ANTIOXIDANT STATUS OF TYPE 2 DIABETIC PATIENTS WITH AND WITHOUT NEPHROPATHY ATTENDING FEDERAL MEDICAL CENTER KEFFI, NASARAWA STATE

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ABSTRACT
Background: Oxidative stress originating from hyperglycaemia is involved in the pathogenesis of diabetic nephropathy. Antioxidants play important role in ameliorating the effect of oxidative damage caused by free radical.
Aim: The aim of the present study was to evaluate antioxidant levels in type 2 diabetic patients with nephropathy in the region and to compare it with those of type 2 diabetics without nephropathy and healthy non-diabetic controls.
Methodology: One hundred and twenty-two (122) participants consisting of (60) diabetic test subjects and (62) apparently healthy control subjects were recruited with their consent. The diabetic subjects were further categorized into two groups (32) with type 2 diabetics with nephropathy (DWN), and (28) type 2 diabetics without nephropathy (DWON)) and were compared with (28) apparently healthy non-diabetic controls.
Fasting blood glucose (FBG) and glycated haemoglobin (HbA1c), serum creatinine (SCr) and random urine proteins (UPr) were assayed by routine laboratory methods. Serum antioxidant levels (superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), vitamin E (VE) and vitamin A (VA)), were measured using standard commercial reagent kits. Statistical package for the social sciences (SPSS version 23) was used for data analysis.
Results: The results were expressed as mean ± SD and Pearson correlation was analysed to ascertain the relationship among variables. There was significant increase seen in FBG, HbA1c, SCr, eGFR and SOD and decrease in GPX, CAT and Vitamin E in type 2 diabetic patients with and without nephropathy as compared with control population, whereas vitamin A level was unaltered amongst the groups. Negative correlation was seen between eGFR and SOD enzyme activity in diabetic group, while CAT and vitamin E showed strong positive correlation to eGFR in the diabetic groups. Duration of disease was found to be more in the diabetic with nephropathy than in diabetic without nephropathy. Mean age did not show any significant difference among the groups (p=0.0732).
Conclusion: The present study observed that the change in level of antioxidants of diabetic subjects is more severe than in apparently healthy subjects and higher in DWN than DWON. Present study may suggest the benefits of antioxidants in combating the free radicals of oxidative stress which is a major contributor in the pathophysiology of diabetic nephropathy.

INTRODUCTION
Diabetic nephropathy is characterized by gradual and progressive alterations in vascular system of the renal tissue due to chronic hyperglycaemia (Wu *et al*. 2014).

Experimental and clinical studies suggest an association between hyperglycaemia, oxidative stress, and diabetic complications (Elmarakby *et al.* 2014). According to Bunza and Alhassan, (2019), four molecular mechanisms are implicated for their role in causing diabetic complications; which include: increased polyol pathway influx, increased formation of advanced glycation end-products (AGEs), activation of protein kinase C uniforms, and increased hexosamine pathway activity. Oxidative stress activates the pathogenic pathways which exacerbate insulin resistance leading to diabetes and its complications including nephropathy, retinopathy, neuropathy and cardiovascular disease (Bunza and Alhassan, 2019). Oxidative stress encountered at the level of the renal tissue causes renal damage (Ramchandra *et al*. 2012).

In normal cells there is an intricate pro-oxidant and antioxidant balance. In oxidative stress, this balance shifts towards pro-oxidants. If the oxidant species production is increased with concomitant prolonged and massive stress, it results in serious cell damage. To overcome these consequences, cells have antioxidant defense systems (SOD, GPX, CAT, Vitamin E and Vitamin A) which scavenge the free oxygen radicals and suppress free radical chain and resultant cell damage. (Shaw *et al.* 2010).

Altered antioxidant status arising from chronic hyperglycaemia is implicated in the genesis of diabetic complications, more especially diabetic nephropathy (Shin *et al.* 2014). Oxidative stress has been implicated as an important pathophysiological process leading to diabetes mellitus and its associated complications including nephropathy, resulting in high morbidity and mortality rates. Interestingly, Oxidative stress is recognized as a common product of many pathways involved in pathogenesis of diabetic nephropathy, triggering a chain of reactions leading to injury, and vice versa. Majid, (2013) therefore, has indicated that inhibiting oxidative stress and blocking the various pathways, might have a positive effect in slowing down disease progression to diabetes nephropathy especially in diabetics.

