. Diabetes mellitus is not only a multifarious disease but linked with many other obstacles including obesity, nephropathy, retinopathy and atherosclerosis etc. Therefore, it could be speculated that significant damage to DNA of diabetic patients particularly in old age might have added effect on DNA-damage. The comet assay clearly described the damaging of DNA strands as shown in fig. It has also been speculated that occurrence of type 2 diabetes mellitus can be linked with higher levels of oxidative stress due to hyperglycemia as a consequence of glycoxidation and sorbitol system activation as well as from limitations of hexose manophosphate thrust leading to a significant diminish in glutathione production (Rachek *et al*., 2007).

Broedbaek *et al.* (2011) reported DNA damage in diabetic patients and indicated that 8-hydroxy-2-deoxyguanosine (80HdG) which can be synthesized by numerous different ROS as well as hydroxyl radicals, singlet oxygen, peroxyl radicals and perooxynitrite to produce modification to DNA bases in addition to strand breaks.

Therefore, it could be concluded from this study that accumulation of this products of oxidative agent(s) as indicated in Table 4 have caused the damage to DNA, Hb, proteins and lipid profiles of young and old diabetic patients.

ACKNOWLEDGEMENT

All authors are thankful to HEC for its appreciable cooperation to complete this work. This work is a part of PhD thesis of Shazia Anwer Bukhari who conducted this work under the supervision of Prof. Dr. Saeed Ahmad Nagra and partial work was conducted with the collaboration of corresponding author and other co-authors during her PhD research.

REFERENCES

Adonis S and Rohit A (2010). Vitamin E and Cardiovascular Disease. *Am. J. Ther.*, **17**(3): 56-65.

Adrian CE, Cardile V, Crascì L, Caggia S, Dugo P, Bonina F and Panico A (2012). Protective effects of an extract from Citrus bergamia against inflammatory injury in interferon-gamma and histamine exposed human keratinocytes. *Life. Sciences.*, **90**(25-26): 968-974.

Avissar N, Slemmon JR, Palmer IS and Cohen HJ (1991). Partial sequence of human plasma GPx and immunologic identification of milk glutathione peroxidase as the plasma enzyme. *J. Nutr.,* **121**: 1243-1249.

Baldi S, Innocenti M, Frascerra S, Nannipieri M, Lippi A, Rindi P and Ferrannini E (2012). Effects of hemodialysis and vitamin E supplementation on low-density lipoprotein oxidizability in end-stage renal failure. *J. Nephrol.,* **26**(3): 549-555.

Balogh E, Bereczky Z, Katona E, Kőszegi Z, Édes I, Muszbek L and Czuriga I (2012). Interaction between homocysteine and lipoprotein (a) increases the prevalence of coronary artery disease/myocardial infarction in women: A case-control study. *Throm. Res.*, **129**(2): 133-138.

Bieri JG, Tolliver TJ and Catignani GL (1979). Simultaneous determination of alpha-tocopherol and retinol in plasma or red cells by high pressure liquid chromatography. *Am. J. Clin. Nutr.,* **32**: 2143-2149.

Crook M (1993). The determination of plasma or serum sialicacid. *Clin. Biochem.*, **26**(1): 31-33.

Diez JJ, Sanchez P and Iglesias P (2011). Prevalence of thyroid dysfunction in patients with type 2 diabetes. *Exp. Clin. Endocr. Diabetes.,* **119**(4): 201-207.

Duncan DB (1955). Multiple Range and Multiple F Tests. *Biometrics,* **11**(1): 1-42.

Giacco F and Brownlee M (2010). Oxidative stress and diabetic complications. *Circ. Res.,* **107**: 1058-1070.

Halim EM and Mukhopadhyay AK (2006). Effect of *Ocimum sanctum* (Tulsi) and vitamin E on biochemical parameters and retinopathy in streptozotocin induced diabetic rats. *Indian J. Clin. Biochem.,* **21**(2): 181-188.

