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Investigation of oxidative stress in nephropathy: Diabetic Vs Non-Diabetic Profile

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Chronic kidney disease (CKD) is associated with a high risk of cardiovascular disease (CVD) development, whereas, diabetes mellitus is a major etiology of CKD leading to end stage renal disease. Oxidants and antioxidants imbalance plays an important role in the disease progression both in diabetes and CKD. The study was planned to assess oxidative stress alone in CKD and diabetic CKD. A total of 100 patients were enrolled in the study meeting defined criteria after explaining purpose and taking consent, categorized into two groups of Normoglycemic CKD and diabetic CKD with equal sample size along with 50 healthy counterparts. Personal and family history was recorded on a structured questionnaire. Fasting blood samples were collected in heparinized tubes and plasma/hemolysates were prepared for biochemical estimation of catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH), malonaldehyde (MDA), glucose and creatinine. Mean values were compared using SPSS17 at alpha level 0.05. Male gender, anemia and use of chewable/smoking tobacco were the prevailed findings. Whereas, an increase in the oxidative stress of both patients' groups was observed, that was more pronounced in the diabetic nephropathy. The mean percent change for CAT was found (-14%) though remained statistically non-significant, while for GSH (-22%, $p<0.05$), SOD (-32%, $p<0.05$), MDA (49%, $p<0.05$). The marked increase in oxidative stress along with diabetes mellitus may be one of the significant contributors that accelerate the CKD progression to ESRD as compare to normoglycemic CKD.

Keywords: Chronic kidney disease, Diabetic Nephropathy, Oxidative stress, Antioxidant Enzymes

INTRODUCTION

Diabetes mellitus is a group of metabolic diseases caused by defects in insulin action, insulin secretion, or both. It is a chronic disease which elevates risk of hypertension, abnormalities of lipoprotein metabolism, renal disease, cerebrovascular, peripheral arterial disease, and atherosclerotic cardiovascular disease (American Diabetes Association, 2019), and is the single most important cause of the renal abnormality leading to kidney failure (Nasri and Rafieian-Kopaei, 2015).

Diabetic nephropathy (DN) is a clinical syndrome assessed by the presence of elevated urinary albumin excretion (UAE) or proteinuria greater than 0.5 gm/24 hours in the absence of other renal diseases commonly called overt nephropathy, clinical nephropathy, proteinuria, or macroalbuminuria (Gross et al., 2005). Approximately 20-30% of diabetic patients with type 1 or type 2 develop the condition of diabetic nephropathy, but in type 2 diabetes, a considerably smaller fraction of these patients progress to end-stage renal disease (ESRD) (American Diabetes Association, 2019).

Hyperglycemia via metabolic derangements like elevated oxidative stress, activation of protein kinase C (PKC)-mitogen-activated protein kinases

(MAPKs), renal polyol formation, accumulation of advanced glycation end products, as well as such hemodynamic factors as elevated intraglomerular pressure, systemic hypertension, and inappropriate activation of the renin-angiotensin system (RAS) have been responsible for the progression and development of diabetic nephropathy (Cao and Cooper, 2011).

The normal kidney due to its increased metabolic activity produces a significant amount of oxidative stress which is balanced by a huge antioxidant system (Vasavada and Agarwal, 2005). Oxidative stress and ROS both are playing an important role in the development and progression of diabetic microvascular as well as macrovascular complications via two mechanisms, i.e., the metabolic modifications of target tissue molecules, and the alterations in the renal hemodynamics, which then lead to the diabetic nephropathy (Vasavada and Agarwal, 2005; Hunjoo and Kim., 2008; Josephine et al., 2008) although the intracellular signal transduction pathways regulated by reactive oxygen species (ROS) remains unclear.

In diabetic nephropathy, the nitroso-oxidant balance transfers toward a pro-oxidant condition that leads to the vascular and tissue injury. This oxidative damage progresses associated with worsening condition of diabetic nephropathy (Vasavada and Agarwal, 2005).

