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Impact of hyperglycaemia on molecular markers of oxidative stress and antioxidants in type 2 diabetes mellitus

ABSTRACT

Introduction. The pathogenesis of type 2 diabetes mellitus (T2DM) is strongly linked to oxidative stress mainly caused by chronic hyperglycaemia. The present study investigates the association between hyperglycaemia with oxidative stress markers, antioxidants and lipid profile.

Materials and methods. The case-control study involved two groups, T2DM patients (n = 83) and age and sex matched controls (n = 81). Serum levels of various molecular markers malondialdehyde (MDA), reactive oxygen species (ROS) and nitric oxide (NO), superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), vitamin C, total antioxidant capacity (TAC) and lipid parameters total cholesterol, triglycerides, low density lipoprotein (LDL) and high density lipoprotein (HDL) were measured using spectrophotometric assays. Results were analysed to compare and correlate glycaemic levels with these molecular markers.

Results. T2DM patients had a higher body mass index (BMI) and body fat percentage. 2 hour blood glucose, glycated haemoglobin A_{1c} % (HbA_{1c}), total cholesterol, triglycerides and LDL were higher in diabetics, HDL was found to be lower in diabetics than in controls. Mean levels of enzymatic and non-enzymatic antioxidants SOD, CAT, GSH, vitamin C and TAC were significantly lower while oxidative stress markers NO, ROS and MDA were higher in T2DM patients. NO showed a positive correlation (r = 0.3993, p < 0.0001) whereas TAC showed a negative correlation with glycaemia (r = –0.4796, p < 0.0001).

Conclusions. Poor glycaemic control in T2DM causes elevated ROS and NO levels with increased lipid peroxidation and lowered antioxidant capacity. MDA and NO being the major risk factors could be used as a parameter along with antioxidants to assess oxidative stress in T2DM patients. (Clin Diabetol 2019; 8, 4: 215–222)

Key words: total antioxidant capacity, nitric oxide, malondialdehyde, type 2 diabetes mellitus, hyperglycaemia

Introduction

Type 2 diabetes mellitus or non-insulin dependent diabetes mellitus (NIDDM) is a metabolic syndrome of multiple etiology characterised by chronic hyperglycaemia resulting from disturbances in glucose and lipid metabolism. Diabetes causes an array of microvascular and macrovascular complications and stroke with an increasing incidences worldwide. The various diabetic complications, β-cell dysfunction and worsening of glycaemic control is linked to increased reactive oxidative stress (ROS) and reactive nitrosative stress (RNS) owing to increased production of free radicals such as the nitric oxide, superoxide radical, hydrogen peroxide and the hydroxide radical and free radical induced lipid peroxidation and a deficiency in the antioxidant defence mechanisms. The lipid peroxidation of tissues which is primarily caused due to increased ROS, is thought to play an important role in the development of atherosclerosis and other microvascular complica-

tions [1]. During the development of the disease, hyperglycemia causes increase in production of ROS in different tissues by forming advanced glycation end products. Hyperglycaemia-induced mitochondrial superoxide production is the sole underlying mechanism by which it induces cellular damage. The antioxidant defence network maintains the mitochondrial level of ROS within balanced concentrations. However, in hyperglycemia environment, enhanced glucose flux through glycolysis and Krebs cycle causes an overdrive of mitochondrial electron transport chain (ETC) resulting in mitochondrial dysfunction and increased ROS formation [2]. Excessive levels of ROS leads to cellular dysfunction, altered cell cycle, altered cell-signalling, increased inflammation and also is linked to development of insulin resistance, impaired metabolic pathways, diabetes and cardiovascular disorders (CVD) through dysfunction of insulin secretion and metabolism. Antioxidant defence mechanisms involve both enzymatic and non-enzymatic strategies. Common antioxidants include vitamin A, C and E, non-enzymatic antioxidant and cofactor GSH (L-γ-glutamyl-L-cysteinylglycine) and the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). They work in synergy with each other and against different types of free radicals [3]. The impairment of the endogenous antioxidant defence system is produced in many ways during chronic hyperglycaemia.

Lipids with unsaturated double bonds are highly susceptible to damage by free radicals, this process is commonly known as lipid peroxidation and it has deleterious effects in the biological system and it has been strongly linked to diabetes pathogenesis and complications. Malondialdehyde (MDA) is formed as an end product of lipid peroxidation. Elevated MDA levels show adverse physiological consequences which include altering cell membrane structural integrity, inactivating membrane bound enzymes and cell surface receptors. MDA is involved in foam cell formation which leads to atherosclerosis and other cardiovascular diseases. The determination of MDA is an important parameter to evaluate in vivo lipid peroxidation [4]. Nitric oxide (NO) is a gaseous free radical synthesized as a by-product from L-arginine to L-citrulline conversion by the action of nitric oxide synthase (NOS). Endotoxins and cytokines produce an inflammatory response which expresses NOS II, which can generate excess endogenous NO over long periods further aggravating inflammatory and ischemic processes. NO plays a role in angiogenesis, neurotoxicity and is involved in mediating diabetic retinal vascular injury which suggests the possible role of NO in pathogenesis of diabetic retinopathy (DR) [5]. The collective action of all the antioxidants present in

plasma and other body fluids can be defined as the total antioxidant capacity (TAC) which provides a composed parameter instead of a sum of all antioxidants. Alterations in TAC levels show a significant role in metabolic syndrome (MetS) and gestational diabetes [6].

