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Original Research Article

A Study of Oxidative Stress and Antioxidant Defense in Chronic Kidney Disease in a Tertiary Care Hospital of West Bengal

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ABSTRACT

Background: Chronic Kidney Disease (CKD) is one of the major diseases claiming a sizeable fraction of overall mortality and morbidity every year in our country. Multiple studies have found increased oxidative stress and imbalance in antioxidant defense in CKD patients. Apart from the disease pathology itself, other associated factors, like inflammation, hemodialysis etc. also contribute to increased oxidative stress, which in turn complicate the clinical condition further. There are multitude of markers to measure oxidative stress and antioxidant defense, but it is very difficult to measure overall oxidative stress status accurately and completely. There is also very little data available about the oxidative stress status in CKD patients in the population of West Bengal.

Aims and Objectives: This study aims to measure Total oxidative Stress (TOS) and Total antioxidant defense (TAD) status in CKD patients in a sample population of West Bengal, more efficiently, by a colorimetric method modified and standardized in our laboratory. We also aim to find out the relationship (if any) of the Oxidative stress status in these patients with the severity of the disease

Materials and Methods: The study included 40 CKD patients from OPD and IPD of a tertiary care hospital of Kolkata, West Bengal, and 30 age and sex matched healthy volunteers. Serum Urea, Creatinine, Sodium and Potassium were measured by standardized commercial kits and TOS and TAD were measured by colorimetric chemical methods, modified and standardized in our laboratory.

Results: The TOS was found to be significantly increased (P=0.003) in CKD patients in comparison to healthy control subjects. TAD was also found to be significantly elevated (P<0.001) in the patient group. However, TOS and TAD showed no correlation with serum creatinine (P>0.05) in our study group.

Conclusion: The total oxidative stress does indeed increase in the chronic Kidney Disease, in the population of West Bengal too, and this result reaffirms the similar findings elsewhere in the world. The increase in Total Antioxidant Defense in our study population, contrary to a usual decrease in TAD found with increase in TOS, indicates a compensatory increase as found and suggested by some previous studies. This study did not find any significant relationship of TOS and TAD levels with disease severity, which warrants a more elaborate study with a larger sample size, and taking into account different confounding factors.

Key Words: Total Oxidative Stress, Total Antioxidant Defense, Chronic Kidney Disease

INTRODUCTION

Chronic kidney disease (CKD), is a slow progressive loss of kidney function over several years. A GFR of <60 for more than three months typically indicates chronic kidney disease. [1] There is increased free radical production, antioxidant depletion and changes in content

of lipoproteins in CKD and this abnormality increases in a gradually, with severity of renal dysfunction. [2] Inflammation, also found in CKD, increases the process of oxidant generation even further. Many recent evidences, from both experimental as well as clinical studies, suggest that oxidative stress may be implicated in the pathogenesis of atherosclerosis and some of the complications of ESRD, like dialysis-related amyloidosis, malnutrition and anemia. ROS release itself further increases its generation which is even more augmented by co-release of pro-inflammatory cytokines, angiotensin II and advanced glycation end products etc. [3]

Free radicals have a very short half-life (in seconds); thus it is very difficult to measure them by tests that can be routinely done in clinical settings. So, usually more stable marker molecules are measured to assess oxidative stress. These are molecules that are derived from lipid peroxidation. For example, F2-isoprostane, which is a non-enzymatic, free radical-catalyzed isomer of cyclooxygenase-derived enzymatic products of arachidonic acid, can be found in both urine and plasma. [4] It is a rather stable end product of oxidation of arachidonic acid and can be measured with high sensitivity and specificity. [5] Other different markers of oxidative stress include Malonyldialdehyde, 8-Hydroxy-2′-deoxyguanosine, Dimethyl arginine (ADMA), Acrolein etc.

