RESEARCH ARTICLE

Effect of single hemodialysis session on inflammatory and oxidative stress markers of chronic kidney disease patients

Sabiha Naaz[1], Amina S Zaidi[1], Ayaan Alam[2], Anwar Habib[3], G. A. Khan[4] and Iqbal Alam[1*]

ABSTRACT

Background: Chronic kidney disease (CKD), with its high prevalence, morbidity and mortality, is a significant public health problem. The progression of CKD has been shown to result in inflammation and oxidative stress. Hemodialysis (HD) is the most common renal replacement therapy (RRT) modality for CKD in India. The present study aimed to compare and evaluate the immune modality profile, oxidative marker and biochemical profile of pre- and post-dialysis chronic kidney disease patients. *Material and method*: Depending on inclusion and exclusion criteria, the study included 40 CKD patients, attending HAHC hospital, HIMSR, New Delhi, who were on hemodialysis. The mean age of the patient was 60 ± 15 yrs. Patients undergoing dialysis thrice a week for about 4 hours each session at a flow rate of 250-300 mL/min. Inflammatory and oxidative stress markers were assessed pre- and post-dialysis during any one session of dialysis in recruited CKD patients. *Result*: The dialysis session not only improved the kidney functions, as expected; there were significant decreases in inflammatory and oxidative stress markers after the single dialysis session. In addition, a significant decrease in other serum biochemical marker levels was observed post-dialysis. *Conclusion*: Hence, evident from this study, improvement in inflammatory markers and renal function tests after dialysis suggests that indeed hemodialysis is the most common and better treatment option for chronic kidney disease patients.

Keywords: Chronic kidney disease, Pre-hemodialysis, Post-hemodialysis, Inflammatory stress, Oxidative stress, Sterile inflammatory markers.

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Introduction

Chronic kidney disease (CKD), with its high prevalence, morbidity and mortality, is a significant public health problem.[1] In the 2013 Global burden of disease study, 9,56,200 people were estimated to have died from chronic kidney disease, a 134% increase from 1990, one of the significant rises among the top causes of death.[2] The number of deaths attributable to CKD in India rose from 0.59 million in 1990 to 1.18 million in 2016.[3] It is apparent from the singular rising trend in the prevalence as well as mortality cited above that kidney disease should be a global public health priority. CKD is particularly significant because, worldwide, more than 1·4 million individuals with end-stage renal disease (ESRD) are estimated to receive renal replacement therapy (RRT) with dialysis or transplantation, with 8% annual growth.[4] Because of challenges in access to appropriate healthcare, over 50% of patients with advanced CKD are first seen when the eGFR is < 15 mL/min per 1.73 m^2.[5]

A steep increase in cases of hypertension, diabetes, and other diseases that are risk factors for chronic kidney disease is also driving growth in the prevalence of chronic kidney disease, putting enormous pressure on healthcare resources.[6]

Chronic kidney disease (CKD) is defined as the presence of kidney damage or an estimated glomerular filtration rate (eGFR) less than 60 mL/min/1.73 mt^2, persisting for three months or more, irrespective of the cause.[7] It is a state of progressive loss of kidney function, ultimately resulting in the need for renal replacement therapy (dialysis or transplantation).

High levels of metabolic end-products—the uremic toxins—have become clinically relevant in CKD progression and are tightly related to many CKD-associated complications[8-10] CKD patients tend to suffer from many complications, such as hypertension, cardiovascular diseases, anaemia, metabolic acidosis,[11] altered immune response, mineral and bone disturbances and neurological complications.[12] Among these complications, cardiovascular dysfunctions and infections promoted by an altered immune might be responsible for an increased risk of morbidity and mortality.[13] In these conditions, inflammation and oxidative stress play pivotal roles.[14]

The progression of CKD has been shown to result in inflammation and oxidative stress.[15] CKD patients typically

[1]Department of Physiology, Hamdard Institute of Medical Sciences & Research, Jamia Hamdard, New Delhi, India

[2]Hamdard Institute of Medical Sciences & Research, Jamia Hamdard, New Delhi, India

[3]Department of Medicine, Hamdard Institute of Medical Sciences & Research, Jamia Hamdard, New Delhi, India

[4]Department of Clinical Nutrition, College of Applied Medical Sciences, King Faisal University, Alhasa, KSA.

