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Assessment of Red Cell Fragility by Flow Cytometry and Red Cells Antioxidant Enzymes (Glutathione Peroxidase and Reductase) in End Stage Renal Disease Patients Undergoing Hemodialysis

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ABSTRACT

Background: Anemia is common and multifactorial in hemodialysis (HD) patients. Oxidative damage is known to cause erythrocyte deformability.

Objectives: We aimed to assess the influence of the HD procedure on red cell antioxidant enzymes and red cell fragility using Flow Cytometry (FCM).

Patients and Methods: This is a case-control study involving 70 HD patient and 30 healthy controls; all patients received recombinant erythropoietin (rEPO) and iron. Two blood samples were taken pre-and post-HD for assessment of biochemical and hematological parameters, red cell fragility by flow cytometry and red cell antioxidant enzymes glutathione peroxidase (GSH-Px) and reductase (GSH-Rx).

Results: The mean age of HD patients was 48.42± 14.02 yrs; the causes of ESRD were hypertension 35 (50%), obstructive nephropathy 11 (15.7%), chronic glomerulo-nephritis 7 (10%), diabetes mellitus 6 (8.6%) and unknown etiology 11 (15.7%). GSH-Px and GSH-Rx decreased significantly in post-HD compared to pre-HD and control group (p=0.0001). Percentage of residual red cells in pre-HD and post-HD were significantly lower than healthy control (p=0.0001) with insignificant difference between pre-HD and post-HD group (p=0.6709). Patients were categorized into responder 27 (38.57%) and non-responder to rEPO therapy 43 (61.43%). Non-responders to rEPO therapy had significantly lower percentage of residual red cells and GSH-Px activity compared to responders.

Conclusions: HD aggravates oxidative damage; nevertheless the increased red cell fragility detected in pre-HD patient was not intensified immediately after dialysis (i.e. no acute hemolysis). However; gradual deterioration of RBCs due to mechanical stress cannot be excluded following extracorporeal blood circulation in the dialysis machine. We therefore suggest that antioxidant supplementation might be capable of recovering antioxidant defense in red cells and plasma and thus preventing oxidative damage induced by HD.

Key Words: *RBCs osmotic fragility – Hemodialysis – Flow cytometry – ESRD – Glutathione.*

INTRODUCTION

The pathogenesis of anemia in hemodialysis (HD) patients comprises different mechanisms **[1,2]**. Decreased RBCs lifespan was observed in nearly all HD patients; this may be due to extrinsic (extracorporeal circuit) or intrinsic (uremic milieu) factors that lead to modifications in RBCs membrane composition **[3,4]**.

Patients with End Stage Renal Disease (ESRD) have reduced capacity to handle oxidative stress as indicated by decreased levels of antioxidants (such as glutathione) and increased lipid peroxidation within the RBC membrane which may contribute to shortening of RBC lifespan **[5]**.

There is great evidence that HD patients are in a continuous state of oxidative stress, which may be aggravated by bio-incompatibility of the dialyzer membrane, that may induce formation of Reactive Oxygen Species (ROS) e.g.

H2O2 and superoxide which are neutralized by the extensive RBC antioxidant system involving both non-enzymatic and enzymatic antioxidants like glutathione peroxidase **[6]**. Furthermore, both dialyzable components of uremic plasma and dialysis procedure trigger premature suicidal erythrocytes **[7]**.

The RBC deformability is determined by cell geometry, internal viscosity, rheological properties of the membrane, osmotic pressure, calcium, nitric oxide, temperature, ageing and depletion of adenosine triphosphate. Hence, a slight decrease in RBC deformability causes a significant increase in micro-vascular flow resistance and blood viscosity **[8]**.

Osmotic deformability is frequently measured in RBCs and provides information on their viability, cellular water content, surface area and deformability, particularly in relation to several pathological conditions **[9]**. Therefore we tried to investigate red cell fragility by flow cytometry.

The aim of this study was to assess the influence of the HD procedure on both RBC fragility (using flow cytometry) and red cells antioxidant enzyme GSH-Px and GSH-Rx.

