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Research Article

EVALUATION OF OXIDATIVE STRESS MARKERS IN CHRONIC KIDNEY FAILURES OF SOUTH INDIAN POPULATION

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ABSTRACT
Oxidative stress defines an imbalance between the formation of reactive oxygen species and antioxidants. The existence of oxidative stress and higher incidence of cardiovascular diseases (CVD) in association with uraemia is proven from studies on Chronic Kidney Disease (CKD) patients. Non traditional risk factors like oxidative stress are being given special emphasis to explain high incidence and identification of new therapeutic interventions. Excess Reactive oxygen Species levels have been implicated to damage DNA, lipids, proteins etc., It may also affect the cells of host, particularly at the inflammation site contributing to proteinuria observed in Chronic Kidney Disease patients. The uremic status, oxidant and antioxidant levels were assessed in the present study. This prospective observational study was conducted for nine months. Patients meeting the study criteria were included. Malonyldialdehyde (MDA), glutathione-S-transferase (GST), Protein thiols, Total proteins, Serum urea, creatinine, albumin and Haemoglobin levels were estimated using suitable methods. Study recruited 108 Chronic Kidney Disease patients, divided into three groups namely, patients without haemodialysis (54), patients with haemodialysis (54) and control population (50). Serum urea, creatinine, MDA and GST levels were found to be significantly increased ($P<0.0001$), and total proteins, albumin, proteinthiols, and Haemoglobin levels were found to be significantly decreased in Chronic Renal Failure patients compared to normal controls ($P<0.0001$). Our study confirms the presence of oxidative stress in Chronic Kidney Disease patient population. Our study also emphasises the need for anti-oxidant therapy in CKD patients.
KEY WORDS: Cardiovascular Diseases, Glutathione-S-transferase, Malonyldialdehyde, Oxidative stress, Total proteins, Uraemia.

INTRODUCTION

Enhanced oxidative stress has been well established in uraemia.[1,2] Oxidative stress is defined as the tissue damage resulting from an imbalance between an excessive generation of oxidant compounds and insufficient anti-oxidant defence mechanism.[3] It has been proposed to play a role in cardiovascular Diseases (CVD) and infectious diseases, cancer, diabetes, anaemia, and neurodegenerative pathology. The incidence of these diseases has been found to increase in uraemia[4,5] with cardiovascular disease being the major cause of death in patients affected by chronic renal failure (CRF).[6] The risk of CRF patients having a cardiovascular event has been reported to be 3–5 times higher than in the general population.[7,8] Non-traditional risk factors for CVD such as oxidative stress, are being given special emphasis not only to explain the high incidence of CVD, but also to identify new targets for therapeutic interventions.[6]

The production of Reactive Oxygen Species (ROS) is a natural process; the phagocyte oxidant generation system which includes both polymorphonuclear neutrophils (PMNs) and monocyte-macrophage cells is based on the inducible production of ROS.[6,9] The phagocytes produce ROS to perform physiological processes, such as killing bacteria etc.[10] ROS and inflammation are deeply interrelated, as different oxidant free radicals are generated by phagocytic cells in response to inflammatory stimuli.[6] ROS are further released together with proinflammatory cytokines, which in turn amplify oxidant generation.[11] The excess levels of ROS have been implicated in the damage to DNA, lipids, proteins, and the cell's carbohydrate content.[6] It may also affect the cells of host organisms, particularly at the sites of inflammation, contributing to proteinuria as observed in CRF patients.[1,12] ROS are highly reactive compounds with a half-life of only seconds; therefore, there *in vivo* measurement to assess oxidative stress is generally not feasible. Instead, lipids, proteins, carbohydrate, and nucleic acid have lifetimes ranging from hours to weeks after being modified by ROS, which makes them ideal markers of oxidative stress.[13] The total thiol status in the body, especially thiol (-SH) groups present on protein are considered as major plasma antioxidants *in vivo* and most of the SH-groups are present over albumin and are major reducing groups present in our body fluids.[13,14] During lipid peroxidation, unstable hydroperoxides resulting from peroxyl radical-dependent chain reactions involving unsaturated fatty acyl moieties later break down to smaller and more stable products like Malonyldialdehyde (MDA) or thiobarbituric acid-reactive substances (TBARS), which are considered to be oxidative stress markers.[6] And this MDA enhances the atherosclerosis formation. Glutathione S-transferase (GST) comprises a multigene family of proteins involved in the metabolism of many disease-causing electrophilic substrates and it protects the cells against oxidative stress.[15] It also plays a role in the detoxification of organic hydroperoxides.[16] Different GST subclasses are localized to specific parts of body.[17] α-GST is localized to specific parts of the renal tubule (proximal) and is readily released into the urine during injury, therefore, it is considered to be an excellent biomarker for proteinuria.[18]

