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Original Article

Antioxidant Enzymes status in South Indian Hemodialysis patients

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ABSTRACT

Free radicals occupy a pivotal role among the atherosclerosis explanations.An imbalance in favor of pro-oxidants can lead to the oxidation of macromolecules resulting in tissue injury.27 patients of ESRD on maintenance haemodialysis were included and blood samples were collected at the start of haemodialysis (prehd), at 1 hr, 2hrs, 3hrs and at the end of the dialysis procedure (post HD).Plasma MDA estimated as TBARS (Thiobarbituric acid reactive substance),erythrocyte Super Oxide Dismutase (SOD), Glutathione reductase, Glutathione Peroxidase (GPx),Catalase Continuous Spectrophotometric rate determination. A significant decrease in malondialdehyde levels (p=0.017) were observed which however on correction for clearance, SOD, GR and Catalase showed an increase in activity (p<0.001) as a result of dialysis. A significant decrease in malondialdehyde levels (p=0.017) were observed which however on correction for clearance, as shown in Figure: 21 A was found to be increased (p=0.001).No change in GPx activity (p=0.704) was observed as a result of dialysis. Hemodialysis per se has been suggested to induce oxidative stress, with reactive oxygen species being generated on the surface of dialysis membranes by activation of polymorphonuclear leukocytes. Ongoing oxidative stress present in the patients on MHD may play a pathophysiological role in the development of cardiovascular disease. HD is mainly responsible for free radical production as well as non-enzymatic antioxidant losses.

1. Introduction

Free radicals occupy a pivotal role among the atherosclerosis explanations. OS can be viewed as a disturbance in the balance between oxidant production and antioxidant defense. An imbalance in favor of pro-oxidants can lead to the oxidation of macromolecules resulting in tissue injury. While there are many types of free radicals, oxygen free radicals are particularly important because of the involvement of oxygen in many metabolic processes [1]. MDA is widely used as a marker of oxidative stress in many studies. MDA, a water-soluble low molecular weight LPO product, is partially excreted via urine under normal conditions. However, it is not known exactly what proportion of MDA formed in the body is eliminated by the kidney.

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Free radicals occupy a pivotal role among the atherosclerosis explanations. OS can be viewed as a disturbance in the balance between oxidant production and antioxidant defense. An imbalance in favor of pro-oxidants can lead to the oxidation of macromolecules resulting in tissue injury. While there are many types of free radicals, oxygen free radicals are particularly important because of the involvement of oxygen in many metabolic processes [1]. MDA is widely used as a marker of oxidative stress in many studies. MDA, a water-soluble low molecular weight LPO product, is partially excreted via urine under normal conditions. However, it is not known exactly what proportion of MDA formed in the body is eliminated by the kidney.
Antioxidants are the substances that protect the tissues from free radical attack by preventing free radical formation, by blocking chain reaction or by repairing the oxidatively damaged bio-molecules. Intracellular enzymatic antioxidants are Superoxide Dismutase (SOD), Catalase and Glutathione. Main non-enzymatic cellular antioxidant is reduced glutathione (GSH). Glutathione reductase (GR), Antioxidant enzymes such as catalase, superoxide

dismutase and glutathione peroxidase maintain a reducing tone within cells. Catalase is a common enzyme found in the blood and in most living cells that catalyzes the decomposition of hydrogen peroxide into water and oxygen. It is a tetramer of four polypeptide chains, each over 500 amino acids long [2]. Glutathione peroxidase (GPx) is a selenium-containing enzyme whose blood level is a good indicator of the selenium status of the animal; occurs in a plasma form, an enzyme with specificity for phospholipids, and an intracellular form. Glutathione reductase (GR) is a flavin enzyme involved in the defense of the erythrocyte against hemolysis. A partial deficiency occurs relatively frequently but is due to a deficiency of riboflavin. Superoxide dismutase plays a key role in catalyzing the dismutation of O_2. - to O_2 and H_2O_2. Catalase or GPx then remove the hydrogen peroxide formed. GPx catalyzes the reduction of H_2O_2 and a wide range of organic hydroperoxides (ROOH) to water and corresponding alcohols (ROH), respectively. They are specific for glutathione (GSH) as the reducing substrate.