Materials and methods
Subjects and study design

The present study was conducted in Department of Studies in Zoology, University of Mysore, Karnataka, India during the period of January–July 2018. The study conformed to the Helsinki declaration and was conducted with the approval of the Institutional ethical and research committee. 83 T2DM patients were recruited from an outpatient department of medicine of a primary health centre. For comparison, 81 age and sex matched healthy controls were randomly selected and recruited. Diagnosis for T2DM was done according to World Health Organisation (WHO) recommendations for the diagnostic criteria for diabetes and intermediate hyperglycaemia (2006). Inclusion criteria for T2DM patients included age group of 25–60, 2 hour blood glucose $\geq$ 11.1 mmol/L and treatment with hypoglycaemic drugs. Exclusion criteria included patients using any kind of vitamin supplements, lipid lowering drugs, antioxidant supplements, anti-inflammatory drugs, pregnant and lactating women, alcoholics, smokers and individuals with tobacco and other drug addiction, past history of chronic illness like tuberculosis, autoimmune disorders, endocrine disorders, patients with type 1 DM and any patient who did not meet the inclusion criteria were excluded from study group. Criteria for controls were based on previous medical and family history of diabetes, free of any metabolic disorders and other major diseases and 2 h blood glucose < 11.1 mmol/L.

Assessment of biochemical parameters

Venous blood samples was drawn to analyse 2 hour blood glucose and the serum was used to analyse HbA$_{1c}$ and lipid parameters. The serum was stored at −20°C for further analysis of molecular markers. All the biochemical estimations were done with the spectrophotometer Varioskan LUX multimode microplate reader instrument (ThermoFisher Scientific, USA). 2 h glucose in blood was estimated by GOD-POD technique. The lipid profile estimation was done using Arkray (Arkray healthcare, Mumbai, India) lipid kit. Serum total cholesterol (TC) was estimated by CHOD-PAP method. Serum HDL was estimated with the HDL precipitation reagent and a HDL standard using the CHOD-PAP kit. Triglycerides (TG) was estimated using GPO-PAP technique. LDL was measured indirectly using the Friedewald formula [7].

Assessment of obesity parameters

The BMI were calculated by dividing weight in kilograms by the square of the height in metres [kg/m^2]. The body fat percentage were measured using OMRON HBF-306 body fat monitor. The Asian Indian criteria of obesity and overweight was used to define the BMI in the subjects (normal BMI: 18.0–22.9 kg/m^2, overweight: 23.0–24.9 kg/m^2, obesity: > 25 kg/m^2) [8].

Molecular markers analysis

The total serum proteins were measured by Lowry method using bovine serum albumin as the standard. SOD activity was measured spectrophotometrically using Fridovich et al. [9] method where photo reduction of riboflavin leading to nitrite formation was measured at 543 nm. Serum Glutathione (GSH) was measured by its reaction with 5-5' dithiobis (2-nitrobenzoic acid) (DTNB) to give a yellow coloured complex whose absorbance is measured at 412 nm [10]. CAT activity was measured spectrophotmetrically by following the oxidation of H_2O_2 at 240 nm. The decomposition of H_2O^2 can be followed directly by decrease in absorbance at 240 nm. The difference in absorption per unit time is the measure of catalase activity [11]. Vitamin C in serum was determined by dinitrophenyl hydrazine method where ascorbic acid is oxidized to form a coloured complex which is measured at 520 nm. TAC was determined spectrophotometrically through the formation of phosphomolybdenum complex. 100 μL of serum was mixed with 100 μL of 5% trichloroacetic acid (TCA) to precipitate the proteins. The sample was centrifuged and the supernatant was reacted with 1ml of TAC reagent (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) which was incubated at 95°C for 90 minutes. The samples were cooled to room temperature and the absorbance of the aqueous solution was read at 695 nm against a blank [12]. The lipid peroxidation product, MDA, was measured by using thiobarbituric acid reactive substances (TBARS). MDA reacts with thiobarbituric acid at 100°C in an acidic medium to give a pink coloured complex. The colour intensity of the MDA-TBA complex was measured at 535 nm by using a spectrophotometer [13]. Serum NO was measured using the Griess reaction using a modified method of Grisham et al. [14]. Equal volumes of the serum sample and freshly prepared Griess reagent were incubated at 37°C for 10 minutes. A stable decomposition product (NO_2–) formed the coloured complex and its absorbance was measured at 540 nm using a spectrophotometer. ROS was measured fluorometrically using the dichlorofluorescindiacetyl (DCFDA) oxidation method by determining the hydrogen peroxide concentrations present in serum [15]. ROS oxidises DCFDA into a fluorescent compound dichlorofluoroscein (DCF). The fluorescence emitted was determined using a fluorometric reader (Varioskan LUX multimode microplate reader) at an excitation wavelength of 525 nm and an emission wavelength of 488 nm.