In the present study, we aim to assess oxidative stress by determining relevant antioxidant levels and oxidative stress markers in CRF patients compared Haemodialysis patients, Non-Haemodialysis patients to normal controls.

MATERIALS AND METHODS

The study was carried out on 54 CRF patients, who were not on haemodialysis therapy, 54 CRF patients who were on haemodialysis therapy and on 50 healthy controls. The causes

of CRF were,chronic glomerulonephritis (60 cases), diabetic nephropathy (40 cases), and unknown (eight cases), and all of them were on conservative management. None of the patient groups received any form of antioxidant medication; all of them were on renal diet (50g protein and 5g salt/day). The healthy controls were not on any kind of prescribed medication or dietary restrictions. Informed consent was taken from all subjects involved in the study and the study was approved by the Institutional Review Board. Blood samples (5mL) were drawn into plain vacutainers from the antecubital veins of healthy controls and CRF patients. The blood was allowed to clot for 30min and centrifuged at 2000 *g* for 15min for clear separation of serum.

Biochemical Determination

Disodium hydrogen phosphate(Qualikems Fine Chemicals, India),(DTNB)-5, 5'-dithio bis (2-nitrobenzoic acid -))HiMedia Laboratories Ltd., Mumbai,india,Reduced Glutathione(Loba Chemie Ltd., Mumbai, India), Methanol(Finar Chemicals Ltd, India),Sodium dihydrogen phosphate(Qualikems Fine Chemicals, India),Thiobarbituric acid (TBA)Molychem, Mumbai, India,Trichloracetic acid(Finar Chemicals Ltd, India),Disodium EDTA-HiMedia Laboratories Ltd., Mumbai, India,(CDNB) -1-chloro-2, 4-dinitrobenzene-HiMedia Laboratories Ltd., Mumbai, India, Total protein Kit(Kamineni life sciences Pvt. Ltd, Hyderabad, India.)Albumin Kit(Tranasia bio-medical Ltd, baddi, solan, Himachal Pradesh),Creatinine Kit(Tranasia bio-medical Ltd, baddi, solan, Himachal Pradesh),Urea Kit(Tranasia bio-medical Ltd, baddi, solan, Himachal Pradesh).

Thiol assay

The reaction mixture containing 900 µL 2 mM Na_2EDTA in 0.2 M Na_2HPO_4, 20 µL 10 mM DTNB in 0.2 M Na_2HPO_4, and 100 µL serum was incubated at room temperature for five min; the absorbance was read at 412 nm. Appropriate sample and reagent blanks were prepared simultaneously and the respective absorbance was noted. Corrected absorbance values were used to calculate serum protein thiol content using a molar extinction coefficient of 1600/M/cm and values were expressed as µM. The calibration curve was produced using GSH dissolved in phosphate-buffered saline (PBS).[19]

GST assay

One mL of reaction mixture containing 850 µL of 0.1M phosphate buffer, pH 6.5, 50 µL 20 mM CDNB (1-chloro 2,4-dinitrobenzene), and 50 µL 20 mM GSH was preincubated at 37° C for 10 min. Reaction was started by adding 50 µL serum and GST activity was assayed kinetically. Reaction was followed at one minute intervals for five minutes by measuring the absorbance at 340 nm. GST was determined by using a molar extinction coefficient of 9.6/mM/cm and GST activity was expressed as IU/L.[20,21]

MDA assay

The reaction mixture containing 1 mL 0.67% thiobarbituric acid (TBA), 500 µL 20% tricarboxylic acid (TCA), and 100 µL serum was incubated at 100° C for 20 min and centrifuged at 12,000 rpm for 5 min. The absorbance of the supernatant was read at 532 nm and MDA concentration was determined by using a molar extinction coefficient of 1.56×10^5/M/cm and the values were expressed as µM.[22]

Total Protein Assay: (BIURET METHOD).

