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Impact of Hepatitis C Virus Infection on Neutrophil Oxidative Burst Function in Hemodialysis Patients

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Polymorphonuclear leukocyte (PMN) functions have been studied extensively in hemodialysis (HD) patients; however, results are contradictory and the mechanisms that modulate phagocytosis and oxidative burst are not completely understood. Hepatitis C virus (HCV) is a frequent complication of HD that may be associated with disturbed PMN function; however, the impact of HCV infection on neutrophil oxidative burst function in HD patients is unknown. We investigated Neutrophil oxidative burst function in 24 HD patients (15 HCV-positive and, 9 HCV-negative patients) before and after dialysis. HCV-RNA was detected by RT-nested PCR while, quantitative measurement of oxidative burst function was assessed by flowcytometry. Neutrophil Oxidised burst function was significantly diminished in HD patients as comapred to controls (*P*=0.001, oxidised PMN (%); *P*=0.02 mean flueresnce intensity, MFI), and in pre-dialysis as compared to post-dialysis samples (oxidised PMNs (%): 60.5 ± 3.2 *vs.* 72.1 ± 3.9, *P*=0.02); (MFI: 352 ± 42 *vs.* 500 ± 50, *P*=0.03). Alteration in Neutrophil oxidative burst function in the pre-dialysis samples was significant in HCV-positive patients as compared to HCV-negative patients (oxidized PMNs (%): 50 ± 2.9 *vs.* 63 ± 5.1, *P*= 0.02); (MFI: 291 ± 31 *vs.* 438 ± 64, *P*=0.006). Marked reduction in *E. coli* induced burst in pre-dialysis samples compared to post-dialysis was found in HCV-positive when compared to HCV-negative patients (oxidized PMNs (%): 50 ± 2.9 *vs.* 74.8 ± 4.7, *P*=0.001), (MFI: 291 ± 31 *vs.* 493 ± 63, *P*=0.002). In conclusion, a possible role of concomitant HCV infection in alteration of Neutrophil oxidative burst function is highly suggested.

Polymorphonuclear leukocytes (PMN) are the first line of defense against foreign invaders and the major cell type involved in most acute and chronic inflammatory diseases (Witko-Sarsat *et al.,* 2000). The high frequency of bacterial infections in end stage renal disease patients suggests that PMN dysfunction may be involved in the immune deficiency observed in this population (Sarnak & Jaber., 2000).

Neutrophil phagocytic functions have been studied extensively in hemodialysis (HD) patients; however, results are contradictory and the mechanisms that modulate phagocytosis and oxidative burst during dialysis are not completely understood. Some reports suggested that both uremia and dialysis modality may interfere with PMN function (Sardenberg *et al.,* 2006). Incubation of PMN with uremic plasma or with different hemodialysis membranes increased their apoptosis. In addition, PMN harvested from uremic patients showed a reduced ability to generate superoxide in response to stimuli as well as impaired phagocytosis, chemotaxis and degranulation (Cendoroglo *et al.,* 1999). However, according to others chronic renal insufficiency does not impair PMN phagocytosis or oxidative burst. To the contrary, it enhances receptor-mediated oxidative burst (Ward & McLeish, 1995).

A high prevalence of HCV infections in patients undergoing HD has been described worldwide. The Egyptian population is a unique and rich population to study HCV infection due to the significant high prevalence of HCV infection, the poorly defined epidemiology, the co-infection with other blood-born viruses (HBV, GBV-C and

TTV) and, the dominance of genotype 4 (Hassoba *et al.,* 1997; Hassoba *et al.,* 2003)

Assessment of PMN oxidative burst function in HCV infected patients revealed a significantly higher H_2O_2 production by PMA-stimulated cells in HCV-infected patients compared to controls (Toro *et al.,* 1998). In addition, a positive correlation between H_2O_2 production and serum ALT and AST was found in the HCV-infected patients (Toro *et al.,* 1998). On the other hand, others reported a negative correlation between neutrophils oxidative burst and liver enzymes (ALT and AST) in liver cirrhosis (Panasiuk, *et al.,* 2005). These results suggested that a functional alteration in PMN cells is manifested in this chronic viral infection (Toro *et al.,* 1998).

Since concomitant blood born viral infections might be one of the fundamental requisites to remedy the problems related to impaired neutrophil functions in HD, the present study was designed to evaluate the potential influence of HCV infection on the oxidative burst function of PMNs in HD patients.