To counteract the damaging effects of these oxidants, endogenous antioxidant defense mechanisms are in place. There are two lines of enzymatic (like Catalase, Superoxide Dismutase, Glutathione peroxidase etc) and non-enzymatic antioxidant defense (Like Vitamin A, C, E, Glutathione, Uric Acid etc) within the cell.

Multiple studies have shown significant imbalance in pro-oxidant and antioxidant activities in patients with renal dysfunction. [6,7] High plasma 8-isoprostane levels signifying increased oxidative stress has even been found in the early stages of CKD. Some studies also suggest that as the disease progresses, so does the level of oxidative stress and correlates significantly and inversely with the level of glomerular filtration rate (GFR). [8] Similar results are found, using different markers of oxidative stress like malonyldialdehyde, glutathione peroxidase, and oxidized serum albumin. [9,10]

Indirect confirmation that oxidative stress is especially important in renal dysfunction comes from several studies. Some of the randomized trials in the general population, such as the Heart Protection Study and Heart Outcomes and Prevention Evaluation study measured effect of exogenous antioxidant vitamins in CKD patients, but none found any significant advantage. [11,12] However, in a different study in CKD patients having hemodialysis and with history of cardiovascular disease, use of high-dose oral vitamin E was seen to be significantly reducing cardiovascular events. [13]

This increased oxidative stress in CKD can be explained by common characteristics found in CKD patients, like advanced age, diabetes and renal hypertension etc. End stage renal disease patients, also have dietary restriction of fresh fruits and vegetables (to avoid hyper-kalaemia), which lower their levels of vitamin C intake and thus predispose them further to oxidative stress. [14] Moreover, hemodialysis causes further more generation of ROS due to activation of polymorphonuclear neutrophils and also causes loss of antioxidant vitamins through the dialysis process itself and thereby increase oxidants and reduces antioxidant level. [15,16]

Inflammation, malnutrition, chronic volume overload and autonomic dysfunction also contribute significantly in generating increased oxidative state. Activation of polymorphonuclear neutrophils and increased levels of different mediators of inflammation such as CRP, IL-6, TNF-α and fibrinogen are often found in CKD, which suggest that CKD is a low-grade inflammatory process in itself. [17] Activation of polymorphonuclear

neutrophils, generates myeloperoxidase, which triggers ROS activation. A recent study found serum myeloperoxidase to be significantly associated with both inflammation and mortality in CKD patients under hemodialysis. [18]

Still, there is a significant dearth of data about the oxidative stress status in Chronic Kidney Disease in the population of West Bengal. Simple but dependable and comprehensive methods are also needed to asses overall oxidative stress and antioxidant defense status, so that they can be routinely measured in these patients. This study aims to bridge that gap.

MATERIALS AND METHODS

The study was done at NRS Medical College & Hospital, Kolkata, West Bengal, in the Department of Biochemistry, from April to June 2016, with prior permission of institutional ethics committee. A total of 70 study subjects were included in this study, of which 40 individuals were patients suffering from chronic renal failure, attending Nephrology OPD or admitted to nephrology IPD of the institution. 30 age and sex matched healthy volunteers were included as control subjects.

Collection of samples:

Blood samples of both renal failure patients and healthy volunteers were taken after overnight fasting. 5ml of blood samples were collected in clot vial using the aseptic measures. The samples were allowed to stand for 1 hour and then centrifuged at 2500 rpm for 3mins for separation of serum. Finally the serum was allequoted, tests done and stored at -20^{o} C.

Estimation of Serum Urea by Berthelot method: Using standardized commercial kit by clinical chemistry Autoanalyser.

Estimation of serum Creatinine by JAFFE's method: Using standardized commercial kit by clinical chemistry Autoanalyser.

Estimation of sodium and potassium: by Electrolyte Analyzer (Ion Selective Electrode)

Estimation of total oxidative stress (TOS) in plasma:

It is a simple colorimetric test based on the principle of Free Oxygen Radical Test (FORT) which is modified and then standardized in our laboratory. [19-22] The crude non-lyophilized form of the chromogen originally used in FORT test is very unstable, so we used is N, N-dimethyl-p- phenylenediaminesulphate as chromogen.

