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Role of oxidative stress and enzymatic antioxidants status in diabetic nephropathy (DN) patients- in western Uttar Pradesh

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Abstract

Metabolic abnormalities in type 2 diabetic patients can increase the generation of free radicals which leads to oxidative stress. This leads to disturbances in insulin secretion and action leading to diabetic complications like nephropathy etc. The free radical burden is normally detoxified by various endogenous and exogenous antioxidants. The study aims to find the relationship between oxidative stress and enzyme antioxidants in DN. This study was carried out at Lala Lajpat Rai Memorial Medical College, Meerut, U. P. from 2021-22. This was a type of case-control study. Total 150 subjects of age and sex matched were enrolled in 03 groups. Healthy control and the cases divided into 2 groups of diabetes without and with nephropathy. Malondialdehyde (MDA) in plasma, *superoxide dismutase* (SOD) and *catalase* (CAT) were analyzed in hemolysate. The results observed were presented in mean ± SD. Controls and cases were compared using "t-tests" and ANOVA. The correlations were determined by the Pearson's correlation "p" value<0.05 considered significant. Comparison of various circulatory parameters found a significant difference between cases as compared to controls at $p < 0.05$. Correlation of eGFR with *catalase* in DN group p=0.01 was found to be significant. A correlation of SOD with *catalase* was found to have a positive correlation in DN. We conclude that with the severity of disease oxidative stress was increased and enzyme antioxidants decreased. In patients of DN catalase were found a significant correlation with eGFR, UAE, SOD etc. but we did not find significant association between MDA and SOD or CAT in western U. P.

Keywords: Oxidative stress, enzyme antioxidants, diabetes mellitus, and diabetic nephropathy

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1. Introduction

Diabetes mellitus (DM) and its complications are the major cause of morbidity and mortality worldwide [1]. The global prevalence of diabetes in adults was reported 9% (463 million adults) and 77 million adults have diabetes in India as per data estimated in 2019. The prevalence of diabetes and its associated complications is gradually increasing with increasing of its risk factors such as sedentary lifestyle, eating fast food or packed food, working environment and mental stress which results in chronic hyperglycemia, hypertension, metabolic and genetic alterations [2]. DN is the major cause of kidney disease and affects ~30-40% of type 2 diabetes mellitus (T2DM) patients. It is defined by increased urinary excretion of albumin/protein without any other renal diseases and increases the risk of death. Chronic hyperglycemia and genetic alterations are the risk factors causing metabolic, biochemical, and vascular abnormalities in diabetic complications [3]. Metabolic disorders characterized by hyperglycemia which is the result of insulin resistance or beta cell dysfunction [4]. During glucose metabolism and energy production, generation of free radicals such as $\bullet O^{-2}$, •OH, •NO etc., which are also known as reactive oxygen or nitrogen species (ROS or RNS) occurs in excess leading to **oxidative stress**. Endogenous or exogenous antioxidants reduce ROS and detoxify them by neutralization. In case of

hyperglycemia, excess production of ROS from energy generation pathway i.e., electron transport chain (ETC) imbalances antioxidant status in the body [5]. Oxidative stress causes morphological and functional changes in cell proteins, membrane lipids and nucleic acids by which metabolic signal transduction impairment occurs. This leads to disturbances in insulin secretion and action and ultimately leads to diabetic complications like retinopathy, neuropathy, and nephropathy etc. As mentioned above, lipids are damaged by ROS/RNS and produce hydroperoxides, e.g., malondialdehyde (MDA). It is used as a biomarker for oxidative stress [6]. Endogenous enzymes which act as antioxidants to protect against free radicals are *superoxide dismutase (SOD)*, and *catalase (CAT)*, etc. These are responsible for the detoxification of superoxide radicals to hydrogen peroxide and ultimately to water and molecular oxygen in the cell [7]. This study was focused on the metabolic generation of excess free radicals and detoxification by enzyme antioxidants. Furthermore, we can find out the relationship between oxidative stress and enzyme antioxidants in diabetes with or without nephropathy that will be helpful in the management as well as prevention of diabetic complications.