Statistical analysis

The subjects were grouped into T2DM (n = 83) and controls (n = 81). The difference in various parameters like age, body mass index, diabetes duration, glycaemic levels, lipid profiles and molecular markers were tested for significance by comparing the two groups using student t-test and the relationship of family history with DM was assessed using chi-square test. The association between hyperglycaemia and diabetes duration with various clinical factors were assessed using linear regression analysis. BMI and Age were entered in the multiple regression analysis model to determine its association with glycaemic status. Pearson's correlation coefficient was used to determine the relationship between glycaemic levels and serum NO and TAC levels. All the data were entered in a Microsoft excel spreadsheet. Statistical analysis was performed using IBM SPSS version 23.0 (SPSS, Chicago, IL, USA) and GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). Data are reported as mean ± standard deviation (mean ± SD). The level of significance for all tests was set at $p < 0.05$.

Results

Individuals with T2DM were older compared to non-diabetics ($p < 0.0001$). T2DM patients also had a higher rate of diabetes in family history. The BMI ($p < 0.0001$) and body fat percentage ($p < 0.0001$) were higher in T2DM when compared to the controls. The average DM duration for the whole sample was 9.0 ± 3.1 years, females had a higher DM duration than males (Table 1). The diabetic patients in the study were either undergoing treatment with metformin only (n = 33) or no treatment at all (n = 50). There was no statistically significant difference observed in the levels of various parameters between the treatment and non-treatment groups. The 2-hr blood glucose ($p < 0.0001$), HbA_{1c}% ($p < 0.0001$), total cholesterol ($p < 0.0001$), triglycerides ($p < 0.001$) and LDL ($p < 0.019$) were higher in diabetics when compared to controls while serum HDL ($p < 0.002$) was found to be lower in diabetics than in controls (Table 2). Mean levels of enzymatic and non-enzymatic antioxidants SOD ($p = 0.045$), CAT ($p < 0.0001$), GSH ($p < 0.0001$), vitamin C ($p < 0.001$) and total antioxidant capacity ($p < 0.0001$) were significantly lower in T2DM patients compared

Table 1. Demographic characteristics of the patients

Parameter	Total (n = 164)	Type-2 diabetics (n = 83)	Controls (n = 81)	p
Age (years)	48.1 ± 8.8	50.0 ± 8.3	46.1 ± 8.9	0.0001
Sex ratio (M/F)	99/65	50/33	49/32	0.9092
Family history of T2DM (yes/no)	44/120	32/51	12/69	0.0007
Diabetes duration in years	–		–	0.0001
a. Male		a. 9.6 ± 3.7		
b. Female		b. 8.6 ± 2.5		
BMI [kg/m^2]	27.0 ± 4.1	28.1 ± 4.1	25.9 ± 3.9	0.0001
Body fat (%)	31.3 ± 6.6	33.1 ± 5.8	29.5 ± 6.9	0.0001

Data presented as mean ± SD. T2DM — type 2 diabetes mellitus; BMI — body mass index

Table 2. Biochemical parameters

Parameter	Type 2 diabetics	Controls	95% CI	p
2-hr blood glucose [mmol/L]	14.25 ± 4.46	6.29 ± 1.21	6.94, 8.96	0.0001
HbA$_{1c}$ (%)	10.57 ± 2.80	5.57 ± 0.76	4.36, 5.63	0.0001
Total cholesterol [mg/dL]	159.72 ± 38.69	117.48 ± 32.21	31.32, 53.14	0.0001
Triglycerides [mg/dL]	151.37 ± 48.89	126.0 ± 30.59	10.09, 40.64	0.002
LDL [mg/dL]	58.72 ± 21.40	45.85 ± 30.30	2.26, 23.47	0.019
HDL [mg/dL]	44.44 ± 9.53	48.66 ± 9.31	1.10, 7.33	0.002
Total serum proteins [mg/dL]	6.45 ± 3.80	8.95 ± 3.31	1.37, 3.62	0.0001

Data presented as mean ± SD. HbA$_{1c}$ — glycated haemoglobin; LDL— low density lipoprotein cholesterol; HDL — high density lipoprotein cholesterol; 95% CI — 95% confidence intervals; CI here indicates difference between two population means lies between lower CI and upper CI