MATERIAL AND METHODS

Study design and subjects:

A cross-sectional case-control study was carried out at the dialysis unit in Qena-General Hospital, Qena-Upper Egypt-Egypt, seventy patients on maintenance HD, (34 males and 36 females) and thirty age and sex matched healthy volunteers (21 males and 9 females), who were free of kidney disease and with normal hematological and biochemical values, did not receive any medication known to interfere with the studied variables, served as control. All participants provided an informed consent. The study was approved by the ethical committee in Qena Faculty of Medicine.

Exclusion criteria:

Patients with autoimmune disease, pregnancy, malignancy, hematological disorders, infections, HBV or HCV and HIV positivity, severe hyperparathyroidism, blood loss or transfusion and known cause of anemia (e.g., haemoglobinopathies) were excluded.

All patients attended the dialysis unit 3 times per week; each dialysis session lasted for 4 hours, using bicarbonate dialysate solution with polysulphone F7/F9 membrane dialyzers (Bio-140; Dialife SA, Taverne, Switzerland).

The medication taken by patient's population includes: Anti-hypertensive drugs (calcium antagonists, angiotensin converting enzyme inhibitors), phosphate binders (calcium carbonate), folic acid, vitamin C and vitamin B6, recombinant erythropoietin (rEPO) Epoetin therapy by the subcutaneous route and iron by intramuscular injection by the end of HD.

Classification of the patients, as responders or non-responders to rEPO therapy, was performed in accordance with the European Best Practice Guidelines which defines resistance to rEPO as a failure to achieve target Hb levels of (11-12g/dL) with maintained doses of rEPO **[10]**.

The following characteristics were observed or calculated: Age, gender, duration of dialysis, weight, height, Body-Mass Index (BMI) and blood pressure measured using standard mercury sphygmomanometer. The average of two blood pressure readings, recorded at an interval of 5min., was used for analysis.

Blood samples:

Seven ml venous blood was taken twice from HD patients, pre-HD and post-HD on a midweek dialysis sessions. The pre-HD sample was drawn from arteriovenous fistulas at insertion of the arterial needle, before heparinization of the line and after an overnight fasting period and before the patient was being connected to the dialysis machine and the post-HD sample just prior to patient disconnection, and blood samples were taken once from the control subjects.

Blood samples were collected in EDTA, heparin and plain vacutainer tubes. EDTA tube was used for Complete Blood Count (CBC) using cell dyne-1800 (Abbott diagnostics, USA), and for flow cytometry RBCs osmotic fragility assessment. Plain tube blood was allowed to clot and then centrifuged at 3000rpm for 10

minute and serum separated for assessment of urea, creatinine using Cobas c311 automated chemistry analyzer (Roche diagnostics, Germany). The heparinized tube was centrifuged immediately at 2700 X g for 10min. at 4ºC, and the plasma was separated from the packed RBC. RBCs were washed 3 times with cold saline, and the buffy coat carefully aspirated from the surface of the pellet, then red cell lysate prepared, aliquoted in 1ml cryo-tubes and stored at –80ºC for later analyses of GSH-Px and GSH-Rx.

GSH-Px and GSH-Rx activity were measured using kits provided by Cayman Chemical Michigan-USA; cat no 703102 and 703202 respectively according to manufacturer instructions. The activity of GSH-Px was indirectly measured by a coupled reaction with GSH-Rx. Oxidized glutathione (GSSG), produced upon reduction of hydroperoxide by GSH-Px, is recycled to its reduced state by GSH-Rx and NADPH and the oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340nm at 37ºC using the Humalyzer 2000 analyzer. A standard curve was prepared by using the standard provided in the kit, and the value for each sample was read from the curve. GSH-Px activity precision: The intra-assay coefficient of variation was 5.7%. GSH-Px assay range 50-344nmol/min/ml and the GSH-Rx activity intra-assay coefficient of variation was 3.7%; GSH-Rx assay range 20-255 nmol/min/ml.

Flow-Cytometry Osmotic Fragility Test (FCM OF): In this test a hemolysis inducing agent, deionized water, is spiked to a red cell suspension during acquisition, and the count of red cells is measured sequentially in real-time using FCM according to the method described by Won and Suh **[11]** with a modification in the acquisition time.