Harris E, Macpherson H, Vitetta L, Kirk J, Sali A and Pipingas A (2012). Effects of a multivitamin, mineral and herbal supplement on cognition and blood biomarkers in older men: A randomised, placebo-controlled trial. *Human Psychopharm. Clin. Exp.,* **27**(4): 370-377.

Hideaki K, Gang H, Ki-Ho S, Suzuma K, Bonner-Weir S, Sharma A and Weir GC (2001). Activation of the hexosamine pathway leads to deterioration of pancreatic β-cell function through the induction of oxidative. *J. Biolog. Chem.,* **276**(23): 31099-31104.

Hope and Hope T (2005). Trial Investigators. Effects of long-term vitamin E supplementation on cardiovascular events and cancer. *JAMA.,* **293**: 1338 -1347.

Isidro ML, Penín MA, Nemina R Cordido F (2007). Metformin reduces thyrotropin levels in obese, diabetic women with primary hypothyroidism on thyroxine replacement therapy. *Endocrine*, **32**: 79-82.

Islam NS, Begum P and Ahsan M (2005). Serum micro-nutrient (α-tocopherol, retinol, copper, zinc) status of immunosuspressed rats. *Mal. J. Nutr.,* **11**: 69-73.

Kaplan M, Michael A and Tony H (2012). Oxidative stress and macrophage foam cell formation during diabetes mellitus induced atherogenesis: Role of insulin therapy. *Pharmacol. Therap.,* **136**(2): 175-185.

Lee CH, Kuo SW, Hung YJ, Hsieh CH, He CT, Yang TC, Lian WC, Fan SC and Pei D (2005). The effect of testosterone supplement on insulin sensitivity, glucose effectiveness and acute insulin response after glucose load in male type 2 diabetics. *Endocr. Res.,* **31**(2): 139-148.

Madhur G and Suresh C (2006). Proxidant and antioxidant status in patients of type II diabetes mellitus with IHD. *Ind. J. Clin. Biochem.,* **21**(2): 118-122.

Maeda Y, Inoguchi T, Takei R, Sawada F, Sasaki S, Fujii M, Kobayashi K, Urata H, Nishiyama A and Takayanagi R (2010). Inhibition of chymase protects against diabetes-induced oxidative stress and renal dysfunction in hamsters. *Am. J. Physiol Renal Physiol.,* **299**: 1328-1338.

Maia AL, Kim BW, Huang SA, Harney JW and Larsen PR (2005). Type 2 iodothyroninedeiodinase is the major source of plasma T3 in euthyroid humans. *J. Clin. Invest*, **115**(9): 2524-2533.

Maneesh M, Jayalakshmi H, Singh TA and Chakrabarti A (2006). Imapired hypothalamic-pituitary-gonadal axis function in men with diabetes mellitus. *Ind. J. Clin. Biochem.,* **21**(1): 165-168.

Martin FL, Cole KJ, Williams JA, Millar BC, Harvey D, Weaver G, Grover PL and Phillips PH (2000). Activation of genotoxins of DNA-damage species in exfoliated breat milk cells. *Mutat. Res.,* **470**: 115-124.

Maskarinec G, Erber E, Grandinetti A, Verheus M, Oum R, Hopping BN, Schmidt MM, Uchinda A, Juarez DT, Hodges K and Kolonel LN (2009). Diabetes incidence based on linkages with health plans: The multiethnic cohort. *Diabetes,* **58**(8): 1732-1738.

Mullin GE (2011). Vitamin A and Immunity. *Nutr. Clin. Pract.,* **26**(4): 495-496.

Omaye S, Turnbul JD and Savberlich HE (1979). Ascorbic acid analysis. II. Determination after derivatisation with 2.2. dinitrophenylhydrazine. Selected methods for determination of ascorbic acid in animal cells, tissues and fluids. *Methods Enzymol.,* **62**: 7.

