

Oxidative stress in patients with diabetes mellitus

Abstract

Oxidative stress in a biological system is the shift in the homeostasis between oxidants and antioxidants in favour of oxidants. It is suggested to be playing a key role in onset and development of complication of Diabetes Mellitus. The present study aimed to assess the oxidative stress and compare the antioxidant enzyme status in patients with Type 2 Diabetes and paired controls. Total study population consisted of 33 subjects with Diabetes and 10 controls from both male and female gender incorporated for the study. Plasma Malondialdehyde level, an indicator of oxidative stress and antioxidative enzyme system including Erythrocyte Superoxide dismutase, catalase and Glutathione Peroxidase were estimated. Subjects with diabetes had significantly higher plasma malondialdehyde as compared to controls. Antioxidant enzymes levels were found to be lower in diabetic patients as compared to controls. In conclusion, hyperglycemia increases production of free radicals leading to increased lipid peroxidation. The antioxidant enzyme system weakens in diabetic patients compared to controls. It was noted that patients with good glycemic control had less oxidative stress compared to patients with poor control.

Keywords: oxidative stress, diabetes mellitus, malondialdehyde, superoxide dismutase, glutathione peroxidase

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Introduction

The global burden of diabetes was reported to be 382million in 2013 and the number is expected to rise to more than 592million in less than 25years. Majority of them aged between 40 and 59years and 80% of them belong to low and middle income countries. In India, 65.1million People aging between 20-79years were reported to be diabetic in 2013.¹ Diabetes is a metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both.² Chronic hyperglycemia manifests into macro and microvascular complications of diabetes leading to mortality.³ Oxidative stress is considered to be causative factors for development and complications of diabetes.⁴ It is defined as the state in which the balance between oxidants and antioxidant system in the body is lost in favour of oxidants. We cannot leave without oxygen. Molecular oxygen is essential in the process of mitochondrial respiration for complete metabolism of glucose and other substrates during ATP production. Oxidative phosphorylation results in generation of free radical superoxide from 0.4 to 4% of consumed oxygen.⁵ These free radicals such as superoxide, hydroxyl radical, hydrogen peroxide, nitric oxide, peroxynitrite etc are highly unstable due to presence of unpaired electrons, which may pair with other electrons, resulting detrimental effect. To sustain life, removal of these reactive oxygen species is essential and various antioxidant systems exists in the body to maintain homeostasis.⁶ These antioxidant systems are mainly composed of in vivo antioxidative enzyme system and in vitro dietary source of antioxidants. Anti-oxidative enzymes include superoxide dismutase (SOD) which catalyses the dismutation of superoxide to hydrogen peroxide.⁷ Glutathione peroxidase and catalase further converts hydrogen peroxide to water in cytosol and peroxisome, thereby protecting the cells from overproduction of free radicals.⁸ Dietary antioxidants such as tocopherol and vitamin C prevent chain breaking reaction of lipid peroxidation in all cell membranes.

High blood glucose levels in diabetes patients has shown to increase the generation of reactive oxygen species through various

mechanisms. Nishikawa T⁹ reported that hyperglycemia leads to increase mitochondrial production of superoxide in vascular endothelial cells.⁹ The high glucose level may lead to Autoxidation of glucose, enhanced nonenzymatic glycation and activation of diacylglycerol protein kinase C pathway and also activation of Polyol pathway.¹⁰ Studies have shown alterations in antioxidant enzyme system of diabetic patients,¹¹ increased lipid peroxidation, a determinant of coronary artery diseases and other microvascular complications of diabetes.¹² In the present study attempt have been made to assess and compare marker of oxidative stress, plasma Malondialdehyde and antioxidant enzyme essays of erythrocyte Superoxide dismutase, Catalase and Glutathione peroxidase in type 2 diabetes patients with paired control subjects. Effect of factors such as type of treatment, duration of diabetes, glycemic control and gender on oxidative stress were examined.

Materials and methods

Subject profile

The study was conducted at outpatient department of Endocrinology at Bai Yamuna Anand Nair Charitable Hospital, Mumbai, India, where people mainly from low socioeconomic group usually get the treatment. The patients were identified as Diabetic based on American Diabetes Association guidelines. Total thirty three male and female Type 2 Diabetes Mellitus patients aged between 45 to 65years, attending Diabetes clinic were screened in the study, using inclusion criteria of participants treated with Oral hypoglycemic drugs; insulin therapy or a combination of both along with Medical nutrition therapy. Glycosylated haemoglobin less than 8.5percent were enrolled to avoid extreme deviations in oxidative stress. Participants with morbid obesity, Body Mass Index >35Kg/m², consuming multivitamins or mineral supplements, pregnant, lactating mothers were excluded. All participants were non-smokers, and free of established diabetes complications, liver, kidney, thyroid diseases, cancer, and autoimmune disorders.