Patients and Methods

Patients

The study was approved by the Institutional Review Board (IRB) of Suez Canal University. The study included 24 hemodialysis patients (HCV-positive, n=15, HCV-negative, n=9). Ten blood donors served as a normal control. The hemodialysis patients were dialyzed for 4 hours thrice weekly with hemophane membrane (a non-complement activating dialyzer). Patients with diabetes, acute infections, blood transfusion in the past month, hepatitis B infection, active immunological disease (systemic lupus erythematosus, rheumatoid arthritis), immuno-suppressive therapy, previous transplantation, or a history of malignancy were excluded from the study.

Methods

1- The dialysis Session

Standardized dialysis session was carried out with the same vogue for all patients. Blood samples were obtained directly from the arteriovenous fistula just prior to the start and at the end of a 4-hr mid-week dialysis session.

2- Clinical and Basic Laboratory Data

Baseline demographic and clinical data were recruited from patients' hospital records. These included; age, gender, cause of renal failure, history of schistosomiasis and duration of dialysis therapy. Basic laboratory data included hemoglobin levels; total and differential white cell and counts, serum levels of ALT, AST, albumin, total protein, and prothrombin time.

3- Diagnosis of HCV infection

HCV-RNA was extracted using QIAamp HCV kit (Qiagen ltd, Stanford, CA, USA) and, amplified by RT-nested PCR using primers specific to the highly conserved 5' noncoding region region as previously described (Cristiano *et al.,* 1991).

4- The Oxidative Burst Test

Quantitative assessment of granulocyte oxidative burst was carried out using Bursttest Kit (ORPEGEN Pharma, Heidelberg, Germany). The test allows the quantitative determination of leukocyte oxidative burst in heparinized whole blood. It contains unlabeled opsonized bacteria (*E. coli*), phorbol 12-myristate 13-acetate (PMA) and the chemotactic peptide N-formyl-Met-Leu-Phe (fMLP) as stimulants, dihydrorhodamine (DHR) 123 as a fluorogenic substrate and necessary reagents. It determines the percentage of phagocytic cells which produce reactive oxidants (conversion of DHR 123 to R 123) and their enzymatic activity (amount of R 123 per cell). The analyses were performed within 4 h of sampling. One hundred microliter of heparinized whole blood was mixed and pipetted into the bottom of four 5 mL tubes. Samples were incubated on ice for 10 minutes in order to cool down to 0^{o}C before adding the bacteria. To assess activation, the four tubes (each contained 100 µl heparinized whole blood) were prepared. In the 1^{st} tube, 20 µL of pre cooled and well mixed opsonized (non-labeled) *E. coli* suspension (1 x 10^9 bacteria/mL) were added. In the 2^{nd} tube, 20 µl of the washing solution were added as a "negative control". In the 3^{rd} tube, 20 µL of the fMLP (N-formyl-Met-Leu-Phe) was added as chemotactic low physiological stimulus "low control" while, in the 4^{th} tube, 20 µL of the PMA (phorbol 12-myristate 13-acetate), as a strong activator (high control) was added. All samples were incubated for 10 min at 37°C water bath. Oxidation was performed by adding 20 µL of a fluorogenic substrate solution (dihydrorhodamine (DHR) 123) and vortexing thoroughly followed by incubation for 10 min at 37.0°C water bath. Precisely at the end of the incubation time all samples simultaneously were taken out of the water bath. Erythrocytes were removed using pre-warmed (20-25°C) 1x lysing solution for 20 min. Solution was spinned down (250 g, 5 min, 2-8°C), and supernatant was discarded leaving approximately 100 µL. Samples were washed with 3 mL of washing solution, centrifuged (250 g, 5 minutes, 2-8°C) and the supernatant was decanted leaving approximately 100 µL. To discriminate and exclude aggregation artifacts of bacteria and/or cells in cytometric flow analysis; 200 µL DNA staining solution was added, vortexed and incubated for 10 min on ice in the dark and, cell suspensions were measured within 30 min.

5- Flowcytometric Analysis and Data Evaluation

In stimulated granulocytes, nonfluorescent DHR 123 underwent conversion to fluorescent rhodamine (R) 123 registered in the flowcytometer. 10,000- 15,000 leukocytes per sample were analyzed and, were distinguished from bacteria by their DNA staining. The percentage of cells having produced reactive oxygen metabolites (recruitment) were analyzed as well as their mean fluorescence intensity (amount of cleaved substrate). Cells were analyzed using fluorescence automated cell sorter (FACS Calibur) with CellQuest software (Becton Dickinson, San Jose, CA).