Hyperglycemia in diabetic nephropathy not only produces excessive ROS but also reduces anti-oxidative mechanisms in certain susceptible cell populations, such as, mesangial cells, glomerular epithelial cells, and proximal tubular epithelial cells directly via auto-oxidation and glucose metabolism and indirectly via generation of AGEs and their receptor binding. Thus, oxidative stress is considered as a common pathogenic factor for diabetic complications including nephropathy (Josephine et al., 2008).

As intracellular ROS are not properly able to measure in diabetic kidneys, according to many studies elevated lipid peroxidation of unsaturated fatty acid and oxidized DNA [8-hydroxydeoxyguanosine (8-OHdG), an oxidized purine residue in DNA] in the diabetic kidneys of patients and animals and in the urine, plasma, and renal proximal tubules of diabetic patients and animals with albuminuria, a glomerular injury marker in diabetes, indirectly indicating that ROS are increased in diabetic kidneys (Sharma et al., 2007).

In this context, the present study was aimed to assess the burden of oxidative stress in CKD patients perpetuated with the presence of diabetes mellitus as compare to non-diabetic CKD and healthy counterparts.

MATERIALS AND METHODS

The cross sectional study was randomized, observational, and conducted in collaboration with Department of Nephrology, Jinnah Post Graduate Medical Centre (JPMC), Karachi. A sample of 50 Hyperglycemic patients diagnosed with Nephropathy was included with a Normoglycemic group of another 50 patients of Nephropathy visiting OPD and enrolled for the study if otherwise meet the criteria and showed up their consent to share their history, allow to collect blood sample and was assured to keep the privacy.

The inclusion criteria includes both male and female subjects above the age of 18, having diabetes mellitus with FBG level equal to or more than 126 mg/dL (7.0 mmol/l), already receiving at least one antidiabetic medication, having a diastolic BP 70 to 90 mmHg and/or systolic BP 110 to 130 mmHg, Albumin-to-Creatinine ratio (ACR) ≥ 0.3, Protein-Creatinine ratio (PCR) ≥ 0.5, Glomerular Filtration Rate (MDRD formula) ≤ 80, Serum Creatinine ≥ 1.6 mg/dL for Males, or ≥ 1.4 mg/dL for Females, Glucosylated hemoglobin A1c (HbA1c) ≤ 12%, and non-pregnant, non-lactating women.

The exclusion criteria encompass subjects below age of 18, pregnant or lactating women, having a renal transplant or on dialysis, having renal disease other than diabetic nephropathy, obstructive uropathy (uncorrected and hemodynamically significant), having known non-diabetic kidney disease, and history or evidence of acute renal failure within 6 months prior to randomization visit.

Age matched 50 healthy volunteers are also included for local reference adjustment who are disease free and did not have any major risk factor.

A detail questionnaire was filled in by same interviewer on the basis of responses collected from subjects to gather information on various aspects of family and personal history of different diseases, total time period of becoming diabetic, economic status, life style, concerned habits such as tobacco consumption, exercise, use of alcohol or any other addiction, etc. Their heights and body weights along with blood pressure were measured following standard methods.

By using a monovette system of blood collection, blood samples from the antecubital

vein were taken before breakfast for determining the blood parameters. One ml of anticoagulated blood containing sodium EDTA was used for hematological analysis. The remaining anticoagulated blood was separated into plasma by centrifugation for 10 min at 1,000x g at +4°C. Five ml of blood was used for the detection of superoxide dismutase, catalase, glutathione reductase, urea, creatinine and malondialdehyde. Blood samples either plasma or hemolysates were stored at -80°C for the assessment of antioxidants (SOD, catalase, and GSH), oxidant (MDA), and renal efficiency (by urea and creatinine).

Blood samples were collected by venous puncture in heparinized tubes and the plasma was separated. After centrifugation, the buffy coat was removed and the packed cells were washed three times with 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 15 min at 2°C.

Fasting blood glucose and serum creatinine, urea were determined using fully automated clinical chemistry analyzer. While Superoxide Dismutase, Catalase, Glutathione Reductase, and Malondialdehyde were estimated following already described standard procedures.

