ORIGINAL RESEARCH

Study Of Oxidative Stress Status In Type 2 Diabetic Patients

S. Rajen Singh,[1] Davina Hijam,[2] Abhishek Dubey,[1] Ningthoujam Omita Devi,[1] Sungdirenla Jamir,[1] Chubalemla Longkumer,[1] Kangabam Nandinibala,[1] Th. Premchand Singh,[3] Waikhom Gyaneshwar Singh[4]

ABSTRACT

Introduction: Oxidative stress is associated with the patho-physiology of diabetes mellitus. Severe oxidative stress may cause cell damage which can lead to decreased pancreatic beta-cell function.

Aim: To estimate the levels of antioxidant glutathione peroxidase (GPX), superoxide dismutase (SOD) and lipid peroxidation marker i.e. malondialdehyde (MDA) in type 2 DM patients with and without microvascular complications and to compare the results with that of healthy individuals. To assess the correlation between oxidative stress and diabetes in relation to metabolic control.

Methods: This study was conducted on 60 type 2 DM patients (Thirty with microvascular complications and another thirty without microvascular complications) attending Diabetic Clinic and Medicine ward RIMS, Imphal. Another 30 age and sex matched healthy volunteers were taken as controls. Erythrocyte Glutathione Peroxidase, Superoxide Dismutase and Malondialdehyde levels were estimated spectrophotometrically (Beckman DU 640 Spectrophotometer using commercially available kit RANSEL, Randox Lab Ltd, UK).

Results: The mean ± SD MDA levels of type 2 DM patients with microvascular complications (6.08 ± 0.47 µmol/l) were found to be significantly higher (P<0.000) than those without complications (4.01±0.22 µmol/l) and controls (2.79±0.27 µmol/l). A significant decrease in GPX and SOD was observed in type 2 DM with microvascular complications as compared to those without complications and controls (19.18 ± 0.97 U/gHb, 24.07 ± 0.88 U/gHb, 29.48 ± 0.92 U/gHb respectively for GPX and 578.98 ± 25.78U/gHb,799.94± 55.39 U/gHg and 1146.48+61.05U/gHg respectively for SOD). There was a significant negative correlation between GPX and HbA1C and a positive correlation between Malondialdehyde and HbA1C which was highly significant (P<0.01).

Conclusion: These findings strongly confirmed that diabetic patients are susceptible to oxidative stress and poor glycaemic control had an association with free radical-mediated lipid peroxidation. The results suggest that patients with hyperglycemia and oxidative stress present a high risk for development of diabetic complications and need early intervention.

Key words: Oxidative stress, Type 2 DM, microvascular complications, Malondialdehyde, HbA1C

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[1]Postgraduate Trainee, [2]Assistant Professor, [4]Former Head of Department, Department of Biochemistry, [3]Professor, Department of Medicine, Regional Institute of Medical Sciences, Imphal, Manipur, India,

Corresponding author: Dr. Davina Hijam, Assistant Professor, Department of Biochemistry, Regional Institute of Medical Sciences, Imphal, Manipur, India

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia and associated with increased free-radical activity. The chronic hyperglycemia alters the normal functioning of various organs. Organs specially the eyes, kidneys, nerves, heart, and blood vessels may undergo long term damage and dysfunction.[1, 2] Evidences suggest that worldwide Diabetes mellitus (DM) is one of the most common non-communicable diseases. Over 80% of its carriers are living in low and middle-income countries.[3] There are considerable evidences that many of the biochemical pathways which generates

reactive oxygen species (ROS) are activated by hyperglycemia which ultimately leads to oxidative stress.[5] In hyperglycemia oxidative stress occurs prior to the clinically evident late complications. Increased rate of free radical production and/or impaired antioxidant mechanisms are responsible for increased oxidative stress. Root cause of insulin resistance, impaired glucose tolerance and β-cell dysfunction is increased oxidative stress in a variety of tissues. Increased ROS, protein glycation, advanced glycated end products formation and activation of polyol pathway increase oxidative stress. Normally minute quantities of free radicals are formed and these free radicals are rapidly scavenged. Enzymes like Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx) comprise natural celular defence mechanism against these free radicals. Malondialdehyde (MDA), a marker of lipid peroxidation has been found in RBC membrane of diabetic patients. It is widely accepted as a marker in the development and progression of diabetes and its complication. Free-radical activity causes lipid peroxidation which leads to the development of microvascular (retinopathy, nephropathy, neuropathy) complications of diabetes.