The red cell suspension was prepared by a two-step dilution with normal saline. In the first step, 20-30µL blood was diluted with 1.1mL of normal saline. The volume of blood taken was calculated based on the following formula so that the same number of red cells would be present in each tube:

$$\text{Blood volume (ML)} = 130 \div (\text{Number of red cells}/\mu L)/10^6$$

In the second step, 10µL from the first diluted blood was added to the flow cytometry tube that already contained 1.1mL of normal saline. This second diluted blood was the final red cell suspension ready for flow cytometry acquisition and analysis.

Acquisition: Forward Scatter Characteristics (FSCs) and Side Scatter Characteristics (SSCs) were acquired via linear amplification on the FACS caliber flow cytometer using cell Quest soft wear (Becton Dickinson, San Jose, CA).

A time/FCS acquisition plot, in which eight identical regions were demarcated, was created to acquire events based on time over a period of ~102.4sec. This dot plot showed dots moving from left to right, assuming time is the X parameter; one region took ~11s. The FACS tube containing the thoroughly mixed final red cell suspension was added at the sample injection port. After the first region passed (R1) during acquisition (the area through which the cells pass), the tube was removed without ending acquisition and 0.9ml Deionized Water (DW) was added and acquisition was again continued up to the eighth region. The acquisition was stopped either when a 200000 events were acquired or when 102.4sec passed. It took ~2min to run one tube.

Analysis: The event count per single region was chosen as a parameter reflecting the number of residual red cells in each region of the time/FSC plot prior to and after DW spiking. The average of the total number of events in the last two regions (R7) and (R8) were used for calculation of the percentage % of residual red cells Fig. (1).

The degree of osmotic hemolysis was expressed as "% residual red cells", i.e. the percentage of residual red cells divided by the number of red cells initially present, and was calculated based on the following formula:

$$\%\ \text{Residual red cells} = \frac{\text{Mean event count of last two regions (R7 and R8)}}{\text{Event count of first region (R1)}} \times 1.1/2 \times 100\%$$

1.1/2.0 is the multiplying factor for correction as it is assumed that the first region is

also in a diluted state for comparison with the remaining regions which are diluted by spiking with 0.9mL DW. Increased osmotic fragility is indicated by a low percentage of residual red cells. The FCM-OF was proofed to be valid for screening for red cell membrane disorders **[12]**.

Calculations were done for estimated glomerular filtration rate (eGFR) **[13]** and the percentage blood urea nitrogen reduction ratio (URR %), to assess the intensity of dialysis; inadequate dialysis was defined as a reduction in URR% of less than 65% after a dialysis session **[14]**.

Statistical data:

Data analysis was performed using SPSS version 22 software. Paired-sample and independent-sample two tailed student *t*-tests and Chi-Square were applied where appropriate. Data are expressed in mean, standard deviation and percentage and parametric or non-parametric tests were used for comparison of data. Pearson correlation coefficient was used to explore the relationship between quantitative variables. A *p*-value of less than 0.05 was considered significant.

RESULTS

Baseline demographic characteristics and clinical data of the studied HD patients are presented in (Table 1).

Laboratory data of the studied group are presented in (Table 2).

Correlations between % residual red cells and other clinical and laboratory parameters in pre-and post-HD patients are shown in (Table 3).

Comparison between responder and non-responder to rEPO therapy in pre-HD patients is presented in (Table 4).

No correlation between % of residual red cells and other clinical or laboratory parameters in pre-HD patient was encountered except for a mild significant positive correlation with eGFR (r=0.417, p=0.031) and mild negative correlation with patient age (r=–0.453, p=0.018) in responders as well as mild significant positive correlation with MCHC (r=0.347, p=0.023) in non-responders.

Table (1): Demographic and clinical data of 70 end stage renal disease patients under hemodialysis.

Parameter	ESRD patients
Gender:	
Male: Female	34:36
Age:	
Mean ± SD (yrs)	48.42±14.02
Median (Range)	50 (21-80)
Dialysis duration (month):	
Mean ± SD	31.086±26.49
Median (Range)	144 (6-24)
Pre-HD systolic blood pressure (mmHg):	
Mean ± SD	145.7±18.3
Median (Range)	150 (110-180)
Pre-HD diastolic blood pressure (mmHg):	
Mean ± SD	97.8±11.27
Median (Range)	100 (70-120)
BMI (kg/m^2):	
Mean ± SD	21.5±6.04
Median (Range)	21.1 (11.3-37.8)
Blood urea nitrogen reduction ratio (URR%):	
Mean ± SD	59.74±14.51
Median (Range)	61.33 (16.48-86.43)
<65% URR: No (%)	43 (61.43%)
>65% URR: No (%)	27 (38.57%)
Cause of ESRD:	*No (%)*
Hypertension	35 (50%)
Obstructive nephropathy	11 (15.7%)
Chronic glomerulonephritis	7 (10%)
Diabetic nephropathy	6 (8.6%)
Unknown	11 (15.7%)

BMI : Body Mass Index.
URR : Urea Nitrogen Reduction Ratio.