Several extracellular antioxidants such as proteins (Transferrin, lactoferrin, albumin, ceruloplasmin), and urate prevent free radical reaction in the body sequestering transition metal ions by chelation in plasma. Albumin, bilirubin, and urate may also scavenge free radicals directly. Furthermore, plasma has a considerable peroxyl radical scavenging ability, which is mainly determined by its content of ascorbic acid. Our aim is to study the intradialytic changes in Oxidative stress and Antioxidant enzymes due to a single haemodialysis session in MHD patient.

2.Material and Methods:

For MDA it is determined as Thiobarbituric acid reactive substances (TBARS)[3]. Free MDA, as a measure of lipid peroxidation, was measured spectrophotometrically as TBA reactive substances after precipitating the proteins with trichloroacetic acid (TCA). 500 µl of plasma and 500 µl of saline were mixed with 1 ml of 24% TCA and centrifuged at 2000 rpm for 20minutes. To 1.0ml of the supernatant [protein free filtrate] , 250 µl of TBA reagent was added. The test tubes were covered with rubber cork and kept in boiling water bath for 1 hour. The tubes were then removed and cooled under tap water. 500 µl of n-Butanol was added and vortexed for 1 minute. Later the mixture was centrifuged and the upper butanol layer was read at 532 nm using Perkin Elmer Lamda 1.2 spectrophotometer. The concentration of the samples were derived from the standard curve (Figure: 13) treating the standards in the same way as protein free filtrate. For SOD[4] Based on the ability of SOD to inhibit the oxidation of adrenaline. Take 1 ml of hemolysate, 500 µl Ethanol and 250 µl Chloroform to add test standard and blank. The test tubes were centrifuged for 10 minutes at 4000rpm and the supernatant was used for further analysis. 500 µl Supernatant to all the tubes,Carbonate Buffer 1.500 µl to test and blank 1.900 µl add into the standard add 400 µl of Adrenaline to the test and blank After the addition of adrenaline the formation of adrenochrome in the next 4 minutes was recorded at 470 nm.

For Glutathione Reductase[5] add FAD solution 0.1 ml in with FAD tube, and distilled water 0.1 ml add into without FAD tube and add in to both tubes Phosphate buffer 2.0 ml,EDTA Solution 0.05 ml,Dilute hemolysate 0.1 ml,Oxidized GSH 0.1 ml. The contents were equilibrated at 37°C for 8 minutes and add in both the tubes Reduced NADP solution 0.1 ml. The decrease in absorbance was measured at 340 nm over a 10 min period to obtain Δ A340 in the presence and absence of FAD. For Glutathione peroxidase[6] Add Hemolysate 1 ml,Transformation solution 500 µl to test and blank tubes.The test tubes were incubated for 10 min at room temperature,and add both the tubes Carbonate Buffer 100 µl,GSR 100 µl,GSH 100 µl,NADPH 100 µl.The test tubes were incubated at 37oC for 10 min and add T-butylhydroperoxide 100 µl for both tubes. The linear decrease in NADPH absorption was recorded at 340 nm. For Catalase[7] 1:500 (10ul in 5 ml) dilution of hemolysate was made with phosphate buffer. And the reagents were added in to the test and sample tubes Assay Buffer 500 µl,20mM H2O2 500 µl and sample10 µl add into the test tube and 10 µlin to blank tube.

2.1Statistical Analysis: The descriptive statistics were expressed as mean ± SD. Analysis of variance (ANOVA) was done to study the changes in each parameter during the haemodialysis session, taking pre-HD as 100%.

3.Results:

Baseline characteristics of ESRD patients are given in Table: A. The total number of patients with ESRD studied was 27 of these sixteen were males and eleven were females. Age range was 31-75 years (mean age: 50.4+2.06Years).

Table A: Baseline characteristics of the ESRD patients.