Current evidence has demonstrated the important role oxidative stress plays in the pathogenesis of diabetes mellitus which may diminish the antioxidant defense system of the body, thereby increasing the oxidative load (Manna *et al*., 2015). Studies have associated low concentration of antioxidants with increased risk of diabetes complications in individual patient with diabetes (Sankhla, 2012; Ceriello, 2016). Antioxidants are found to normalize many parameters of oxidative stress and delay development of nephropathy in diabetes. The present study is undertaken to investigate the status of antioxidants superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), vitamin A and vitamin E, in type-2 diabetic patients with and without nephropathy. However, there is paucity of research work to support the role of antioxidants in preventing diabetic nephropathy in human beings (Majid, 2013). Hence the need for more extensive research that would explore the beneficial role of these antioxidants in preventing diabetes nephropathy.

Hence, this study became imperative as it tends to add to the body of already existing knowledge by helping the health-care givers, patients, researchers as well as the general public, gain adequate knowledge regarding the beneficial role of the antioxidants investigated in this study. Also the possible outcome of this study, could help in the diagnostics and therapeutic management of patients with diabetes and nephropathy.

MATERIALS AND METHODS.

Study Design

This is a comparative case-controlled study.

Study Population:

Subjects recruited into the study are those attending the general outpatient departments (GOPD) of medicine and endocrinology unit at the Federal Medical Centre Keffi Nasarawa State, Nigeria.

The study recruited a total of one hundred and twenty-two (122) subjects for this study using random sampling method. A total of sixty (60) patients with type 2 diabetes were recruited for this study about twenty-six (26) males and thirty-four (34) females.

The diabetic patients were further categorized into two groups: diabetics without nephropathy (DWON) (28) and diabetics with nephropathy (DWN (32). All the subjects with type-2-diabetes recruited for the study, were further subjected to other laboratory tests and were also examined by a Nephrologist for diabetic nephropathy.

Similarly, sixty-two (62) age and sex-matched(twenty-eight (28) males, and thirty-four (34) females), apparently healthy individuals served as controls. They were drawn from those who attended routine health check-ups at the hospitals, friends, colleagues, as well as relatives.

Data Collection

Ethical approval was sought from research and ethical committee of Federal Medical center, keffi, Nasarawa state. Data was collected after a questionnaire form was administered to the consented participant after signing of informed consent form.

Sample Collection and Processing

After an overnight fast, 10 ml of venous sample was drawn from participants. 2ml was added into fluoride oxalate tubes for plasma glucose analysis. 3ml of the venous blood collected into Ethylene-Diamine-Tetra–acetic Acid (EDTA) tubes and analyzed for glycated haemoglobin., 5 ml of venous blood samples were added into plain tubes and allowed to clot for about 5 minutes after which serum samples were separated by centrifuging at 3000 rpm for 5 minutes. Superoxide dismutase (SOD) and glutathione (GPx), levels were measured using appropriate assay test kits on spectrophotometer (Vitros 380) at 505 nm and 340 nm respectively. Catalase (CAT) was measured at 405 nm, vitamin E at 533 nm and vitamin A at 620 nm wavelengths respectively. Serum creatinine was estimated using the patient's serum and estimated glomerular filtration rate (eGFR) was calculated. Random urine samples for urine protein determination, were collected into plain urine containers and analyzed immediately upon collection.

Biochemical Assays

Plasma glucose estimation was estimated by GOD_PAP enzyme colorimetric method using Trinder's method (1969). Glycated haemoglobin (HbA1c) assay was carried out using fluorescent immunoassay (FIA) technique. Urine microalbumin estimation was done using the Finecare Microalbumin rapid test which is based on principle of fluorescence immunoassay technology. Serum creatinine estimation was by Jaffe colorimetric-kinetic method by (Murray, *et al*. 1984). Catalase assay was done using the colorimetric measurement by Goth method (1991). Superoxide dismutase assay was done using Hydroxylamine method (Wooliams *et al. 1*983). Glutathione peroxidase assay was done using colorimetric method by Paglia and Valentine (1967). Vitamin E assay was by colorimetric measurement and Vitamin A assay was by colorimetric measurement using Trifluoroacetic acid (TFA) method as described by Neeld and Pearson (1963).