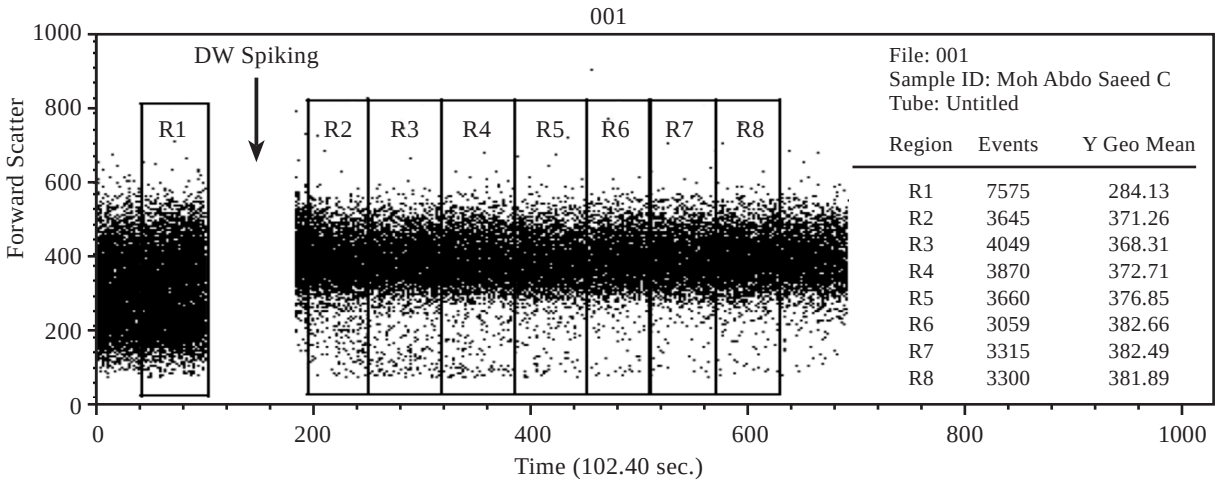

Fig. (1A): The FSC/time plot of red cells from a healthy [% residual red cells 98%].

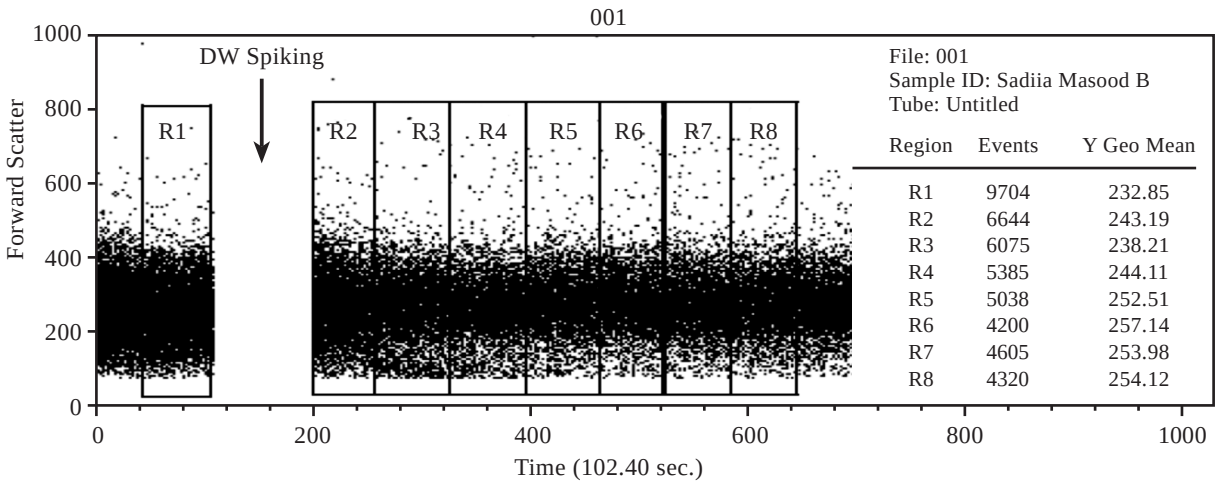

Fig. (1B): The FSC/time plot of red cells [% residual red cells was 83.6% before HD].

