LIPID PEROXIDATION AND ANTIOXIDANT ENZYME LEVELS IN TYPE 2 DIABETICS WITH MICROVASCULAR COMPLICATIONS

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SUMMARY - Serum levels of total cholesterol, triglycerides, lipoproteins, lipid peroxides (TBARS) and erythrocyte antioxidant enzyme activities were measured in 105 non insulin dependent diabetic patients, among whom 38 had microvascular complications (MVC) of diabetes. All the diabetic patients had higher concentrations of glycated hemoglobin (HbA1) compared to controls (10.51 ± 2.42% vs 6.31 ± 0.85% P < 0.001). Significant increase of serum triglycerides (TG), total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) and a significant decrease of high density lipoprotein cholesterol (HDL-C) were observed in the diabetic patients compared to controls (TG: 2.31 ± 0.9 mmol/l vs 1.53 ± 0.48 mmol/l P < 0.001; TC: 5.94 ± 1.4 mmol/l vs 4.3 ± 0.85 mmol/l P < 0.001; LDL-C: 3.96 ± 1.33 mmol/l vs 2.39 ± 0.8 mmol/l P < 0.001; VLDL-C: 0.46 ± 0.2 mmol/l vs 0.3 ± 0.09 mmol/l P < 0.001). TBARS concentrations were significantly increased in the diabetic group compared to those in controls (TBARS: 8.8 U/g Hb/min vs 2.55 ± 0.18 mmol/l P < 0.001). Erythrocyte catalase (CAT) activity was increased and Glutathione peroxidase (GPx) activity was decreased in diabetic patients compared to controls, but no significant change in Superoxide dismutase (SOD) activity was observed in diabetic patients (CAT: 104.94 ± 37.1 KU/g Hb vs 85.8 ± 23.6 KU/g Hb, P < 0.01; GPx: 30.7 ± 9.7 U/g Hb/min vs 40.84 ± 12.3 U/g Hb/min, P < 0.001; SOD: 2.4 ± 1.2 U/mg Hb/min vs 2.55 ± 0.84 U/mg Hb/min, P < NS). In comparison with the diabetic group without MVC, the diabetic group with MVC had decreased GPx and SOD activities, while no difference was observed between these two groups regarding CAT activity (CAT: 106.3 ± 39.9 KU/g Hb vs 103 ± 34.9 KU/g Hb, P = NS). TBARS concentrations were significantly increased in the group with MVC compared to the group without these complications, indicating a positive relationship between TBARS and MVC of diabetes (7.05 ± 1.23 mmol/l vs 6.3 ± 1.02 mmol/l, P < 0.001). Serum triglycerides, LDL and VLDL cholesterol concentration were significantly higher in diabetics with MVC than in diabetics without the complications (TG: 2.7 ± 0.98 mmol/l vs 2.13 ± 0.82 mmol/l, P < 0.01; LDL-C: 4.45 ± 1.3 mmol/l vs 3.67 ± 1.3 mmol/l, P < 0.02; VLDL-C: 0.53 ± 0.19 mmol/l vs 0.43 ± 0.16 mmol/l, P < 0.01), and the serum levels of TC in the group with MVC showed a positive correlation with their lipid peroxide levels (r = 0.368, P < 0.001). The increase in TBARS and the decreased GPx and SOD activities in diabetics with MVC in this study indicate that these factors may contribute to the occurrence of micro vascular complications in NIDDM patients.

Key-words: non Insulin Dependent Diabetes Mellitus (NIDDM), thio Barbicatic acid reactive substances (TBARS), antioxidant enzymes, catalase, glutathione peroxidase, superoxide dismutase.