2. Materials and methods

This was a case-control study, a total of 150 subjects were registered in 3 groups (50 subjects in each group). Group I: Healthy control of age and sex matched, Group II: diabetes without nephropathy and Group III: diabetes with nephropathy. History, physical examination, and various routine biochemical investigations were done to rule out other causes of nephropathy.

Inclusion criteria: Male and female between 30-75 years of age for all groups, screening of T2DM and DN were done as per the guidelines of American Diabetes Association (ADA)[7]. T2DM subjects were negative for urinary protein. DN subjects were diagnosed based on the following criteria: normo-albuminuria (< 30 mg/24 h), microalbuminuria, (30 to 300 mg/24 h) and macroalbuminuria or nephropathy (> 300 mg/24 h) along with fasting blood sugar (≥ 126 mg/dL).

Exclusion criteria: Pregnant or lactating women, non-diabetic kidney disease, hypertension**,** polycystic ovarian syndrome**,** any drug-causing nephropathy**,** or any cause of nephropathy other than T2DM. The present study was done after ethical approval from the institutional committee of Lala Lajpat Rai Memorial Medical College, Meerut, Uttar Pradesh and written informed consent were taken from each subject. (Reference No. S-1/2019/9263 Dated: 16.12.2019)

Laboratory Investigation: A total of 3.0 mL of peripheral blood samples was collected from the patients as well as controls. From which 1.5 mL blood was collected in an EDTA vial for HbA1c and plasma/lysate preparation and 1.5 mL blood in a plain vial for biochemical parameters. Estimations of blood glucose, Liver (serum protein and serum ALT) & Kidney (serum urea and serum creatinine) function tests also Urinary albumin excretion (UAE) of a 24-hour Urine sample have been performed using Selectra Pro M/Selectra XL Autoanalyzer (ELITECH Group). HbA1c was performed on a D-10 HbA1c Analyzer (Bio-Rad). Estimated Glomerular filtration rate (eGFR) was calculated by CKD-EPI Creatinine equation (2021) given by National Kidney Foundation is as follows and classification shown in Table 1 [8]

Estimated GFR (creatinine) = $142 * \min(Scr/K, 1)^{\alpha} * \max(Scr/K, 1)^{-1.200} * 0.9938^{Age} * 1.012$ [if female]

Where: Scr = standardized serum creatinine in mg/dL, K = 0.7 (females) or 0.9 (males), α = -0.241 (female) or -0.302 (male), min (Scr/K, 1) is the minimum of Scr/K or 1.0, max (Scr/K, 1) is the maximum of Scr/κ or 1.0 and Age (years).

Plasma and Lysate separation: Whole blood was collected in an EDTA vial for plasma and lysate preparation. 1.0 mL of whole blood from EDTA vial is transferred to a 2.0 mL Eppendorf tube and centrifuged at 2,500 rpm for 15 minutes at 25-30 °C for plasma separation. Separated plasma after centrifugation was then transferred to a 2.0 mL Eppendorf tube for malondialdehyde (MDA) analysis The RBC pellet which remained intact in the bottom of the tube was used for lysate preparation.

The activity of malondialdehyde (MDA) was analysed in plasma by the Ohkawa *et. al*. Method [9]. Endogenous enzyme antioxidant, *superoxide dismutase (SOD) and catalase (CAT)* were analysed in lysate *by* McCord, & Fridovich *method* [10] and *Aebi method* [11] respectively *on MULTISCAN GO with SkanIt Software (Installation code: 73617) (Cat. No.51119300,* Thermo Fisher Scientific, Japan).

Statistical Analysis: The statistical analysis was done employing online software Statistical Package for the Social Sciences (SPSS) 16.0 version (SPSS Inc., Chicago, Illinois, USA). The results observed were presented in mean ± SD. various circulatory parameters of controls and cases were compared using independent sample "t-tests" and ANOVA for continuous variables. The bivariate correlations between quantitative variables were determined by the Pearson's correlation. A "p" value less than 0.05 & 0.01 was considered statistically significant.

