

Antioxidant status and lipid peroxidation in type II diabetes mellitus with and without complications

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(Received 18 September/15 December 1995; accepted 20 December 1995)

1. This study was conducted on 467 cases of non-insulin-dependent diabetes mellitus and 180 healthy controls. Lipid peroxidation products in plasma and erythrocytes were assayed as thiobarbituric acid reactive substances, along with the erythrocyte antioxidant enzymes, namely superoxide dismutase, catalase and glutathione peroxidase. In addition, scavenger vitamins A, C and E and reduced glutathione levels in blood were also measured.
2. Lipid peroxidation was significantly raised within the first 2 years of diagnosis, and superoxide dismutase, catalase, reduced glutathione and vitamins C and E were significantly lowered.
3. These changes were correlated with the duration of the disease and were of a higher magnitude with the development of complications.
4. The results suggest that the antioxidant deficiency and excessive peroxide-mediated damage may appear early on in non-insulin-dependent diabetes mellitus, before the development of secondary complications.

INTRODUCTION

An imbalance in the antioxidant protective mechanism leading to oxygen stress in the cells is being identified as a common factor in diabetes mellitus and several other disorders. Toxic oxygen-derived products are generated in all aerobic cells [1] and include superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-), the latter being the most lethal. Superoxide damages DNA [2], inactivates enzymes [3], oxidizes hormones [4] and perturbs membranes [5].

Reactive oxygen species are increasingly formed in diabetes mellitus by the auto-oxidation of glucose and glycosylated proteins [6]. Hyperglycaemia leads to activation of the sorbitol pathway and contributes to the formation of triose phosphate and its auto-oxidation which results in two reactive species, α -oxaldehyde and H_2O_2 [7]. Gerli et al. [8] hypoth-

esized that oxygen free radicals may explain the long-term complications of diabetes mellitus, and Baynes [9] postulated a general pathway involving the sequence of events by which oxidative stress leads to tissue damage. According to this process, incomplete scavenging of reactive radicals leads to oxidation of cellular lipids, proteins, nucleic acids and glycoconjugates. The resulting damage at the cellular level is observed as oxidation, fragmentation and cross-linking. Evidence of lipid peroxidation [10] has been observed in a number of secondary complications in diabetes mellitus [11]. Vitamins A, E and C, carotene and glutathione (GSH) provide antioxidant defences by their ability to exist in reversible oxidized and reduced forms [12, 13]. The enzymes superoxide dismutase (SOD; E.C.1.15.1.1), which detoxifies superoxide radical, and catalase (E.C.1.11.1.6) and glutathione peroxidase (GPx; E.C.1.11.1.9), which act on H_2O_2 and hydroperoxide respectively, serve as the endogenous antioxidants.

The present study was undertaken to assess the lipid peroxidation and antioxidant status in non-insulin-dependent diabetes mellitus (NIDDM) and their correlation to glycaemic control, duration of diabetes and the development of secondary complications. There has not been a detailed study relating the increased oxidative stress and plasma lipid peroxidation in NIDDM with and without complications. This study deals with the role of lipid peroxidation in the development of secondary complications.

MATERIALS AND METHODS

Patients

Blood samples were obtained under fasting conditions from 467 cases of NIDDM attending the Diabetes Research Center and M.V. Diabetes Specialties Center in Madras. Blood glucose control, dietary advice, monitoring and therapeutic measures for complications (if any) were carried out by the

Key words: antioxidant enzymes, antioxidant vitamins, diabetes mellitus, lipid peroxidation

Abbreviations: GPx, glutathione peroxidase; NIDDM, non-insulin-dependent diabetes mellitus; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances

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Table 1. General data of the population studied and their classification. Abbreviations: NEU, neuropathy; RET, retinopathy; CTR, cataract; IHD, ischaemic heart disease. Statistical significance **P* < 0.001 compared with control subjects