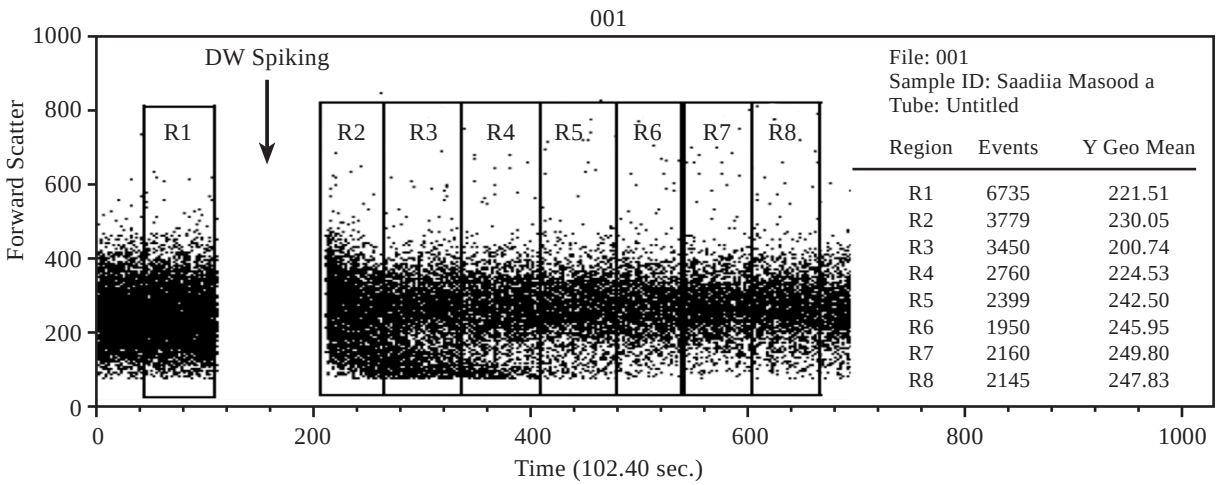

Fig. (1C): The FSC/time plot of red cells [% residual red cells was 58% after HD].

*: DW indicates deionized water.

Fig. (1): Flow cytometry FSC/time plot for % red cell acquisition and analysis form: A) Healthy control; B) A case before hemodialysis; and C) A case after hemodialysis.

Table (2): Comparison of laboratory data between end stage renal patients and controls.