3. Results and Discussions

Results showed in Table 2 that there were no significant differences between mean ages of subjects among groups independently or combined. Sex difference was found significant only between diabetic group without nephropathy and healthy controls. Duration of diabetes between diabetic without and with nephropathy groups found significant at p-value = 0.00. The mean of blood sugar, glycated hemoglobin (HbA1c), serum urea, serum creatinine, eGFR and Urinary albumin excretion (UAE) was found significantly higher in Group III (diabetes with nephropathy) as compared to Group II (diabetes without nephropathy) and Group I (Healthy Controls) respectively p = 0.00. The mean of plasma MDA and enzymatic anti-oxidants such as *SOD & CAT* activities in lysate were found statistically significant ($p < 0.05$ & $p<0.01$) among study groups, as shown in Table 1 S. No. 13, 14, 15. Screening for normal liver function test serum protein and serum *Alanine transaminase* (ALT) did not find significant difference among groups independently or combined except between Group I and Group III (Table 2 S. No. 7, 8). Duration of diabetes in patients of Diabetic Nephropathy has shown a significant positive correlation with urinary albumin excretion (UAE) and *Catalase* (CAT) as shown in Table 3 r = 0.458** and 0.296* respectively. Serum Urea with SOD (r

= -0.290*) in Group II and serum Urea with eGFR, UAE and CAT (r = -0.544**, 0.392**, 0.310* respectively) in diabetic nephropathy group were found a significant correlation as shown in Table 3 (S. No. 3). There was a strong negative correlation between serum creatinine and eGFR r = -0.532** & -0.819** and a positive correlation between serum creatinine and UAE (r = 0.460** & 0.470*) in both case groups diabetes without nephropathy as well as diabetic nephropathy as shown in Table 3(4). eGFR has shown a negative correlation with UAE (r- value = -0.385**) in patients of diabetes without nephropathy and a significant negative correlation of eGFR with UAE (r = -0.461**) and with SOD (r = -0.32*) were found significant in patients of diabetic nephropathy. We have not found any significant correlation of plasma MDA either with *SOD* or *Catalase* in both case groups. But we have found a significant positive correlation of *SOD* with *Catalase* in diabetic nephropathy group r = 0.297* as shown in Table 3.

In this study, malondialdehyde (MDA) was found significantly higher in study groups as compared to control subjects independently and in between all groups. A Gujarat based study found that plasma MDA was higher in study groups as compared to controls [12]. Iranian study found no significant difference between study groups [13]. A Varanasi, Eastern U. P. based study found an increase in oxidative stress in ESRD patients and DN and T2DM with the progression of disease [14]. Sharma M. *et al*. (2016) Delhi based study found that MDA level was higher in DN subjects as compared to T2DM and healthy control [15]. Activities of SOD and *Catalase* have been found significantly lower in DN subjects as compared to T2DM subjects & healthy controls. A Gujarat based study found a decrease in *SOD* levels in the study group as compared to controls [12]. A study found that significant increase in MDA & *catalase* but a decreased activity of *SOD* in T2DM subjects with or without nephropathy as compared to controls and in DN as compared to T2DM [16]. It was reported that *SOD* activity in T2DM was lower when compared to healthy individuals [7]. In the present study with the severity of disease oxidative stress was increased and enzyme antioxidants decreased. In patients of diabetic nephropathy, we have found a significant correlation of *catalase* with SOD but there was no significant correlation of MDA either with *superoxide dismutase or catalase* in western U. P. In a study, it was found that the excessive production of reactive oxygen species (ROS) results in lipid peroxidation with a corresponding decrease in the activities of antioxidant enzymes in diabetic nephropathy [16]. Antioxidant systems are essential in balancing redox homeostasis. A main part of the antioxidant defense is performed by enzymes e.g., *superoxide dismutase (SOD) and catalase (CAT) etc. and* other non-enzymatic structures, including vitamin E, vitamin C, and various biochemical mechanisms for ROS detoxification. For example, the disintegration of hydrogen peroxide by *SOD* and *CAT*, ultimately results in molecular oxygen and water [17]. Treatment that can stabilize oxygen metabolism and regulate oxidative stress can delay the development of diabetic complications. Therefore, antioxidant therapy might play a crucial role to reduce the risk of the progression of type 2 diabetes and its complications. Cochrane database systematic review showed that therapy with human recombinant superoxide dismutase, vitamin E, acetylcysteine and coenzyme Q was not very much satisfactory in decreasing the risk of diabetic nephropathy [18, 19]. There are some limited results found with disputes of antioxidant therapy, which could be useful in preventing diabetic complications.