Principle: The test is based on the ability of transition metals, such as iron, to catalyze the breakdown of hydroperoxides into derivative radicals, which are then preferentially trapped by the buffered chromogen (N,N-dimethyl- p-phenylenediamine-sulphate) and develop a coloured product which can be colorimetrically measured.

R-OOH + Fe2+ →R-O• +OH- +Fe3+

R-OOH + Fe3+ →R-OO• + H++Fe2+

RO.+ ROO. + 2CrNH2 →R-O• + R-OO• + [Cr-NH2+.]

Standardization and assay of total oxidative stress (TOS): Standard curve was prepared using different dilutions of hydrogen peroxide (H_2O_2) (in milli-mole per liter) and difference of absorbances between 6th and 4th minute were measured at 505 nm (increase in absorbance noted maximum in this period).

Estimation of total antioxidant defense (TAD) in plasma:

It is also a simple colorimetric test based on the principle of Free Oxygen Radical Defense (FORD) test. This test is also modified and standardized in our laboratory. [19,21,22]

Principle: In presence of acidic buffer (pH = 5.2), and an oxidant (FeCl3), the chromogen (N, N-dimethyl- p-phenylenediaminesulphate) forms a stable and colored radical cation that can be colorimetrically measured at 505 nm. Antioxidant compounds in the sample reduce the radical cation of the chromogen, and thus quench the color, and produce a discoloration of the solution, measured as decrease in absorbance, which is proportional to their concentration. Standard

curve is produced using Trolox (6-hydroxy-2, 5,7,8-tetramethylchroman-2-carboxylic acid), a derivative of vitamin E that can be used as an antioxidant. Test samples were compared with the standard curve.

Chromogen (no color) + Fe2+ + H+ → chromogen·+ (purple)

Chromogen·+ (purple) + AOH → chromogen (no color) + AO

An end-point analysis of the colorimetric reaction is done at 505 nm and at 37°C.

Standardization of total antioxidant defense (TAD) assay: Standard curve of TAD by modified FORD assay was prepared by using different dilutions of trolox, between 4_{th} and 6_{th} minute. The difference in absorbance between 4_{th} and 6_{th} minute at 505 nm of different dilutions of trolox were measured and plotted to get the calibration curve.

The results are expressed as Trolox equivalents (mmol/l).

STATISTICAL METHODS:

Data were expressed as mean ± standard deviation(SD), Comparison of data was done using unpaired two-tailed student's t-test and Pearson's correlation for Gaussian distribution and Mann Whitney U test and Spearman's correlation for non- Gaussian distribution and P<0.05 was considered as significant. Statistical analysis was done using Microsoft Office Excel-2007 and SPSS Statistics version 2017.

RESULTS

The mean and SD values of the parameters measured, ie, Serum Urea, Creatinine, Na+, K+, TOS and TAD values are listed in Table 1

Table 1: Comparison of different parameters in CRF patients (case) and Healthy controls subjects

PARAMETER	CASE	CONTROL
UREA (Mean±SD) In mg/dL	150.2±47.7	37.2023±7.85340
CREATININE (Mean±SD) In mg/dL	5.48±1.61	0.82±0.23
SODIUM (Mean±SD) In mmoL/L	131.9±10.7	134.6667±2.63050
POTASSIUM (Mean±SD) In mmoL/L	4.8±1.4	4.1467±.51511
TOS (Mean±SD) (mmoL/L-equivalent of H2O2)	43.5±21.6	31.1±8.2
TAD (Mean±SD) (mmoL/L-equivalent of Trolox)	627.5±402.6	176.6±159.4

The Urea and creatinine levels were predictably, found to be significantly higher in CRF patients, compared to healthy control subjects (P <0.001) by Independent –T test.