Parameter	ESRD patients		Controls (No=30)	*p*-value
	Pre-HD (No=70)	Post-HD (No=70)		
Urea (mg/dl)	128.3±39.2** 54-254 123.5	51.3±26.3 16-141 48	27.4±7.9 15-46 26	0.0001[a]* 0.0001[b]* 0.0001[c]*
Creatinine (mg/dl)	11.07±3.14 4.74-21.28 10.54	5±2.17 1.88-12.38 4.66	1.06±1.3 0.56-1.8 0.8	0.0001[a]* 0.0001[b]* 0.0001[c]*
EGFR (mL/min/1.73m^2)	4.7±1.7 2-10 4	13.14±5.9 4-32 11	>60	0.0001[a]* 0.0001[b]* 0.0001[c]*
Hb (g/dl)	10.5±2.4 3.9-17.3 10.4	10.7±2.6 3.9-18.7 10.3	13.3±1.3 12-17.7 12.85	0.0001[a]* 0.0001[b]* 0.6831[c]
MCV (fl)	88.39±6.79 68.9-110 87.55	87.47±7.28 68-106 86.2	87.13±6.39 77-102.2 86.8	0.3908[a] 0.8245[b] 0.0371[c]*
MCH (pg)	27.78±2.2 21.3-33.7 27.55	27.88±2.44 21-35.4 27.7	28.2±2.14 27-32.8 28	0.3671[a] 0.5227[b] 0.5176[c]
MCHC (g/dl)	31.42±0.76 29.5-32.9 31.5	31.86±0.84 29.5-33.8 32	32.37±0.68 31.1-34.2 32.3	<.00001[a]* 0.0040[b]* 0.00001[c]*
RDW %	14.7±1.4 12-19.6 14.5	14.8±2.03 12.3-18.8 14.6	14.6±1.15 12.1-17.4 14.6	0.7513[a] 0.5995[b] 0.6853[c]
Platelets count X 10^9/L	219.4±66.7 113-420 203	213.9±71.6 122-493 200	304.6±78.9 165-467 288	0.0001[a]* 0.0001[b]* 0.7773[c]
WBCs X 10^9/L	6.76±2.5 3-15.6 5.85	7.2±3.1 2.4-19.9 6.1	6.0±1.8 3.7-9.4 6.0	0.1437[a] 0.0636[b] 0.3884[c]
Absolute neutrophil count X 10^9/L	4.0±2.082 1.170-1.2324 3.423	4.658±2.914 0.462-1.612 3.640	3.351.7±1.866 3.257-7.392 3.270	0.1447[a] 0.026[b]* 0.1265[c]
Absolute lymphocyte count X 10^9/L	1.790.7±0.453 1.020-2.880 1.761	1.587.7±0.566 0.574-2.700 1.616	1.981.8±0.568.6 0.616-2.960 1.932	0.0697[a] 0.0019[b]* 0.041[c]
Absolute Mid cells count X 10^9/L	0.926±0.488 0.225-2.604 0.792	0.860±0.474 0.270-2.587 0.728	0.557±0.095 0.370-0.801 0.553	0.000014*[a] 0.000662*[b] 0.391532*[c]
Red cell GSH-Px (nmol/min/ml)	37.47±7.59 22-48 37.5	26.833±6.15 15-36 36	270.3±75.79 124-432 233	0.0001[a]* 0.0001[b]* 0.0001[c]*
Red cell GSH-Rx (nmol/min/ml)	21±4.89 14-35 21	14.3±3.96 9-22 15	169.93±43.32 103-298 138	0.0001[a]* 0.0001[b]* 0.0001[c]*
% residual red cells	62.62±28.15 18-120 62.45	64.5±24.5 21-131 64.1	91±24.8 57-133 90	0.0001* 0.0001* 0.6709

ESRD: End Stage Renal Disease.
HD : Hemodialysis.

* : Significant.
**: Mean ± SD, range, median.

a: Pre-HD vs. controls.
b: Post-HD vs. controls.
c: Pre-HD vs. post-HD.

Table (3): Pearson correlation between % residual red cells and other clinical and laboratory parameters in end stage renal disease patients pre-and post-Hemodialysis (HD).

Variable	% residual red cells in ESRD patients			
	Pre-HD		Post-HD	
	r	*p*-value	*r*	*p*-value
Dialysis duration (month)	–0.002	0.986	–0.051	0.675
Systolic blood pressure (mmHg)	–0.167	0.167	–0.074	0.543
Diastolic blood pressure (mmHg)	–0.118	0.331	–0.055	0.651
Urea (mg/dl)	0.002	0.986	–0.157	0.194
S. Creatinine (mg/dl)	0.068	0.575	–0.153	0.663
eGFR (mL/min/1.73m^2)	0.023	0.850	0.139	0.251
Hb (g/dl)	0.216	0.072	0.261	0.261
Hct (%)	0.180	0.135	0.249	0.376
MCHC (g/dl)	0.295*	0.0131*	0.240*	0.045*
Mid cells absolute count X 10^9/L	–0.155	0.200	–0.259*	0.030*
Absolute lymphocyte count X 10^9/L	–0.276*	0.0207*	–0.063	0.604
Platelets count X 10^9/L	–0.152	0.209	–0.097	0.4243
Red cell GSH-Px (nmol/min/ml)	–0.0996	0.4149	–0.2384*	0.0468*
Red cell GSH-Rx (nmol/min/ml)	0.0841	0.4888	–0.1396	0.2511

*: Significant weak correlation.

Table (4): Comparison between responder and non-responder to rEPO therapy in end stage renal disease patients pre-hemodialysis.