Table 1: Classification of estimated Glomerular Filtration Rate (eGFR) as per National Kidney Foundation

S. No	Classification	Estimated GFR (mL/min/1.73 m^2)	Remark
1	Grade 1	≥ 90	Normal or high
2	Grade 2	60-89	Mildly decreased
3	Grade 3a	45-59	Mildly to moderately decreased
4	Grade 3b	30-44	Moderately to severely decreased
5	Grade 4	15-29	Severely decreased
6	Grade 5	< 15	Kidney failure

Table 2: Comparative analysis of Circulatory Parameters between 3 Study Groups

S. No.	Parameters	Controls (GROUP I) Mean ± SD	Diabetes without Nephropathy (GROUP II) Mean ± SD	Diabetes with Nephropathy (GROUP III) Mean ± SD	Comparison between groups (P-Value)			
					I & II##	I & III ##	II & III ##	I, II, & III #
1	**Age (in years)**	51.02 ±9.04	52.14±7.80	53.70±9.55	0.25	0.07	0.18	0.31
2	**Sex (male) n**	35	20	26	**0.00****	0.06	0.22	**0.01***
3	**(females) n**	15	30	24				
4	**Duration of Diabetes (in years)**	Nil	3.05±1.99	10.42±3.66	-	-	**0.00****	-
5	**FPG/RBS (mg/dL)**	88.87±17.47	212.41±98.97	256.93±72.76	**0.00****	**0.00****	**0.00****	**0.00****
6	**HbA1c (%)**	5.06±0.54	7.80 ±1.07	9.09±2.75	**0.00****	**0.00****	**0.00****	**0.00****
7	**Serum Protein (g/dL)**	6.81±0.76	6.88 ±0.69	7.17±1.31	0.31	**0.04***	0.08	0.13
8	**Serum *ALT* (IU/L)**	33.62±13.88	36.40±17.36	45.86±42.71	0.18	**0.02***	0.07	0.07
9	**Serum Urea (mg/dL)**	29.17±8.08	32.63±7.73	71.93±39.21	**0.01****	**0.00****	**0.00****	**0.00****
10	**Serum Creatinine (mg/dL)**	0.82±0.11	0.92±0.11	1.94±0.75	**0.00****	**0.00****	**0.00****	**0.00****
11	**e GFR (CKD-EPI 2021) (mL/min/1.73m^2)**	101.22±10.69	85.32±12.45	40.78±15.45	**0.01****	**0.00****	**0.00****	**0.00****
12	**Urine albumin excretion (UAE) (mg/24 h)**	17.11±2.76	20.13±3.51	409.70±201.16	**0.00****	**0.00****	**0.00****	**0.00****
13	**Plasma MDA (nmol/mL)**	21.26±12.06	49.39±37.21	62.38±36.73	**0.00****	**0.00****	**0.04***	**0.00****
14	**Lysate *SOD* (U/mg/mL Protein)**	0.29±0.15	0.23±0.10	0.19±0.08	**0.00****	**0.00****	**0.02***	**0.00****
15	**Lysate *CAT* (U/min/mg/mL Protein)**	16.32±3.14	14.31±3.99	9.94±6.07	**0.00****	**0.00****	**0.00****	**0.00****

