

Analysis of malondialdehyde and ferric reducing ability of plasma in type 2 diabetes mellitus patients in Lucknow city, India

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ABSTRACT

Background: The incidence and prevalence of type 2 diabetes mellitus (T2DM) is increasing due to obesity and sedentary lifestyles. Increased oxidative stress leads to oxidative damage of biomolecules and decreased antioxidant capacity.

Aim: The study was designed to determine the malondialdehyde (MDA) and ferric reducing ability of plasma (FRAP) in T2DM patients at IIMS&R Hospital, Lucknow.

Method: The oxidative stress was analyzed in T2DM patients as MDA. The total antioxidant capacity was estimated as the FRAP in T2DM patients and control subjects.

Results: The level of MDA was observed to be higher in T2DM patients (4.84 ± 1.09) than controls (2.20 ± 0.85) ($p < 0.05$). The FRAP was lower among T2DM patients (509.46 ± 126.36) than controls (895.62 ± 179.92) ($p < 0.05$).

Conclusion: MDA was found to be higher in T2DM, and the FRAP level was lower in T2DM patients compared to controls, indicating increased oxidative stress and a decreased antioxidant level in T2DM patients.

Keywords: oxidative stress, antioxidant, ferric reducing ability of plasma, FRAP, malondialdehyde, MDA, type 2 diabetes mellitus

Introduction

Diabetes mellitus (DM) is a polygenic syndrome characterized by elevated fasting blood sugar caused by a relative or absolute insulin. Diabetes mellitus (DM) is a polygenic illness marked by high fasting blood sugar due to a relative or absolute insulin insufficiency. The incidence and prevalence of diabetes mellitus are on the rise due to the aging population, obesity, and sedentary lifestyle. Diabetes mellitus, one of the leading causes of disability and mortality, affects approximately 195 million individuals of various ages worldwide. Diabetes is of two types, i.e., type 1 (insulin-dependent diabetes mellitus, T1DM) and type 2 (noninsulin-dependent diabetes, T2DM) [1].

Diabetes is a major cause of kidney failure, nerve damage, heart attacks, and strokes, and the leading cause of adult blindness and amputation. Diabetes mellitus varies from one individual to another, but is determined by the patient's health and diet [2]. It is a chronic disease caused by inheritance of genes responsible for insulin insufficiency or pancreatic insulin deficiency. One of the most prevalent consequences of diabetes is hyperglycemia. Progressive insulin deficiency, longer duration of diabetes, and glycemic management all raise the risk of hyperglycemia in patients with T2DM [3]. Hyperglycaemia may increase the vascular changes leading to unconsciousness, coma, and even death [4]. Patients with T2DM have greater blood sugar

fluctuations than normal subjects [5]. In T2DM, the fluctuations in blood sugar were positively correlated with the development of coronary artery disease (CAD), and chronic persistent hyperglycemia damages the endothelium of blood vessels.

Patients with diabetes mellitus have been reported to have a correlation between increased oxidative stress and lowered levels of physiological antioxidants. An antioxidant prevents the generation of oxidizing species, which would otherwise react with the antioxidant rather than the substrate [6, 7]. Many studies have found a positive correlation between serum lipids, lipoproteins, lipid peroxides and erythrocyte antioxidant enzymes (catalase, glutathione peroxidase, and superoxide dismutase) in T2DM patients. Diabetic complications develop with increased activity of free radical-induced damage and accumulation of lipid peroxidation products. Oxidative stress can be measured by estimating the lipid peroxidation in the body. Malondialdehyde (MDA) acts as a marker of oxidative stress when there is an imbalance between the production and depletion of antioxidants. Malondialdehyde (MDA) is a stable product of lipid peroxidation and marker of oxidative stress which occur when there is an imbalance between production and scavenging of free radicals. Free radicals react with polyunsaturated fatty acids (PUFA) to form peroxides, thus degrading lipids and releasing MDA products [8].