Table 3. Oxidative stress and antioxidant parameters

Parameter	Type 2 diabetics	Controls	95% CI	p
SOD [units/mg protein]	0.0602 ± 0.108	0.1008 ± 0.128	0.001, 0.080	0.045
CAT [nmol H$_2$O$_2$ oxidised/min/mL]	0.0284 ± 0.0298	0.0481 ± 0.0350	0.01, 0.03	0.0001
GSH [nmol/mL]	23.316 ± 0.584	24.593 ± 1.120	0.705, 1.849	0.0001
Vitamic C [μmol/L]	31.001 ± 8.693	55.992 ± 18.691	15.957, 34.025	0.0001
TAC [μmol α-tocopherol/L]	119.977 ± 44.103	160.793 ± 60.302	18.985, 62.649	0.0001
Serum NO [μmoles/L]	72.170 ± 26.021	47.022 ± 23.607	17.539, 32.757	0.0001
ROS [mmol DCF formed/min/mg protein]	0.474 ± 0.241	0.388 ± 0.128	0.025, 0.147	0.007
Serum MDA [nmol/mg protein]	0.304 ± 0.178	0.178 ± 0.141	0.076, 0.177	0.0001

Data presented as mean ± SD. SOD — superoxide dismutase; CAT — catalase; GSH — reduced glutathione; TAC — total antioxidant capacity; NO — nitric Oxide; ROS — reactive oxygen species; MDA — malondialdehyde; 95% CI — 95% confidence intervals; CI here indicates difference between two population means lies between lower CI and upper CI

to controls. Oxidative stress markers NO (p < 0.0001), ROS (p = 0.007) and MDA (p < 0.0001) were higher in T2DM patients when compared to controls (Table 3).

Linear regression analysis showed a positive association between hyperglycaemia and oxidative stress markers, BMI, total cholesterol, triglycerides and LDL and negative association with antioxidant levels and HDL (Table 4). There was no correlation observed between diabetes duration with different molecular markers and lipid profile, a positive association between diabetes duration and glycaemic levels, BMI and fat % whereas a negative association with TAC was observed (Table 5). Multiple regression analysis was used to test if age and BMI significantly affects the glycaemic status in T2DM patients. The results of the regression indicated the two predictors explained 7.3% of the variance [R^2 = 0.073, F(2,161) = 6.34, p = 0.002]. It was found that BMI significantly affected glucose

Table 4. Association of hyperglycaemia with different parameters

Parameter	*R²	Slope	95% CI	F	p
Age	0.0194	0.2395 ± 0.1335	−0.0220, 0.5011	3.221	0.0746
BMI	0.0328	0.1461 ± 0.0623	0.0239, 0.2682	5.497	0.0203
Fat %	0.0316	0.2285 ± 0.0993	0.0337, 0.4231	5.290	0.0227
Cholesterol	0.1629	3.233 ± 0.5758	2.105, 4.362	31.53	0.0001
HDL	0.0952	−0.666 ± 0.1731	−1.006, −0.3276	14.84	0.0002
LDL	0.0642	1.482 ± 0.5601	0.3699, 2.595	7.003	0.0094
TG	0.0564	2.072 ± 0.8391	0.4058, 3.739	6.099	0.0152
CAT	0.0675	−0.0017 ± 0.0004	−0.0026, −0.0007	11.74	0.0008
SOD	0.0347	−0.0048 ± 0.0021	−0.0090, −0.0006	5.143	0.0248
GSH	0.1552	−0.1022 ± 0.0324	−0.1673, −0.0370	9.918	0.0027
Vitamin C	0.1298	−0.3521 ± 0.1479	−0.6517, −0.0525	5.667	0.0224
TAC	0.1558	−5.009 ± 1.138	−7.268 to −2.750	19.37	0.0001
ROS	0.0497	0.0090 ± 0.0033	0.002512, 0.01551	7.384	0.0074
NO	0.1304	1.945 ± 0.3945	1.172 to 2.718	24.30	0.0001
MDA	0.0500	0.0074 ± 0.0026	0.0023, 0.0125	8.055	0.0052

*Linear regression analysis — goodness of fit. 95% CI — 95% confidence intervals; BMI — body mass index; TG: — triglycerides; LDL — low density lipoprotein cholesterol; HDL — high density lipoprotein cholesterol; SOD — superoxide dismutase; CAT — catalase; GSH — reduced glutathione; TAC — total antioxidant capacity; NO — nitric oxide; ROS — reactive oxygen species; MDA — malondialdehyde

