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Oxidant-antioxidant status, high sensitive C-reactive protein and homo cysteine levels in type 2 diabetic patients with and without microalbuminuria

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ABSTRACT

High levels of high sensitive C-reactive protein (hs-CRP), homocysteine (Hcy) and oxidative stress are known to be associated with premature vascular disease in type 2 diabetes mellitus (DM). The aim of this study was to estimate homocysteine levels and oxidant-antioxidant status and to determine the relationship between them in type 2 diabetic patients with and without microalbuminuria. The study population consisted of 75 subjects [age and sex-matched] divided into three groups. Fasting blood samples were obtained from 50 diabetic patients (25 with and 25 without microalbuminuria) and 25 healthy subjects. The level of blood glucose, HbA1C, urea, creatnine and serum lipids were significantly higher in the patients with microalbuminuria compared with patients without microalbuminuria and control subjects, comparatively higher level in patients with microalbuminuria. On the other hand HDL-C was found to be significantly lower in patients with microalbuminuria. The level of hs-CRP and Hcy levels were found to be significantly higher in patients with microalbuminuria compared with patients without microalbuminuria and healthy controls. The level of plasma thiobarbituric acid reactive substances (TBARS) was markedly increased and the level of enzymic and non-enzymic antioxidant was significantly decreased in the patients with microalbuminuria compared with patients without microalbuminuria and control subjects. The present study highlights the occurrence of lipid peroxidation and possible breakdown of antioxidant status in patients with microalbuminuria. Decreased antioxidant levels, increased lipid peroxidation and increased hs-CRP and Hcy levels were observed in patients with microalbuminuria.

1. Introduction

Diabetes mellitus is a serious and costly disease with micro and macrovascular complications representing the leading causes of morbidity and mortality associated with the condition. According to the World Health Organization, diabetes affects more than 170 million people worldwide, and this number will rise to 370 million by 2030 [1]. About one third of those affected will eventually have progressive deterioration of renal function. Among these complications, diabetic nephropathy affects 40% of type 1 and 10% of type 2 diabetic patients [2]. The first clinical sign of renal dysfunction in patients with diabetes is generally microalbuminuria (a sign of endothelial dysfunction that is not necessarily confined to the kidney) that develops in 2 to 5 percent of patients per year. Patients with diabetes mellitus are at high risk of cardiovascular disease and the risk is further increased when they are complicated with nephropathy [3].

High plasma or serum total homocysteine (tHcy) concentration is a risk factor for atherothrombotic diseases, which has recently come under increased scrutiny [4]. A recent prospective study showed that the plasma tHcy concentration was a significant predictor of six- year all-cause mortality risk, both in patients with normoalbuminuria and those with microalbuminuria. It is possible that alterations in free radical activity and hyperhomocysteinaemia may be important in the pathogenesis and high prevalence of cardiovascular disease in microalbuminuric type 2 DM [5]. There has been no previous detailed study relating the plasma lipid peroxidation (i.e. malondialdehyde [MDA] production) and homocysteine in type 2 DM with and without microalbuminuria [6,7].

Formation of lipid peroxides by the action of free radicals on unsaturated fatty acids has been implicated in the pathogenesis of atherosclerosis and vascular diseases [8]. Diabetic patients have an increased incidence of vascular disease, and it has been suggested that free radical activity is increased in diabetes. Mechanisms that contribute to increased oxidative stress in diabetes may include not only increased non-enzymatic

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glycosylation and autoxidative glycosylation but also metabolic stress resulting from changes in energy metabolism, alterations in sorbitol pathway, changes in the level of inflammatory mediators, the status of antioxidant defense systems and localized tissue damage results from hypoxia and ischemic reperfusion injury. Increased levels of the products of oxidative damage to lipids have been detected in serum of diabetic patients, and their presence correlates with the development of complications [9,10].

The most important free radicals that cause oxidative stress are superoxide, hydroxyl radical and hydrogen peroxide. In human erythrocytes there are antioxidant enzymes together with cytoplasmic radical scavengers directed against free radicals in order to protect erythrocytes. One of the cytoplasmic radical scavengers that can reduce free radicals is reduced glutathione (GSH). Glutathione peroxidase (GPx) catalyses the reduction of peroxide. It is generally believed that the protective effect of GSH against the oxidative breakdown of lipids is mediated through GPx by the reduction of endogenously formed hydroperoxides of unsaturated fatty acids to hydroxyl derivatives. Although the pathogenic mechanism of vascular complications in type 2 DM is very complex, free radical reactions induced by reactive oxygen species are thought to be one of the possible factors involved [10]. The increased risk of cardiovascular disease in individuals with microalbuminuria is only partly due to a higher prevalence of established risk factors such as DM. The pathophysiological basis of the association between microalbuminuria and underlying generalized vascular injury may be endothelial dysfunction [11].

