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 A1c % (HbA1c), 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-
The collective action of all the antioxidants present in retinal vascular injury which suggests the possible role neurotoxicity and is involved in mediating diabetic ischemic processes. NO plays a role in angiogenesis, long periods further aggravating inflammatory and NOS II, which can generate excess endogenous NO over produce an inflammatory response which expresses from L-arginine to L-citrulline conversion by the action is a gaseous free radical synthesized as a by-product evaluation 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].

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 ≥ 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 HbA1c 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/m²). 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/m², overweight: 23.0–24.9 kg/m², obesity: > 25 kg/m²) [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-S²’ dithiobis (2-nitrobenzoic acid) (DTNB) to give a yellow coloured complex whose absorbance is measured at 412 nm [10]. CAT activity was measured spectrophotometrically by following the oxidation of H₂O₂ at 240 nm. The decomposition of H₂O₂ 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 (NO³⁻) formed the coloured complex and its absorbance was measured at 540 nm using a spectrophotometer. ROS was measured fluorometrically using the dichlorofluorescin diacetate (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), Hba₁% (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
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 [R² = 0.073, F(2,161) = 6.34, p = 0.002]. It was found that BMI significantly affected glucose
levels ($\beta = 0.207$, $p = 0.007$) as well as age ($\beta = 0.184$, $p = 0.017$). 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 $\beta$-cell dysfunc-
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.

Acknowledgement
The authors would like to thank Nithin B., Sowmya K.L. and Sudeep Pinto for helping with sample collection and data compilation. The authors thank Molecular Reproductive and Human Genetics Lab, DoS in Zoology and Institute of Excellence staff, University of Mysore for providing the instrumentation facilities to carry out the above work.

Conflict of interest
The authors declare no conflict of interest.

REFERENCES