**Table 5. Association of diabetes duration with different parameters**

Parameter	*R²	Slope	95% CI	F	p
Glucose	0.5537	1.092 ± 0.1089	0.8745, 1.309	100.5	0.0001
BMI	0.0472	0.2940 ± 0.1467	0.0015, 0.5864	4.015	0.0484
Fat %	0.059	0.4664 ± 0.2070	0.05392, 0.8789	5.078	0.0269
Cholesterol	0.0185	1.730 ± 1.399	−1.059, 4.519	1.529	0.2199
HDL	0.0055	−0.2809 ± 0.4835	−1.248, 0.6861	0.3375	0.5635
LDL	0.0243	1.390 ± 1.393	−1.425, 4.205	0.9960	0.3243
TG	0.0158	2.718 ± 3.391	−4.135, 9.570	0.6425	0.4276
CAT	0.0057	−0.0007 ± 0.0010	−0.0029, 0.0014	0.4694	0.4952
SOD	0.0386	−0.0091 ± 0.0057	−0.0206, 0.0024	2.495	0.1193
GSH	0.0398	−0.0866 ± 0.0699	−0.2284, 0.0551	1.535	0.2231
Vitamin C	0.0382	−0.0712 ± 0.0842	−0.2483, 0.1057	0.7161	0.4085
TAC	0.2906	−8.923 ± 2.323	−13.64, −4.208	14.75	0.0005
ROS	0.0139	0.0059 ± 0.0064	−0.0069, 0.0187	0.8504	0.3601
NO	0.0048	0.5972 ± 0.9477	−1.292, 2.486	0.3971	0.5304
MDA	0.0095	0.0058 ± 0.0069	−0.0080, 0.0198	0.7128	0.4012

*Linear regression analysis — goodness of fit. 95% CI — 95% confidence intervals; BMI — body mass index; TG — triglycerides LDL — low density lipoprotein cholesterol; HDL — high density lipoprotein cholesterol; SOD — Superoxide dismutase; CAT — Catalase; GSH — reduced glutathione; TAC — total antioxidant capacity; NO — nitric oxide; ROS — reactive oxygen species; MDA — malondialdehyde

levels (β = 0.207, p = 0.007) as well as age (β = 0.184, p = 0.017). Glycaemic levels showed a significant negative correlation with total antioxidant status (r = −0.4796, p < 0.0001) (Figure 1A), whereas glycaemic levels showed a significant positive correlation with oxidative stress marker NO (r = 0.3993, p < 0.0001) (Figure 1B).

Discussion

Oxidative stress has focus interest in various clinical research in recent times. There is a growing evidence connecting the action of oxidative stress to the pathogenesis and complications in diabetes mellitus and many other diseases. Oxidative stress plays a role in pathogenesis of insulin resistance and β-cell dysfunc-
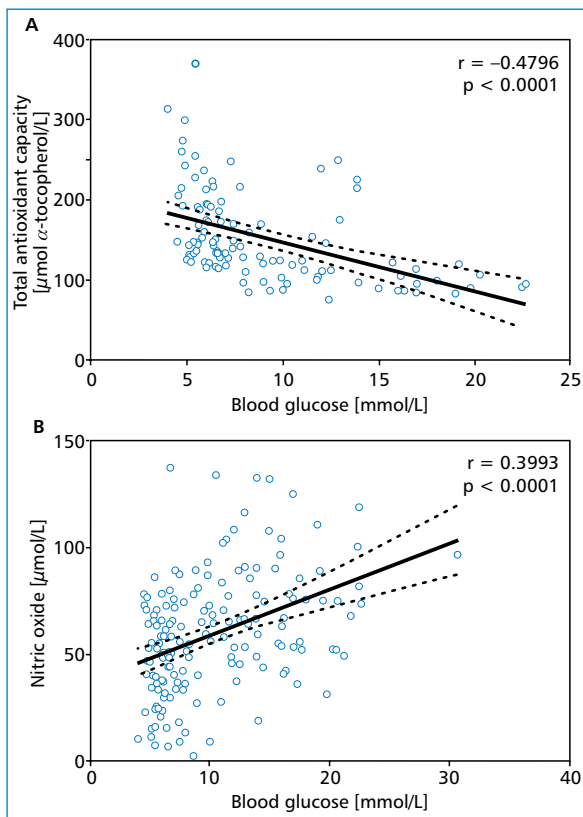
Figure 1A. Correlation between total antioxidant capacity (TAC) and blood glucose; **B.** Correlation between serum nitric oxide (NO) and blood glucose

tion, caused by dysregulation of cell homeostasis and metabolism [4]. Hyperglycaemia is the principal metabolic alteration which is associated with diabetes mellitus, and increased glycaemic levels in bodily fluids has been implicated to increase oxidants, cause cellular damage, vascular dysfunction and pathogenesis of vascular disease.