The activity of superoxide dismutase was assayed by the method of Kakkar et al., (1984) that is based on NADH-phenazine methosulphate and amino blue tetrazolium formazan inhibition. Briefly 0.01 M (pH 7.0)-phosphate buffer mixed with hemolysate followed by 2 M H_2O_2 addition. Reaction was terminated by dichromate-acetic acid reagent (5%potassium dichromate and glacial acetic acid were mixed in 1:3 ratio) after incubation for 90 seconds. End reaction color is extracted into n-butanol layer and read at 520nm.

The activity of catalase was measured using method of Sinha (1972) based on reduction of Dichromate in acetic acid to chromic acetate, then heated in presence of H_2O_2 forming per chromic acid. The chromic acetate as end product was then measured at 590 nm. The activity of catalase was expressed as U/ml for plasma (U- µmoles of H_2O_2Utilised / second).

The reduced glutathione was measured by the method of Beutler and Kelley (1963) based on the development of yellow color when 5,5'- dithio-bis-2-nitrobenzoic (DTNB) is added to compound containing sulphydryl groups. The color developed was read at 412 nm in spectrophotometer. The values were expressed as mg/dl for plasma.

Levels of malondialdehyde (MDA) was estimated by thiobarbituric acid reactive substances (TBARS) by the method of Buege and Aust, 1978. MDA reacts with thiobarbituric acid (TBA) to form pink colored adduct, TBA-MDA adduct at 535nm. The MDA was calculated using of MDA, 1.56 * 105 M-1 cm 1. MDA was expressedΣmolar extinction coefficient (nmol/ ml of plasma).

Data was expressed as Mean ± S.D. and statistical significance was evaluated applying Student 't' test using statistical software (SPSS17®) for mean comparisons at α=0.05.

RESULTS

A total 150 subjects took part in the study, among those 50 had CKD with normoglycemic condition, whereas other 50 were the patients of CKD with hyperglycemic condition. While 50 healthy individuals with no personal history of diabetes, hypertension, or any other cardiac problem and having normal body weight and possessing no addiction, other way comparable to CKD patient groups, were added as control.

Mean age of participants in heathy control, non-diabetic CKD and DN was 40±7, 45±3.5 and 48±5 respectively. The control group is free from any sort of addiction, smoking, supplement intake or not taking any medicine while oral tobacco consumption was 36% in CKD and 42% in DN group, smoking was reported 25% and 32% in groups respectively. None of the patient is clinically established dyslipidemia or on lipid lowering medication.

The level of catalase was found to be decreased in normoglycemic CKD in comparison with the healthy control but the mean change was statistically non-significant, whereas the levels of catalase was also found decreased in the hyperglycemic group of CKD patients as compared to the healthy control and the mean change was significant ($p<0.05$). While the inter-group comparison of normo and hyperglycemic groups remained insignificant though we found a further reduction in the later group (Table 1, figure 1).

The mean percent reduction in the levels of catalase was found 7% in normoglycemic CKD, 20% in diabetic CKD with reference to the control group values (figure 1).

The levels of glutathione reductase found to be decreased in CKD alone group as compared to the healthy control and the mean change was statistically significant ($p < 0.05$), whereas the levels of glutathione reductase was also decreased in the diabetic group as compared to

healthy control ($p < 0.01$), (Table 1).

The mean percent reduction in the levels of glutathione reductase was found 20% in normoglycemic CKD. 38% in diabetic CKD with reference to the control group values, while the studied patients group showed 22% reduction with reference to normoglycemic (Figure 1).

The levels of superoxide dismutase found to be decreased in normoglycemic as compared to the healthy control but the mean change was statistically non-significant, whereas the levels of superoxide dismutase decreased in the hyperglycemic as compared to the healthy control and the mean change was significant ($p < 0.01$). While the inter-group comparison of the normo and hyperglycemic group was also remained significant ($p < 0.05$) but a less reduction was found in the later group (Table 1).

The mean percent reduction in the levels of superoxide dismutase was found 16% in normoglycemic CKD. 44% in diabetic CKD with reference to the control group values, while DN patients group showed 32% reduction with reference to normoglycemic (Figure 1). The levels of malondialdehyde increased in normoglycemic CKD as compared to the healthy control, whereas the levels of malondialdehyde was also increased in hyperglycemic CKD ($p < 0.01$). While the inter-group comparison of the normo and hyperglycemic group remained significant ($p < 0.05$) (Table 1).