There are numerous methods to determine the total antioxidant capacity of human biological samples or serum/plasma, e.g., the FRAP assay that uses the reduction of Fe^{3+} tripyridyltriazine to Fe^{2+} tripyridyltriazine to produce a blue-colored intermediate Fe^{2+} -TPTZ which can be determined in a redox-linked colorimetric method. The increased oxidative stress initiates the cascade of free radical reactions i.e., generation of reactive oxygen species (ROS) resulting in increased lipid peroxidation of plasma membranes, hence causing an increase in oxidative damage. The formation of free radical with antioxidant deficiency in T2DM patients is increased over time due to prolonged hyperglycemic leading to diabetic complications such as diabetic nephropathy, retinopathy, neuropathy, peripheral

arterial disease and atherosclerosis. Therefore, the diabetic complications may be prevented by supplementing the antioxidant rich components in diet to improve the intrinsic antioxidant system.

Methods

This case-control study was approved by Institutional Ethical Committee of the IIMS&R, Integral University, Lucknow (IEC/IIMS&R/2018/25). We enrolled 63 T2DM patients and 63 healthy controls after collecting their medical and family histories and receiving their informed consent. This study was conducted over a period of six months at the Department of Biochemistry, Integral Institute of Medical Sciences and Research (IIMS&R), Integral University, Lucknow.

Measurement of blood sugar

The fasting blood sugar (FBS) and post-prandial blood sugar (PPBS) was estimated by the glucose oxidase/peroxidase method [9]. Glucose is oxidized by glucose oxidase (GOD) into gluconic acid and hydrogen peroxide. In the presence of peroxidase (POD), hydrogen peroxide is oxidized the chromogen 4-amino antipyrine/phenolic compound to a red compound. The intensity of the red is proportion to the glucose concentration and was measured at 505 nm (489-530 nm). The absorbance (A) of the samples was measured at 505 nm, and the concentration of glucose in the sample was estimated using the following equation:

$$\text{Glucose (mg/dl)} = \frac{A_{\text{sample}}}{A_{\text{standard}}} \times \text{concentration of standard (100 mg/dl)}$$

Estimation of MDA by TBARS method

Malondialdehyde (MDA) was estimated by the method of [10]. Plasma was deproteinized and the precipitate was treated with thiobarbituric acid at 90°C for 1 hour [11]. The pink colour formed gives the measure of thiobarbituric acid reactive substance (TBARS) which was measured at 530 nm using a UV-Visible double beam spectrophotometer (Systronics-2205, Systronic India Ltd. Gujarat, India). The MDA concentration was calculated according

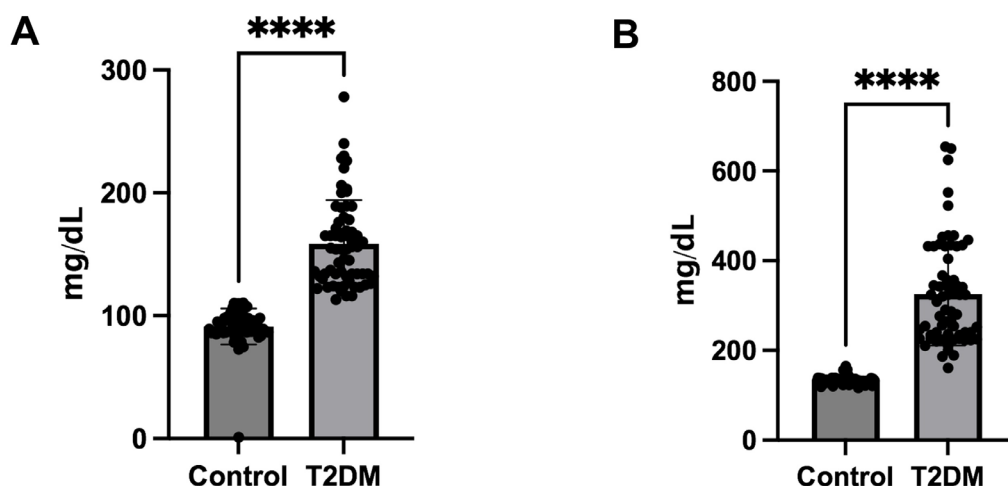


Figure 1. Comparison of blood sugar between control subject and T2DM patients. (A) fasting blood sugar (FBS), (B) post-prandial blood sugar (PPBS)

to the following formula: $\text{MDA } (\mu\text{mol/l}) = \frac{A \times 532 \text{ nm} \times 1.75}{0.156}$