This study reveals a detailed information on the impact of hyperglycaemia on oxidative stress status in T2DM patients. Present study shows a two-fold higher blood glucose as well as HbA_{1c} levels in diabetic compared to normal subjects, which is an indication of worsening glycaemic control. Hyperglycemia increases the levels of free radical generation which activates several pathways involved in pathogenesis of complications of diabetes: polyol pathway, increased AGE formation and activation of protein kinase C (PKC) isoforms [16]. This ultimately leads to irreversible damage of biomolecules like proteins, lipids and nucleic acids and loss of its functional ability [17]. Dyslipidaemia is a major risk factor for CVD in diabetes mellitus. It is characterised by high total cholesterol, serum triglyceride concentrations, high LDL cholesterol and lowered HDL

cholesterol concentrations. Dyslipidaemia is attributed to free fatty acid flux secondary to insulin resistance [18]. Data from table 2 shows a considerable increase in total cholesterol, serum triglycerides and LDL levels and a significant decrease in HDL levels in this study indicating a dyslipidaemia condition in the diabetic patients although the LDL and triglyceride parameters in diabetics do not exceed the American Diabetes Association 2011 guidelines for lipid goals [19]. Dyslipidaemia in diabetes have the ability to generate oxidative stress which possibly leads to the development of macroangiopathy causing endothelial dysfunction and atherosclerosis.

The study also showed a significant drop in both enzymatic and non-enzymatic antioxidants. There was a drop in serum CAT activity which was in agreement with the findings of Pasaoglu et al. [20]. Several studies on serum and erythrocyte SOD levels have shown increased, decreased as well as unchanged enzyme levels. In this study there was a decrease in SOD activity. This finding is in accordance with Kesavulu et al. We observe a decrease in CAT activity and consequently SOD activity. A possible explanation for the fall in SOD activity could be linked to glycation of the SOD enzyme in serum due to hyperglycaemic condition. Decrease in SOD activity consequently leads to decrease in CAT activity as both the enzymes function in unison to neutralise superoxide ion to water and oxygen molecule [21]. Another possible explanation for the fall in CAT activity could be due to downregulation of serum CAT due to catalase gene mutations caused by elevated hydrogen peroxide levels [22]. The study also observed decrease in serum GSH levels which was consistent with that of Gallou et al. [23]. GSH a key intracellular antioxidant is involved in redox regulation of protein thiols and hydrogen peroxide in mitochondria [21]. Several studies have shown that reduced/oxidized glutathione ratio is lower in diabetics [24]. In hyperglycaemic conditions, glucose is preferentially used in polyol pathway which consumes NADPH that is necessary for GSH regulation by the GR enzyme. Thus, hyperglycaemia indirectly causes GSH depletion. Vitamin C is a powerful dietary antioxidant, it donates electrons thus helping in scavenging free radicals. Vitamin C reduces the overall hyperglycaemic state by reducing blood glucose, reducing glycosylation of proteins and by decreasing the production of sorbitol. Several clinical studies have shown that vitamin C levels in diabetics are lowered and that supplementing with vitamin drugs helps to slightly alleviate the diabetic complications [25]. In this study, the level of vitamin C, is significantly decreased in diabetics. A possible explanation for the low vitamin C levels in diabetics could be linked to increased ascorbic acid

oxidation or impaired regeneration from its oxidized state [21]. There has been limited studies describing the total antioxidant status in T2DM. This study concluded that diabetics had a lower TAC level than controls. The correlation study showed that TAC was negatively correlated with glycaemic levels indicating worsening plasma antioxidant capacity with increased hyperglycaemia in T2DM. Opara et al. observed a decrease in TAC levels in diabetic patients whereas Korkmaz et al. observed increased TAC levels in early diabetic stages and Savu et al. observed an increase in TAC levels synergistically with other antioxidants as well as MDA [25–27]. As the results stand controversial and the mechanisms are poorly understood, further investigation is needed to validate the status of TAC for monitoring antioxidant levels in diabetes.

T2DM is characterised by prolonged and increased intracellular and extracellular ROS generation. In this study ROS levels were studied by determining the peroxide concentrations in serum. The ROS levels in diabetics were slightly higher than in controls although there was no significant difference between them. Free radicals attack membrane phospholipids causing lipid peroxidation and high levels of these oxidised products have been correlated with development of vascular complications. The high levels of MDA in serum can be linked to failure of antioxidant system to curb the deleterious action of free radicals and hence it serves as a reliable stress marker to assess free radical induced tissue damage [7]. The increase in lipid peroxidation reflected by the increase in serum MDA levels in diabetics in the present study are in accordance with previous studies that hyperglycaemia increases lipid peroxidation from overproduction of free radicals in diabetics [20]. The study showed significantly higher levels of serum NO in T2DM patients than in controls. Additionally, NO levels were positively correlated with increasing glucose levels which shows worsening glycaemic control accelerates NO production in serum. These findings are in agreement with Maejima et al. and Ozden et al. [28, 29]. Ozden et al. explains increased NO synthesis is a result of compensatory mechanism due to oxidative stress causing NO inactivation by inhibiting NO mediated endothelial function. Contrary to this study, Ghosh et al. reported decreased serum NO levels in T2DM patients [30]. Elevated serum NO levels have been attributed to pathogenesis of diabetes and diabetic retinopathy, hence assessing NO levels could be an important tool to measure the severity of diabetes in patients.