Increased levels of the products of oxidative damage to lipids have been detected in serum of diabetic patients, and their presence correlates with the development of complications. Hence the present study was undertaken to assess the extent of lipids, hCRP, Hcy, lipid peroxidation and the status of the antioxidant defense system in patients with type 2 diabetes with and without microalbuminuria compared with normal healthy subjects.

2. Materials and Methods

2.1. Study Population

The study population consisted of 75 subjects [age and sex-matched] divided into three groups. Fasting blood samples were obtained from 50 diabetic patients (25 with and 25 without microalbuminuria) and 25 healthy subjects were investigated. The prospective study was carried out at the K.G. Hospital and Post Graduate Medical Institute, Coimbatore, Tamil Nadu, India from June 2008 to January 2010. General health characteristics such as age, sex, smoking status, menopausal status, alcohol consumption, and dietary habits, particularly as related to preference were investigated by a self-administered questionnaire.

Blood samples were collected by venous puncture in heparinized tubes and the plasma was separated by centrifugation at 1000 g for 15 min. After the collection of plasma, the buffy coat was removed and the packed cells were washed thrice with cold physiological saline. A known volume of the erythrocytes was lysed with hypotonic phosphate buffer (pH 7.4). The hemolysate was separated by centrifugation at 2,500 g for 10 min at 2°C. Biochemical estimations were carried out immediately.

2.2. Biochemical investigation

Biochemical including blood glucose, HbA1C, hs-CRP, urea, creatnine, total protein, albumin, total cholesterol, triglyceride, HDL-C and LDL-C were determined using fully automated clinical chemistry analyzer (Hitachi 912, Boehringer Mannheim, Germany). Serum VLDL-C was calculated according to Friedewald et al [12]. Determination of plasma homocysteine was carried out using a kit based on fluorescence polarization immunoassay (Abbott Laboratories, Ltd., USA).

2.3. Lipid Peroxidation

Lipid peroxides were estimated by measurement of thiobarbituric acid reactive substances in plasma by the method of Yagi [13]. The pink chromogen produced by the reaction of thiobarbituric acid with malondialdehyde, a secondary product of lipid peroxidation was estimated. The absorbance of clear supernatant was measured against reference blank at 535 nm.

2.4. Enzymic Antioxidants

Superoxide dismutase (SOD) was assayed utilizing the technique of Kakkar et al [14]. based on inhibition of the formation of nicotine amide adenine dinucleotide, phenazine methosulfate and amino blue tetrazolium formazan. A single unit of enzyme was expressed as 50% inhibition of NBT (nitroblue tetrazolium) reduction/min/mg protein.

Catalase (CAT) was assayed colorimetrically at 620 nm and expressed as µmoles of H_2O_2 consumed/min/mg protein as described by Sinha [15]. The reaction mixture (1.5 ml, vol) contained 1.0 ml of 0.01 M phosphate buffer (pH 7.0), 0.1 ml of erythrocyte lysate and 0.4 ml of 2 M H2O2. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3).

Glutathione peroxidase (GPx) activity was measured by the method described by Rotruck et al [16]. Briefly, reaction mixture contained 0.2 ml of 0.4 M Tris-HCl buffer pH 7.0, 0.1 ml of 10 mM sodium azide, 0.2 ml of homogenate (homogenized in 0.4 M, Tris-HCl buffer, pH 7.0), 0.2 ml glutathione, 0.1 ml of 0.2 mM H_2O_2. The contents were incubated at 37°C for 10 min. The reaction was arrested by 0.4 ml of 10% TCA, and centrifuged. Supernatant was assayed for glutathione content by using Ellmans reagent (19.8 mg of 5, 5'-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate). Reduced glutathione (GSH) was determined by the method of Ellman [17]. 1.0 ml of supernatant was treated with 0.5 ml of Ellmans reagent and 3.0 ml of phosphate buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm.