Payama K, Johanc S, Stanley F, Johan J, Fredrike L, Ingmarf J and Peter NM (2012). Combined effects of brachial pulse pressure and sialic acid for risk of cardiovascular events during 40 years of follow-up in 37 843 individuals. *J. Hypertension.,* **30**(9): 1718-1724.

Peralta EA, Brewer AT, Louis S and Dunnington GL (2009). Vitamine increases biomarkers of estrogen stimulation when taken with tamoxifen. *J. Surg. Res.,* **153**(1): 143-147.

Pfister R, Sharp SJ, Luben R, Wareham JN and Khaw KT (2011). Plasma vitamin C predicts incident heart failure in men and women in European Prospective investigation into cancer and Nutrition–Norfolk prospective study. *Am. Heart J.,* **162**(2): 246-253.

Reynolds AC, Dorrian J, Liu PY, Van Dongen HPA and Wittert GW (2012). Impact of five nights of sleep restriction on glucose metabolism, Leptin and Testosterone in young adult men. *PLoS ONE*. 7(7): e41218. doi:10.1371/journal.pone.0041218.

Schulze MB, Schulz M, Heidemann C, Schienkiewitz A, Hoffman K and Boeing H (2007). Fiber and magnesium intake and incidence of type 2 diabetes: A prospective study and meta-analysis. *Arch. Intern. Med.,* **167**(9): 956-965.

Song Y, Cook NR, Albert CM, Van Denburgh M and Manson JE (2009). Effects of vitamins C and E and b-carotene on the risk of type 2 diabetes in women at high risk of cardiovascular disease: A randomized controlled trial. *Am. J. Clin. Nutr*., **90**: 429-437.

Steel RGD and Torrie JH (1997). Principles and procedures of statistics. McGraw Hill Book Company Inc. New York.

Suksomboon N, Poolsup N and Sinprasert S (2011). Effects of vitamin E supplementation on glycaemic control in type 2 diabetes: Systematic review of randomized controlled trials. *J. Clin. Pharm. Therap.,* **36**(1): 53-63.

Sulaiman GM, Ahmed AH, Al-Amiery, Mohammed AA and Al-Temimi AA (2012). The Effect of cherry sticks extract on the levels of glycoproteins in alloxan-induced experimental diabetic mice. *Ann. Clin. Lab. Sci.,* **42**: 34-41.

Suleiman M, Aronson D, Asleh R, Kapelovich MR, Roguin A, Meisel SR, Shochat M, Suleiman A, Reisner SA, Markiewicz W, Hammerman H, Lotan R, Levy NS and Levy AP (2005). Haptoglobin polymorphism predicts 30-day mortality and heart failure in patients with diabetes and acute myocardial infarction. *Diabetes*, **54**: 2802-2806.

Sun VI, Larry W, Oberley and Ving U (1988). A simple method for clinical assay of superoxide dismutase. *Clin. Chem.*, **34**(3): 497-500.

Tiano JP and Jarvis FM (2012). Importance of oestrogen receptors to preserve functional β-cell mass in diabetes. *Nature Reviews Endocri. aop.*, doi:10.1038/nrendo. 2011.242.

Uyanik MH, Albayrak A, Odabasoglu F, Karakus E, Ozden K, Polat B, Yayla M, Karamese M, Albayrak A and Yazgi H (2012). Effects of diabetes on cytokines and oxidative organ injury in a rat model of sepsis. *Cellular Molec. Bio.,* **58**: 1623-1631.

Varki NM and Varki A (2007). Diversity in cell surface sialic acid presentations: Implications for biology and disease. *Lab Invest*, **87**: 851-857.

Williams DB, Wan Z, Frier CB, Bell RC, Field CJ and Wright DC (2012). Dietary supplementation with vitamin E and C attenuates dexamethasone-induced glucose intolerance in rats. *AJP. Regu. Physiol.* **302**(1): 49-58.

Wintergerst KA, Rogers ES and Foster MB (2011). Hyperthyroidism presenting with hyperglycemia in an adolescent female. *J. Pedi. Endocr. Metab.,* **24**(5-6): 385-387.