Sodium chloride diluent 0.9% W/V: Dissolve 4.5g sodium chloride in about 400ml of distilled water and then make up to 500ml with distilled water. stable at room temperature (25-35^{0}C). Make a fresh solution once in 6 months. **Biuret reagent**: Dissolve 4.0g sodium hydroxide in about 400 ml of distilled water. Add 4.5 g sodium potassium tartrate. Mix to dissolve. Then add 1.5g copper sulphate followed by 4.5g potassium iodide. Transfer the solution into a 500 ml volumetric flask and make up to the mark with distilled water. Store in a tightly stoppered polyethylene bottle at room temperature (25-35^{0}C). Stable for 6 months. Make Pipette into test tubes labeled Blank (B), Standard (S), and Test (T) and Mix well and incubate at 370 for min. Read absorbance of standard (S) and Test (T) against Blank (B) at 555 nm.

Calculations

$$\text{Total Protein (gm/dl)} = \frac{\text{Absorbance of (T)}}{\text{Absorbance of (S)}} \times 5.5 \text{ (Standard Conc)}$$

Normal range: 6.5-8.5 gm/dl.

Parameters determined using automated assays

In CRF patients and healthy controls, serum total protein levels were determined using Biuret method, albumin level was measured by the bromocresol green dye-binding method, urea by the urease-glutamate dehydrogenase method, and creatinine by Jaffe's method using a Clinical Chemistry Autoanalyzer (Hitachi 912).

STATISTICAL ANALYSIS

The results were expressed as mean ± standard deviation (SD). $P < 0.0001$ was considered to be statistically significant. Statistical analysis was performed using the Graph pad prism. Independent sample t-test was used to compare mean values and Pearson's correlation was applied to correlate the parameters.

RESULTS

A Total number of 108 patients were enrolled into the study, out which 54 were grouped as undialysed patient and 54 were grouped as Haemodialysed patients, 50 Healthy human volunteers were grouped as control. Among 54 dialysis treated patients 33 were males and 21 were females.

As shown in Tables Serum urea, creatinine, MDA and GST levels were found to be significantly increased (P<0.0001), and total proteins, albumin, protein thiols, and Haemoglobin levels were found to be significantly decreased in Chronic Renal Failure patients compared to normal controls (P<0.0001).

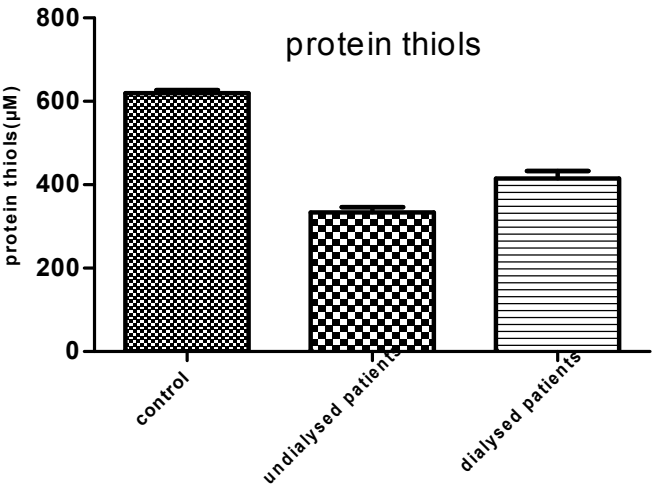
Table.1: Demographic and clinical characteristics of chronic renal failure patients (dialysed treated & undialyzed patients) and normal controls (Mean±SEM)