***Corresponding author:** Iqbal Alam, Department of Physiology, Hamdard Institute of Medical Sciences & Research, Jamia Hamdard, New Delhi, India. Email: iqbalasc@yahoo.com

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suffer from chronic inflammation[16] and have severely impaired anti-oxidative systems, which worsen progressively with the degree of renal failure.[17] Inflammation and oxidative stress are crucial as defense mechanisms against infections, but, if not properly regulated, they may initiate several deleterious effects, such as cytokine overproduction and an increase in pro-inflammatory and oxidative stress mediators.[18] Oxidative stress is frequently observed in CKD/ESRD and is a non-traditional risk factor for all causes of mortality.[19,20] Thus, treating inflammation and oxidative stress is of primary importance in the uremic syndrome. Hemodialysis (HD) is the most common renal replacement therapy (RRT) modality in India.[19] A 2018 estimate put the number of patients on chronic dialysis in India at about 1,75,000, giving a prevalence of 129 per million population.[20] The number of HD stations in India was estimated at 12,881 in 2018.[21] However, despite the preference for HD as a significant mode of RRT, the associated risks of unregulated inflammation and oxidative stress in CKD patients have not been extensively documented. There are very few studies on the effect of acute sessions of hemodialysis on inflammatory and oxidative stress in chronic kidney disease patients.

The present study aims to study inflammatory biomarkers (Tumour Necrosis Factor α, Interleukin 6 and High mobility group box 1), to estimate the level of oxidative stress markers (superoxide dismutase and malondialdehyde enzymes), to test nitric oxide (NO) concentration in blood, and to evaluate hematologic and biochemical profile (ALP, AST, creatinine, urea, TLC and Platelets) of chronic kidney disease patients pre and post-dialysis condition.

Material And Method

The present cross-sectional study was conducted in the Department of Physiology, HIMSR, in collaboration with the Department of Medicine, HAHC Hospital, Jamia Hamdard, New Delhi, on chronic kidney disease patients on hemodialysis, after approval from the Ethical Committee of HIMSR. A total of 40 subjects were taken, depending on the inclusion and exclusion criteria.

The inclusion criteria of chronic kidney disease patients for the present study were patients with diagnosed CKD on maintenance hemodialysis, patients aged more than 18 years and those who gave informed consent were included in the study. The exclusion criteria of these patients were pregnancy and lactation and the presence of any other chronic inflammatory disease except diabetes mellitus, hypertension, autoimmune disorder, malignancy or known hematological disorder.

After obtaining informed consent from Chronic kidney patients on hemodialysis attending Medicine OPD at HAHC hospital, New Delhi, at the onset of the study, a proforma was filled by the patients having detailed information about their general health status, history of present and past illness, family history, history of medication. After that 3 mL of blood sample was collected in 200 µl in sodium citrate tubes at any one hemodialysis session using standard aseptic precautions. Both the samples were centrifuged at a speed of 3000 rpm at 40°C for 10 minutes to separate the serum from the blood cells. Then the serum was pipetted and stored at -80°C till use. In both the pre and post-dialysis samples of chronic kidney disease patients on hemodialysis tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6) and high mobility group box 1 (HMGB 1) estimation were done to evaluate inflammation and superoxide dismutase (SOD), nitric oxide (NO) and malondialdehyde (MDA) enzymes levels to assess the level of oxidative stress.

TNF-α, IL-6 AND HMGB 1

ELISA was performed using plasma samples. Briefly, 50 µL of plasma was incubated with an equal volume of coating buffer [0.5M carbonate buffer (pH 9.6)] in an assay plate overnight at 4°C. Nonspecific binding was blocked by 5% BSA in the same buffer. The samples were then washed with PBS containing 0.05% Tween 20 and incubated for 2 hours with diluted primary antibody (TNF-α, IL-6 and HMGB 1) in blocking buffer (1:500). The samples were next washed and incubated with diluted respective secondary antibody-HRP (1:2000) in the same buffer for 2 hours. After washing, the samples were incubated with p-nitrophenyl phosphate (1 mg/mL) in a carbonate buffer containing 10 mM $MgCl_2$. The development of color was assessed at 450 nm. The reaction was stopped by adding 50 µL of 1 M NaOH. Results were representative of three separate experiments and expressed as means ± Standard deviations.[22]

Superoxide Dismutase

This method utilizes the inhibitory effect of superoxide dismutase enzyme on the auto-oxidation of pyrogallol [23]. The reaction mixtures were prepared in various concentrations of standards, tests and controls. The assay mixture of 3 mL volume consisted of 100 µL each of 1-mmol/l EDTA and 1-mmol/l Diethylene triamine pentetic acid (DTPA) in air equilibrated tris HCl buffer (50 mmol/l, pH = 8.2). To this, 100 µL of standards (with varying concentrations of SOD enzyme) or test sera was added. In controls, neither the test sample nor the standard was added to the assay mixture to obtain uninhibited auto-oxidation of pyrogallol. Finally, 100 µL volumes of 0.2 mmol/l pyrogallol was added to all vials to start the reaction. After 10 seconds, a change in absorbance at 420 nm was recorded in a spectrophotometer at every 10-second intervals for a period of 4 minutes. The average change in the absorbance per minute was calculated, and percentage inhibition in standards and test samples was calculated.