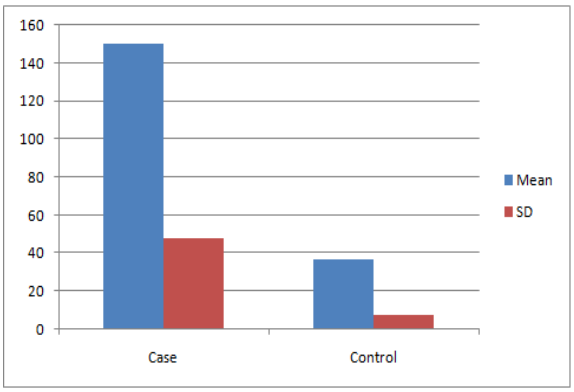

Figure 1: comparison of Urea values (Mean and SD) between CRF patients and control subjects

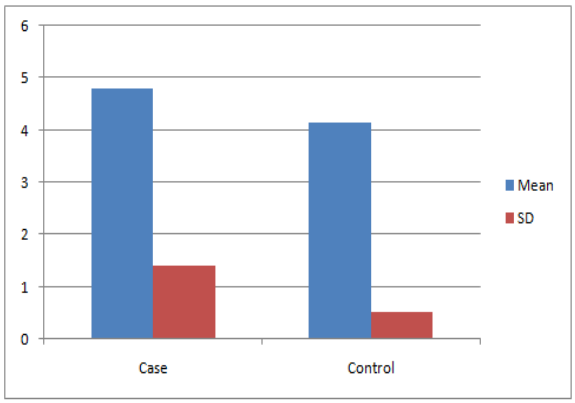

Figure 2: comparison of Creatinine values (Mean and SD) between CRF patients and control subjects

No statistically significant difference were found (P>0.05) in sodium and potassium values when compared between CRF patients and healthy control subjects.

Both Total oxidative stress (TOS), as well as total antioxidant defense (TAD) levels, when measured, were found to be higher in the CRF patients in comparison to control subjects. But the values were found to be widely dispersed, (Table-1) both in patients and control subjects, due to wide range of physiological factors that can affect these parameters. When compared by non-parametric Mann-Whitney test, both TOS and TAD were found to be significantly higher (P<0.05) in the patients.

Table 2: Comparison of TOS and TAD between CRF patients and control subjects by "Mann Whitney test"

Parameter	Z value	P Value
TOS	-2.933	-5.667
TAD	0.003	<0.001

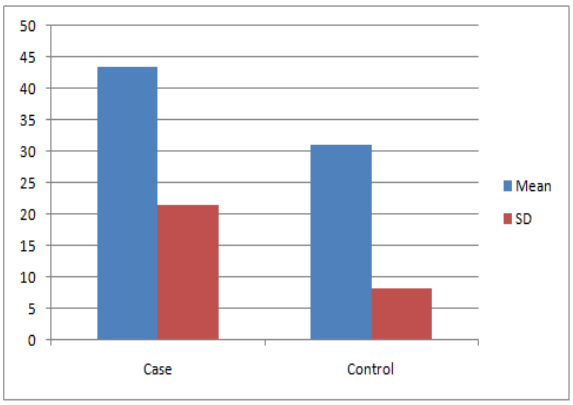

Figure 3: comparison of TOS levels (Mean and SD) between CRF patients and control subjects

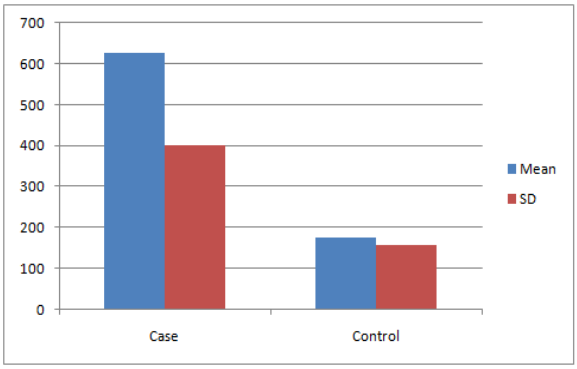

Figure 4: comparison of TAD levels (Mean and SD) between CRF patients and control subjects

TOS and TAD levels did not show any statistically significant (P>0.05) correlation with serum creatinine or Urea levels in our study.