Statistical Analysis

This was performed using the software SSPS 11.0 for Windows. Values were expressed as mean ± SEM, and percentages as appropriate. Continuous variables were compared using Independent sample T test, while paired samples were compared using paired sample T-test with a 95% confidence interval. Kruskal-Wallis test was used to compare the three groups (pre-dialysis, post-dialysis and control). *P* values of 0.05 or less were regarded as significant.

Results

Demographic and laboratory characteristics of HD patients are demonstrated in table (1).

Table 1. Demographic and Laboratory Characteristics of the Hemodialysis Patients.

Variables	Values
Age (yrs) $	43 ± 15
Gender (Male: Female)	13: 11
Duration on dialysis (months)$	36 ± 2.4
Positive history of schistosomiasis@	9 (37.5%)
Neutrophil count (×1000/mm^3)$ pre-dialysis	4.1 ± 1.5
Neutrophil count (×1000/mm^3)$ post-dialysis	4.0 ± 0.9
Prothrombin time (seconds)$	12.7 ± 0.6
HB level (gm/dl) $	10.5 ± 2
ALT (U/L) $	49 ± 25
AST (U/L) $	42 ± 30
T. protein (gm/dL) $	6.9 ± 0.5
Albumin (gm/dL) $	3.7 ± 0.3
HCV RNA (positive) @	15 (62.5%)
HCV duration (yrs) $	11 ± 2

$= mean ± SEM; @= percentages of the total population.

Oxidative Burst Function in HD vs. Normal Control

Oxidative burst function was compared between HD patients (befor and after dialysis) and normal control. Percentage of oxidised neutrophils as well as MFI after *E. coli* stimulation were significantly different among the three tested samples (oxidised PMNs (%): 60.5 ± 3.2 *vs.* 72.1 ± 3.9 *vs.* 75.7 ± 1.3, *P*=0.001), (MFI: 352 ± 42 *vs.* 500 ± 50 *vs.* 450 ± 42, *P*=0.02) for pre-dialysis, post-dialysis and control group respectively. Percentage of PMA-induced oxidized neutrophils as well as MFI were not statistically different among the three groups (Table 2).

Effect of Dialysis on Neutrophils Oxidative Burst Function

Oxidative burst functions were compared before and after dialysis in the total HD population as well as in HCV-positive and HCV-negative subgroups. Incubation of neutrophils with *E. coli* induced significant decrease in oxidative burst in pre-dialysis samples compared to post-dialysis (oxidized PMNs (%): 60.5 ± 3.2 *vs.* 72.1 ± 3.9, *P*=0.02). In addition, neutrophils with oxidative burst (MFI of rhodamine 123) were statistically lower in pre-dialysis samples compared to post-dialysis samples (MFI: 352 ± 42 *vs.* 500 ± 50, *P*=0.03). *In vitro* stimulation with the strong activator PMA causes production of reactive oxidants in neutrophils that was comparable in both pre-dialysis and post-dialysis samples (Table 2).

Table 2. Neutrophil Oxidative Burst Activity in Hemodialysis Patients (Pre- and Post-Dialysis).

Oxidative burst function	Pre-dialysis N=24	Post-dialysis N=24	Control N=10	*P**	$P^{\$}$
After PMA stimulation:					
Oxidized neutrophils (%)	87.5 ± 3.2	86.1 ± 3.8	92.5 ± 2.2	NS	NS
Mean Fluorescence Intensity (MFI)	829 ± 123	678 ± 76	823 ± 52	NS	NS
After E. coli stimulation:					
Oxidized neutrophils (%)	60.5 ± 3.2	72.1 ± 3.9	75.7 ± 1.3	0.001	0.02
Mean Fluorescence Intensity (MFI)	352 ± 42	500 ± 50	450 ± 42	0.02	0.03
After fMLP stimulation:					
Oxidized neutrophils (%)	3 ± 0.3	3 ± 0.3	3.7 ± 0.2	NS	NS

Data are presented as mean ± SEM, NS= not significant

PMA= phorbol 12-myristate 13-acetate; fMLP= N-formyl-Met-Leu-Phe

*P**= comparison between the 3 groups (Kruskal-Wallis H test)

$P^{\$}$= pre-dialysis samples vs. post-dialysis (Independent sample T-test)