The amount of enzyme required to inhibit the auto-oxidation of pyrogallol by 50% is defined as one unit of SOD activity. Accordingly, the enzyme activity in different standard concentrations was expressed in units. A standard graph was plotted, with the percentage of inhibition versus SOD enzyme activity in described units. The activity of the SOD enzyme in each test serum was calculated by using this standard graph.[23]

MDA-Thiobarbituric Acid Reactive Substances (TBARS) estimation

Lipid peroxidation (LPO) was estimated using the method of Wright *et al*., (1981). In a 0.05 mL serum sample, 0.58 mL phosphate buffer (0.1M, pH 7.4), 0.2 mL ascorbic acid (100 mM) and 0.02 mL ferric chloride (100 mM) were added. The total volume was 1-mL. The reaction mixture was incubated at 37˚C in a water bath for 1-hour. The reaction was stopped by adding 1-mL 10% trichloroacetic acid. Following the addition of 1-mL 0.67% thiobarbituric acid, all the tubes were placed in a boiling water bath for 20 minutes and then shifted to a crushed ice bath before being centrifuged at 2500 g for 10 minutes. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the optical density of the supernatant at 535 nm using a spectrophotometer (Hitachi) against a blank using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.[24]

Nitric Oxide Assay

The synthesis of NO is assessed by using the conversion rate of oxyhemoglobin to methemoglobin through NO using a scanning spectrophotometer (Lambda 35, Perkin-Elmer, Norwalk, CT) a. Sample plasmas were added to a reaction mixture containing Krebs buffer (pH 7.4) with 15 mM oxyhemoglobin, 10 mM L- arginine, and 240 nM insulin in a total volume of 2.5 mL for 45 minutes at 37 °C while the mixture is constantly stirred. The NO content was quantitated by recording the spectral changes in the reaction mixture due to the conversion of oxyhemoglobin to methemoglobin, i.e., a decrease in the absorbance at 575 and 630 nm maxima on a standard curve constructed by using pure commercial (> 99% pure) NO in 0.9% NaCl under identical conditions. The amount of NO in the reaction mixture was confirmed by using an independent chemiluminescence technique.

Protein Estimation

Protein reacts with Folin' Ciocalteu phenol reagent and give a colored complex. The color so formed is due to the reaction of alkaline copper with protein as, in the Biurets test, and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein.

Statistical Analysis

Statistical analysis was performed using GraphPad software. Continuous variables were expressed as Mean ± Standard deviation (SD) or range, and qualitative data were expressed in percentages. A paired sample t-test was used for comparison of means between pre- and post-dialysis. A p-value of <0.05 was considered statistically significant.

Results

The Study has been conducted on 40 patients who are suffering from chronic kidney disease (CKD) on hemodialysis. Among 40 CKD patients, 25 were males and 15 were females attending HAHC Hospital. The mean age of these patients was 60 ± 15 yrs. The mean duration of hemodialysis was 31 ± 2 months. Regarding vascular access, all had an arteriovenous fistula and used a polysulfane membrane dialyzer. Patients were on maintenance HD (4 h/session, 3 sessions/wk) (Table 1).

Out of 40 patients, 23 patients were hypertensive, 5 patients had type 2 diabetes mellitus and 12 patients had both hypertension and type 2 diabetes mellitus. (Figure 1)

There was a significant decrease in inflammatory markers levels *i.e*. TNF-α, IL-6 and HMGB 1 after dialysis when compared to pre-dialysis samples, as shown in Figure 2.

There was a significant decrease in oxidative stress markers levels i.e. NO, Superoxide dismutase levels, after dialysis when compared to pre-dialysis samples. There was a decrease in malondialdehyde post-dialysis but was not significant as shown in Figure 3.

It was observed that there was a significant decrease in serum urea and creatinine levels in post-dialysis samples as compared to pre-dialysis samples (Figure 4).