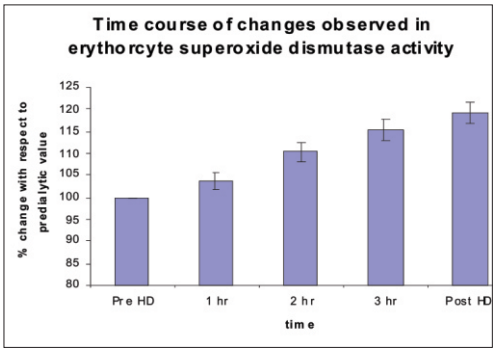
Parameter	Mean + SEM
Age (Years)	50.4+2.06
Height (centimeters)	159.0+1.51
Weight (Kilograms)	55.3+6.15
Hemoglobin (gram/dl)	9.6+0.36
Packed cell volume (%)	29.9+1.23
Calcium(mg/dl)	9.4+0.07
Phosphorus(mg/dl)	4.6+0.25
Alkaline Phosphatase (IU/L)	178.7+21.0
Uric acid (mg/dl)	5.1+0.17
Total bilirubin (mg/dl)	0.84+0.06
Direct bilirubin (mg/dl)	0.17+0.03
SGOT (IU/L)	34.8+4.84
SGPT (IU/L)	30.8+4.64
Total protein (gram/dl)	7.2+0.10
Albumin (gram/dl)	3.6+0.06
Cholesterol (mg/dl)	147.6+0.61
Triglycerides (mg/dl)	115.1+7.84

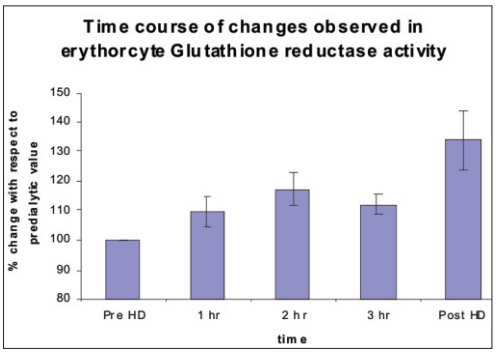
Table-B: Showing the mean ± SEM of the oxidative stress markers studied

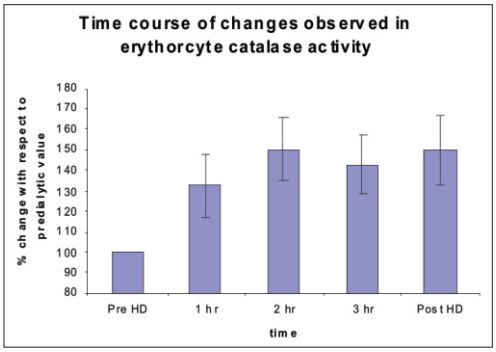
Malondialdehyde (µmol/lit)	5.53±0.71	4.14±0.49	3.76±0.47	2.97±0.37	2.60±0.34	↓ 0.017
Malondialdehyde (µmol/mg of creatinine)	0.07± 0.01	0.08± 0.01	0.08± 0.01	0.07± 0.01	0.08± 0.01	↑ 0.001
Gpx (Units/Gm of Hb)	13.47±1.60	13.44±1.99	11.36±1.45	13.69±1.93	10.84±1.39	No change 0.704
CAT (Units/mg of Hb)	4544.26±491.01	5259.98±478.7	5595.36±381.0	5236.43±384.772	5624.83±525.533	↑ 0.001
GR (Units/mg of Hb)	0.99±0.03	1.05±0.034	1.14±0.06	1.11±0.05	1.26±0.04	↑ 0.001

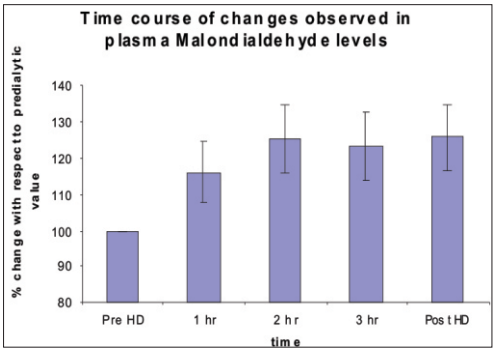
SOD- Superoxide dismutase,GPX-glutathione peroxidase, CAT-catalase, GR- Glutathione reductase.