Research tools

A pretested questionnaire was used for interviewing participants about demographic profile such as age, gender. Diabetes related information, including duration of diabetes; present glycemic control status, type of treatment. BMI, Waist to Hip ratio and waist circumference were assessed. Biochemical parameter such as Glycosylated Haemoglobin was recorded.

Antioxidant status assessment

Sample collection: 10ml blood was drawn from each participant after an overnight fast of 12hours by venipuncture using a disposable needle and syringe and needle under aseptic conditions. Out of which 5ml blood was collected in each EDTA tube (Labtech disposal) for the estimation of erythrocyte superoxide dismutase, erythrocyte catalase, and erythrocyte glutathione peroxidase and plasma malondialdehyde.

Sample preparation: The samples were centrifuged at 2000rpm for 10mins to separate the plasma. The buffy coat was washed three times with cold saline, and was hemolysed by adding ice cold ultrapure water to yield a 50% hemolysate. Aliquots of hemolysate were stored at -70°C till analysis.

Antioxidative status analysis: Estimation of erythrocyte superoxide dismutase was measured by using McCord JM et al.¹³ Glutathione peroxidase and catalase activity of the hemolysate was estimated using method by Beutler E et al.¹⁴ The preparation of hemolysate for catalase activity was done using method by Beutler E et al.¹⁴ Plasma

malondialdehyde level was measured using method by Stock J et al.¹⁵

Statistical analysis

Statistical analysis of the data was done using prism pad graphics by applying unpaired T test of independent variables. Mean and standard deviations were calculated for demographic and anthropometric profile.

Results and discussion

Demographic profile

Mean age of the control and diabetic subjects was 49.9years and 50.8years respectively. Among controls, 6 subjects were female and 4 were male. Among the diabetic population, 24 subjects were male and 9 were female.

Anthropometric assessment

The mean body mass index of diabetic subjects was $24.9 \pm 3.87 \text{ kg/m}^2$. The control subjects were enrolled on the basis of similar BMI, which was noted to be $24.31 \pm 3.79 \text{ kg/m}^2$. The mean waist circumference of diabetic subjects was higher (87.93cm) as compared to controls (82.3cm). Waist to hip ratio was higher among Diabetic participants (0.95) compared to control subjects (0.93) (Table 1). Similar observation was reported by A. Kamath¹⁶ of higher prevalence of central obesity among diabetic patients. Clinical studies of Vazquez G¹⁷ have shown stronger association of central obesity with diabetes than general fat.

Table 1 Anthropometric measure of diabetic and control subjects

	Body mass index(Kg/m ²)	Waist Circumference(cm)	Waist to hip ratio
Controls (n=10)	24.9±3.87	82.3±8.14	0.93
Diabetic (n=33)	24.31±3.79	87.93±9.10	0.95

Antioxidative status

The HbA1c level reflects the mean glucose concentration over last 8-12weeks approximately and provides a much better indication of long-term glycemic control. The mean HbA1c level was found to be higher ($7.573 \pm 0.09\%$) in subjects with diabetes compared to control subjects ($5.480 \pm 0.09\%$). The marker of lipid peroxidation, Plasma malondialdehyde (MDA), was found to be significantly higher ($P=0.01$) in patients with diabetes ($1.920 \pm 0.09 \text{ nm/ml}$) as compared to control subjects ($1.450 \pm 0.09 \text{ nm/ml}$). This increase in plasma malondialdehyde level indicates increased oxidative stress in participants with Type 2 diabetes mellitus (Figure 1,2). Patients with type 2 diabetes mellitus were found to have statistically significant ($P<0.01$) reduction($1.118 \pm 0.05 \text{ Units/gHb}$) in mean erythrocyte superoxide dismutase enzyme as compared to controls ($1.501 \pm 0.05 \text{ Units/gHb}$). Erythrocyte glutathione peroxidase level was also found to be significantly ($P<0.01$) low ($13.13 \pm 0.49 \text{ Units/gHb}$) in patients with diabetes as compared to controls ($32.93 \pm 3.53 \text{ U/gmHb}$). Similar trend was observed with erythrocyte catalase level with significant ($P<0.01$) decrease ($0.8600 \pm 0.04 \text{ Units/gHb}$) in diabetes subjects as compared to controls ($1.701 \pm 0.09 \text{ Units/gHb}$) (Figure 3-5).