Subjects	Healthy controls (n = 180)	NIDDM complications			
		Group 1 (n = 200)	Group 2 (n = 89)	Group 3 (n = 103)	Group 4 (n = 75)
Duration of diabetes					
Range		8 months to 5 years	2 to 13 years	5 to 15 years	11 to 36 years
Mean ± SD		2 ± 1	6 ± 4	9 ± 4	18 ± 2
Age (years)	50 ± 9	43 ± 9	50 ± 9	51 ± 8	55 ± 7
Males (%)	65	72	55	60	60
BMI (kg m ⁻²)	23 ± 5	23 ± 13	26 ± 9	26 ± 8	24 ± 6
Lactovegetarian (%)	57	28	35	40	28
Fasting blood glucose (mmol l)	4.4 ± 0.5	9.5 ± 3.7*	11.0 ± 3.6*	15.3 ± 6.0*	16.5 ± 4.0*
HbA _{1c}	4.6 ± 0.7	9.7 ± 1.7*	10.3 ± 1.6*	12.4 ± 2.3*	13.6 ± 2.6*
Complications	None	None	NEU = 30 IHD = 14 RET = 30	NEU, RET, IHD = 23 IHD, RET = 27 RET, NEU = 24 CTR, IHD = 14 CTR, NEU = 15	Albuminuria = all IHD, RET = 37 NEU = 20 IHD, CTR, NEU, RET = 18

medical and paramedical staff in the centres. Family history, age, height, weight, the nature of any complications and the number of years since the onset of the disease were obtained from the records (Table 1). Criteria for classification were based on the WHO study group report (1985). All patients were under a suitable therapeutic regimen at the time of blood sampling. Informed consent was obtained from all the participants of the study and the protocol was approved by the ethical committee of the centres. The control group comprised 180 healthy volunteers from Madras University's administrative staff and the staff and family members of IGCAR township at Kalpakkam, India. The two groups as a whole were similar in age and sex distribution. They were examined and found to be free from disease.

Retinopathy in the patients was confirmed by fundal examination, the presence of microaneurysms, soft exudates and intra-retinal haemorrhages, and photography. Ischaemic heart disease was established by ECG findings and history of myocardial infarction. Neuropathy was established by the absence of foot pulses, ankle and brachial Doppler pressures and symptoms. Nephropathy was considered to be present if there was proteinuria of 1 g l in the urine samples.

Biochemical parameters assayed

Blood haemoglobin [14], glucose [15] and HbA_{1c} [16] were assayed in blood samples collected with EDTA (1 mg ml) sodium salt as anticoagulant. The remaining blood was used for separating the cells and isolation of cell membrane by the method described earlier [17]. Antioxidant enzymes SOD and GPx were assayed in the haemolysate [18, 19], while catalase was assayed in the membrane [20].

Vitamin A [21] and vitamin E [22] were estimated in the plasma, vitamin C [23] and GSH [24] were assayed in the whole blood. Lipid peroxidation was assayed in plasma and erythrocytes according to the method of Yagi [25] as thiobarbituric acid reactive substances (TBARS).

Statistical analysis

After obtaining the data on 467 patients, it was observed that the deviations in the antioxidant status from normal were not linked to the nature of the complications but to the complexities and the duration of diabetes and glycaemic control assessed by HbA_{1c}; for instance, the antioxidant status in retinopathy did not differ from that observed in ischaemic heart disease. The 467 cases of NIDDM were therefore broadly classified according to the number of complications. Group 1 included 200 cases with no symptoms of any secondary complications, group 2 included 89 cases with a single complication, group 3 included 103 cases with two or more complications without albuminuria and group 4 included 75 cases positive for albuminuria with symptoms of multiple complications. The biochemical parameters are expressed as mean ± SD for each group. Statistically significant deviations in the patients compared with the control subjects were arrived at using analysis of variance.

RESULTS

The general data on the population studied are given in Table 1. The age in the control group is comparable to that in the overall group of NIDDM cases studied. There was no gross obesity in the population studied, as can be seen from their body mass index. It can be seen in Table 1 that there is a

Table 2. Lipid peroxidation in plasma and erythrocytes, and antiperoxidative enzyme activities in diabetes mellitus and healthy control subjects. Values are expressed as mean \pm SD for 'n', number of cases studied. Statistical significance: * $P < 0.001$ in diabetes compared with healthy control subjects.

