

Original Research Article

Total antioxidant status in type 2 diabetes mellitus with diabetic nephropathy

Heena Singla*, Gitanjali Goyal, Cheenu Garg, Kajal Bhalla

Department of Biochemistry, Guru Gobind Singh Medical College, Faridkot, Punjab, India

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*Correspondence:

Dr. Heena Singla,

E-mail: heenasingla76@yahoo.com

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ABSTRACT

Background: Diabetes mellitus has emerged as one of the most common health hazard all over the world. Diabetic nephropathy is the most challenging long term complication of Type 2 Diabetes mellitus and microalbuminuria is the earliest marker of diabetic nephropathy. In diabetes, chronic hyperglycemia and deranged lipid profile lead to excess generation of free radicals. The increased oxidative stress plays a major role in pathogenesis of diabetic complications, including diabetic nephropathy. There are many naturally occurring antioxidant enzymes in our body. Diabetes has multiple effects on protein levels and activity of these antioxidant enzymes. This further augments the oxidative stress. There are many non-enzymatic antioxidants in our body which include vitamins A, C, E and trace minerals like copper, zinc, manganese and selenium.

Methods: The study was done on a total of 150 subjects. Group A comprised of 60 Type 2 diabetic patients with diabetic nephropathy, Group B comprised of 60 Type 2 diabetic patients without diabetic nephropathy and Group C comprised 30 healthy controls. Total antioxidant status, microalbuminuria and glycosylated haemoglobin were measured.

Results: In present study, authors found that total antioxidant status is drastically reduced in all diabetic patients, and it was found to be further low in patients with diabetic nephropathy. This decrease was found to be directly proportional to the degree of diabetic nephropathy, as measured by the levels of microalbuminuria.

Conclusions: Timely institution of antioxidant supplementation therapy may emerge as a promising measure in delaying the onset and progression of diabetic complications, especially diabetic nephropathy.

Keywords: Diabetic nephropathy, Microalbuminuria, Oxidative stress, Total antioxidant status

INTRODUCTION

Diabetes mellitus (DM) in all its heterogeneity has taken the central stage as one of the ultimate medical challenges. In 2018, there were more than 500 million prevalent cases of type 2 diabetes worldwide. The prevalence was comparable between low income and high income countries. The total number of people with diabetes is projected to rise from 171 million in 2000 to

366 million in 2030. The prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030.¹

At present, India leads the world with largest number of diabetic subjects. It has been termed 'diabetes capital of the world'. According to WHO, India had 69.2 million people living with diabetes in 2015. This number may increase to 98 million by 2030. This expected increase

over next 15 years would be due to ageing, urbanization, change in diet and physical activity.²

The most important demographic change contributing to increased diabetes prevalence across the world appears to be increase in proportion of people over 65 years of age. Improved survival may contribute to increasing prevalence of diabetes. Obesity is thought to be the primary cause of type 2 diabetes, especially in people who are genetically predisposed to the disease.³

Diabetic complications are the major causes of morbidity and mortality in patients with diabetes. Diabetic nephropathy is one of the major long-term complication of diabetes mellitus and has emerged as a leading cause of End Stage Renal Disease. Data from Diabetes Control Association has established that chronic hyperglycemia is a major initiator of diabetic microvascular complications which include diabetic nephropathy, retinopathy and neuropathy. Increased glycosylated haemoglobin, duration of diabetes and systolic blood pressure were independently associated with diabetic nephropathy.⁴ Diabetic nephropathy has been established to be among the leading causes of renal failure.

Both macrovascular and microvascular complications cause significant morbidity and mortality among diabetic patients, the predominant of which include coronary artery disease and diabetic nephropathy.^{5,6} Prevalence of overt nephropathy in Indian diabetics was 2.2%, while microalbuminuria was present in 26.9%.⁷

In diabetes, glucose processing uses a variety of diverse metabolic pathways; hence, chronic hyperglycemia can induce multiple cellular changes leading to complications in diabetic patients.