Characteristics	Normal controls (n=50)	Undialysed CRF patients. (n=54)	Dialysed CRF patients. (n=54)
1.Age (years)	48.86±1.4	50.03774±1.469	44.73585±1.2637
2.Sex(M/F)	48/12	37/17	33/21
3.Blood urea (mg/dL)	40.0978± 0.5386	66.12189±1.2396	60.67925±1.5804
4.Serum creatinine (mg/dL)	1.0368± 0.0233	2.8367±0.1210	4.7849±0.1212
5.HB%	15.0348± 0.1493	10.1690±0.2665	9.2716±0.2225
6.UrineAlbumin (mg/dL)	15.48± 0.6453	257.5472±22.6613	2000

Table.2: Serum oxidative markers and antioxidants in normal controls and patients with undialysed and dialysed chronic renal failure (Mean±SEM)

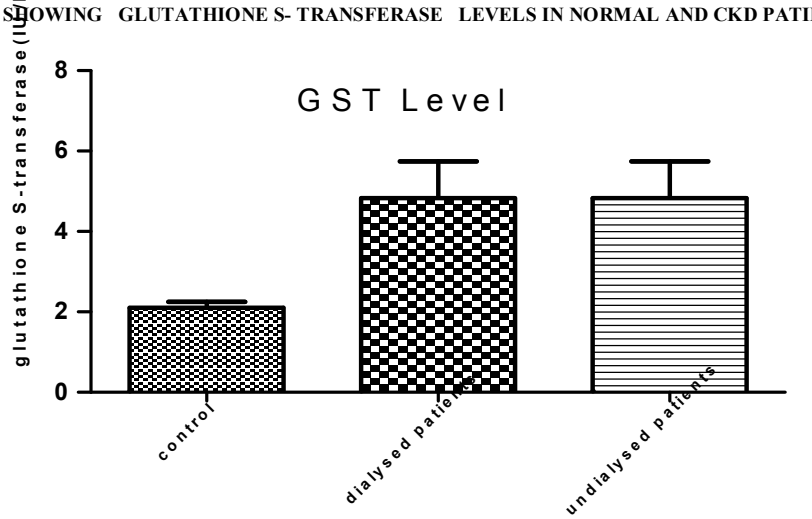
Biomarkers	Normal controls (n = 50)	Undialysed patients (n = 54)	Dialysed patients (n = 54)	Significance (p value) Control v/s patients
1. Protein thiols (µM)	619.4302± 7.7	334.5581±12.3906	415.2615±17.5829	P<0.0001
2.GST (IU/L)	2.1028± 0.1439	6.6107±1.4942	4.8298±0.9039	P<0.0001
3. MDA (µM)	1.3175± 0.1457	2.64383±0.1947	6.129283±0.3469	P<0.0001
4.Total protein (g/dL)	8.171± 2.960	4.899± 0.50944	6.869±0.601	P<0.0001

Figure 1: GRAPH SHOWING PROTEIN THIOLS LEVELS IN NORMAL AND CKD PATIENTS.



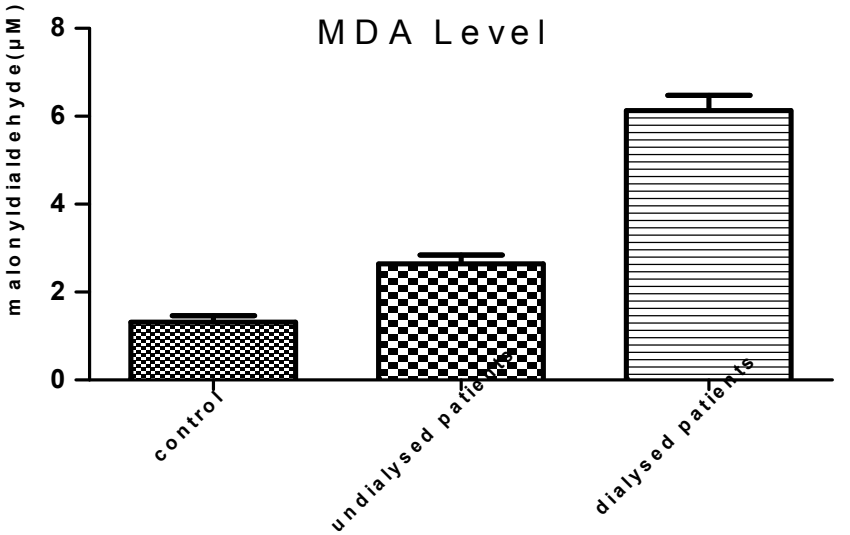
The protein thiol levels were normal in the control population (Mean **619.4302± 7.7,** Table 2). There was a significant decrease in blood levels of CRF patients (*P*<0.0001) and mean significance value is (P< 0.05) compared to that of controls.