Factors affecting oxidative stress in diabetes

Gender: Significant difference in oxidative stress as represented by plasma malondialdehyde level, between diabetic and normal group was noted. But when the diabetic group was stratified on gender basis, it was observed that there was no gender specificity (male-

$1.913 \pm 0.10 \text{ nanomoles/ml}$, female- $1.939 \pm 0.2357 \text{ nanomoles/ml}$). It was noted that Erythrocyte superoxide dismutase and glutathione peroxidase enzymes levels were marginally higher in male subjects as compared to female however the difference was again not statistically significant. Erythrocyte catalase level was found to be $0.87 \pm 0.22 \text{ unit/gm Hb}$ for male as compared to $0.81 \pm 0.27 \text{ unit/gm Hb}$ for female subject (Table 2). Therefore it can be concluded that these antioxidant enzyme systems although are significantly lower in diabetic condition but are not gender specific. Based on these findings male and female diabetic subjects were studied as one group to further study effect of other factors.

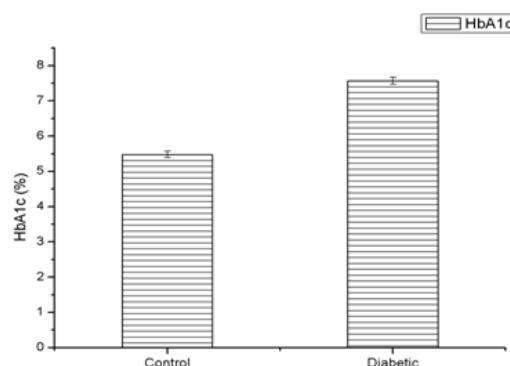
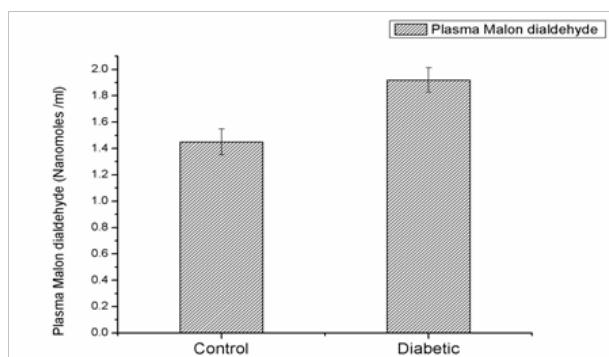
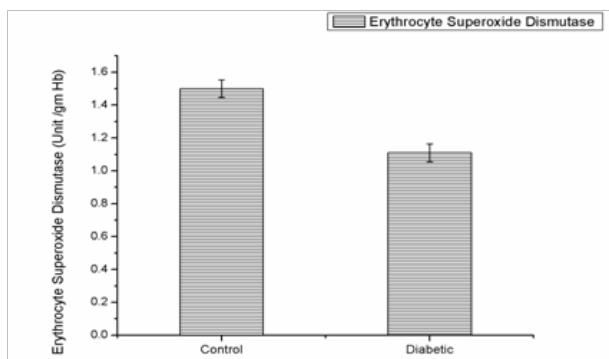
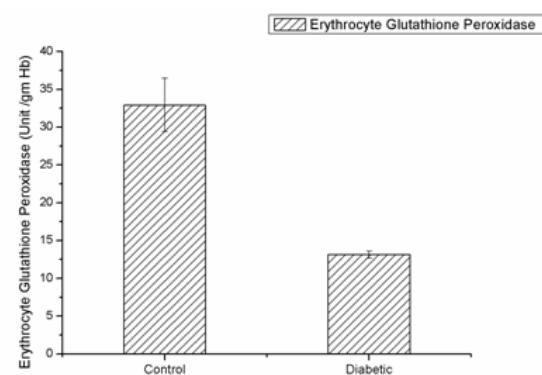
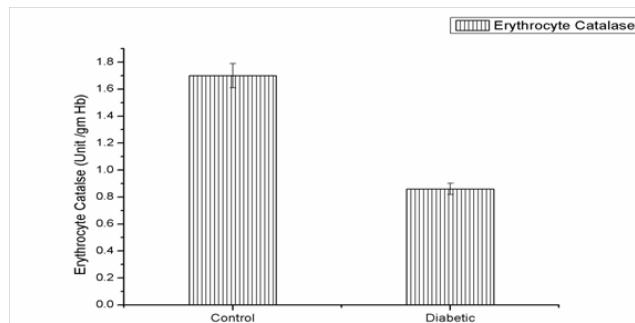


Figure 1 Comparison of Glycosylated haemoglobin among control and diabetic subjects.