DISCUSSION

In our study both urea and creatinine were significantly elevated in the CKD patients, as obvious due to the renal dysfunction (Table 1). We found a significant elevation in the TAD levels (p<0.001) in the CKD patients compared to control.

In our study, we found total oxidative stress levels to be significantly increased (p=0.003) in the patients (43.5±21.6) in comparison to the controls (31.1±8.2). A gradual increase in oxidative stress markers is found in kidney disorders. It appears that ROS increase in CKD occurs gradually as renal function deteriorates, and as glomerular filtration rate declines. [8,10] In a study done on rats, it has been found, that plasma malonyldialdehyde values are accompanied by increases in renal malonyldialdehyde levels, which suggests that plasma ROS levels can be assessed to get an idea about ROS generation in kidneys. [23] Increase in NAD(P)H oxidase activity has been found in patients with renal insufficiency, [24,25] even in early stages of CKD, which suggests increased ROS generation itself may contribute to the oxidative stress in CKD. [26]

It is not clear, whether antioxidant defense becomes overwhelmed and further increases oxidative stress in CKD, as a variety of results have been reported. [8,27] In a recent study, evaluating oxidative stress status in patients with CKD, no differences were observed in the plasma total antioxidant capacity (TAC), which remain stable with renal function loss even in presence of gradual increase of oxidative stress. Reduction in TAC level was in fact observed only in end- stage renal failure patients. [8]

Along with an expected increase in the TOS levels in the CKD patients, in our study, the TAD levels in the CKD patients (627.5±402.6) have also been found to be significantly increased in comparison to healthy subjects (176.6±159.4). Some of the previous studies also had similar results with increase in antioxidant levels as the oxidative stress increased and the disease progressed. [8,28,29]

This may indicate a compensatory increase in the antioxidant defense in face of the increased burden of oxidative stress. A recent study found greater serum uric levels to be associated with elevated TAS levels in individuals with atherosclerosis, [28] suggesting, that increased levels of Uric acid, which acts as an antioxidant in plasma, may be a compensatory mechanism to counteract oxidative damage. Similar results of compensatory increase in antioxidant levels in conditions of elevated oxidative stress were found in recent studies in heart

failure patients, and brain tissue of Alzheimer's disease. [29,30]

Some of the previous studies have shown a direct correlation with the stage of renal failure with the oxidative stress levels. Some studies found an inverse relation between Oxidative stress and GFR in patients with CKD. [9,10,31] In a study on 55 pre-dialysis patients it was found that serum oxidized albumin levels increased progressively along with progression of stages of CKD. [10] However, no correlation between eGFR and levels of reduced thiols, F2- isoprostanes, and carbonyl radicals were found by Oberg et al. [32] It was suggested, that other factors also contribute towards the increase in oxidative stress in these patients. These factors include age, diabetes, malnutrition, chronic inflammation, and factors associated with the hemodialysis itself. [33,34]

In our study, though, we did not find any significant correlation (P>0.05) between the TOS and TAD levels with the serum creatinine levels of the CRF patients. This may be due to the other confounding factors like diabetes, age, other inflammatory conditions, and history of dialysis, which we did not include in our analysis.

CONCLUSION

Our study re-affirms the previous findings elsewhere in the world, that oxidative stress and antioxidant defense status in the body indeed get severely altered in patients of Chronic Renal Failure, in the eastern Indian population too. Unlike some of the previous studies, we did not find any significant correlation of oxidative stress and antioxidant status with the severity of the disease. A more elaborate study, with a larger number of study population and analyzing effects of different confounding factors individually has to be done in future, to establish the relationship of oxidative stress and antioxidant status in CRF patients in our population.

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