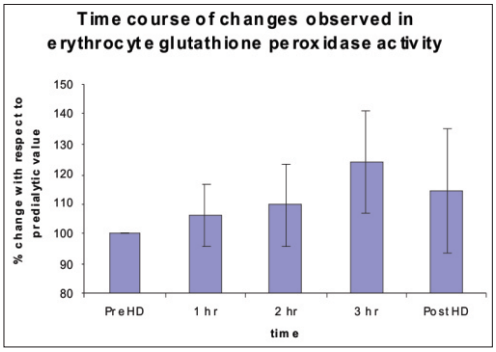
Figure 1: Showing Time course of changes in Antioxidant enzymes during a 4 hr dialysis session with polysulphone membrane. The data was collected hourly, and converted to percentages with predialytic value taken as 100%. The results are presented as mean±SEM (n=27). A. Changes in erythrocyte SOD , B. Changes in erythrocyte catalase,C. Changes in erythrocyte GPx , D. Changes in erythrocyte GR.E. Changes in MDA levels. SOD-Superoxide dismutase , GPx-Glutathione peroxidase, GR-Glutathione reductse











The mean ± SEM of the oxidative stress markers studied with results of analysis of variance for repeated measures along with significance of changes observed due to a haemodialysis session is shown in Table: B. As shown in Table: B, a significant decrease in malondialdehyde levels (p=0.017) were observed which however on correction for clearance, as shown in Figure: 1 A was found to be increased (p=0.001). The antioxidant enzyme especially, SOD, GR and Catalase showed an increase in activity (p<0.001) as a result of dialysis as shown in Figure 1 A,B and D respectively. However, no change in GPx activity (p=0.704) was observed as a result of dialysis.

4.Discussion

Hemodialysis per se has been suggested to induce oxidative stress, with reactive oxygen species being generated on the surface of dialysis membranes by activation of polymorphonuclear leukocytes [8-12]. It has been well documented that even a single session of hemodialysis significantly increases lipid peroxides and decreases antioxidants[13-16]. Ongoing oxidative stress present in the patients on MHD may play a pathophysiological role in the development of cardiovascular disease. Indicators of oxidative stress have often been evaluated in ESRD patients. Of these malondialdehyde (MDA), a low molecular weight end product of lipid hydroperoxide decomposition, lipid hydroperoxides which represents the hydroperoxide fraction of the plasma lipids [17], oxidized LDL [18], oxidant modification of amino acid residues like nitrotyroxine, 3 – chlorotyrosine and breath ethane are often measured. Other markers include F2-isoprostanes which results from non-enzymatic free radical-induced peroxidation of arachidonic acid. MDA is the most often measured index of lipid peroxidation. Results with regard to MDA levels in haemodialysis are conflicting with some studies showing an increase[19,20] while others reporting a decrease in MDA levels to normal after dialysis due to its clearance [21-22].

There are varying reports on the erythrocyte activities of enzymes glutathione peroxidase and superoxide dismutase. Some studies reported an increased erythrocyte glutathione peroxidase activities and no change in level of erythrocyte superoxide dismutase enzymes due to haemodialysis [23-26] . Balashova et al [27] showed no change of activities of erythrocyte glutathione peroxidase and a decreased activity of erythrocyte superoxide dismutase in haemodialysis patients. Salamunic et al [28] reported that the mean catalytic concentrations of glutathione peroxidase were decreased that of superoxide dismutase were increased in patients before haemodialysis when compared with control values. The mean catalytic concentrations of superoxide dismutase returned to control range, while those of glutathione peroxidase decreased further when compared to control values. Kose et al [29] reported an increased erythrocyte glutathione peroxidase and superoxide dismutase activities in the postdialysis group when compared with predialysis group. But there was a decrease in the activity of these enzymes in postdialysis group when compared with control group. Martine-Mateo et al [30] found that the enzyme activity increased by 20% post HD when compared with the pre- HD value. Roxborough et al [31] found that after HD plasma GSH-Px activity increased and reached the value observed in the control group. Both teams thought that the decreased activity might be attributed, at least in part, to the inhibition caused by ligands or toxic agents of endogenous nature. These inhibitors are most likely removed by dialysis. Catalase is the other enzyme which can act on H2O2. Mimic-Oka J et al [25] found low catalase activity in hemodialysis patients. Sommerburg etal [32] demonstrated increase in catalase activity as a result of dialysis.