Increased oxidative stress plays a major role in the development of diabetic complications. Oxidative stress is excess formation and/or insufficient removal of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). These species (ROS and RNS) are unstable and highly reactive.⁸ These reactive oxygen species include free radicals such as superoxide (O_2^-), hydroxyl (OH), peroxy (RO_2), hydroperoxy (HRO_2) as well as non-radical species such as hydrogen peroxide (H_2O_2) and hydrochloric acid (HOCl). Reactive nitrogen species include free radicals like nitric oxide (NO) and nitrogen dioxide (NO_2), nitrous oxide (HNO_2) and alkyl peroxynitrates (RONOO).

These free radicals play important role in the pathogenesis of diabetic cardiovascular complications.⁹ Many biochemical pathways strictly associated with hyperglycemia, as in diabetics, such as glucose auto-oxidation, polyol pathway and protein glycosylation can increase the production of free radicals. The reducing sugars and proteins undergo sequential glycosylation and

oxidation reactions to form glyco-oxidation products in diabetics.

Under normal conditions, O_2^- is quickly eliminated by antioxidant defense mechanisms. These include manganese dependent superoxide dismutase (Mn-SOD) and glutathione peroxidase (GSH-Px) in the mitochondria, copper dependent superoxide dismutase (Cu-SOD) in the cytosol, and catalase in lysosomes. Expression and activity of these enzymes are drastically reduced in diabetes.¹⁰

Diabetes has multiple effects on protein levels and activity of these enzymes. This further augments oxidative stress by causing a suppressed anti-oxidant defense response. Also, production of one molecule of reactive oxygen species may lead to the production of other such molecules through radical chain reactions.

Reactive oxygen species can stimulate oxidation of low-density lipoprotein (LDL) to form oxidised LDL particles (ox-LDL). So, these ox-LDL particles cannot be recognized by the LDL receptors. These are taken up by scavenger receptors in macrophages leading to foam cell formation and atherosclerotic plaques.¹¹

The superoxide radical O_2^- can activate several damaging pathways in diabetes including accelerated formation of advanced glycation end products (AGE), polyol pathway and hexosamine pathway. All of which have been proven to be the causative factors in the pathogenesis of microvascular and macrovascular complications of diabetes. The susceptibility of diabetics to micro- and macrovascular complications may be a function of imbalance between this oxidative stress and endogenous anti-oxidant status in body.

There are non-enzymatic antioxidants also in our body which include vitamin A, C and E; trace elements like copper, zinc, manganese and selenium. These also include cofactors like folic acid, vitamin B1, B2, B5 and B12, Glutathione and carotenoids.¹² Vitamin E is an important fat-soluble vitamin that prevents lipid peroxidation.

Loss of SOD activity in RBCs is a function of duration of diabetes. SOD, inhibited by glycosylation, is lowered in poorly controlled diabetes mellitus. A further decrease in SOD activity has been observed in NIDDM patients who developed nephropathy.¹³

A study has suggested the central role of oxidative stress in development of diabetic nephropathy and beneficial effects of antioxidants in renal injury owing to diabetes.¹⁴ Increased oxidative stress usually occurs when the available supply of body's antioxidants is insufficient to handle and neutralize these free radicals.¹⁵

The normal kidney generates a substantial amount of oxidative stress due to its high metabolic activity, and this

is balanced by an extensive anti-oxidant system. But in pathological states, such as hyperglycemia, oxidant balance shifts towards a pro-oxidant state that accelerates tissue and vascular injury. This oxidative damage progresses concomitant with worsening glucose metabolism, vascular dysfunction and kidney disease. Accordingly, strategies to reduce oxidative stress in diabetes mellitus may exert favourable effect on the progression of diabetic nephropathy.