Subjects	GPx, lysate (μ g GSH utilized min^{-1} mg Hb $^{-1}$)	SOD, lysate (IU mg Hb)	Catalase (μ mol H ₂ O ₂ consumed 10^{12} cells min $^{-1}$)	Plasma TBARS (nmol/dl)	Erythrocyte TBARS (nmol/g Hb)
Healthy controls (n = 180)	5.7 \pm 0.4	3.3 \pm 0.3	4.2 \pm 0.4	187 \pm 30	4.04 \pm 0.3
Group 1 (n = 200)	7.2 \pm 0.4*	2.6 \pm 0.3*	4.01 \pm 0.3*	312 \pm 30*	9.0 \pm 0.1*
Group 2 (n = 89)	8.0 \pm 0.3*	2.6 \pm 0.2*	3.7 \pm 0.5*	332 \pm 20*	10.5 \pm 0.32*
Group 3 (n = 103)	8.6 \pm 0.4*	2.5 \pm 0.2*	3.5 \pm 0.4*	361 \pm 17*	11.23 \pm 0.4*
Group 4 (n = 75)	10.6 \pm 0.5*	1.4 \pm 0.1*	3.2 \pm 0.4*	431 \pm 8*	11.94 \pm 0.7*
Correlation coefficients					
HbA _{1c}	0.4310	-0.3759	-0.4725	0.4282	0.4470
Duration of NIDDM	0.7413	-0.6609	-0.7169	0.6970	0.7211

Table 3. Antioxidant vitamins and GSH levels in NIDDM and healthy control subjects. Values are expressed as mean \pm SD for 'n', number of cases studied. Statistical significance: * $P < 0.001$ in diabetes compared with healthy control subjects.

Subjects	Vitamin A (mmol/l plasma)	Vitamin E (mmol/l plasma)	Vitamin C (mmol/l blood)	GSH (μ mol/l blood)
Healthy controls (n = 180)	2.3 \pm 0.18	24.8 \pm 2.3	60.0 \pm 5.5	54.0 \pm 3.1
Group 1 (n = 164)	2.2 \pm 0.11*	21.1 \pm 1.5*	48.8 \pm 2.3*	48.1 \pm 7.5*
Group 2 (n = 72)	2.1 \pm 0.16*	20.1 \pm 1.1*	41.2 \pm 3.4*	44.2 \pm 7.4*
Group 3 (n = 57)	2.2 \pm 0.15*	19.9 \pm 1.1*	40.2 \pm 4.1*	45.5 \pm 2.2*
Group 4 (n = 34)	2.0 \pm 0.08*	18.7 \pm 1.4*	29.9 \pm 6.3*	39.2 \pm 3.5*
Correlation coefficients				
HbA _{1c}	-0.1226	-0.3677	-0.4806	-0.3478
Duration of NIDDM	-0.2739	-0.3087	-0.5974	-0.4622

progressive increase in the values of HbA_{1c}, in correlation with the duration of diabetes and as the associated number of secondary complications increase. The lower frequency of females in this study group may not necessarily reflect a lower incidence of disease in the female population, but may be due to the social inequalities in Indian society. The incidence of consumption of meat, eggs and fish is higher among those with diabetes mellitus. Blood glucose control (as seen from fasting blood glucose and HbA_{1c} measurements) is poor in the groups with complications.

The erythrocyte antiperoxidative enzyme activities (SOD, catalase and GPx) are shown in Table 2. It can be seen that the activity of GPx is significantly elevated whereas catalase and SOD activities decreased in diabetic subjects within the 2 years of the onset of the disease and the 'r' values show a negative correlation with the duration of the disease. Lipid peroxidation in plasma and erythrocytes is significantly elevated in NIDDM (Table 2) and appears to be associated with the multiplicity of complications and duration of diabetes mellitus. TBARS in plasma and erythrocytes correlate signifi-

cantly with the duration of disease.

Blood levels of antioxidant vitamins are shown in Table 3. Plasma vitamin A levels remain unaffected in NIDDM. The levels of vitamin E (α -tocopherol) and vitamin C are significantly lowered and the lowest levels observed were associated with severe complications including albuminuria. Levels of vitamin C show inverse correlations with both glycaemic control and the duration of diabetes. GSH levels were reduced by 11% in diabetes mellitus without complications and further depletion was seen with the progression of the disease. The lipid peroxidation and antioxidant data are illustrated in Fig. 1 and are reported as the percentage of healthy group.