There is conflicting evidence on the fate of GR in patients on dialysis. McGrath and colleagues [33] demonstrated a low level of GR in patients on peritoneal dialysis but not in patients on hemodialysis. In contrast, Ceballos-Picot and colleagues [34] reported increased GR activity in the erythrocyte of patients on hemodialysis. Superoxide dismutase, glutathione peroxidase and catalase, together with glutathione, form the main line of defense against ROS in erythrocytes. There was an increase in SOD activity ($P = 0.001$) as a result of dialysis session as shown in Figure: 1 A. Increase in superoxide anion generation is due to the blood membrane interaction during dialysis. Increase in concentration due to increased synthesis is unlikely to occur during the short course of dialysis insult (<6 hrs.). The increase observed is mostly due to its increased activity in defense against superoxide radicals. This is in agreement with Rico et al [35] who have reported an increase in erythrocyte SOD activity. Some earlier studies found a decrease in erythrocyte SOD as a result of a dialysis session, which was attributed to the presence of an activating factor in uremic plasma [36]. This was later reported to be due to clearance of copper and zinc during dialysis (37). However, Mc. Grath et al [33] and Cavdar et al [38] found no change in SOD activity as a result of haemodialysis. As shown in Fig 1 B, a significant increase in catalase ($P = 0.001$) activity was observed as a result of dialysis in the present study. However, low catalase activity was reported by Mimic– Oka J et al [25]. Increase in formation of H_2O_2 as a result of dismutation of superoxide radicals by SOD causes increased activity of GPX & catalase. However, due to the difference in Km, the contribution of GPX & catalase in detoxification of H_2O_2 is different. GPX acts at low H_2O_2 concentration whereas catalase plays a role when GPX pathway reaches saturation. An increase in catalase activity observed might be due to low levels of GPX observed in dialysis patients coupled with increased generation of hydrogen peroxide above the capacity of GPX [39-41].

In the present study, no significant change in GPx activity were observed as a result of dialysis ($p=0.704$) (Fig. 1 C). Data regarding GPX activity in HD are conflicting. Some studies have reported an increase in GPX activity [42] while others have reported no change [33, 38]. Selenium is an essential component of gluthathione peroxidase and loss of selenium during dialysis has been suggested to be the cause of reducing the activity of GPx [33].

As shown in Fig.1D, a significant increase in GR activity was observed ($P = 0.001$). This is in agreement with Biasioli et al [42] and Mc. Grath et al [33]. The pentose phosphate pathway supplies NADPH required by GR to generate reduced Glutathione. It is known that this pathway is impaired in the uremic state leading to a decreased content of reduced Glutathione in erythrocytes which is required for GPX activity. The increased GR activity suggests that there may be a transient improvement in the pentose phosphate pathway because of improvement in the uremic state due to dialysis. However, variations occur in the impairment of Pentose Phosphate Pathway and this possibly explains the difference observed in the enzyme activity, in different studies. Increased GR activity has been suggested to be a defensive mechanism by which glutathione availability is preserved [43].

5.Conclusion

HD is mainly responsible for free radical production as well as non-enzymatic antioxidant losses. This suggests that HD, far from improving oxidative stress, worsens the same. Diverse

mechanisms might account for increased oxidative stress, including antioxidant deficiency, neutrophil activation during dialysis, and chronic inflammation. Degranulation of neutrophils passing through the dialyzer may also play a role. Increase in MDA activity of erythrocyte SOD, GR, and CAT show a net oxidative imbalance as a result of dialysis session. Hence it can be concluded that a single session of HD adds to the OS of uremic state.

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