Glutathione-S-transferase (GST) activity was determined spectrophotometrically by the method of Habig et al [18]. The reaction mixture contained 1.0 ml of 0.3 mM phosphate buffer (pH 6.5), 0.1 ml of 30 mM 1-chloro-2, 4-dinitrobenzene (CDNB) and 1.7 ml of double distilled water. After preincubating the reaction mixture at 37°C for 5 min, the reaction was started by the addition of 0.1 ml of homogenate and 0.1 ml of glutathione as substrate. The absorbance was followed for 5 min at 340 nm. Reaction mixture without the enzyme was used as blank. The activity of GST is expressed as µM of GSH-CDNB conjugate formed/min/mg protein using an extinction coefficient of 9.6 mM^{-1} cm^{-1}.

2.5. Statistical analysis

All data were expressed as mean ± SD. The statistical significance was evaluated by Student's t test using Statistical Package for the Social Sciences (SPSS Cary, NC, USA) version 10.0.

3. Results

Information about the investigated characteristics is shown in Table 1. The mean age limit was 49 ± 13.4 in diabetic patients with microalbuminuria, 44 ± 10.2 in diabetic patients without microalbuminuria and 42 ± 9.3in control subjects.

The decrease body mass index in diabetic patients with microalbuminuria (22.5 ± 6.9kg/m2) compared with control subjects (32.7 ± 6.7kg/m2) was statistically significant. On the other hand there is no difference body mass index in patients with microalbuminuria compared with patients without microalbuminuria.

Table 2 shows the levels of biochemical investigation in control subjects, diabetic patients with and without microalbuminuria. The level of blood glucose, HbA1C, urea, creatnine and serum cholesterol, triglyceride, very low density lipoprotein (VLDL) and low density lipoprotein (LDL) were significantly higher in the patients with microalbuminuria compared with patients without microalbuminuria and control subjects. On the other hand proteins and HDL-C was found to be significantly lower in patients with microalbuminuria. The level of hs-CRP and Hcy levels were found to be significantly higher in patients with microalbuminuria compared with patients without microalbuminuria and healthy controls. The above-mentioned biochemical parameters were found to be more significantly altered in diabetic patients with microalbuminuria compared with diabetic patients without microalbuminuria subjects.

Table 1. Demographic characteristics of different study group.

Parameter	Control subjects	Diabetic patients without microal -buminuria	Diabetic patients with microal -buminuria
Total number of subjects (n)	25	25	25
Sex (male)	100 %	100 %	100 %
Smokers (%)	10%	20%	25%
Alcohols (%)	7%	15%	15%
Mean age (mean ± SD; years)	42 ± 9.3	44 ± 10.2^{NS}	49 ± 13.4*‡
Body mass index (kg/m2)	32.7 ± 6.7	30.8 ± 7.1^{NS}	22.5 ± 6.9*‡

Values are given as mean ± S.D from 25 subjects in each group. * Diabetic patients with microalbuminuria compared with control subjects; ‡ Diabetic patients with microalbuminuria compared with diabetic patients without microalbuminuria; NS - Non significant; p<0.05 considered statistically significant.

Table 2. Comparison of biochemical changes in control subjects and diabetic patients with and without microalbuminuria.

Parameter	Control subjects	Diabetic patients without microal -buminuria	Diabetic patients with microal -buminuria
Blood glucose (mg/dl)			
Fasting	98 ± 10	185 ± 22***	285 ± 37***‡
Postprandial	129 ± 23	243 ± 20***	453 ± 30***‡
HbA1C (%)	3.7 ± 1.2	7.1 ± 1.72***	14.3 ± 3.01***‡
Urea (mg/dl)	19 ± 5.1	21 ± 5.0^{NS}	49 ± 12***‡
Creatnine (mg/dl)	0.6 ± 0.32	0.7 ± 0.9 ^{NS}	1.3 ± 0.9***‡
Total protein (g/dl)	7.3 ± 1.1	7.1 ± 1.0 ^{NS}	5.7 ± 1.0***‡
Albumin (g/dl)	5.0 ± 1.2	3.9 ± 0.7*	2.5 ± 0.6***‡
Total cholesterol	152 ± 19	198 ± 16 ^{NS}	235 ± 22**‡
Triglyceride (mg/dl)	120 ± 18	165 ± 22*	320 ± 40***‡
HDL-C (mg/dl)	47 ± 8.6	32 ± 12.0*	27 ± 6.2***‡
LDL-C (mg/dl)	59 ± 12	92 ± 12**	176 ± 17***‡
VLDL-C (mg/dl)	25 ± 7.5	34 ± 6.6*	64± 12*‡
hs-CRP (mg/dl)	0.45 ± 0.07	1.42 ± 0.27***	4.28 ±1.31***‡
Homocystein (µmole/L)	9.8±3.84	13.7 ±4.26*	17.02 ± 6.13***‡