The present study has been taken up to access the co-relation between oxidative stress and diabetes mellitus and development of its complication, as no such study conducted in Manipur is available. This study has been carried out with an aim to estimate serum lipid peroxidation marker, malondialdehyde (an oxidant) and antioxidant enzyme (glutathione peroxidise and superoxide dismutase) in type 2 diabetes mellitus patients with and without micro-vascular complication and to compare with that of healthy individuals. And also, to assess the correlation between oxidative stress and diabetes in relation to metabolic control.

MATERIAL AND METHODS

A study was conducted in Department of Biochemistry in collaboration with Department of Medicine in Regional Institute of Medical Sciences, Imphal during a period of October 2011 to April 2013. Study was approved by the institutional review board and all participants gave written informed consent**.** The study group comprised of sixty cases (selected randomly from the diabetic patients who visited the Regional Institute of Medical Sciences) which were divided into two groups. (i) Thirty confirmed type 2 diabetic patients without complication who were newly diagnosed or under treatment for diabetes mellitus with oral hypoglycemic agents and/or diabetic diet. (ii) Thirty confirmed type 2 diabetic patients with microvascular complication under treatment, who were coming from different areas of Manipur and attending Diabetic clinic or Medical OPD or admitted in the Medicine ward of Regional Institute of Medical Science Hospital, Imphal. Thirty age and sex matched apparently healthy individuals from general population in Imphal, Manipur were selected as controls.

All cases and controls were aged 18 years and above. Each individual enrolled in study underwent a detailed history, clinical examination and laboratory examination designed for the study. Type 2 DM patients with and without complication were diagnosed on the basis of history, physical examination, biochemical investigations and according to revised criteria for diagnosing DM issued by consensus panel expert from the National Diabetes Group and World Health Organization.[6]

Thirty confirmed cases of type 2 diabetes mellitus cases that came to RIMS Hospital for treatment were included in this study. Patients with hormonal disorder, cancer, renal failure, transplant rejection, chronic disease, macrovascular complication and hepatic failure were excluded from study. No patients were on antioxidant therapy

Five ml of venous blood was collected from each individual after an overnight fasting of twelve hours. Of this 3.5 ml was collected in sterilized heparinized vial for estimation of MDA, SOD, GPX and Hemoglobin concentration. Half ml was collected in EDTA vial for estimation of Glycocylatyed Hemoglobin (HbA1C) and 1 ml was kept for routine test.

HbA1C was estimated by Fast Ion Exchange Resin Separation Method as described by Goldstein DE, Little RR & Widdmayer HM[7] using kit from HUMANE, Wiesbaden, Germany. Urine was examined for microalbuminuria using

MICRAL-TEST an ACCU-CHECK product distributed by Roche Diagnostics 201, Boulevard Armand-Frappier, Canada. For determination of Hemoglobin (Hb) Cyanmethemoglobin method[8] assay was done using DIAGNOUR Hemoglobin (Cyanmethemoglobin) kit manufactured by RFCL Limited, India. The activity of Superoxide Dismutase (SOD) in erythrocytes was assayed Spectrophotometrically using Beckman DU 640 Spectrophotometer[9] and a commercially available kit RANSOD, Randox Lab Ltd, Crumen, UK. Glutathione Peroxide(GPX)[10] and Plasma total Malondialdehyde (MDA)[11] were assayed spectrophotometrically using Beckman DU 640 Spectrophotometer and commercially available kits of RANSEL, (Randox Laboratories Ltd, UK) and Bioxytech,(Oxis International, Inc. Portland, USA) respectively.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS version 16. Data were expressed in Mean ± SD. Statistical tests like χ2-test, independent t-test, ANOVA (F-test) and correlation coefficient 'r' were applied whenever found suitable and necessary. The P-value less than 0.05 was considered significant.

RESULTS

Table 1 shows basic profile of study population. It shows that there were 30% males and 70% females in all the three groups studied. But P-value ($P > 0.05$) indicates this difference is insignificant and sex composition of one group is almost similar to that of other group. Among the group of cases with complication maximum numbers of cases were with nephropathy (46.67%). After this 26.67% cases were of both nephropathy and retinopathy. Few patients were on antihypertensive therapy. No patient was taking insulin in both the group of diabetic patients.

Table 2 shows the comparison of numerical parameters considered in the present study in terms of their mean and standard deviation (mean ± SD) amongst the groups. Age of diabetic patient with complication was found to be significantly ($P < 0.05$) older (56.07 years) than the age of diabetic patients without complications (51.03 years). Also the patients of diabetes with complication have a longer duration of illness than those without complication.

Mean HbA1C for the group of cases with complication was found to be highest (9.10%). For the group without complication it was lower (7.32%) than group with complication but it was higher than control group (5.21%). The difference was highly significant statistically ($p < 0.05$) indicating a poor control of diabetes.