The regression analysis shows hyperglycaemia to be strongly associated with obesity and CVD-risk parameters showing a positive association with BMI, body fat, total cholesterol and LDL. It also showed a strong positive association with MDA and NO levels and a negative association with CAT, GSH, vitamin C and TAC levels. In contrast, diabetes duration and oxidative stress parameters and lipid profile showed no correlation. This finding is consistent with the results of Aouacheri et al. [31]. The positive association between diabetes duration with glycaemic levels and negative association with TAC in the analysis indicates prolonged hyperglycaemic conditions greatly reduces the antioxidant capacity of cells, leading to oxidative stress. This study shows that poor glycaemic control in T2DM elevates oxidative and nitrosative factors, decreases antioxidants and alters lipid profile all of which are strongly linked to diabetic and vascular complications.

Conclusions

This study shows the importance of monitoring levels of antioxidants in parallel with NO and MDA along with the usual glycaemic and lipid markers in T2DM which could be useful to assess the degree of oxidative stress present and provide important identification cues for patients to undergo antioxidant treatment since administration of antioxidants is found to be effective only in selective group of patients with depleted natural antioxidants and elevated oxidative stress levels. Some of the limitations of this study are the small patient population to assess the different parameters and the need to perform assays on protein carbonyls to further understand the extent of damage caused by oxidative stress in T2DM. More clinical studies are needed on larger patient populations to understand the underlying physiological effects of ROS, RNS and different antioxidants in T2DM and the potential of using these parameters to determine oxidative stress in the patients.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

1. I V, M H. Introductory Chapter. In: Lushchak V, editor. Oxidative Stress — Molecular Mechanisms and Biological Effects. InTech. 2012.

2. Brand MD. The sites and topology of mitochondrial superoxide production. Exp Gerontol. 2010; 45(7–8): 466–472, doi: 10.1016/j.exger.2010.01.003, indexed in Pubmed: 20064600.

3. Feher J, Csomos G, Vereckei A. Control of Free Radical Reactions in Biological Systems. In: Free Radical Reactions in Medicine. Berlin, Heidelberg: Springer Berlin Heidelberg. 1987: 11–17.

4. Noberasco G, Odetti P, Boeri D, et al. Malondialdehyde (MDA) level in diabetic subjects. Relationship with blood glucose and glycosylated hemoglobin. Biomed Pharmacother. 1991; 45(4–5): 193–196, indexed in Pubmed: 1932603.

5. Niwa K, Takizawa S, Kawaguchi C, et al. Expression of inducible nitric oxide synthase immunoreactivity in rat brain following chronic hypoxia: effect of aminoguanidine. Neurosci Lett. 1999; 271(2): 109–112, indexed in Pubmed: 10477114.

6. Papapetropoulos A, García-Cardeña G, Madri JA, et al. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. J Clin Invest. 1997; 100(12): 3131–3139, doi: 10.1172/JCI119868, indexed in Pubmed: 9399960.

7. Warnick GR, Knopp RH, Fitzpatrick V, et al. Estimating low-density lipoprotein cholesterol by the Friedewald equation is adequate for classifying patients on the basis of nationally recommended cutpoints. Clin Chem. 1990; 36(1): 15–19, indexed in Pubmed: 2297909.

8. Misra A, Chowbey P, Makkar BM, et al. Concensus Group. Consensus statement for diagnosis of obesity, abdominal obesity and the metabolic syndrome for Asian Indians and recommendations for physical activity, medical and surgical management. J Assoc Physicians India. 2009; 57: 163–170, indexed in Pubmed: 19582986.

9. Beyer WF, Fridovich I. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal Biochem. 1987; 161(2): 559–566, indexed in Pubmed: 3034103.

10. Moron M, Depierre J, Mannervik B. Levels of glutathione, glutathione reductase and glutathione s-transferase activities in rat lung and liver. Biochimica et Biophysica Acta (BBA) — General Subjects. 1979; 582(1): 67–78, doi: 10.1016/0304-4165(79)90289-7.

11. Cakmak I, Horst W. Effect of aluminium on lipid peroxidation, superoxide dismutase, catalase, and peroxidase activities in root tips of soybean (Glycine max). Physiologia Plantarum. 1991; 83(3): 463–468, doi: 10.1111/j.1399-3054.1991.tb00121.x.

12. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal Biochem. 1999; 269(2): 337–341, doi: 10.1006/abio.1999.4019, indexed in Pubmed: 10222007.

13. Bernheim F, Bernheim MLC, Wilbur KM. The reaction between thiobarbituric acid and the oxidation products of certain lipides. J Biol Chem. 1948; 174(1): 257–264, indexed in Pubmed: 18914082.