Pre-hemodialysis parameter	Responder 27 (38.57%)	Non-responder 43 (61.43%)	*p*-value
Male: Female	16:11	18:25	0.1563
Dialysis duration (month)	32.41±21.35** 7.0-84 (24)	25.84±24.59 6-96 (24)	0.2598
Hb (g/dl)	12.97±1.54 11-17.3 (12.7)	9.06±1.52 3.9-10.9 (9.3)	<0.00001*
MCHC (g/dl)	31.67±0.55 30.7-32.9 (31.8)	31.26±0.82 29.5-32.9 (31.3)	0.0259*
RDW (%)	14.49±1.16 12-16.6 (14.4)	14.77±1.28 12.6-17.5 (14.5)	0.6297
% URR	61.62±12.98 36.2-86.43 (61.38)	58.52±15.45 16.48-77.05 (61.29)	0.3676
Platelets count X 10^9/L	188.42±45.18 138-282 (193)	230.14±75.87 113-420 (219)	0.0362*
WBCs X 10^9/L	5.976±1.387 3.6-10.9 (5.4)	7.26±2.77 3.0-15.6 (6.5)	0.0401*
Absolute neutrophil count X 10^9/L	3.352±1.279 1.200-6.867 (3.29)	4.407±2.373 1.170-12.324 (3.894)	0.0494*
Absolute lymphocyte count X 10^9/L	1.715±0.457 1.080-2.739 (1.744)	1.839±0.449 1.020-2.880 (1.776)	0.1230
Absolute Mid cells count X 10^9/L	0.812±0.407 0.225-2.289 (0.696)	1.015±0.473 0.576-2.604 (0.810)	0.0712
Red cell GSH-Px (nmol/min/ml)	36.70±7.48 22-48 (37)	37.23±7.76 21-47 (37)	0.3904
Red cell GSH-Rx (nmol/min/ml)	21.07±5.05 14-35 (21)	20.88±4.72 14-33 (21)	0.9064
% residual red cells	70.78±30.21 18-112.8 (74.6)	57.51±25.56 18.5-119.9 (49)	0.0337*

Urea nitrogen reduction ratio (URR %). *: Significant. **: Mean ± SD, range, (median).

DISCUSSION

HD is a common therapeutic strategy for patients with ESRD. This procedure promotes a complex biological response when the patient's blood interacts with the artificial HD membranes **[3]**. It is known that the mechanical properties of RBC change with age and mechanical stress in the extracorporeal circuit accelerates the aging process and thus might reduce the lifespan of RBC. This reduced lifespan is treated with erythropoietin which does not only increase red cell production but also increase RBC lifespan **[15]**.

RBCs of patients undergoing HD are more susceptible to oxidative stress. These factors may contribute to the shortened survival of RBC **[16]**. The increased oxidative stress levels in patients undergoing HD may promote the oxidation of surface phosphatidylserine, thereby further contributing to phagocytic removal of RBC **[17]**.

In the present study; patients under HD were more anemic than controls, and non-responder were more anemic than responder patients. Both pre-and post-HD the Hb concentration and hematocrit were significantly decreased compared to controls; this reflects the extent of reduction of the circulating red cell mass. On the other hand, RBCs count, Hb, MCH, MCHC and RDW showed insignificant increase while WBCs count, neutrophils significantly increased in post-HD compared to pre-HD. This is in agreement with a previous study **[18]**. In contrast another study **[19]** showed statistically significant increase in RBCs count, Hb, Hct, RBCs indices in ESRD patients in post-HD compared to pre-HD. This was attributed to the fact that pre-HD patients are usually hyper volemic and the values of RBCs count, Hb levels are lower and the increase in post-HD may be associated with a translocation of RBCs from the splanchnic circulation in order to compensate the hypovolemic stress during dialysis **[20,21]**.

The increase in MCHC determines cytoplasmic viscosity; loss of erythrocyte water in hypertonic media leads to a local increase in RBC cytoplasmic viscosity resulting in increased MCHC and loss of deformability **[8]**.

In the present study; there was no significant difference between pre-and post-HD group compared to control in WBCs count. This is in agreement with a previous study **[19]**.