Table 1: Comparison of Oxidative Stress Parameters between healthy individuals and CKD patients either Non-diabetic or Diabetic

	GROUPS		
PARAMETERS	**Healthy Control (n=50)**	**Non-diabetic CKD (n=50)[1]**	**Diabetic CKD (n=50)[1, 2]**
CAT (U/ml)	53.32 ± 4.5	48.4 ± 2.4 ^{N}	41.6 ± 2.9 $^{*, N}$
GSH (mg/ml)	25.6 ± 3.1	20.4 ± 1.8*	15.9 ± 1.4 $^{**, *}$
SOD (U/mg Hb)	3.25 ± 0.29	2.7 ± 0.31^{N}	1.84 ± 0.35 $^{**, *}$
MDA (µmol/L)	1.33 ± 0.20	1.96 ± 0.25 ^{N}	2.88 ± 0.38 $^{**, *}$

Mean values compared at standard alpha 0.05
1: As compared with healthy control
2: As compared with Non-diabetic CKD
*: p<0.05
**: p<0.01
N: Non-significant (statistically)

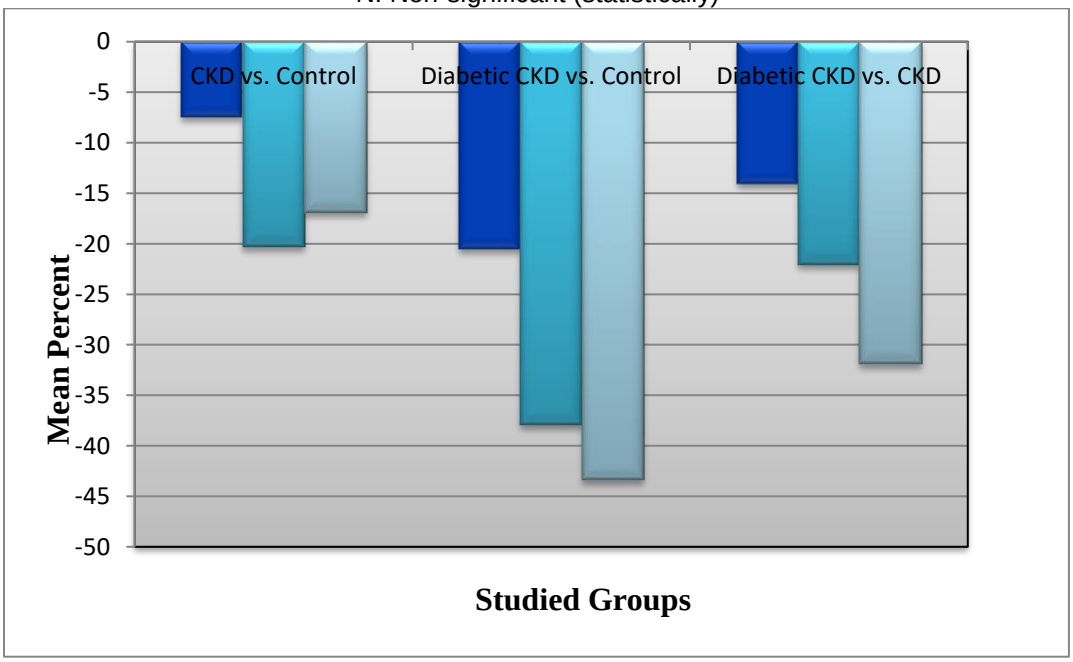

Figure 1: Mean Percent reduction in the antioxidant enzyme levels in non-diabetic CKD patients and Diabetic CKD as compared with Non-CKD healthy control groups.
CAT: catalase GSH: reduced glutathione SOD: superoxide dismutase

DISCUSSION:

Diabetic Nephropathy, especially as one of the complication of Diabetes mellitus, is rising globally. Diabetic Nephropathy has come out as the leading cause of ESRD, requiring dialysis. DN is a major cause of premature death in diabetic patients, predominantly from CVD, the prevalence of which is about 15-fold greater in patients with diabetic nephropathy (Stephen, 2010). Between 20%-40% of diabetic patients eventually emerge nephropathy, even though the reason is unknown that why not all diabetic patients develop this complication.