RÉSUMÉ - Peroxydation lipidique et statut des enzymes antioxydantes chez des diabétiques de type 2 avec microangiopathie. Les taux sériques de cholestérol total, triglycérides, lipoprotéines, peroxydases lipidiques (TBARS) et les activités enzymatiques antioxydantes érythrocytaires ont été mesurées chez 105 diabétiques non insulino-dépendants, parmi lesquels 38 étaient atteints de microangiopathie. Tous les patients présentaient des taux d'hémoglobine glyquée (HbA1) plus élevés que les témoins (10.51 ± 2.42 % vs 6.31 ± 0.85 % P < 0.001). Une augmentation significative des triglycérides (TG), du cholestérol total (TC) du cholestérol LDL (LDL-C) et du cholestérol VLDL (VLDL-C) et une diminution significative du cholestérol HDL (HDL-C) étaient observées chez les diabétiques par rapport aux témoins (TG: 2.31 ± 0.9 mmol/l vs 1.53 ± 0.8 mmol/l P < 0.001; TC: 5.94 ± 1.4 mmol/l vs 4.3 ± 0.85 mmol/l P < 0.001; LDL-C: 3.96 ± 1.33 mmol/l vs 2.39 ± 0.8 mmol/l P < 0.001; VLDL-C: 0.46 ± 0.2 mmol/l vs 0.3 ± 0.09 mmol/l P < 0.001; HDL-C: 8.8 U/g Hb/min vs 5.14 ± 0.61 mmol/l P < 0.001). Une élévation significative des taux de TBARS sériques était observée chez les diabétiques par rapport aux témoins (TBARS: 8.8 U/g Hb/min vs 5.14 ± 0.61 mmol/l P < 0.001). L’activité catalase érythrocytaires (CAT) était augmentée et l’activité glutathion peroxidase (GPx) était diminuée chez les patients diabétiques par rapport aux témoins, tandis que l’activité superoxide dismutase (SOD) n’était pas significativement modifiée (CAT: 104.94 ± 37.1 KU/g Hb vs 85.8 ± 23.6 KU/g Hb, P < 0.01; GPx: 30.7 ± 9.7 U/g Hb/min vs 40.84 ± 12.3 U/g Hb/min, P < 0.001; SOD: 2.4 ± 1.2 U/mg Hb/min vs 2.55 ± 0.84 U/mg Hb/min, P < NS). Par comparaison aux diabétiques sans microangiopathie, les patients avec microangiopathie présentaient des activités GPx et SOD diminuées, tandis qu’aucune différence n’était notée entre les deux groupes pour l’activité CAT (CAT: 106.3 ± 39.9 KU/g Hb vs 103 ± 34.9 KU/g Hb, P = NS). Les concentrations de TBARS étaient significativement augmentées dans le groupe avec microangiopathie par rapport au groupe indemne de microangiopathie, indiquant une relation positive entre les TBARS et la microangiopathie (7.05 ± 1.23 mmol/l vs 6.3 ± 1.02 mmol/l P < 0.001). Les taux sériques de triglycérides, LDL et VLDL cholestérol étaient significativement plus élevés chez les diabétiques avec microangiopathie que chez les patients sans complications (TG: 2.7 ± 0.98 mmol/l vs 2.13 ± 0.82 mmol/l, P < 0.01; LDL-C: 4.45 ± 1.3 mmol/l vs 3.67 ± 1.3 mmol/l, P < 0.02; VLDL-C: 0.53 ± 0.19 mmol/l vs 0.43 ± 0.16 mmol/l, P < 0.01), et la relation de TC dans le groupe avec microangiopathie étaient positivement corrélés avec les peroxydiques lipidiques (r = 0.368, P < 0.001). L’augmentation des TBARS et la diminution des activités GPx et SOD chez les diabétiques avec microangiopathie suggèrent que ces facteurs pourraient contribuer au développement des complications microvasculaires du diabète de type 2.

Mots-clés : diabète non insulino-dépendant, thio Barbicatic acid reactive substances (TBARS), enzymes antioxydantes, catalase, glutathion peroxidase, superoxide dismutase.
Diabetes mellitus has been shown to be a state of increased free radical formation. The increased production of reactive oxygen species (ROS) has been attributed to protein glycation [1, 2] and (or) glucose auto-oxidation due to a hyperglycemic environment [3]. An impaired radical scavenger function has been linked to altered activity of enzymatic and non enzymatic free radical scavengers [4].

Lipid peroxidation of cellular structures, a free radical-induced activity, is thought to play an important role in ageing, atherosclerosis and late complications of diabetes mellitus. The elevated levels of Poly Unsaturated Fatty Acids (PUFAs) in RBC membranes of NIDDM increase lipid peroxidation of RBC membranes, due to an excessive production of ROS and decreased levels of glutathione [5]. By-products of lipid peroxidation such as conjugated dienes and TBARS are increased in RBC membranes of diabetic animals [6, 7]. There are reports of increased serum conjugated dienes in elderly diabetic patients with complications [8] and increased lipid peroxides in blood plasma of type 2 diabetic patients with macro vascular complications [9] and micro vascular complications [10]. The reports about the status of antioxidants and antioxidant enzymes in diabetic patients are very contradictory, both increases and decreases of antioxidant activity have been reported [7, 11-13].

Most of the studies on the relationship between lipid peroxidation and vascular complications of diabetes involved diabetics with macrovascular complications [9, 14-16], and there are very few reports on the role of free radical-induced lipid peroxidation and alterations in the activities of antioxidant enzymes in microvascular complications of diabetes [10, 17]. Hence the present study was undertaken to assess the relationship between the lipid peroxidation, antioxidant enzymes and occurrence of micro vascular complications in NIDDM patients.