Data analysis

IBM statistical package for the Social Sciences (SPSS Inc, Chicago, IL, USA version 23) was used for data analysis. Data were expressed as mean ± SD (standard deviation) for normally distributed data. The statistical significance of differences between the means of quantitative variable across groups (control, diabetics without nephropathy (DWON) and diabetics with nephropathy DWN) was evaluated by Student t-test and the one- way analysis of variance (ANOVA) test where appropriate. Tukey HSD (Honest Significant Difference) as post-hoc test for variables with normal distribution. Correlation statistics were compared with Pearson coefficients. P-value less than or equal to 0.05 were taken to be significant.

RESULTS AND DISCUSSION

Results Table 1: Demographic Profile of Diabetics with and without Nephropathy and Control groups

Parameter	Control n = 28	DWON n =28	DWN n = 32	p = values
FBG (mmol/l)	4.60±1.20	8.20±2.60	12.10±2.90	0.0001*
HbA1c (%)	4.10±1.30	8.30±2.60	8.90±1.30	0.0001*
Creatinine(umol/L)	1.10±0.20	1.20±0.26	2.70±0.57	0.0001*
eGFR(kg/ml/mins)	92.20 ± 20.53	72.50 ± 14.40	32.6 ± 7.80	0.0001*
Urine protein (g/dl)	15.30 ± 0.98	15.40 ± 0.81	214.80 ± 101.4	0.0001*

Note: DWON (Diabetics Without Nephropathy)
DWN (Diabetics With Nephropathy)

Table 2:Biochemical parameters of the Diabetics with and without Nephropathy and Control group

Parameter	Control n = 28	DWON n =28	DWN n = 32	Total (%) n = 88	p – values
Sex:					
Male	n =13 (14.20%)	n = 11 (12.00%)	n = 15 (17.04%)	0.329	
Female	n = 15 (17.04%)	n = 17 (19.30%)	n = 17 (19.30%)	0.621	
Age (Years)	35.30± 6.76	36.50 ± 7.18	38.52 ± 8.66		0.073
Duration of Diabetis (years)	N/A	5.20 ± 1.00	8.00 ± 1.50		0.0001*
Weight (Kg)	68.60±9.60	65.40±5.40	63.2±5.30		0.0001*
Height (cm)	170.00 ± 8.70	169.40 ± 6.60	170.30 ± 5.40		0.249
BMI (Kg/m^2)	25.09 ± 3.90	23.03 ± 1.70	21.07 ± 2.80		0.0001*

Table 3:Antioxidant parameters of the Diabetics with and without Nephropathy and Control groups

Antioxidants	R	p- value
SOD(U/ml)	0.015 (0.912)	0.001
GPx (U/ml)	0.294 (0.024)	0.05
CAT(U/ml)	0.128 (0.334)	0.001
VE(ug/ml)	-0.068 (0.680)	0.001
VA(ug/ml)	-0.132 (0.317)	0.001

Note: DWON (Diabetics Without Nephropathy)
DWN (Diabetics With Nephropathy)

Table 4. Correlation between HBA1c, and the antioxidants SOD, GPX, CAT, VE and VA amongst the combined diabetic groups. (DWON and DWN).

HBA1c and Antioxidants

Parameter	Control n = 28	DWON n =28	DWN n = 32	p – values
SOD(U/ml)	I59.02±8.20	174.65±7.40	179.56±6.90	0.0001*
GPx (U/ml)	52.41±3.50	44.48±4.10	38.88±26.50	0.0001*
CAT(U/ml)	45.40±3.60	34.09±4.90	20.09±5.80	0.0001*
VE(ug/ml)	6.97±0.85	6.22±0.89	4.48±0.99	0.0001*
VA(ug/ml)	42.41±7.90	41.69±6.77	42.35±7.60	0.921

Note: DWON (Diabetics Without Nephropathy)
DWN (Diabetics With Nephropathy)

Table 5. Correlation between eGFR, and the antioxidants SOD, GPX, CAT, VE and VA amongst the combined diabetic groups. (DWON and DWN).

eGFR and Antioxidants

Antioxidants	r	p-value
SOD(U/ml)	-0.709 (0.000)	0.001**
GPx (U/ml)	0.137 (0.296)	0.001
CAT(U/ml)	0.727 (0.000)	0.001**
VE(ug/ml)	0.367 (0.004)	0.001**
VA(ug/ml)	-0.046 (0.729)	0.001

Note: DWON (Diabetics Without Nephropathy), DWN (Diabetics With Nephropathy)

DISCUSSION

Oxidative stress is implicated as one of the major pathways thought to be involved in the pathogenesis of diabetic nephropathy and its complications, all of them originating from hyperglycemia (Bunza and Alhassan 2019).