Figure 2: GRAPH SHOWING GLUTATHIONE S- TRANSFERASE LEVELS IN NORMAL AND CKD PATIENTS.



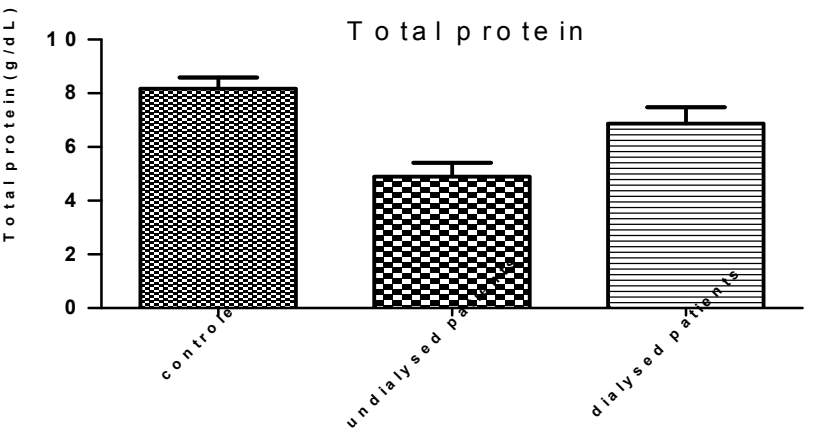
The GST levels were normal in control population (Mean **2.10288± 0.1439,** Table 2). There was a significant increased in blood levels of CRF patients compared to that of controls (*P*<0.0001) and mean significance value is (P< 0.05).

Figure 3: GRAPH SHOWING MALONYLDIALDEHYDE (MDA) LEVELS IN NORMAL AND CKD PATIENTS.



The MDA levels were normal in control population (Mean **1.3175± 0.1457,** Table 2). There was a significant increased in blood level of CRF patients compared to that of controls (*P*<0.0001) and mean significance value is (P< 0.05).

Figure 4: GRAPH SHOWING TOTAL PROTEIN LEVELS IN NORMAL AND CKD PATIENTS.



Total protein levels were normal in all the subjects (**Mean 8.17186± 2.960,** Table 2). There was a significant decrease in total protein of CRF patients (*P*<0.0001) compared to that of controls.

DISCUSSION

Enhanced oxidative stress has well established in uraemia. Oxidative stress defines an imbalance between the formation of that enhanced oxidative stress reactive oxygen species (ROS) and antioxidative defence mechanisms. It has been proposed to play a role in cardiovascular (CVD), infectious diseases, cancer, diabetes, anaemia, and neurodegenerative diseases.

Cardiovascular disease (CVD) being the major cause of death in patients affected by chronic renal failure (CRF).[23] Non traditional risk factors for CVD such as oxidative stress are being given special emphasis not only to explain the high incidence of CVD, but also to identify new targets for therapeutic interventions. The risk of CRF patients having a cardiovascular event has been reported to be 3-5 time higher in undialyzed patients and 10-20 times higher in dialysis treated patients than in the general population.[24,25]

The production of ROS is a natural process; the phagocyte oxidant generation system which includes both polymorphonuclear nutrophils (PMNs) and monocytes-macrophage cells is based on the inducible production of ROS. The phagocytes produce ROS to perform physiological processes, such as killing bacteria etc. ROS and inflammation are deeply interrelated, as different oxidant free radicals are generated by phagocytic cells in response to inflammatory stimuli. ROS are further released together with proinflammatory cytokines, and the level of ROS have been implicated in the damage to DNA, lipids, proteins and the cells carbohydrates content. It may also affect the cells of host organisms, particularly at the sites of inflammation, contributing to proteinuria as observed in CRF patients.[26]

ROS are highly reactive compounds with a half-life of only few seconds; therefore, their *in vivo* measurements to assess oxidative stress are generally not feasible. Instead, lipids, proteins, carbohydrates, and nucleic acids have lifetimes ranging from hours to weeks after being modified by ROS, which makes them ideal markers of oxidative stress.[25]

Proteinuria is a well established finding in CRF patients and uraemia causes Total protein catabolism in chronic renal failure patients.