Estimation of TAC by FRAP assay

The FRAP assay uses antioxidants as reductants in a redox linked colorimetric method employing a reduced oxidant, Fe(III) [12]. Reduction of a ferric tripyridyltriazine complex to ferrous (2,4,6-tripyridyl-s-triazine) i.e., colourless ferric (III) to blue ferrous (II) can be monitored by measuring absorbance at 593 nm. The absorption readings are related to the reducing power of the electron-donating antioxidants present in the test compound. The FRAP assay was a modified assay of the ferric reducing/antioxidant power, and ascorbic acid concentration (FRAPSC) [8] for the evaluation of antioxidants status, total reducing (antioxidant) power, absolute concentration of ascorbic acid, and relative contributed of ascorbic acid to total antioxidant power of the sample [6]. The reagents used for the FRAP assay are: TPTZ (2,4,6-tripyridyl-s-triazine), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ dilute HCl, acetate buffer, ferrous sulphate. The procedure to perform the FRAP assay was as follows. The above reagents were added into a glass tube (100 μl sample, 900 μl distilled water and 2 ml FRAP reagent). For the blank sample, 2 ml FRAP reagent was taken (1 ml distilled water and 2 ml FRAP reagent). The samples were mixed thoroughly and transferred into a cuvette. The samples were read

at 593 nm using a spectrophotometer. The values of FRAP was obtained by using the equation:

$\text{FRAP value of sample } (\mu\text{M}) = \frac{A (\text{sample}) \times \text{FRAP value of standard } (\mu\text{M})}{A (\text{standard})}$

The concentration of FRAP was calculated from the standard curve of Absorbance (A) vs Fe^{2+} concentration. For calibration, aqueous solutions of known Fe ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) concentration in the range of 100-1000 $\mu\text{mol/L}$ were used, and the values were expressed as $\mu\text{mol/L Fe}^{2+}$. Hence, the FRAP assay measures the reducing power and the antioxidant potential of various compounds.

Statistical analysis

The statistical analysis was applied using Prism Grappad 9.0 (San Diego, US). The mean \pm SD of all quantitative clinical parameters were calculated in T2DM patients and healthy controls. The p-values was calculated by the student unpaired t-test and $p < 0.05$ were considered as statistically significant. The correlation was determined by using the Karl's Pearsons correlation coefficient.

Results

Figure 1 shows the fasting blood sugar (FBS) and post-prandial blood sugar (PPBS) of T2DM patients and controls. The FBS was significantly ($p = 0.0001$) higher in T2DM patients (158.66