In the present study, there was significant increase in the fasting plasma glucose and HbA1c in diabetic patients as compared to the control population. This could be indicative of excessive glycosylation of hemoglobin due to increased plasma glucose or hyperglycemia (Tafavi *et al*. 2011). Chronic hyperglycemia results in oxidative stress by simulating reactive oxygen species (ROS) and reactive nitrogen species(RNS) production (Ohno *et al*. 2005), which attacks lipids present in plasma, mitochondria and endoplasmic reticulum membranes and cause peroxidation (Dandona *et al*. 1996).

The present study observed a decrease in enzyme activity of catalase in diabetic group compared with the control group. This is in line with the findings of Babu *et al*. (2015) that reported a decrease in catalase activity in diabetic patients compared with the control. The reason for the decrease might be due to oxidative stress which causes levels of circulating antioxidants to fall due to increased consumption (MacRury *et al*. 1993). Glycation of the antioxidant enzymes which reduces their capacity to detoxify oxygen radicals may also be an important cause for observed reduced catalase activity in the diabetic subjects (Inah *et al*. 2012)

The present study observed a decrease in the activities of glutathione peroxidase enzyme (GPX) in the diabetic groups compared with the non-diabetic control group. This corroborated with the findings of other studies Manal *et al*. (2016) and Moorkth *et al*. (2017). The reason for the decrease may be due to glycation of the antioxidant enzymes which reduces their capacity to detoxify oxygen radicals. In addition, the enzymes may be used to counteract the effects of increased peroxides which accompanies renal damage (Zachara *et al*. 2006). Moreover, the presence of toxin complexes in the blood of the diabetic patients may inhibit the activities of the enzymes (Suresh and Annam, 2013). Finally, the renal proximal tubular epithelial cells are the main source of these enzymes, therefore, any challenge to the renal function could result in lower enzyme production (Zachara *et al*. 2006). Hyperglycemia causes an increase in reactive oxygen metabolites and their derivatives (Maritim, 2005). Glutathione (GSH) is an important substrate for GPX for the conversion of hydrogen peroxide to water and oxygen. Therefore, GSH deficiency causes increase in oxidative stress (Vanderjagt *et al.* 2001).

The present study observed an increase in SOD enzyme activity in the diabetic groups as compared with the control group. This corroborated with the findings of Ramchandra *et al*. (2012); Ganjifrockwala *et al*., (2016), all reported an increase in SOD enzyme activity in diabetic group as compared with apparently healthy control group.

However, this is in contrast with studies by Sayed *et al*., (2013) who reported a decrease in SOD enzyme activity in diabetic patients when compared with healthy control group. They also reported that the increase was seen more in patients with complications as compared to patients without complications, which is similar to the present study finding In the present study, we have also observed more increase in SOD enzyme activity in diabetic nephropathy patients compared to diabetics without nephropathy.

Extracellular superoxide dismutase is one of the three isoforms of SOD found in serum. It is a secretory glycoprotein with an affinity for heparin-like substances and is the main enzymatic scavenger of superoxide in the extracellular spaces. 99% of the enzyme is found bound to heparin sulphate proteoglycans in vascular walls and to a lesser extent in the interstitium and 1% is in circulation. There is equilibrium between the plasma phase and endothelium phase (Sayed *et al.* 2013). The change in plasma SOD enzyme activity could be due to changes in expression of SOD or tissue binding of SOD (Aldachi *et al*. 2004). The reason for increase in SOD enzyme activity in the patients with nephropathy in this study could be due to, increased expression of the enzyme as a compensatory mechanism in response to increased oxidative stress (Ramchandra *et al.* 2012). It may also be due to a reduced tissue binding of SOD as a result of glycation of SOD, which reduces the affinity of this enzyme to heparin without affecting its enzyme activity. In diabetes, the proportion of glycated SOD is higher than healthy control (Bandeira *et al*. 2012). Also another reason could be decrease in tissue binding of SOD due to reduced heparin sulphate leading to an increase in plasma SOD levels. This reason is more evidence based as there have been reports that in diabetic nephropathy, heparin sulphate is reduced in glomerular basement membrane, proportional to degree of proteinuria and damage to glomerulus and can also reduce the ability of membrane glycocalyx to bind SOD (Kimura *et al*. 2003). In the present study, we have also observed more increase in SOD enzyme activity in diabetic nephropathy patients compared to diabetics without nephropathy.