Diabetic patients without complication had significantly lower ($P < 0.05$) activity of GPX and SOD than in control subjects. Patients with Microvascular complication (MVC) had significantly lower activity of GPX and SOD than the group of diabetic patients without complication. Lipid peroxides (malondialdehyd) levels were observed to be significantly higher in diabetic patients with MVC compared to diabetic patients without these complication or controls.

Table 3 shows the difference of Mean ± SD of the parameters between groups of cases with and without complication using t-test. Age of patients of diabetes with complication was found to be significantly higher than that of patients without complication. Also duration of illness was more in the group of patients with complication ($p < 0.05$). There was significant increase of HbA1C in diabetic with MVC showing a poor control of blood sugar. A further significant decrease in GPX and SOD activity was observed in the diabetics with MVC indicating more antioxidant deficiency. Plasma MDA concentration was significantly higher in diabetic patients with complication indicating more lipid peroxidation with production of more ROS leading to more oxidative stress.

Table 4 shows the relationship between the parameters considered and HbA1C in the group of cases without complication by using correlation coefficient 'r'. Correlation of age, duration of diabetes with HbA1C was statistically insignificant ($P > 0.05$). The indirect correlation for glutathione peroxidase (U/gHb) with glycated hemoglobin was highly significant ($p<0.001$). A negative correlation with superoxide dismutase and positive correlation with malondialdehyde were not significant.

Table 5 shows the relationship between the

parameters considered and HbA1C in the group of cases with complication by using correlation coefficient 'r'. Correlation of age, duration of diabetes with HbA1C was not significant statistically ($P > 0.05$). Glutathione peroxidase has a negative correlation with HbA1C which is significant at $p < 0.05$. Correlation with SOM was indirect but insignificant. Malondialdehyde had a positive correlation with HbA1C which was highly significant $p < 0.01$.

Multiple regression on group of diabetic patients without complication showed that GPx is independently related to HbA1C percent. ($R^2 = 0.410$, $P < 0.01$). Multiple regression on group of diabetic patients with complication showed that Malondialdehyde is independently related to HbA1C percent ($R^2 = 0.340$, $P < 0.05$).

DISCUSSION

In the present study, Type 2 Diabetes Mellitus was found to be more predominant in females which constitute about 70% of the cases in both the groups of diabetic patients. Only 21% in both the groups of diabetic patients were males. This finding is consistent with the statement that T2DM is more common in women.[12,13]

It was also observed that mean age of T2DM were 51.03±6.90 and 56.07±7.79 for cases without and with complication respectively.

The decreased GPX activity in diabetic patients without and with MVC in this study 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.[21] In diabetic patients low Glutathione content is found, which can directly explain the low activity of GPX.[22] By acting as a co-substrate for Glutathione Peroxidase activity, Glutathione functions as a direct free-radical scavenger [23] Enzyme inactivation could also contribute to low GPX activity. glucose control is associated with complications. Evidences suggest that hyperglycemia plays a role in generation of reactive oxygen species (ROS). These ROS ultimately increase oxidative stress in a variety of tissues.[14] Increase in the production of free radicals can occur due to many biochemical pathways tightly associated with hyperglycemia e.g., glucose autoxidation, polyol pathway, prostanoid synthesis and protein glycation .[15]

In this study, there was significant difference of blood glucose control as indicated by mean HbA1C (9.10%, 7.32% and 5.21%) among two

Parameters		**Group**			**$\chi 2$**	**P-Value**
		Diabetes Without Complication	Diabetes With Complication	Controls		
Sex	Male	9(30)	9(30)	10(33.33)	0.104	0.949
	Female	21(70)	21(70)	20(66.67)		
Complications	Nephropathy		14(46.67)			
	Nephropathy+ Retinopathy		8(26.67)			
	Neuropathy+ Retinopathy		3(10)			
	Neuropathy		3(10)			
	Retinopathy		2(6.67)			
Drugs	OHA	22(73.33)	20(66.67)		3,095	0.213
	OHA+AH	6(20.00)	10(33.33)			
	NO DRUGS	2(6.67)	-			
	INSULIN	-	-			

Table:1 - Group wise distribution of basic profile among the study population

Parameter	Control	T2DM without complication	T2DM with complication	F	P-value
	Mean ±S.D	Mean±S.D	Mean±S.D		
Age (years)	46.20 ± 9.93	51.07±7.79	56.07 ±7.79	10.575	0.000
Duration of diabetes		4.50±1.52	8.50±3.58	107.024	
GPX (U/gHb)	29.48 ± 0.92	24.07±0.88	19.18 ± 0.97	920.90	0.000
SOD (U/gHb)	1146.48+61.05	799.94±55.39	578.98+25.78	986.865	0.000
MDA (µmols/l)	2.79 ± 0.27	4.01+0.22	6.08 ± 0.47	715.13	0.000