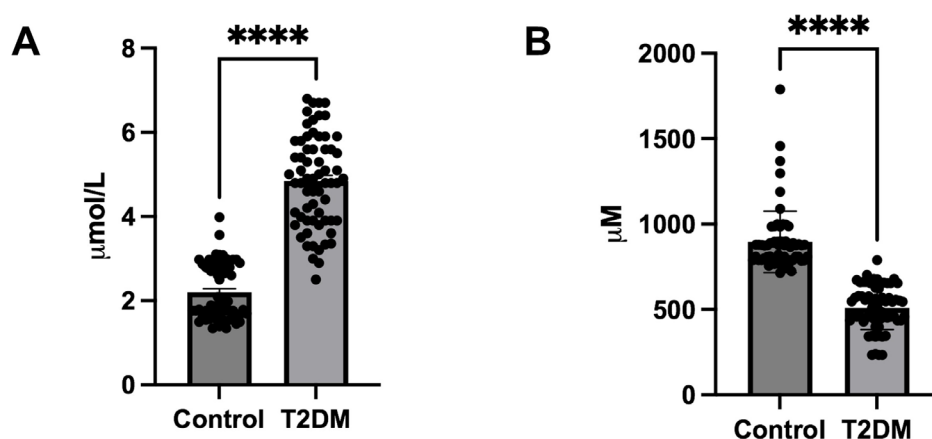


Figure 2. Comparison of MDA and FRAP level between control subject and T2DM patients. (A) MDA level, (B) FRAP level

± 35.38) compared to controls (91.22 ± 29.32). Similarly, the PPBS was significantly ($p < 0.0001$) higher among the T2DM patients (325.55 ± 114.55) compared with control group (132.98 ± 8.84).

Figure 2 shows the malonaldehyde (MDA) and ferric reducing ability of plasma (FRAP) of T2DM patients and controls subjects. The MDA was significantly ($p = 0.0001$) higher in T2DM patients (4.84 ± 1.09) compared to controls (2.20 ± 0.85). In our study, it was found that the mean value of MDA was significantly increased in diabetic group as compared to nondiabetic patients.

In contrast to this, the total antioxidant capacity was reduced which was estimated by the FRAP. The level of FRAP was found to be lower (509.46 ± 126.36) in T2DM patients as compared to control subjects (895.62 ± 179.92), and the difference was statistically significant ($p=0.0001$). The decreased FRAP in T2DM patients indicates the reduced antioxidant power or the reduced total antioxidant capacity (TAC).

These results indicate that MDA is a good marker of lipid peroxidation, serum levels of MDA were significantly increase in T2DM patients and depletion of antioxidant defences appear early in T2DM, before the development of secondary complications. There is a significant positive correlation between the MDA level and FBS and a significant negative correlation between PPBS and FRAP in T2DM patients.

Discussion

The development of diabetes complications, such as diabetic nephropathy, retinopathy, neuropathy, peripheral neuropathy vascular disease, and atherosclerosis, is also significantly influenced by the accumulation of free radicals in T2DM patients with an antioxidant deficiency. The link between increased oxidative stress and decreased level of physiological antioxidants has been found in patients with DM [13]. Diabetic complications develop with increased activity of free radical-induced lipid peroxidation and accumulation of lipid peroxidation products. Oxidative stress can be measured by estimating the lipid peroxidation in the body. MDA acts as a marker of oxidative stress when there is an imbalance between the production and depletion of antioxidants. There is a significant positive correlation between the MDA level and FBS in T2DM patients. This study confirms increased oxidative stress in DM compared to nondiabetic counterparts and emphasizes the importance of these markers for early diagnosis and therapeutic intervention. In the early stages of T2DM, the antioxidant defense system counters the effects of increased free radicals. However, in the later stages, the balance between the generation of free radicals and antioxidant defense is impaired due to decreased antioxidant activity. In DM, there is oxidative stress because of the excessive generation of free radicals caused by persistent hyperglycemia. The

simultaneous decline of antioxidant defense systems can lead to the damage of cellular organelles and enzymes, increased lipid peroxidation, and the development of complications of diabetes mellitus [14].

This study showed an increase in serum MDA concentrations in diabetic subjects, which agrees with previous studies [15]. Earlier reports by Sato et al. [16] showed an increased level of thiobarbituric acid-reactive substances (TBARS), a marker of lipid peroxidation, in the blood samples of DM patients. TBARS indirectly measures lipid peroxidation in erythrocyte antioxidant enzyme activity in diabetic patients [17, 18]. Free radicals react with polyunsaturated fatty acids (PUFA) to form peroxides, thus degrading lipids and releasing MDA products. MDA is a stable product of lipid peroxidation, which is measured as an index of lipid peroxidation [8]. Many studies have found a positive relationship between serum lipids, lipoproteins, lipid peroxides, and erythrocyte antioxidant enzymes (catalase, glutathione peroxidase, and superoxide dismutase) in T2DM patients. Our study found that the level of MDA was significantly increased in the diabetic group compared to nondiabetic patients.