The present study, observed a decreased level of antioxidant Vitamin E, and no significant change in the level of Vitamin A in the diabetics when compared with non-diabetic subjects. This corroborated with the findings of Maria *et al*., (2018), who noted that antioxidants decrease the oxidative damage caused by free radical by reacting with free radicals or indirectly by inhibiting the activity or expression of free radicals (Rani and Mythili, 2014). Vitamin E is a free radical chain- breaking antioxidant in the lipid phase and is found to be helpful in reducing the damaging effects of free radical on the structural and functional components of cells and vascular walls (Ashor *et al.* 2015).

Vitamin A is activated when there is low oxygen tension within the cells. However, both vitamins have been found to normalize many parameters of oxidative stress and delay development of complications in diabetics (Kuroki *et al*., 2003). The reason for decrease in Vitamin E and unaltered Vitamin A levels in the patients with diabetics with or without nephropathy in this study could be due to, oxidative stress which causes levels of circulating antioxidants to fall due to increased consumption (MacRury *et al*. 1993). Other factors that have been associated with low levels of plasma antioxidant vitamins include low intake of antioxidant –rich foods (particularly fruits and vegetables), poor health status, cigarette smoking, and low physical activity (Ahmad *et al*. 2003). The activity of Vitamin A as antioxidants also depends on their interaction with other antioxidants, such as vitamins E and C (Jian *et al*. 2010)).

The present study observed a strong negative correlation between SOD levels and estimated glomerular function (eGFR) which is a scale of measurement for assessing the integrity of the glomerulus.

Furthemore, the present study observed, a strong positive correlation between Vitamin E and Glomerular function (eGFR is a marker for measuring the glomerular integrity). This is in line with the findings of Gaede *et al* (2001) that reported that Vitamin E (680 mg/day) significantly improved renal function in Type 2 diabetes.. However, Ceriello, (2016) reported that Vitamin E failed to show beneficial effect on diabetic nephropathy.

However, Vitamin E showed very weak negative correlation to HBA1c (glycemic control). This is in line with the findings of Bursell *et al.*, (1999), that noted that oral Vitamin E treatment normalized elevated baseline creatinine clearance in diabetic patients with diabetic without inducing a significant change in glycemic control in an 8-month randomized double-masked placebo-controlled crossover trial. The present study catalase was observed to show strong positive correlation to eGFR. This is in line with the findings of Inah *et al.* (2012).

Catalase plays an important role in protecting the kidney from diabetic stress through maintaining peroxisomal and mitochondrial fitness. Therefore, it's deficiency might hasten renal injury (Inah *et al*. 2012). GPx and VA showed very weak positive and negative correlations respectively with eGFR. This in contrast with the findings of Beisswenger *et al*., (2005) who determined a negative correlation between low levels of GPx and glomerular function. We observed a weak positive relation between Glutathione peroxidase (GPX) and glycated haemoglobin (HBA1c). This is indicative of the degree of renal dysfunction and oxidative stress This corroborates the findings of Mohammad *et al*., (2008). VE and VA showed very weak negative correlations to HbA1c. This is similar to the findings of Sankhla *et al*., (2012). SOD, CAT, showed very weak positive correlations to glycemic control (HBA1c). Development of diabetic nephropathy is accelerated by poor glycemic control and the elevated glucose level, increases the superoxide ion. (William *et al*. 1993). However, conflicting results on the effects of antioxidant supplementation on glycemic control have emerged from previous studies (Sarmento *et al*. 2013; Xu *et al*. 2014).

CONCLUSION

Thus, the present study observed an increase in the level of SOD and decreased level of GPx and CAT and VE in the diabetic subjects with or without nephropathy and unaltered levels of VA. This increase in SOD level and increase in GPx , CAT and VE were found to be more in the diabetic subjects with nephropathy than diabetic without nephropathy. This implies the beneficial roles the antioxidants play in inhibiting the progression to End-Stage-Kidney-Disease.

RECOMMENDATIONS

Firstly; the study recommends that evaluation of antioxidant status should be included as part of routine check-ups in the diagnosis and management of patients with diabetes, as this will help to detect diabetics complications early before it progresses to end-stage-kidney-disease (ESKD).

Secondly; Investigation of other antioxidants or diets that help to improve activity of SOD, GPX and Catalase in kidney should be encouraged and thirdly; regular consumption of antioxidant-rich foods should be encouraged particularly amongst the diabetics as this can help, boost their antioxidants levels.

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