Values are given as mean ± S.D from 75 subjects in each group

* Diabetic patients with microalbuminuria compared with control subjects (p<0.05, p<0.01, p<0.001), * Diabetic patients without microalbuminuria compared with control subjects (p<0.05, p<0.01, p<0.001, NS-Not significant) ‡ Diabetic patients with microalbuminuria compared with diabetic patients without microalbuminuria (p<0.001).

Table 3 shows the levels of plasma TBARS and enzymatic antioxidant status in SOD, CAT, GSH, GPx, and GST in control subjects, diabetic patients with and without microalbuminuria. Lipid peroxidation indicated by plasma TBARS level was significantly higher in diabetic patients with microalbuminuria when compared to diabetic patients without microalbuminuria and control subjects. The decrease in the levels of erythrocyte enzymatic antioxidant status in SOD, CAT, GSH, GPx and GST in diabetic patients with microalbuminuria when compared to diabetic patients without microalbuminuria and control subjects were statistically significant. On the other hand the level of lipid peroxidation and erythrocyte enzymatic antioxidant profile were found to be more significantly altered in diabetic patients with microalbuminuria compared with patients without microalbuminuria subjects.

Table 3. shows the levels of plasma TBARS and enzymatic antioxidant status in SOD, CAT, GSH, GPx, and GST in control subjects, diabetic patients with and without microalbuminuria.

Parameter	Control subjects	Diabetic patients without microal -buminuria	Diabetic patients with microal -buminuria
TBARS (nmol/ml)	3.56 ± 0.78	5.89 ± 0.65**	7.57 ± 3.52*‡
SOD (U^A)	4.91 ± 0.66	3.74 ± 0.47**	1.24 ± 0.15*‡
CAT (U^B)	80.5 ± 7.75	75.8 ± 7.50*	58.3 ± 9.90*‡
GSH (mg/dl)	53.42 ± 8.52	45.43 ± 7.75*	29.23 ±7.3*‡
GPx (U^C)	12.15 ± 2.69	10.93 ± 0.85*	3.53 ± 0.85*‡
GST (U^D)	3.90 ± 0.55	2.42 ± 0.45*	1.02 ± 0.45*‡

Values are given as mean ± S.D from 75 subjects in each group * Diabetic patients with microalbuminuria compared with control subjects (p<0.001); * Diabetic patients without microalbuminuria compared with control subjects (*p<0.05, **p<0.01);‡ Diabetic patients with microalbuminuria compared with diabetic patients without. microalbuminuria(p<0.001).

A–One unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction/min/mg Hb.
B – µmol of H_2O_2 consumed/min/mg Hb
C – µmol of GSH consumed/min/mg Hb.
D – µmol of CDNB–GSH conjugate formed/min/mg Hb.

4. Discussion

Diabetes mellitus is a complex and multifactorial disease indulging severe insulin dysfunction in conjunction with gross abnormalities in glucose homeostasis, lipid and protein metabolism. The metabolic dysregulation associated with diabetes causes secondary pathophysiologic changes in multiple organ systems that impose a heavy burden of morbidity and mortality from macrovascular and microvascular complications [19]. Oxidative stress plays an important role in chronic complications of diabetes and is postulated to be associated with increased lipid peroxidation. The present study was examine the changes in both extra and intracellular antioxidants and oxidant status in diabetic patients. In the present study, we noticed

a marked increase in HbA1C level in diabetic patients, which could be due to excessive glycosylation of hemoglobin. Diabetes is also grossly reflected by profound changes in protein metabolism and by a negative nitrogen balance and loss of nitrogen from most organs. Increased blood urea production in diabetes may be accounted for by enhanced catabolism of both liver and plasma proteins [20]. The diabetic hyperglycemia induces elevation of the plasma levels of urea and creatinine, which are considered as significant markers of renal dysfunction. The decrease in total protein and albumin may be due to microproteinuria and albuminuria, which are important clinical markers of diabetic nephropathy, and/or may be due to increased protein catabolism.