There are several methods to determine the total antioxidant capacity (TAC) of human biological samples or serum/plasma, e.g., the FRAP assay that uses the reduction of Fe^{3+} tripyridyltriazine to Fe^{2+} tripyridyltriazine to produce a blue-colored intermediate Fe^{2+} -TPTZ which can be read at 593 nm. The antioxidants used in the FRAP assay are required as reductants in a redox-linked colorimetric method. The FRAP assay does not need specialized equipment or skills for the critical control of limiting and reaction conditions [19]. Metal ions such as iron and copper participate in the Fenton or Fenton-like reaction to generate oxidizing species causing oxidative damage to biomolecules [20].

The generation of oxygen free radicals occurs first with the reduction of ferric to ferrous iron and then by the Fenton reaction with ferrous iron, catalyzing the breakdown of hydrogen

peroxide (H_2O_2) to hydroxyl radical ($\text{OH}\bullet$) and hydroxide ion (OH^-) (Haber-Weiss reaction). Another reaction known as the Haber-Weiss reaction or superoxide-driven Fenton reaction between H_2O_2 and superoxide reaction ($\text{O}_2^{\bullet-}$) in the presence of iron or copper ions leads to the formation of hydroxyl radical ($\text{OH}\bullet$). The Fenton reaction results in the generation of OH^- and $\text{OH}\bullet$ by a reaction between Iron (II) (Fe^{2+}) and H_2O_2 [21]. In the Haber-Weiss reaction, OH^- and $\text{OH}\bullet$ are produced by the reaction of H_2O_2 and $\text{O}_2^{\bullet-}$ catalyzed by iron [22]. The Haber-Weiss cycle is a two-step reaction, the ferric ion reduces to a ferrous ion via reaction with superoxide, which in turn, reacts with H_2O_2 to form OH^- and $\text{OH}\bullet$, converting ferrous back to ferric ion [23].

An effective antioxidant may reduce the oxidative damage caused by $\text{OH}\bullet$ from Fenton or Fenton-like reactions through a different mechanism such as blocking or inhibiting the reaction that produces ROS, scavenging or quenching ROS, converting them into harmless or non-toxic species. To scavenge $\text{OH}\bullet$, the antioxidant must react with it before the radical reacts with biomolecules. This requires the presence of antioxidants in close proximity to the radical and a higher reactivity and concentration than those of the biomolecules [20]. Antioxidants remove the peroxidation product, and α -tocopherol (vitamin E) inhibits lipid peroxidation in the presence of vitamin C leading to protection against cardiovascular disease [24, 25]. The enzymatic antioxidants, superoxide dismutase, glutathione peroxidase, catalase, glutathione reductase, glutathione, and vitamins A, E, and C are usually measured to assess the antioxidant status in blood. The foods of plant origin not only provide us with antioxidant vitamins (e.g., vitamin C, vitamin E, or provitamin A) but also a complex mixture of other natural substances with antioxidant capacity. Some phytochemicals also provide nutrients and antioxidants for protection against oxidative stress [13, 26-32] which may reduce the risk of diabetes and cardiovascular complications.

Conclusion

These findings demonstrate the increased oxidative stress in T2DM patients as measured by MDA was higher in DM. Moreover, the TAC is reduced in T2DM patients than healthy controls. The increased oxidative stress is caused by the increased production of reactive oxygen species, which leads to lipid peroxidation of plasma membranes and oxidative damage. The present study shows that persistent hyperglycemia in T2DM patients activates cellular and tissue damage by oxidative stress, but the compensatory mechanisms for defense against the ROS to normalize oxidative stress was not achieved in T2DM patients.

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Conflict of interest

The authors declare that there is no conflict of interest.

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