**Figure 2** Plasma malondialdehyde (MDA) of control and diabetic subjects.**Figure 3** Erythrocyte superoxide dismutase level of control and diabetic subjects.**Figure 4** Erythrocyte glutathione peroxidase level of control and diabetic subjects.**Figure 5** Comparison of erythrocyte catalase level among control and diabetic subjects.**Table 2** Antioxidative status across gender in diabetic patients

	MDA(nanomoles/ml)	SOD(Unit/g Hb)	Catalase(Unit/g Hb)	Glutathione peroxidase(Unit/gHb)	HbA1c(%)
Controls(n=10)	1.45±0.09	1.50±0.05	1.70±0.09	32.93±3.53	5.48±0.09
Male(n=24)	1.91±0.10	1.18±0.060	0.87±0.22	13.2±2.9	7.51±0.11
Female(n=9)	1.93±0.23	0.94±0.10	0.81±0.27	12.39±2.38	7.73±0.16
P value	0.9059	0.0483	0.7069	0.3751	0.32

Treatment modalities: Based on type of treatment prescribed for glycemic control such as oral hypoglycemic agents (OHA) or insulin therapy, the patients were divided in two groups. The patients treated with insulin had prominent lower lipid peroxidation (1.65 ± 0.54 nanomoles/ml) as compared to patients treated with oral hypoglycemic drugs (1.94 ± 0.53 nanomoles/ml). But the differences

were not statistically significant ($P = >0.05$). The erythrocyte levels of all antioxidative enzymes receiving insulin therapy was found to be marginally higher as compared to patients being treated with oral hypoglycemic agents (Table 3). The mean glycosylated haemoglobin level was found to be $7.58 \pm 0.10\%$ in patients on insulin as compared to patients treated with oral hypoglycemic drugs $7.43 \pm 0.38\%$.

Table 3 Comparison of antioxidative status based on Type of treatment

	MDA(nanomoles/ml)	SOD(Unit/g Hb)	Catalase(Unit/g Hb)	Glutathione peroxidase(Unit/gHb)	HbA1c(%)
Controls(n=10)	1.45±0.098	1.50±0.05	1.70±0.09	32.93±3.53	5.48±0.09
OHA(n=30)	1.94±0.53	1.1±0.31	0.84±0.24	12.73±2.73	7.58±0.10
INSULIN(n=3)	1.65±0.54	1.28±0.23	1±0.15	13.13±1.03	7.43±0.38
P value	0.3918	0.3402	0.2813	0.9987	0.65

Body Mass Index (BMI): There was no significant difference in plasma malondialdehyde level in patients with normal body mass index (between $18.5-22\text{kg}/\text{m}^2$) and patients with Body mass index between $22.0-30.0\text{Kg}/\text{m}^2$ (Table 4). Patients with normal BMI in the enrolled subjects had a higher plasma malondialdehyde (1.97 ± 0.13 nanomoles/ml) as compared to subjects with BMI more

than normal (1.89 ± 0.13 nanomoles/ml). The statistically insignificant differences observed in normal subjects may be indicating BMI may not have a direct impact on oxidative stress. The difference in other antioxidant enzymes in both the group was not statistically significant. The HbA1c was marginally higher in subjects with normal BMI than in subjects with BMI more than normal cut off.

Table 4 Comparison of antioxidative status based on body mass index

	MDA(nanomoles/ml)	SOD (Unit/g Hb)	Catalase(Unit/g Hb)	Glutathione peroxidase(Unit/gHb)	HbA1c(%)
Controls(10)	1.45±0.09	1.50±0.05	1.70±0.09	32.93±3.53	5.48 ±0.09
Diabetic with Normal BMI(12)	1.97±0.13	1.31±0.06	0.86±0.08	12.81±0.83	7.6 ±0.17
Diabetic with BMI> 22–30kg/m ² (21)	1.89±0.13	1.00±0.06	0.85±0.04	13.32±0.63	7.54 ±0.12
P value	0.39	0.004	0.87	0.63	0.33

Duration of diabetes: Diabetic subjects were grouped based on duration of diabetic condition into (less than 5years and more than 5years of identification. Plasma malondialdehyde levels were higher in patients with diabetes for more than 5years (2.06± 0.27nanomoles/ml) as compared to group with diabetes for less than 5years

(1.88±0.58nanomoles/ml). Similarly erythrocyte superoxide level, erythrocyte glutathione peroxidase and catalase all have the similar trend of but not significant higher level in diabetes patients with duration more than 5years (Table 5).