**PATIENTS AND METHODS**

The study group consisted of 105 patients with type II (non insulin dependent) diabetes mellitus, among whom 38 patients had microvascular complications (MVC) such as diabetic retinopathy and nephropathy. Diabetic retinopathy was diagnosed by an ophthalmologist by fluorescein angiography and color fundoscopy and diabetic nephropathy was diagnosed on the basis of persistent proteinuria (>0.5 g/24h) and elevated levels of blood urea and creatinine. Diabetics who had macro vascular complications such as coronary heart diseases and peripheral vascular diseases along with MVC were excluded from the study. All the diabetic patients selected for this study were non alcoholics, non smokers and were on irregular treatment for diabetes, and none of the study subjects were on antioxidant therapy or on lipid lowering drugs. Forty one age- and body weight-matched non smoking, non alcoholic healthy individuals with no family history of diabetes were studied in parallel as a control group. Informed consent was obtained from all the study subjects and the study was performed according to the local medical ethical guidelines. The clinical details of the study subjects are given in Table I.

The diabetic patients were divided into two groups according to the existence or not of the microvascular complications of diabetes.

Fasting venous blood samples were collected from all the patients and control subjects, for the measurement of blood glucose, lipids, lipoproteins, lipid peroxides and erythrocyte antioxidant enzyme (Catalase, Glutathione peroxidase and Superoxide dismutase) activities. Blood glucose, serum Triglycerides (TG), total cholesterol (TC) and HDL cholesterol levels were measured by using the standard methods [18-21], LDL-cholesterol and VLDL cholesterol were calculated by using Friedewald formula [22]. Glycemic control was assessed by measuring glycated haemoglobin (HbA1), by the procedure of Eross et al., [23].

<table>
<thead>
<tr>
<th>TABLE I. Clinical details of the study subjects.</th>
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<tbody>
<tr>
<td><strong>Non diabetic controls</strong></td>
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<tr>
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</tr>
<tr>
<td>Number (n)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
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<tr>
<td>Age (years)</td>
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<tr>
<td>BMI (Kg/m²)</td>
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<td>Duration of diabetes (Years)</td>
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<td>Fasting blood glucose (mmol/l)</td>
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**P < 0.05 compared to diabetic patients without MVC.**

**P < 0.001 compared to non diabetic controls.**
Plasma levels of Malondialdehyde (MDA), a marker of lipid peroxidation was measured as Thiobarbituric acid reactive substances (TBARS) by the method of Yagi et al., [24] modified by Griesmacher et al., [16]. The percentage of recovery of MDA was 96.4%. The inter and intra assay of coefficient of variation of this method were 5.2% and 3.5%, respectively. In this method, addition of glucose up to 15 mM did not significantly influence the TBARS levels.

Erythrocyte catalase (CAT) activity was measured by the method of Aebi [25], which is based on the decomposition of H₂O₂ by catalase. The decrease in absorbance at 240 nm was measured at 20°C and CAT activity was expressed as K units per gram of Haemoglobin. Glutathione peroxidase (GPx) activity in erythrocytes was measured according to the method of Wendel [26] and Superoxide dismutase (SOD) activity in the haemoglobin free supernatant was measured by Misra and Fridovich method [27]. Haemoglobin free supernatant was prepared by the method of Concetti et al., [28].

Statistical analysis – Results are expressed as mean ± Standard deviation, differences between groups were considered significant when the p value determined by the unpaired Student’s ‘t’ test was less than 0.05. Pearson correlation analysis was used to test the correlation between various parameters and considered significant when p value was less than 0.05.

RESULTS

Laboratory data for controls and diabetic patients are given in Table II. The HbA1 levels of diabetic patients were significantly higher than those of the controls indicating a poor control of diabetes. The HbA1 levels were significantly higher in diabetic group with MVC than those without complications. All the diabetic patients had significantly higher concentrations of TG, TC, LDL and VLDL cholesterol and lower concentration of HDL cholesterol than in control subjects. Diabetic patients with MVC had higher levels of TG, VLDL and LDL cholesterol than the other group of diabetic patients. Significantly increased levels of lipid peroxides (TBARS) were observed in the diabetic patients compared to controls. The TBARS levels were significantly higher in the diabetic patients with MVC than in diabetics without these complications (Table III).

Correlation analysis revealed a significant positive correlation between TBARS and HbA1 in the diabetic patients (r = 0.42, P < 0.001). A significant positive correlation was also observed between TC and TBARS in all the diabetic subjects (r = 0.368, P < 0.001).