Oxidative stress also appears to play a pivotal role in the development of diabetic nephropathy. Mostly, metabolic activity within the nephron generates a large amount of ROS which are counterbalanced by an enormous number of enzymatic and nonenzymatic antioxidants including catalase (CAT), glutathione reductase (GSH-Red), and superoxide dismutase (SOD) as well as by free radical scavenging systems. The level of these antioxidant enzymes critically affects the susceptibility of different tissues to oxidative stress and is related with the development of diabetic complications including DN. ROS mediate many negative biological effects, such as oxidation of proteins, peroxidation of cell membrane lipids, renal vasoconstriction and damage to DNA. Unluckily, hyperglycemia tips the balance towards generation of ROS; nearly all of them seem to be formed in the mitochondria. The metabolism of glucose via harmful alternate pathways like PKC activation and production of AGEs, in the setting of hyperglycemia also seems slightly dependent on ROS. Hyperglycemia particularly induces OS, even before diabetes becomes clinically manifest. Concentrations of markers of DNA damage induced by ROS have been found in larger amount in patients with more-severe nephropathy.

Moreover, the histological investigation of human kidney biopsy specimens has detected products of glycol-oxidation (combined products of glycation and protein oxidation) and lipoxidation in the mesangial matrix and glomeruli, whereas these lesions are much less common in specimens from individuals without hyperglycemia (Suma et al., 2008).

The previous studies in experimental diabetes have documented that the renal NO levels become reduced as a result of elevated OS, particularly as a result of increased expression of SOD and catalase. The OS is apparent in increased products of mitochondrial oxidative stress and also enhanced production of peroxynitrite in the kidney. OS is implicated as an important mediator in the pathophysiology of diabetic nephropathy. Both hyperglycemia and activation of the RAS play an important role in the production of ROS (Sharma et al., 2007).

Previous research studies have demonstrated a relation between complications of diabetes and elevated oxidative stress. The prospective mechanisms for OS could include multiple factors like the enhanced mitochondrial formation of ROS, reduced protective mechanisms activity, or elevated production of ROS from Amadori products or Schiff bases (Paul et al., 2005).

A large body of documentation indicates that diabetes is a state of enhanced OS, and it has been suggested that oxidants play an important role as causative agents for the major pathways that have been incriminated in vascular complications of diabetes including nephropathy. In DN, hyperglycemia increases the generation of oxidants in glomerular cells, oxidants have various direct biologic effects that are related to DN, as a consequence of that antioxidants decrease the hyperglycemia–induced biologic effects.

There are possibly various pathways for increased production of oxidants. Phagocyte-like NAD(P)H oxidase is a vital source of oxidants in many nonphagocytic cells, including renal cells such as glomerular mesangial cells and tubular epithelial cells. Recent studies have demonstrated the important role of NAD(P)H oxidase and PKC in increased production of oxidants in diabetes and its related complications including DN.

In response to hyperglycemia, mitochondrial metabolism has also been suggested as an important source for the production of oxidants, alike to that proposed in the vascular bed. Oxidants can activate almost all of the known pathways in mesangial cells that have been involved in diabetes, including PKC, MAPKs, TGF-β1, and fibronectin.

Additional support comes from other studies in which antioxidants prevent albuminuria, renal and glomerular hypertrophy, glomerular expression of TGF-β1 and ECM, and activation of PKC in experimental diabetes. Our result is consistent with the result of Moussa, who reported an increase in oxidant (MDA) and decrease in antioxidant (GSH) (Moussa, 2008).

The study limitations are small sample size collected from one center and lack of follow up to further investigate the role and intensity of oxidative stress with advancement of CKD and its relation with blood glucose levels in controlled vs

uncontrolled diabetes mellitus patients.

CONCLUSION

Increased oxidative stress in diabetic nephropathy may be one of the major factor responsible for earlier renal function deterioration as compared to non-diabetic CKD patients.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

AUTHOR CONTRIBUTIONS

GY designed the study, analyzed data, supervised experimental work, reviewed manuscript, SMN performed bench work, NIK collected organized data, SS collaborated in sample collection from patients, LN contributed in final write up. All authors read and approved the final version.

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