Table 5 Comparison of antioxidative status based on Duration of diabetes

	MDA (nanomoles/ml)	SOD(Unit/g Hb)	Catalase(Unit/g Hb)	Glutathione peroxidase(Unit/gHb)	HbA1c(%)
Controls(10)	1.45±0.098	1.50±0.05	1.70±0.09	32.93±3.53	5.480±0.09
Less than 5years(26)	1.88±0.58	1.09±0.31	0.83±0.25	12.73±2.73	7.55±0.11
More than 5years(7)	2.06±0.27	1.2 ±0.28	0.95±0.14	14.59±2.62	7.6 ±0.18
P value	0.45	0.43	0.24	0.12	0.77

Glycemic control: Patients with glycosylated haemoglobin less than 7% (good glycemic control) were compared with patients with haemoglobin more than 7%. Lipid peroxidation was found to be significantly (p=0.03) lower (1.575±0.18nanomoles/ml) in patients with good glycemic control (HbA1c <7.0%) (2.030±0.10nanomoles/ml). There was a marginal difference in HbA1c between patients with

good glycemic control (1.575±0.18nanomoles/ml) as compared to control subjects (1.450±0.09nanomoles/ml). Erythrocyte superoxide dismutase, catalase and glutathione peroxidase levels were marginally low in patients with good glycemic control as compared to patients with glycosylated haemoglobin >7.0% (Table 6).

Table 6 Antioxidative status based on glycemic control in diabetic patients

	MDA(nanomoles/ml)	SOD(Unit/g Hb)	Catalase(Unit/g Hb)	Glutathione peroxidase(Unit/gHb)
Controls (HbA1c <6.5%)	1.45±0.098	1.50±0.05	1.70±0.09	32.93±3.53
HbA1c < 7%(8)	1.57±0.18	1.01±0.06	0.69±0.07	12.62±0.72
HbA1c>7(25)	2.03±0.10*	1.15±0.068	0.91±0.04	13.29±0.61
P value	0.03	0.28	0.02	0.57

Discussion

Oxidative stress results when the generation of free radicals in system exceeds the system's ability to neutralize and eliminate them.¹⁸ Oxidative stress plays a critical role in development of insulin resistance and β - cell dysfunction which are the major mechanisms in the pathogenesis of type 2 diabetes mellitus.¹⁹ Chronic hyperglycemia leads to increased production of reactive oxygen species through various pathways such as glucose oxidation, polyol pathway, Protein kinase C (PKC) activation, formation of advanced glycation end products (AGEs).²⁰ In the present study lipid peroxidation marker plasma malondialdehyde levels were found to be higher in diabetic subjects compared to control subjects indicating increased oxidative stress in diabetes patients. This can be attributed to overproduction of free radicals and dysfunctional enzyme activity observed in diabetes. The susceptibility of erythrocyte membrane to oxidative stress and increased capacity of monocytes to produce superoxide in diabetes are also contributory factors. The results are consistent with both human

and animal studies.^{21,22,23} The activity of erythrocyte superoxide dismutase which forms the first line of defense against free radicals was found to be reduced in diabetes subjects compared to normal in the present study. Similar trend was observed by Song F²⁴ and Bikkad at al.²⁵ Decreased (50%) enzyme activity can be attributed to hyperglycemia induced glycation of enzyme protein²⁶ and increased susceptibility of enzyme to free radical induced inactivation.²⁷ The present results are not in line with findings reported by Godin et al.²³ in which the enzyme activity increased as a compensatory mechanism to combat oxidative stress.

Overproduction of free radicals and possible metabolic interaction

Lowered erythrocyte catalase activity in diabetes subjects in the present study can be related to increased enzymatic glycation. Catalase is one of the regulators of free radical hydrogen peroxide metabolism. Reduced enzyme activity confirms weakened antioxidant

enzyme system in diabetes. Similar finding were reported by Kedziora – Kornatowska KZ.²⁸ Our results are in contrast with findings of Ceriello et al.²⁹ and Selvum et al.³⁰ wherein significantly increased catalase activity in tissues and blood of diabetics was reported compared to controls.