Diabetes has been shown to be associated with numerous thrombotic, atherosclerotic, and cardiovascular diseases. Cholesterol has been singled out as the cause of atherosclerosis. However, other lipids, such as triglycerides and phospholipids, also show similar correlations [21]. In our study, the levels of serum lipids were found to be elevated in diabetic patients.

The abnormally high concentration of serum lipids in diabetes is mainly a result of the increase in mobilization of free fatty acids from peripheral depots, because insulin inhibits the hormone-sensitive lipase. On the other hand, glucagons, catecholamines, and other hormones enhance lipolysis. The marked hyperlipemia that characterizes the diabetic state may therefore be regarded as a consequence of the uninhibited actions of lipolytic hormones on fat depots. The increase and fall in the individual lipoprotein levels is a reflection of the total serum cholesterol levels; that is, the levels of VLDL-C, LDL-C, and HDL-C increase or decrease with the level of total serum cholesterol, and it is their ratio that determines the pathophysiology of lipoprotein metabolism [21-22].

The significant increase in TBARS and decrease in GSH and GPx levels of patients with type 2 DM compared with the control group suggests permanent structural membrane alterations in diabetes, as well as increased production of reactive oxygen species and decreased antioxidants in the circulation [23]. It has been proposed that a diabetics' blood is more prone to lipid peroxidation due to the impaired antioxidant defence system. In fact, oxidative stress is an imbalance between free radical production and lipid peroxidation on one hand, and the antioxidant defence system on another. The pro-oxidant-antioxidant imbalance in diabetes may be due to either acceleration of cellular reactions leading to increased free radical production, such as non-enzymatic protein glycation, glucose oxidation and increased sorbitol pathway, or reduced antioxidant defence potential [24].

This study provides evidence that the imbalanced TBARS and GSH levels are more pronounced in type 2 DM patients with microalbuminuria. Plasma MDA levels correlate with the duration of type 2 DM. Excessive lipid peroxidation in the plasma can arise due to factors favouring the formation of reactive oxygen species. In poorly controlled DM, glucose oxidation through the pentose phosphate pathway leads to excessive formation of NADPH, which in turn can promote lipid peroxidation in the presence of the cytochrome P-450 system. Oxyhaemoglobin in erythrocytes could act like cytochrome P-450 in the presence of NADPH and this could induce increased lipid peroxidation [25,26].

Microalbuminuria is a strong predictor of cardiovascular morbidity and mortality in type 2 DM. The increased risk of cardiovascular disease in individuals with microalbuminuria is only partly due to a higher prevalence of established risk factors such as diabetes, hypertension, smoking and dyslipidaemia. Hyperhomocysteinaemia is another recently recognized risk factor for cardiovascular disease [27]. The present study shows that there is significant increase in homocysteine levels in type 2 DM patients with microalbuminuria compared with control patients. Microalbuminuria is thought to be caused by increased glomerular albumin filtration as a result of decreased glomerular charge selectivity and increased intraglomerular pressurer regulation of which is affected by renal endothelial and mesangial cell function. Mesangial cells have some properties in common with vascular smooth muscle cells [28]. Hyperhomocysteinaemia and hsCRP may induce dysfunction of the vascular endothelium and increase proliferation of vascular smooth muscle cells, possibly by increasing oxidative stress. The present study supports that hyperhomocysteinaemia is significantly related to increasing oxidative stress and decreasing GSH. For type 2 DM patients there was also significant positive correlation between TBARS and homocysteine and a negative correlation between GSH and homocysteine levels. Increased homocysteine levels result in increased risk of atherosclerosis. Increased TBARS levels are due to increased free radical production. Both homocysteine and free radicals oxidize LDL, which results in endothelial damage increasing the risk of atherosclerosis. Thus, increased levels of homocysteine and MDA are associated with increased risk of atherosclerosis.

5. Conclusion

In conclusion, decreased antioxidant levels and increased lipid peroxidation, hs-CRP and homocysteine levels were observed in patients with microalbuminuria. We demonstrate the association of low-grade systemic inflammation, as indicated by elevated hs-CRP and homocysteine levels, with T2D patients in Indian population. These changes may contribute to vascular disease, which is particularly prevalent in type 2 DM patients with microalbuminuria.

Competing interests

The author(s) declare that they have no competing interests.

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