Total protein and albumin levels in serum were decreased in these patients when compared to normal controls. Previous studies (V Kolagal, SA Karanam *et al* 2010) have shown significantly decreased protein thiols in the serum of uremia cases which correlated positively with serum albumin. In agreement with previous studies, our study also shows decreased protein thiols in CRF patients. Oxidants could induce oxidative damage to plasma proteins, giving rise to advanced oxidation protein products (AOPP), levels of which have been reported to be significantly high in CRF patients. Advanced oxidation protein products are potential uremic toxins and mediators of inflammation. Furthermore, serum levels of AOPPs increase with progressive renal failure and are intimately associated with CVD.[25]

The total thiol status in the body, especially thiol (-SH) groups present on protein are considered as major plasma antioxidants *in vivo* and most of the SH-groups are present over albumin and are major reducing groups present in our body fluids. previous studies conducted by V Kolagal, SA Karanam; *et al* 2010 and Tatjana Cvetković; *et al* 2009 has shown low levels of protein thiols, which correlates negatively with levels of AOPPs, a condition termed as "thiol stress" that contributes to the pathogenesis of CVD in this patient population. In our study, we observed a significant decrease in protein-SH levels and they correlated positively with the decrease in paraoxonase activity and albumin levels. Previous study conducted by Prakash et al 2010. Also demonstrated decreased protein-SH level in CRF patients on chronic maintenance hemodialysis; the study also demonstrated positive association between protein-SH and albumin.[25,27,28]

Glutathione S-transferase (GST) comprises a multigene family of proteins involved in the metabolism of many disease-causing electrophilic substrates and it protects the cells against oxidative stress. It also plays a role in the detoxification of organic hydroperoxides. Different GST subclasses are localized to specific parts of body. α-GST is localized to specific parts of the renal tubule (proximal) and is readily released into the urine during injury, therefore, it is considered to be an excellent biomarker for proteinuria. As GST is involved in the detoxification of electrophiles and hydroperoxides, GST level increased in dialysis and undialysed CRF patients due to enhanced oxidative stress. We found significant difference in the plasma levels of GST in CRF patients when compared to healthy controls.[29,30]

During lipid peroxidation, unstable hydro peroxides resulting from unsaturated fatty acyl moieties later break down through series of chain reactions to smaller and more stable products like malonyldialdehyde (MDA) or thiobarbituric acid-reactive substances (TBARS), these are considered to be ideal oxidative stress markers. MDA is a lipid peroxidation product which is formed during oxidation process of polyunsaturated fatty acids (PUFA) by reactive oxygen species. MDA is the sensitive marker of lipid peroxidation. MDA level is significantly elevated in hemodialysis patients when compared to conservatively managed patients. This is in accordance with study of (C.M Loughrey et al. A. Marjani and Talia Weinstein et al 2009).

CONCLUSION

The serum levels of oxidative stress parameters in the Indian chronic kidney disease population are not much explored. We have made sincere efforts to assess few enzymatic and non-enzymatic oxidative stress parameters.There was a significant decrease in the levels of total proteins, haemoglobin and serum albumin of the CRF patients. Our findings emphasize that undialysed patients are at a higher risk when compared to dialysed patients for the thiol stress. Study also found that Indian chronic renal failure patients are having higher levels of MDA, which can increase LDL and induces membrane damages and worsen atherosclerosis. As a result of membrane damages in the renal tubule, increase in GST levels was also found in the study. Uraemia in the CRF patients increases the oxidative burden and our study shows a clear evidence for the use of antioxidants in CRF patients.

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