Table IV shows antioxidant enzymes status in all the study subjects. Among the erythrocyte antioxidant enzymes, catalase activity was significantly increased in all the diabetics compared to controls. But there was no significant difference in the catalase activity between the two groups of diabetic patients.

GPx activity was significantly decreased in the diabetic patients compared to controls, and a further decrease in GPx activity was observed in the diabetics with MVC compared to those without the complications.

Table II. Biochemical parameters in the study subjects.

<table>
<thead>
<tr>
<th></th>
<th>Non diabetic controls</th>
<th>Diabetic patients</th>
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<tr>
<td>Hb A1 (%)</td>
<td>6.31 ± 0.85</td>
<td>10.51 ± 2.42*</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.3 ± 0.85</td>
<td>5.94 ± 1.4*</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.04 ± 0.18</td>
<td>0.81 ± 0.24*</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>2.39 ± 0.8</td>
<td>3.96 ± 1.33*</td>
</tr>
<tr>
<td>VLDL-cholesterol (mmol/l)</td>
<td>0.3 ± 0.09</td>
<td>0.46 ± 0.2*</td>
</tr>
<tr>
<td>Triglycerides (µmol/l)</td>
<td>1.53 ± 0.48</td>
<td>2.31 ± 0.9*</td>
</tr>
<tr>
<td>Lipid peroxides (µmol/l)</td>
<td>5.14 ± 0.61</td>
<td>6.7 ± 1.5*</td>
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</table>

*P < 0.001 compared to non diabetic controls.

Table III. Hb A₁ and levels of serum lipids, lipoproteins and lipid peroxides in the two groups of diabetic patients.

<table>
<thead>
<tr>
<th></th>
<th>Diabetics with MVC</th>
<th>Diabetics without MVC</th>
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<tbody>
<tr>
<td>Hb A₁ (%)</td>
<td>11.63 ± 2.3*</td>
<td>9.74 ± 2.1</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>6.3 ± 1.3</td>
<td>5.76 ± 1.41</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>0.84 ± 0.2</td>
<td>0.79 ± 0.26</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>4.45 ± 1.3***</td>
<td>3.67 ± 1.3</td>
</tr>
<tr>
<td>VLDL-cholesterol (mmol/l)</td>
<td>0.53 ± 0.19**</td>
<td>0.43 ± 0.16</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.7 ± 0.98**</td>
<td>2.13 ± 0.82</td>
</tr>
<tr>
<td>Lipid peroxides (µmol/l)</td>
<td>7.05 ± 1.23*</td>
<td>6.3 ± 1.02</td>
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</table>

* P < 0.001 compared to diabetics without MVC,** P < 0.01 compared to diabetics without MVC,*** P < 0.02 compared to diabetics without MVC.
Table IV. Antioxidant enzyme activities in diabetics, diabetics with MVC, without MVC and Non diabetic controls.

<table>
<thead>
<tr>
<th></th>
<th>Catalase (KU/g Hb)</th>
<th>Glutathione peroxidase (U/g Hb/min)</th>
<th>Superoxide dismutase (U/mg Hb/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non diabetic controls</td>
<td>85.8 ± 23.6</td>
<td>40.84 ± 12.3</td>
<td>2.55 ± 0.84</td>
</tr>
<tr>
<td>Diabetic patients</td>
<td>104.94 ± 37.1**</td>
<td>30 ± 9.7*</td>
<td>2.4 ± 1.2</td>
</tr>
<tr>
<td>Diabetics with MVC</td>
<td>106.3 ± 39.9</td>
<td>25.32 ± 8.4 †</td>
<td>1.83 ± 0.53 †</td>
</tr>
<tr>
<td>Diabetics without MVC</td>
<td>103.0 ± 34.9</td>
<td>34.5 ± 8.8</td>
<td>2.84 ± 1.4</td>
</tr>
</tbody>
</table>

* P < 0.001 compared to non diabetic controls. ** P < 0.01 compared to non diabetic controls. † P < 0.001 compared to diabetics without MVC.

The SOD activity in diabetic patients was not much different from that of non diabetic controls, but a significant decrease in SOD activity was observed in diabetic patients with MVC compared to those without these complications.

** DISCUSSION **

Diabetes mellitus, which is associated with increased free radical activity [1, 2] leads to a higher incidence of atherosclerotic and cardiovascular diseases [29]. Lipid peroxides are thought to be formed by free radicals and may play an important role in the development of atheromatous vascular disease. Several authors have reported increased levels of lipid peroxides in diabetic patients [8, 11, 16], while a few could not find any significant increase in lipid peroxidation in the diabetics [9, 30]. In the present study, serum levels of TBARS were significantly increased in all the type II diabetic patients, when compared with controls.