DISCUSSION

In order to address the correlation between the presence of secondary complications in diabetes and peroxidative damage, the present study was undertaken. This study focuses on the Indian Asian ethnic group for which there are very little data. The other important aspect of this study is the large sample

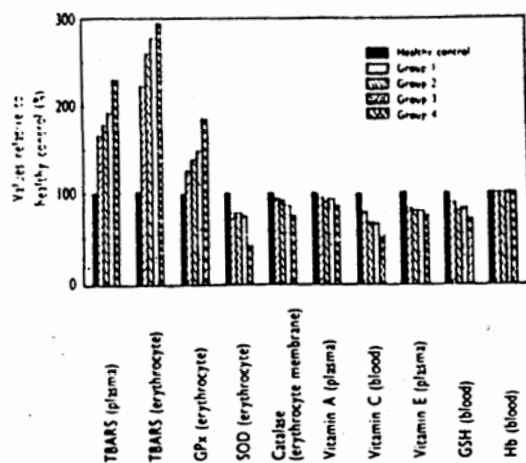


Fig. 1. Lipid peroxidation products (TBARS) in plasma and cells and antioxidant levels in blood in NIDDM. Mean values obtained in the healthy controls are taken as 100%. Group 1, no complications; group 2, with a single complication; group 3, with multiple complications but without albuminuria; group 4, multiple complications with albuminuria. All the parameters assayed with the exception of haemoglobin were statistically significant ($P < 0.001$) compared with controls.

size. Although similar findings have been previously reported [26], the present study is unique in that it reports observations from a large sample size. Glycaemic control (HbA_{1c}) was poor in all the four groups with NIDDM, and the variation within each group was higher with the multiplicity of complications, indicated by the larger SDs (2.3%, and 2.6%, in groups 2 and 3 respectively). Mean HbA_{1c} values in this study of 9.7%, and 10.3%, for groups 1 and 2 respectively were comparable to values of 8.64%, reported by Perriello [27] in Italy, 9.0%, by Groop et al. [28] in Finland and 8.1%, by Lyons et al. [29] in South Carolina.

Plasma TBARS were elevated by 80% in the early stages of diabetes (Table 2 and Fig. 1), with time-dependent progressive increases. Plasma TBARS correlate with the duration of NIDDM. This is an important finding that has not been reported in previous studies and justifies the aim of the present study. Excessive lipid peroxidation in the plasma and cells can arise due to factors favouring the formation of reactive oxygen species. In poorly controlled diabetes mellitus, glucose oxidation through the pentose phosphate pathway leads to the excessive formation of NADPH, which in turn can promote lipid peroxidation in the presence of the cytochrome P-450 system [30]. Oxyhaemoglobin in erythrocytes could act like cytochrome P-450 in the presence of NADPH and this could induce increased lipid peroxidation [31]. Alternatively, inactivation or inhibition of antioxidant enzymes by glycosylation in poorly controlled diabetes mellitus may give rise to increased lipid peroxidation. Evi-

dence of lipid peroxidation has also been observed in a number of diabetic complications [32], i.e. retinopathy, cataract and atherosclerosis. However, it may be noted that high glucose in the circulation may interfere with the assay used for the assessment of lipid peroxidation products. Thus the higher TBARS observed in NIDDM is not solely the result of peroxidative damage.

The excessive peroxidation is also associated with reduced SOD activity (Table 2), vitamin E, C and GSH levels (Table 3). The antiperoxidative enzyme GPx is elevated in NIDDM. There is a positive correlation between the duration of diabetes mellitus and GPx ($r=0.74$) and a negative correlation with catalase ($r=-0.72$). Blood glucose control alone cannot normalize SOD activity in NIDDM. This was shown by Crouch et al. [33] who observed that SOD activity did not reach normal levels by insulin therapy and glycaemic control, while dietary glutathione supplementation normalized SOD levels, but had no effect on blood glucose. Loss of SOD activity in the erythrocytes appears to be a function of the duration of diabetes ($r=-0.67$). A negative correlation was also reported by Hayakawa and Kuzuya [34] between SOD and HbA_{1c} . SOD is inhibited by glycosylation and is lowered in poorly controlled diabetes mellitus. Due to the absence of protein synthesizing machinery in the erythrocytes, the inactivation of SOD by glycosylation may be a dominant factor in the loss of SOD activity observed. Conflicting reports on the levels of GPx have appeared in the literature. Hagglof et al. [35] reported a decrease in GPx in IDDM, while Dohi et al. [36] reported an increase in GPx in the kidney of diabetic rats. Earlier workers have reported an increase in the activity of GPx in the haemolysate and plasma in NIDDM [37, 38]. Erythrocyte GPx is a selenium-dependent enzyme and Gebre-Medhin et al. [39] observed increased levels of plasma selenium in diabetic children. Underwood [40] reported that the selenium levels in tissues are dependent on their intake and a deficiency resulted in lowered GPx activity. It would appear that as an adaptive process of combating excessive peroxidative damage, diabetic tissue retains selenium and thereby increases GPx activity in the cells. However, a selenium turnover study is required to confirm whether a compensatory mechanism sets in for saving tissue selenium levels in diabetes. Dietary factors other than selenium intake also affect GPx activity, as observed with the intake of polyunsaturated fatty acid [41]. Dietary deficiencies of copper and zinc also lead to lipid peroxidation [42]. Zinc deficiency resulted in increased H_2O_2 generation, and in diabetes mellitus it is essential to avoid zinc and other nutrient deficiencies [43]. Both GPx and catalase detoxify intracellular peroxide. Studies on streptozotocin diabetes and diet-restricted rats indicated an inverse relationship between hypoinsulinaemia and tissue antioxidant enzyme levels, and this condition could be reversed