According to a study, formation of free radicals along with antioxidant deficiency in diabetes mellitus increases over time. This plays a major role in the development of diabetic nephropathy, which is an important complication of the disease. The study revealed the importance of determining the antioxidant status in addition to the markers of oxidative stress. It observed further intensification of lipid peroxidation in non-insulin dependent diabetes with obvious nephropathy compared to the group without nephropathy.¹⁶

Diabetic nephropathy is the leading cause of end stage renal disease worldwide and a leading cause of diabetes mellitus related morbidity and mortality. Patients with diabetes currently account for around 28% of ESRD patients in India. Sixty percent of patients with diabetic Nephropathy have type 2 diabetes. Microalbuminuria is the hallmark of diabetic Nephropathy in its earliest stage.¹⁷ It is predictive of development of overt proteinuria. It has well established association with progressive renal disease. Once proteinuria is established, renal function declines inexorably.

Assessment of risk of development of overt nephropathy can be easily done from albumin excretion rate in untimed urine specimen.¹⁸ Diabetic nephropathy can be detected at quite early stage by presence of microalbuminuria, which is defined as urinary albumin excretion rate in the range of 30- 300 mg/day.¹⁹ It is then followed by persistent albuminuria (albumin excretion rate or AER >300 mg/day). So, microalbuminuria was found to be a very useful parameter to detect diabetic nephropathy in its earliest stages. It has also been established as a risk factor for development of overt renal failure.²⁰ It is becoming increasingly recognized as an independent risk factor for cardiovascular disease in patients with hypertension and diabetes, and even in the general population.²¹ A correlation has been reported between obesity and microalbuminuria.²²

Diabetic nephropathy has been found to be more common in patients with family history of diabetes and hypertension.²³ Microalbuminuria is an important clinical marker in patients with diabetes because of its well-established correlation with severity of disease.²⁴

So American Diabetes Association (ADA) had recommended routine screening adults >45 years of age and especially those with BMI >25 Kg/m² for microalbuminuria in diabetics.²⁵

In view of all these considerations, this study was conducted to compare the levels of total antioxidant status in Type 2 diabetics with and without microalbuminuria as well as in healthy controls, and also to find out correlation between the two parameters, if any.

METHODS

Study design

The present study was conducted on a total of 150 subjects, attending the OPD or admitted in indoor wards of Department of Medicine in collaboration with Department of Biochemistry at a tertiary care center. A total of 150 subjects were enrolled in the study. These were divided into following groups:

- Group A - 60 known type 2 diabetic patients with micro-albuminuria
- Group B - 60 known type 2 diabetic patients without micro-albuminuria
- Group C - 30 healthy controls.

The study was approved by institutional thesis and ethical committee. Detailed present and past history of the patients was collected on a proforma which included age , sex, dietary habit, family history, smoking habit, drinking habit, drug intake and level of physical exercise. Informed written consent was taken on the printed proforma.

Exclusion criteria

- Patients with liver disease.
- Patients with Thyroid disorders.
- Pregnant and Lactating mothers, as pregnancy is associated with gross hormonal changes which have effect on lipid profile, and pregnancy is in itself a potentially diabetogenic condition.
- Females on oral contraceptive pills
- Patients who did not give consent for the study.

Informed written consent was taken from all the selected subjects who were enrolled in the study on a printed proforma. After taking their informed consent, blood samples were collected for the routine investigations like Hb, TLC, DLC, Urine C/E, Fasting blood glucose, BUN (Blood Urea Nitrogen), creatinine, bilirubin, SGOT, SGPT, ALP, electrolytes, calcium, uric acid and lipid profile.

Special investigations included measurement of Total anti-oxidant status, glycosylated haemoglobin and microalbuminuria.

The sample was put in a dry, clean and tightly closed vacutainer tube and allowed to clot. Then the serum was separated and tests were performed. Spot urine sample

was collected to perform complete urine analysis and to test for microalbuminuria.

Fasting blood glucose was measured on Beckman Coulter AU 480 fully automated analyser. Renal function tests included BUN (Blood Urea Nitrogen) and serum creatinine. Blood urea was measured by urease method and Serum creatinine was estimated by Jaffe's method, on Beckman Coulter AU 480 fully automated analyser. BUN was calculated from the value of blood urea, using the formula BUN = Blood Urea divided by 2.15.