When biochemical parameters were compared, there was a slight increase in serum AST and ALT levels of the post-dialysis samples as compared to the pre-dialysis samples, though not significant (Figure 5).

Discussion

We have assessed inflammatory stress and oxidative stress in 40 chronic kidney disease patients on maintenance hemodialysis. We have also looked at the effect of acute single hemodialysis sessions on these parameters. The mean age of the patients was 60 ± 15 yrs. The mean duration of hemodialysis was 31 ± 2 months. Regarding vascular access, all had an arteriovenous fistula and used a polysulfane membrane dialyzer. They were on maintenance HD (4 h/session,3 sessions/wk) (Table 1). In this study, among 40 CKD patients, 57% were hypertensive, 30% were having type 2 diabetes mellitus and 13% were both diabetic and hypertensive (Figure 1).

It is evident from this study that inflammatory markers levels of TNF-α, IL-6 and HMGB 1 were significantly decreased in post-dialysis samples when compared to pre-dialysis samples (Figure 2). In the pre-dialysis sample, the increased levels of inflammatory markers TNF-α, interleukin-6 (IL-6) and high mobility group box 1(HMGB 1) are partly responsible for chronic inflammation.[25] In chronic kidney disease patients, higher expression of TNF-α are associated with markers of malnutrition and inflammation and predicts mortality. IL-6 accelerates the progression of chronic kidney disease (CKD) not only by aggravation of kidney injury but also by initiating its complications, especially chronic vascular disease (CVD).[26]

As a potential inflammatory cytokine, HMGB 1 plays multiple roles in the pathogenesis of renal disease. High mobility group box (HMGB) 1, a highly conserved non-histone protein, presents in the nuclei. Extracellular HMGB1 is the prototypic endogenous "danger signal" that triggers inflammation and immunity.[27] Hence, it is evident through this study that a

Tabel 1: Basic characteristics of patients

Total patients	*40 (25 Male and 15 female)*
Age	60 ± 15 yrs
Hemodialysis	31 ± 2 months
Maintenance hemodialysis session	4 hour/session, 3 sessions/week

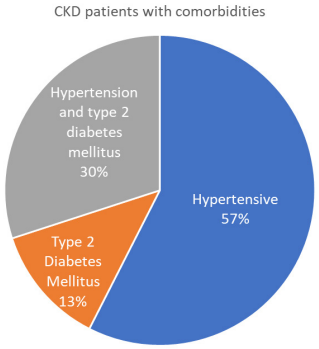

Figure 1: Comorbidities in CKD patients

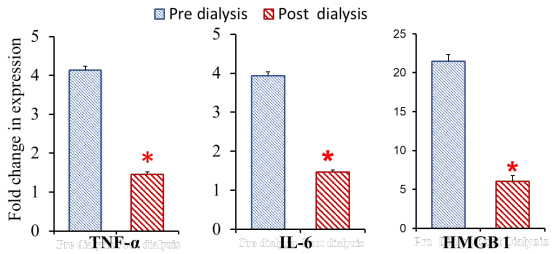

Figure 2: Fold change in expression of inflammatory biomarkers of pre- and post-dialysis samples from chronic kidney disease patients. Each bar represents the mean ± SD, * indicates $p < 0.05$ when both are compared.

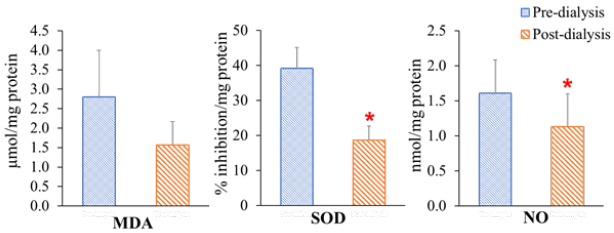

Figure 3: Comparison of serum levels of lipid peroxidation (MDA) and nitric oxide (NO), and serum activity of superoxide dismutase (SOD) from pre- and post-dialysis samples from chronic kidney disease patients. Each bar represents the mean ± SD, * indicates $p < 0.05$ when both are compared.

single hemodialysis session has a very positive effect on reducing these inflammatory markers, which are the main culprits in the progression of chronic kidney disease.