Table:2- Comparison between control and type 2 DM with microvascular complication

Parameter	T2DM without complication	T2DM with complication	t	P
	Mean±SD	Mean±SD		
Age (yr)	51.03 ± 6.90	56.07 ± 7.79	2.647	0.000
Duration of diabetes (yr)	4.50 ± 1.52	8.50 ± 3.58	5.618	0.000
HbA1C (%)	7.32 ± 1.31	9.10 ± 1.37	5.138	0.000
GPX (U/gHb)	24.07 ± 0.88	19.18± 0.97	20.349	0.000
SOD (U/gHb)	799.94±55.39	578.98±25.78	19.805	0.000
MOD (µmols/L)	4.01 ± 0.22	6.08 ± 0.47	21.717	0.000

Table:3- Comparison between Type 2 DM with and without complication

Parameters	Glycated hemoglobin (%)	
	r	P
Age (yr)	-0.248	0.187
Duration of diabetes (yr)	0.118	0.533
Glutathione peroxidase (U/gHb)	-0.637(*)	0.000
Superoxide dismutase (U/gHb)	-0.350	0.058
Malondialdehyde (µmol/L)	0.292	0.118

* Correlation is significant at 0.001 level

Table-4: Correlation between HbA1C (%) and age, duration of diabetes, GPX, SOD & MDA in Type 2 DM without complication

Parameters	Glycated Hemoglobin (%)	
	R	P
Age (Yr)	0.057	0.767
Duration Of Diabetes (Yr)	-0.015	0.396
Glutathione Peroxidase (U/Ghb)	-0.394(*)	0.031
Superoxide Dismutase (U/Ghb)	-0.385	0.447
Malondialdehyde (µMol/L)	0.542(**)	0.002

*Correlation is significant at 0.05 level, ** Correlation is significant at 0.01 level

Table:5- Correlation between HbA1C (%) and age, duration of diabetes, GPx, SOD & MDA type 2 DM with complication

groups of T2DM and control. HbA1C was increased in diabetic without complication compared to controls and a further increase in HbA1C was observed in diabetic patients with MVC compared to controls. The observation shows diabetes with poor blood

The occurance of this process in diabetes could contribute to the elevated levels of plasma peroxides.[16,17]

In this study, there was significant difference in the activity of GPX and SOD among the two groups of T2DM and controls, and a further decrease of GPX and SOD activity was observed in diabetic patients with MVC compared to control and compared to without MVC. The lowest activities were noticed in patients with poor diabetic control. These findings were in agreement with a number of studies[18,19] but contradictory to the findings of Kajanochumpal S et al.[15] In diabetic patients, the autoxidation of glucose results in formation of hydrogen peroxide[16] which inactivates SOD.[20] Therefore the accumulation of hydrogen peroxide may be one of the explanations for decreased activity of SOD in these patients.

. Under severe oxidative stress conditions GPX may be inactivated which is relatively stable enzyme under normal conditions .[24] High glucose condition may cause inactivation of enzyme by its glycation.[25] The low activity of GPX causes accumulation of H_2O_2 in diabetic patients. This could also explain the progressive decrease in SOD in later stages of the diabetes.

Diabetes is usually accompanied by increased production of free radicals.[26] Lipid peroxides are thought to be formed by free radicals and may play an important role in progression and development of MVC. In this study serum levels of MDA were increased in the diabetics compared to controls and a further increase was observed in diabetic with MVC compared to control and compared to patients without MVC. In this study, the correlation between the MDA concentration and HbA1C levels in diabetics is positive but not significant among diabetics without complication but was highly significant in group of diabetics with complications. The hyperglycemia in association with improper blood glucose control observed in these diabetic patients could be the causative factors for the increased production of lipid peroxides as the MDA positively correlated with HbA1C.

In spite of low activities of SOD in both groups of diabetes a negative but nonsignificant correlation between the activities of the enzymes and levels of HbA1C was observed. These findings were similar to the findings of Ruiz et al[27] but not with those of Singhania N etal.[28] There was also a negative correlation between GPX activity and HbA1C both in group of diabetic without complication and with complication which is significant at $p < 0.001$ and $p < 0.05$ respectively. The small size of study population limits its ability to draw a concrete conclusion and a further study with larger sample size is required.

CONCLUSION

The findings of the present study strongly confirm susceptibility of diabetic patients to oxidative stress and that poor glycaemic control is associated with free radical-mediated lipid peroxidation. Patients with MVC have significantly much lower activity of GPx and SOD than the group of diabetic patients without complication. Significantly increased levels of MDA in diabetic patients with microvascular complications suggest that oxidative stress plays an important role in the pathogenesis of microvascular complications.

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