Increased non enzymatic and auto-oxidative glycosylation is one of the possible mechanisms that contributes to the formation of free radicals and free radical-induced lipid peroxidation in diabetes mellitus [2, 3]. Earlier studies on the relationship between the lipid peroxides and glycosylated protein have yielded conflicting results. A few workers have shown a positive correlation between TBARS concentration and the measures of blood glucose control such as HbA1 and fructosamine [31, 32], while several others could not [16, 33]. In this study, we have found a positive correlation between the TBARS concentration and HbA1 levels in diabetics.

Various studies in the past reported conflicting results regarding total and LDL cholesterol levels in NIDDM patients, some reporting elevated LDL cholesterol levels [34, 35] while several others [36, 37] could not find a significant increase in the total and LDL cholesterol levels in diabetics. But in this study we have observed significantly increased levels of total cholesterol and LDL cholesterol levels in the diabetics compared to controls, and among the diabetics, those with micro vascular complications had higher LDL and TG levels compared to those without these complications.

A few studies [16, 31] have reported a positive correlation between cholesterol, triglycerides and TBARS while others [38, 39] could not detect any correlation between TBARS and lipid levels. In this study there was a positive correlation between total cholesterol and TBARS in the diabetic patients while the TG did not correlate with TBARS levels.

Gallou et al., [40] suggested that TBARS appear to be an independent marker of diabetes-induced vascular disease in patients with Type 2 diabetes. Several authors [9, 10, 14-16, 31, 40] have reported increased levels of lipid peroxides in diabetic patients with vascular diseases. In the present study, we have also observed increased TBARS levels in all the diabetics and a much higher increase in diabetics with MVC. The hyperglycemia in association with hyperlipidemia observed in the diabetic patients could be the causative factors for the increased production of OFR’s and lipid peroxides as the TBARS positively correlated with HbA1 and total cholesterol. Nacitarhan et al., [39] also reported the same observation in their study.

The status of antioxidant enzymes in diabetic patients is controversial [7, 11, 12, 41]. A few authors [7, 17, 41] have shown an increased activity of membrane-bound catalase in the diabetic erythrocytes, while others [5, 42] could not detect any change in erythrocyte catalase activity in the diabetic patients. In the present study there was a significant increase in catalase activity in all the diabetic patients. The increased catalase activity in diabetics is an indication of the increased production of peroxide radicals.

A few authors have reported increased GPx activity in RBC of type 2 diabetics [13, 17] but some others have reported decreased GPx activity [41, 43]. In this study, erythrocyte GPx activity was decreased in the diabetic patients compared to controls, and a further decrease in erythrocyte GPx activity was observed in diabetic patients with MVC.
There are reports of both increased [41] and decreased [44] activity of SOD in diabetic patients. However in our study, we did not observe any change in SOD activity in diabetic patients compared to controls. The same has also been observed by Peuchant et al., [5]. The SOD activity was significantly decreased in diabetics with MVC compared to those without these complications.

Non enzymatic glycation of SOD makes the enzyme inactive [45]. The poor glycemic control observed in diabetic patients with MVC in this study may be the reason for the decreased activity of SOD in these patients. In the present study we have also observed no change in SOD activity in diabetic patients without MVC compared to controls, eventhough there was an increase in free radical-induced lipid peroxidation. This could be due to the antioxidant property of glucose, which acts as an antioxidant at higher levels and can scavenge superoxide ions [46]. So there is no need for SOD to increase its activity. But it is not known when and in which circumstances glucose behaves as an oxidant or as an antioxidant.

The decreased GPx activity in diabetic patients with MVC could be due to decreased activity of SOD, which is required for scavenging superoxide radicals. Decreased activity of SOD leads to increased levels of superoxide radicals, which will cause inhibition of GPx [47].

**CONCLUSIONS**

It is very clear from this study that there are abnormalities in the lipoproteins, lipid peroxides levels and antioxidant enzyme activities in the diabetic patients. The increased lipid peroxidation in diabetics could be due to hyperglycaemia, hyperlipidaemia and decreased antioxidant enzyme activities. The increase in TBARS levels inspite of increased CAT activity in diabetes could be due to increased production of Oxygen free radicals (OFRs) in excess of the capacity of CAT to scavenge them. In addition to this there was no correspondence increase in GPx and SOD activities to scavenge the excess OFR’s in diabetes. It appears from this study that lipid peroxides may play a role in the pathogenesis of MVC of diabetes as evidenced by the higher levels of LPO in the diabetics with microvascular complications.

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**REFERENCES**