14. Grisham MB, Johnson GG, Lancaster JR. Quantitation of nitrate and nitrite in extracellular fluids. Methods Enzymol. 1996; 268: 237–246, indexed in Pubmed: 8782590.

15. Black MJ, Brandt RB. Spectrofluorometric analysis of hydrogen peroxide. Anal Biochem. 1974; 58(1): 246–254, indexed in Pubmed: 4825377.

16. Karacay O, Sepici-Dincel A, Karcaaltincaba D, et al. A quantitative evaluation of total antioxidant status and oxidative stress markers in preeclampsia and gestational diabetic patients in 24–36 weeks of gestation. Diabetes Res Clin Pract. 2010; 89(3): 231–238, doi: 10.1016/j.diabres.2010.04.015, indexed in Pubmed: 20537747.

17. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circ Res. 2010; 107(9): 1058–1070, doi: 10.1161/CIRCRESAHA.110.223545, indexed in Pubmed: 21030723.

18. Mooradian AD. Dyslipidemia in type 2 diabetes mellitus. Nat Clin Pract Endocrinol Metab. 2009; 5(3): 150–159, doi: 10.1038/ncpendmet1066, indexed in Pubmed: 19229235.

19. American Diabetes Association. Standards of medical care in diabetes — 2010. Diabetes Care. 2010; 33 Suppl 1: S11–S61, doi: 10.2337/dc10-S011, indexed in Pubmed: 20042772.

20. Pasaoglu H, Sancak B, Bukan N. Lipid peroxidation and resistance to oxidation in patients with type 2 diabetes mellitus. Tohoku J Exp Med. 2004; 203(3): 211–218, indexed in Pubmed: 15240931.

21. Kesavulu MM, Rao BK, Giri R, et al. Lipid peroxidation and antioxidant enzyme status in Type 2 diabetics with coronary heart disease. Diabetes Res Clin Pract. 2001; 53(1): 33–39, indexed in Pubmed: 11378211.

22. Nourooz-Zadeh J, Rahimi A, Tajaddini-Sarmadi J, et al. Relationships between plasma measures of oxidative stress and metabolic control in NIDDM. Diabetologia. 1997; 40(6): 647–653, doi: 10.1007/s001250050729, indexed in Pubmed: 9222643.

23. Gallou G, Ruelland A, Legras B, et al. Plasma malondialdehyde in type 1 and type 2 diabetic patients. Clin Chim Acta. 1993; 214(2): 227–234, indexed in Pubmed: 8472388.

24. Baynes JW. Role of oxidative stress in development of complications in diabetes. Diabetes. 1991; 40(4): 405–412, doi: 10.2337/diab.40.4.405, indexed in Pubmed: 2010041.

25. Opara EC, Abdel-Rahman E, Soliman S, et al. Depletion of total antioxidant capacity in type 2 diabetes. Metabolism. 1999; 48(11): 1414–1417, indexed in Pubmed: 10582550.

26. Korkmaz GG, Konukoglu D, Kurtulus EM, et al. Total antioxidant status and markers of oxidative stress in subjects with normal or impaired glucose regulation (IFG, IGT) in diabetic patients. Scand J Clin Lab Invest. 2013; 73(8): 641–649, doi: 10.3109/00365513.2013.846477, indexed in Pubmed: 24152132.

27. Savu O, Ionescu-Tirgoviste C, Atanasiu V, et al. Increase in total antioxidant capacity of plasma despite high levels of oxidative stress in uncomplicated type 2 diabetes mellitus. J Int Med Res. 2012; 40(2): 709–716, doi: 10.1177/147323001204000235, indexed in Pubmed: 22613434.

28. Maejima K, Nakano S, Himeno M, et al. Increased basal levels of plasma nitric oxide in Type 2 diabetic subjects. Relationship to microvascular complications. J Diabetes Complications. 2001; 15(3): 135–143, indexed in Pubmed: 11358682.

29. Ozden S, Tatlipinar S, Biçer N, et al. Basal serum nitric oxide levels in patients with type 2 diabetes mellitus and different stages of retinopathy. Can J Ophthalmol. 2003; 38(5): 393–396, indexed in Pubmed: 12956281.

30. Ghosh A, Sherpa ML, Bhutia Y, et al. Serum nitric oxide status in patients with type 2 diabetes mellitus in Sikkim. Int J Appl Basic Med Res. 2011; 1(1): 31–35, doi: 10.4103/2229-516X.81977, indexed in Pubmed: 23776769.

31. Aouacheri O, Saka S, Krim M, et al. The investigation of the oxidative stress-related parameters in type 2 diabetes mellitus. Can J Diabetes. 2015; 39(1): 44–49, doi: 10.1016/j.jcjd.2014.03.002, indexed in Pubmed: 25065473.