by insulin administration [44]. The increase in the activity of GPx in diabetes mellitus may be an adaptive or compensatory mechanism developed to deal with the increased generation of free radicals. In diabetes mellitus with secondary complications the system is geared to produce more GPx, and in our study we report that the activity of GPx is increased by 25% in NIDDM without complications and by 85% in NIDDM with multiple complications, when compared with the controls.

The pattern of changes in the antioxidant scavenger levels in blood is interesting. Maximum reduction was seen in vitamin C (50%), while a 25% reduction was observed in the endogenous scavenger GSH (Table 3 and Fig. 1). Vitamin E levels were slowly reduced with duration of diabetes mellitus, while vitamin A levels showed little alteration. Correlation coefficients for vitamins A and E (Table 3) were below -0.37, suggesting that blood levels of the vitamin do not vary in relation to either glycaemic control or duration of the disease. Reduced levels and altered metabolic turnover of ascorbic acid have been reported in several tissues in experimentally induced diabetes [45] and in human diabetes [46]. Sinclair et al. [47] reported reduced concentrations of ascorbic acid and increased oxidative stress in NIDDM (as evidenced by dehydroascorbic acid:ascorbic acid ratios) compared with the controls. Vitamin C has a sparing effect on vitamin E by regenerating the active form of the vitamin [48].

We have observed a progressive decline in the GSH content of erythrocytes with the duration of diabetes mellitus (Table 3). Meister [49] reviewed the metabolism of glutathione and revealed that there was more than one pool of GSH and fluctuations of GSH measured were much more complex than a simple cause and effect relationship. Liver was the major source of extracellular GSH [50], and a sinusoidal carrier-mediated transport of GSH from liver was essential for GSH homeostasis [51]. Starvation leads to a substantial reduction in hepatic GSH in a number of experimental animals [52]. In starvation, hepatic GSH levels were depleted, but parallel reductions were not seen in the heart, lung, intestine or in the erythrocytes [53, 54]. Reduction in the proportion of reduced glutathione by oxidized glutathione led to the characteristic functional imbalances in the glucose-stimulated insulin response in pancreatic islets [55]. The data provided by these authors suggested that alterations in the redox state of thiols in the islet cells, resulting from antioxidant deficiency or reactive oxygen species induced by diabetogenic agents, may lead to reduction in insulin secretion.

In the 467 cases of NIDDM studied, there is evidence for increased levels of circulating reactive oxygen species (as seen by increased peroxidation). Vitamin C, GSH, SOD and catalase deficiencies and increased lipid peroxidation are manifested within 2 years of the detection of NIDDM (Fig. 1). It will be

interesting to assess the antioxidant status in patients susceptible to NIDDM to verify whether antioxidant deficiency is a predisposing factor for the development of glucose intolerance.

Therapeutic measures to increase antioxidants and control lipid peroxidation are warranted for effective control of secondary complications. From the observations reported it may be concluded that antioxidant deficiency and excessive peroxidative damage appear very early on in NIDDM, well before the development of secondary complications.

ACKNOWLEDGMENT

R.K.S. and A.B. acknowledge the graduate assistantships from Lady Tata Memorial Trust and Birla Smarak Kosh respectively

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