The reduction in erythrocyte glutathione peroxidase activity in subjects with diabetes compared to controls in the present study may be attributed to metabolism of excessive glucose by polyol pathway. This pathway utilises NADPH as a hydrogen donor and decreases the NADPH/ NADP⁺ ratio. Increased sorbitol pathway utilises the NADPH leading to decreased regeneration of reduced glutathione (GSH). Failure in regeneration of glutathione (GSH) weakens the antioxidant defense by glutathione peroxidase and decreases its activity. The decrease in SOD activity may lead to increase level of superoxide radicals which will cause the inactivation of GPx increasing free radical damage.³¹ Similar trend was observed by Ruitz C³² and Komosinska-Vassev K.³³

Gender specificity

Studies which have reported gender specific difference among men and women about diabetes mellitus. Women with diabetes are 4 to 6 folds at risk of developing coronary artery disease (CAD) irrespective of their postmenopausal status as compared to men.³⁴ Women with diabetes have a poorer prognosis after myocardial infarction than do men with diabetes.³⁵ But present study did not establish any gender specific difference in antioxidative status. Plasma malondialdehyde levels were found to be low and antioxidative enzymes were marginally high in males compared to female. These findings can be attributed to lower glycosylated haemoglobin level in male as compared to female subjects. Similar trend was observed for gender specific difference in two studies with T1 diabetes mellitus patients.^{36,37}

Treatment modality

In the present study, the treatment modality for 90% of the patient was oral hypoglycemic drug. Only 10% of the patients received a combination of insulin and oral hypoglycemic agents (OHA). Lower lipid peroxidation and higher antioxidative enzyme level was observed in patients treated with insulin and drugs compared to only OHA. This indicates better glycemic control was achieved when insulin was added as treatment modality in diabetes management as indicated by HbA1c levels. However the number of subjects treated with insulin was limited in the present study. UK prospective diabetes study (UKPDS) also supports that patients treated with a combination of insulin and drug therapy are able to have good glycemic control and maintain the target HbA1c cut offs compared to patients treated only on OHA.³⁸

Body mass index: Obesity is often associated with state of increased systemic oxidative stress. Lipid peroxidation is associated with many obesity indices and low systemic antioxidant enzyme defense mechanisms.³⁹ In the present study normal diabetic subjects had higher plasma Malondialdehyde and lower antioxidant enzymes compared to overweight and obese patients. The present findings indicate that hyperglycemia was an independent determinant of oxidative stress in the present subjects irrespective of their anthropometric measurements. Therefore Glycemic control should be monitored critically to reduce and maintain oxidative stress.

Treatment duration

Oxidative stress was insignificantly higher and antioxidant

enzymes were lower among subjects with duration of diabetes for more than 5 years. As the duration of diabetes increases, chronic hyperglycemia leads to increased generation of free radicals and alterations in the antioxidant defense. However, in both the groups, the mean HbA1c was near the target cut- off (<7%) as per American Dietetic association.⁴⁰ Fair glycemic control can be critical factor in development of lipid peroxidation and further complications of diabetes than the duration of diabetes.

Glycemic control

Glycemic control plays a key role in the development of oxidative stress and further increase the risk of long term complications. The present findings indicate that subjects with good glycemic control had lower oxidative stress as compared to subjects with poor glycemic control. Improved glycemic control reduces hyperglycemia thereby reduced glucose influx in polyol pathway. This leads to increased NADH concentration and increased the activity of antioxidant enzyme glutathione peroxidase. Reduced oxidative stress decreases the glycation and inactivation of superoxide dismutase. Therefore good glycemic control plays a key role in development of complications of diabetes.

Conclusion

Oxidative stress plays an important role in onset of diabetes and diabetes related long term complications. All three antioxidative enzyme system activity has been found to be low in diabetic condition. The generation of reactive oxygen species exceeds in diabetes with alteration in antioxidant enzyme activity. Effect of factors such as gender, body mass index, treatment modality and duration of diabetes on oxidative stress and antioxidant defense system was found to be not independent factors. However fair glycemic control reduces lipid peroxidation and improves the antioxidant defense system. The management of diabetes mainly focuses on synchronisation of insulin and carbohydrate level to maintain glycemic control. However, management of diabetes should also focus on dietary management of antioxidants and other phytochemicals to reduce oxidative stress and delay the onset of complications of diabetes.

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None.

Conflict of interest

Author declares that there is no conflict of interest.

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