Liver function tests (which included serum bilirubin, SGOT, SGPT and serum alkaline phosphatase), Serum uric acid, serum electrolytes, serum calcium and lipid profile (which included triglycerides, total cholesterol and HDL cholesterol) were all measured on Beckman Coulter AU 480 fully automated analyser.

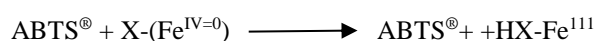
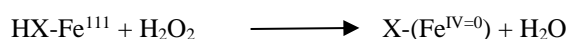
Special investigations

Total antioxidant status

Freshly drawn blood sample was taken. Then it was analysed with Randox total antioxidant status kit using colorimeter.

Assay principle²⁶

ABTS[®](2,2'-Azino-di-{3-ethylbenzthiazoline sulphate}) in the reagent is incubated with a peroxidase (metmyoglobin) and hydrogen peroxide to produce the radical cation ABTS^{®+}. This has a relatively stable blue-green colour. The concentration of Antioxidants in the added sample is directly proportional to their suppression of this blue colour.



where HX - Fe¹¹¹ = Metmyoglobin

X - (Fe^{IV=O}) = Ferrylmyoglobin

ABTS[®] is 2,2'-Azino-di-{3-ethylbenzthiazoline sulphate}.

CAL. Standard

6-hydroxy-2,-5,7,8-tetramethylchroman-2-carboxylic acid. The concentration is not specific. In case of this kit, which was used, it was 2.18 mmol/l.

Sample

Freshly drawn serum was used. Haemolysed samples were avoided. In case of expected delay, samples were stored at 2 to 8° C for upto 36 hours.

For good quality control, perfectly dry and clean cuvettes were used for reconstitution of reagents and for taking readings of blank, standard and samples.

Reference range²⁷

1.30-1.77 mmol/L.

Microalbuminuria

Spot urine sample was collected to test for microalbuminuria. Measurement of microalbuminuria was done using Nycocard reader.

Principle²⁸

It is a solid phase, sandwich format, immunometric assay.

Reference range²⁹

0-20 mg/L.

Measurement of glycosylated haemoglobin

For this test, blood sample was collected in EDTA vial. Measurement was done using Nycocard reader.

Assay principle³⁰

Nycocard HbA1c is a boronate affinity assay.

Reference range³¹

4.8-5.9%.

Statistical analysis

All the results and observations recorded were subjected to appropriate statistical analysis to draw the final conclusions. To represent the data, appropriate tables, bar diagrams, pie charts and scatter plots were made, and final conclusions were drawn.

RESULTS

From the present study following observations were made.

Highly significant difference was found in the values of following parameters among the three groups (Table 1).

Levels of total anti-oxidant status was much lower in diabetic patients with microalbuminuria as compared to diabetic patients without microalbuminuria, who had a bit higher levels of total anti-oxidant status. Total anti-oxidant status was in normal range in healthy controls (p value <0.001).

Table 1: Comparison of important parameters among three study groups.

Parameter	Group A (diabetes with microalbuminuria)	Group B (diabetes without microalbuminuria)	Group C (healthy controls)	p value
BMI(kg/m ²)	25.55±3.70	22.73±2.72	22.73±2.01	<0.001
FBS(mg%)	182.95±62.39	128.82±40.95	99.43±8.77	<0.001
Total cholestrol (mg%)	195.28±41.95	165.68±27.25	161.53±23.82	<0.001
Triglycerides (mg%)	182.03±56.47	140.82±46.04	127.67±36.97	<0.001
LDL- cholestrol (mg%)	119.25±33.62	98.79±20.86	93.60±20.54	<0.001
HbA1c (%)	7.51±2.07	5.68±0.79	5.40±0.25	<0.001
Microalbuminuria (mg/L)	123.15±71.24	16.12±4.46	13.07±0.78	<0.001
Total Anti-oxidant status (μmol/L)	0.708±0.286	1.320±0.284	1.410±0.229	<0.001

*p value <0.001 denoted highly significant difference among the three groups.