In the present study; the absolute lymphocyte counts in the post-HD group were significantly lower than control group with further significant decrease in post-HD compared to pre-HD. This is in agreement with one study **[23]** and in contrast to another **[19]** which found significant increase in WBCs and lymphocyte counts in post-HD when compared to the pre-HD count.

In the present study; the absolute neutrophil counts and mid cell count (mainly monocytes) were significantly higher in post-HD patients than healthy control but with insignificant difference between pre-HD and post-HD. Furthermore, non responders showed significant higher WBC (mainly neutrophils) counts and platelets (as an inflammatory marker) compared to responders. This is in agreement with previous studies **[19,23]**. These data further strengthen previous findings which indicated that HD procedure activates neutrophils and monocytes; although we cannot exclude enhanced inflammatory process particularly in patients not responding to rEPO therapy **[10,24]**.

The present study revealed significant decrease in platelet counts, though still within the normal range, in the pre-and post-HD groups when compared to control group, with insignificant difference between pre-HD and post-HD.

In contrast, significant decrease in platelet count post-HD compared to pre-HD was reported by several studies **[19,25,26]**; this was attributed to platelet's activation and degranulation caused by the use of polysulfone membranes. This finding is in contrast to Yu et al., study **[18]** that reported mild increase in platelet counts after HD.

In post-HD; the mean concentrations of red cell GSH-Px and GSH-Rx were significantly decreased in post-HD compared to pre-HD and control group which could be related to the loss of antioxidant enzymes through the membranes during HD; this is in agreement with previous studies **[27,28]**.

In the present study RBCs FCM-OF analysis established that the percentage of residual red cells from pre-and post-HD group was significantly lower than healthy control but with no significant difference between pre-and post-HD

group. This means that red cell fragility was significantly increased in HD patients but not aggravated by the HD procedure (i.e. no acute hemolysis). However, gradual deterioration of RBCs due to mechanical stress following extracorporeal blood circulation in the dialysis machine cannot be excluded. This is in agreement with previous studies showing that the mechanical properties of RBC were markedly altered in HD patients compared to controls **[29-32]**. The effect of HD may be executed through reduction in spectrin, which is normally associated with a reduction in RBC deformability; reduction in spectrin, is higher in patient's not responding to Epo therapy **[4]**.

Candan et al., **[33]** reported increased red cell osmotic fragility in HD patients due to peroxidation of membrane lipid and increase in Malondialdehyde (MDA) and they found that supplementation with zinc and vitamin C decreases osmotic fragility and MDA in zinc-deficient HD patients. Furthermore, Vlassopoulos et al., **[34]** reported that low RBC osmotic resistance (RBCOR) in HD patients aggravates anemia and raises EPO needs. This is related to the type of dialysis membranes used and L-carnitine supplementation normalizes osmotic resistance in some patients.

In the present study, non-responders to rEPO therapy showed significantly lower Hb, red cell GSH-Px, GSH-Rx activity and % of residual red cells and significantly higher platelets and absolute neutrophil counts compared to responders. This may be attributed to the enhanced inflammatory process observed in non-responder patients and it was explained by pro-inflammatory cytokine's generation from activated T-cells which then promote apoptosis in erythroid progenitor cells in the bone marrow **[35]**.

Gallucci et al., **[36]** concluded that increased oxidative damage of RBC membrane detectable in HD patients is considered a factor of resistance to rEPO.

Mihaljević et al., **[33]** suggested that the administration of rEPO does not affect Red Blood Cell Osmotic Resistance (RBCOR) in HD patients, that RBCOR is not always reduced in those population and that it correlates with a small number of laboratory parameters (serum calcium and hydrogen ions characteristic for the uremic syndrome).

Finally, to our knowledge no previous study investigated red cell fragility in ESRD by flow cytometry; this study was the first to demonstrate a positive correlation between RBC oxidative damage and red cell fragility.

In conclusion, our results showed that HD aggravates oxidative damage. Results of fragility test showed that the red cell fragility is increased in ESRD as detected in pre-HD patients and it is not intensified immediately after dialysis procedure (i.e. no acute hemolysis). However, gradual deterioration of RBCs due to mechanical stress cannot be excluded following extracorporeal blood circulation in the dialysis machine.

We therefore suggest that antioxidant supplementation is capable of recovering antioxidant defense in red cells and plasma thus preventing oxidative damage induced by hemodialysis.

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