The present study also shows a significant decrease in the levels of antioxidant enzymes (SOD and Nitric Oxide) in post dialysis patients' group as compared to pre-dialysis patients groups (Figure 3). Superoxide dismutase (SOD) is the first line of defense against the free radicals. Oxidative stress has been linked to the progression of disease, including chronic kidney disease (CKD). The antioxidant enzymes superoxide dismutase and catalase form the primary defense against reactive species and oxidative stress. Oxidative stress results from increased concentrations of reactive oxygen species and a reduction in antioxidants. Superoxide dismutase (SOD) has potent anti-inflammatory activity. It is a marker of cardiovascular alterations in hypertensive and diabetic patients since changes in serum levels correlate with alterations in vascular structure and function. Treatment of superoxide dismutase (SOD) decreases reactive oxygen species generation and oxidative stress and thus, inhibits endothelial activation. Dyslipidemia, increased oxidative stress and impaired endothelial function may cause a decrease in nitric oxide levels in post-hemodialytic samples.[28,29]

Endothelial cells also contain some superoxide dismutase enzyme, which probably serves to counterbalance the local formation of oxidants.[30] During hemodialysis, the dialysis membrane is subjected to immunologic response by low molecular weight plasma constituents such as IgG and complement components to make the membrane active for granulocytes. Activation of blood granulocytes can increase reactive oxygen species. The oxygen radicals generated may interact with neutrophils and further stimulate ROS production and leading to the imbalance between the production of free radicals and the alteration of the different antioxidant enzymes. This event can increase the LPO level and decrease the SOD activity in hemodialyzed samples.[31]

In this study, it was also observed that lipid peroxidation measured by levels of MDA was decreased in post-dialysis

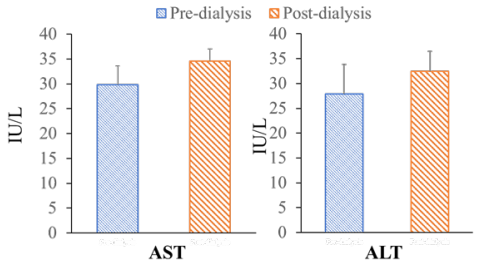

Figure 4: Comparison of serum activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) from pre- and post-dialysis samples from chronic kidney disease patients. Each bar represents the mean ± SD,

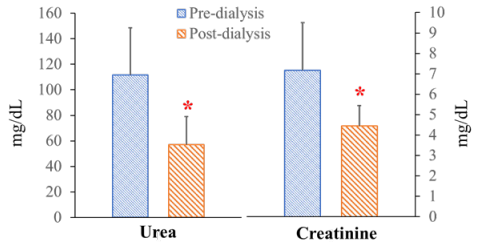

Figure 5: Renal profile of chronic kidney disease patients before and after hemodialysis. Each bar represents the mean ± SD, * indicates $p < 0.05$ when post-dialysis sample is compared with pre-dialysis sample.

levels compared to pre-dialysis levels, though the decrease was not significant. In previous studies, it has been seen that the MDA level is reduced during dialysis due to the removal of uremic toxins, corrections of azotemia and improvement in cardiovascular status through dialysis.[32,33]

The renal function test is analyzed in 40 cases of chronic renal disease (Figure 4). All these cases are on hemodialysis and revealed increased blood urea and creatinine levels. The study observed that the mean level of urea and creatinine significantly decreased in the post-dialysis samples group as compared to the pre-dialysis group. Increased serum creatinine levels in cases of CRF are due to its reduced clearance. Serum creatinine levels are used as a diagnostic test to assess renal function and levels more than 1.5 mg/dl indicate impairment of renal function. In the hemodialysis procedure, the artificial kidney (Dialyzer) is used. After dialysis, the toxin substance is removed resulting in decreased creatinine and urea levels.

This study also observed that the ALP and ALT levels were slightly increased in the post-dialysis samples group compared to pre-dialysis sample groups (Figure 5).

It is well established that the two significant pathological causes of the progression of CKD are inflammatory and oxidative stress. Moreover, the prevalent last resort treatment for CKD is hemodialysis. Improvement in inflammatory markers and renal function test after dialysis suggests that hemodialysis is the most common and better treatment option for chronic kidney disease patients which is the evidence from this study. However, our data also confirm the presence of an increase in oxidative stress because of a decrease in antioxidant defense (SOD enzyme) in HD patients. Due to this adverse effect of HD, demonstrated by our results, clinicians may plan to advise antioxidant therapy. An antioxidant membrane for HD will be a new approach to overrule oxidative stress and inflammation during HD sessions.

The observations made in the study put forward a case for the addition of antioxidants during hemodialysis in the treatment of CKD. Also, regular assessment of these inflammatory and oxidative stress markers in hemodialysis (HD) patients and developing strategies to reduce inflammatory and oxidative stress in these patients, might be beneficial.

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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers' comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.