Table 2: Coefficient of correlation between important parameters in group A subjects (diabetic patients with microalbuminuria).

Parameters	r value	p value**
FBS and HbA1c	+0.589**	<0.001
FBS and microalbuminuria	+0.259*	<0.05
Microalbuminuria and total anti-oxidant status	-0.599**	<0.001
HbA1c and total anti-oxidant status	-0.473**	<0.001
Total cholestrol and total anti-oxidant status	-0.374**	<0.001
Triglycerides and total anti-oxidant status	-0.360**	<0.001

**p value <0.001 denoted highly significant level of correlation between the concerned parameters.

Diabetic patients with microalbuminuria had much higher glycosylated haemoglobin as compared to diabetic patients without microalbuminuria, while the healthy controls had normal glycosylated haemoglobin (p value <0.001).

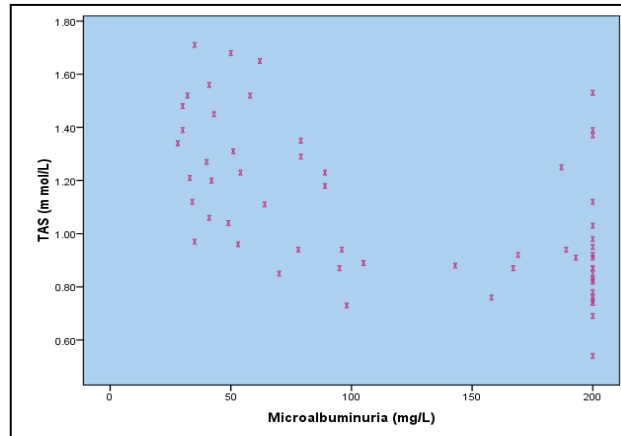
Fasting blood glucose was much higher in diabetic patients with microalbuminuria as compared to diabetic patients without microalbuminuria, who had higher Fasting blood glucose. Healthy controls had normal Fasting blood glucose (p value <0.001).

All the parameters of lipid profile were highly deranged in diabetic patients with microalbuminuria as compared to diabetic patients without microalbuminuria. The healthy controls had normal lipid profile parameters (p value <0.001).

Significant degree of correlations was found between important parameters (Table 2)

There was a strong negative correlation between the levels of microalbuminuria and total anti-oxidant status in Group A subjects (r = -0.599, p <0.001) (Figure 1).

There was a strong negative correlation between glycosylated haemoglobin and total anti-oxidant status in both of the diabetic groups (r = -0.473, p <0.001) (Figure 2).

**Figure 1: Scatter plot showing correlation between microalbuminuria and total anti-oxidant status.**

There was a strong positive correlation between the levels of fasting blood glucose and microalbuminuria in Group A subjects. (r = +0.259, p <0.05). There was a strong negative correlation between Total Cholestrol and total anti-oxidant status in both of the diabetic groups (r = -0.374, p <0.001).

There was a strong negative correlation between Triglycerides and total anti-oxidant status in both of the diabetic groups (r = -0.360, p <0.001).

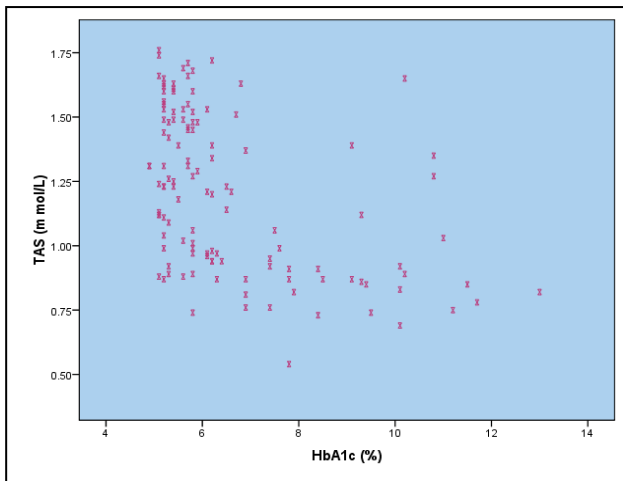


Figure 2: Scatter plot showing correlation between glycosylated haemoglobin and total anti-oxidant status.