*#ANOVA, ##Independent t-test, *p<0.05, **p<0.01, considered statistically significant, HbA1c: Glycosylated Haemoglobin, ALT: Alanine transaminase, MDA: Malondialdehyde, SOD: Superoxide dismutase, CAT: Catalase. The significant p-values are represented in bold.*

Table 3: Correlation analysis between Clinical Parameters among Diabetes Groups without And with Nephropathy

S. No.	Parameters		Diabetes without Nephropathy					Diabetes with Nephropathy				
			eGFR CKD EPI 2021	**UAE**	**MDA**	**SOD**	**CAT**	**eGFR CKD EPI 2021**	**UAE**	**MDA**	**SOD**	**CAT**
1	**Duration of diabetes**	r- value	-0.137	0.098	-0.069	0.074	0.238	-0.240	**0.458****	0.068	0.017	**0.296***
		p-value	0.34	0.49	0.63	0.60	0.09	0.09	**0.00**	0.63	0.90	**0.03**
2	**HbA1c**	r- value	0.178	-0.112	-0.121	-0.115	0.177	0.113	0.042	-0.074	-0.105	0.174
		p-value	0.21	0.43	0.40	0.42	0.21	0.43	0.77	0.60	0.46	0.22
3	**Serum Urea**	r- value	-0.029	0.097	0.013	**-0.290***	0.098	**-0.544****	**0.392****	-0.166	-0.030	**0.310***
		p-value	0.84	0.50	0.92	**0.04**	0.49	**0.00**	**0.00**	0.26	0.83	**0.02**
4	**Serum Creatinine**	r- value	**-0.532****	**0.460****	-0.274	-0.074	0.127	**-0.819****	**0.470****	-0.128	0.081	0.250
		p-value	**0.00**	**0.00**	0.054	0.60	0.37	**0.00**	**0.00**	0.37	0.57	0.07
5	**eGFR CKD EPI 2021**	r- value	1	**-0.385****	0.099	-0.066	0.067	1	**-0.461****	0.168	-0.169	**-0.32***
		p-value		**0.00**	0.49	0.64	0.64		**0.00**	0.24	0.24	**0.01**
6	**UAE**	r- value		1	-0.113	-0.085	0.204		1	0.036	0.149	0.188
		p-value			0.43	0.55	0.15			0.80	0.29	0.191
7	**MDA**	r- value			1	-0.057	-0.057			1	-0.097	-0.117
		p-value				0.69	0.69				0.50	0.41
8	**SOD**	r- value				1	0.016				1	**0.297***
		p-value					0.90					**0.03**

**p<0.05, and **p<0.01, considered statistically significant, HbA1c: Glycated Haemoglobin, ALT: Alanine transaminase, MDA: Malondialdehyde, SOD: Superoxide dismutase, CAT: Catalase. The significant r & p-values are represented in bold.*

4. Conclusions

According to the obtained results on the Diabetes mellitus and diabetic nephropathy are the most common causes of morbidity and mortality worldwide. Hyperglycemia is the result of insulin resistance that causes an increased burden on the cellular and mitochondrial metabolism of glucose and increased production of free radicals (ROS). ROS results in oxidative stress which is a potent marker for damaging cell membrane components like lipids, proteins, and DNA. These could result in mutation and causes alteration in cell signaling or transport mechanisms. To overcome this oxidative stress burden, antioxidants play a crucial role in detoxification through various mechanisms. In the present study, we have found that the oxidative stress parameter MDA is found higher in T2DM and DN subjects as compared to controls with the simultaneous decrease in *catalase* and *superoxide dismutase* in both study groups. We conclude that decreasing oxidative stress by early detection of oxidative stress makers and accordingly antioxidant therapy for the management of diabetic nephropathy to end-stage renal disease could be helpful.

There are some limitations that this study was performed after and during COVID-19 pandemic situation. Further, studies should be performed without the situation of epidemic, pandemic, and any type of chronic complications other than T2DM and DN.

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