DISCUSSION

Oxidative stress has been known to play an important role in the development and progression of diabetic nephropathy. Hyperglycemia has been established as the main cause of complications of diabetes mellitus.¹⁴ Microalbuminuria is an important clinical marker in patients with diabetes because of its well established association with progressive renal disease.¹⁹

According to the present study, total anti-oxidant status was lower in diabetic patients with microalbuminuria as compared to diabetic patients without microalbuminuria, which was further lower as compared to the values of total anti-oxidant status in healthy controls. Oxidative stress resulting from increased generation of reactive oxygen species plays a crucial role in the pathogenesis of diabetic nephropathy.¹⁵

According to the present study, the levels of total antioxidant status had strong negative co-relation with degree of microalbuminuria ($r = -0.589$) in the diabetic patients with microalbuminuria. Activity of anti-oxidant enzymes catalase and SOD (Superoxide dismutase) was significantly lower in diabetic patients with nephropathy as compared to diabetic patients without nephropathy.³²

The same fact is further supported by another study according to which conventional and catalytic antioxidants have been shown to prevent or delay the onset of diabetic nephropathy.¹⁴

Abnormally high levels of free radicals, increased lipid peroxidation and simultaneous decrease in total anti-oxidant status leads to damage of cellular organelles and cell membrane, which ultimately leads to development of complications of diabetes mellitus, including diabetic nephropathy.⁹ Diabetic patients undergo significant oxidative stress as compared to healthy subjects.⁸

According to the present study, higher the blood glucose, lower was the total anti-oxidant status in these patients. The levels of total anti-oxidant status had strong negative co-relation with levels of glycosylated haemoglobin ($r = -0.473$). This is supported by a study according to which free radicals are formed disproportionately in diabetes as a result of glucose auto-oxidation, polyol pathway and non-enzymatic glycation of proteins.¹³

In the present study, we found that diabetic patients with microalbuminuria had more deranged lipid profile (particularly higher triglycerides) as compared to those without microalbuminuria and healthy controls ($p < 0.001$). Significantly increased levels of serum total cholesterol, triglycerides and low density lipoprotein were noticed in the patients with diabetic nephropathy as compared to control subjects. Particularly, the oxidised LDL particles when taken up by scavenger receptors, lead to atherosclerosis. This ultimately leads to diabetic nephropathy.¹¹

According to the present study, the degree of microalbuminuria had strong positive co-relation with levels of fasting blood glucose ($r = +0.259$, $p < 0.05$). Intensive glycemic control delays the onset and progression of diabetic nephropathy, in part through prevention of overproduction of these reactive oxygen species.²⁴

In the present study, in diabetic patients with microalbuminuria, the levels of microalbuminuria correlated with the levels of fasting blood glucose ($r = +0.259$, $p < 0.05$). Microalbuminuria has been shown to have strong correlation with the levels of fasting blood glucose.³³

The poorly controlled diabetics have higher degree of microvascular damage of blood vessels, leading to higher incidence of diabetic microvascular complications, including diabetic nephropathy. These patients have higher degree of microalbuminuria. This microalbuminuria is directly linked to increased oxidative stress and hence, decreased total anti-oxidant status.

Altogether the present study showed that uncontrolled diabetic patients with high fasting blood glucose and microalbuminuria had lower total anti-oxidant levels in comparison with diabetics without microalbuminuria and healthy controls.

CONCLUSION

It appears that in diabetes, antioxidant therapy could alleviate the associated increased oxidative stress. It may emerge as a promising additional therapeutic modality in delaying the onset and progression of diabetic complications, especially diabetic nephropathy. Most importantly, alfa tocopherol therapy especially at high doses, clearly shows a benefit with regard to low-density lipoprotein oxidation in cases of diabetic nephropathy.

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Conflict of interest: None declared

Ethical approval: The study was approved by the Institutional Ethics Committee

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