Effect of HCV Infection on Neutrophils Oxidative Burst Function

Before dialysis, a significant reduction in oxidative burst function after *E. coli* stimulation was observed in HCV-positive patients compared to HCV-negative (oxidized PMNs (%): 50 ± 2.9 *vs.* 63 ± 5.1, *P*= 0.02), (MFI: 291 ± 31 *vs.* 438 ± 64, *P*=0.006). However, this difference was not observed in the post-dialysis samples. In the mean time, a significant reduction in *E. coli* induced burst activity in pre-dialysis samples compared to post-dialysis was observed in HCV-positive compared to HCV-negative patients (oxidized PMNs (%): 50 ± 2.9 *vs.* 74.8 ± 4.7, *P*=0.001), (MFI: 291 ± 31 *vs.* 493 ± 63, *P*=0.002). Oxidative burst induced by either PMA or fMLP stimulation did not differ between HCV-positive and HCV-negative patients. In addition, no correlation was found between MFI of neutrophils oxidative burst after PMA or *E. coli* stimulation and liver function tests (ALT, AST, total protein, albumin, or prothrombin time) (Table 3).

Table 3. Neutrophil Oxidative Burst Activity in Hemodialysis Patients in Relation to HCV Infection

Variables	HCV-positive N=15	HCV-negative N=9	$P^{\$}$
After PMA stimulation:			
Oxidized neutrophils (%)			
Pre-dialysis	87.5 ± 3.9	87.6 ± 5.8	NS
Post-dialysis	88 ± 4.5	83.1 ± 7.1	NS
$P^{\#}$	NS	NS	
Mean Fluorescence Intensity			
Pre-dialysis	834 ± 154	824 ± 217	NS
Post-dialysis	683 ± 93	671 ± 137	NS
$P^{\#}$	NS	NS	
After *E. coli* stimulation:			
Oxidized neutrophils (%)			
Pre-dialysis	50.3 ± 2.9	63.3 ± 5.1	0.02
Post-dialysis	74.8 ± 4.7	68 ± 7.1	NS
$P^{\#}$	0.001	NS	
Mean Fluorescence Intensity			
Pre-dialysis	291 ± 31	438 ± 64	0.006
Post-dialysis	493 ± 63	512 ± 90	NS
$P^{\#}$	0.002	NS	
After fMLP stimulation:			
Oxidized neutrophils (%)			
Pre-dialysis	2.8 ± 0.3	3.3 ± 0.5	NS
Post-dialysis	2.8 ± 0.4	3.5 ± 0.5	NS
$P^{\#}$	NS	NS	

Data are presented as mean ± SEM. NS= not significant
PMA= phorbol 12-myristate 13-acetate; fMLP= N-formyl-Met-Leu-Phe
$P^{\$}$*= HCV positive vs. HCV negative (Independent sample T test).*
$P^{\#}$= pre-dialysis samples *vs.* post-dialysis (*paired sample T test)*)

Discussion

PMNs play a crucial role in host defense against microbial infections. Substantial clinical evidence about profound disturbances in PMN functions in HD patients was reported (Allon *et al.,* 2003). HCV infection is a common infection in HD settings; therefore, its potential influence on the activity of PMN granulocytes from dialysis patients was investigated in this study.

Studies investigating phagocytosis or the production of reactive oxygen species (ROS) in HD patients have produced conflicting results. Decreased, normal, or increased levels of ROS production and phagocytosis have been reported. These discrepancies were attributed to the delicate nature of neutrophils functions measurements, since personal constants and day-to-day variations may lead to large fluctuations in the data. In addition, the different methods used to isolate and measure PMNs function, the ratio of stimulating agents to cells, the type of stimuli, as well as incubation times influence measured neutrophils parameters (Bassoe *et al.,* 2000). This renders a comparison among published results difficult and may explain contradicting results. Importantly, in the present study, PMN function was assessed by flowcytometry using whole blood, and not from isolated and incubated cells.

A common finding in our study is that direct *in vitro* neutrophils stimulation with *E. coli* was more effective than stimulation by PMA. Percentage of oxidised neutrophils after *E. coli* stimulation was significantly lower in both pre-dialysis, post-dialysis samples compared to control. However, MFI after *E. coli* stimulation was not different among the three groups. In the mean time, percentage of PMA-induced oxidized neutrophils as well as MFI were not statistically different among the three groups. However, according to others; respiratory burst activity was diminished in PMA-stimulated PMNs compared to healthy controls and hydrogen peroxide production was normalized by HD (Hirabayashi *et al.,* 1988; Haag-Weber & Schollmeyer, 1989)

Previous studies on the effect of uremia on neutrophils phagocytosis reported discrepant results. A study from 1995 stated that chronic renal insufficiency does not impair PMN phagocytosis and oxidative burst. To the contrary, it enhances receptor-mediated oxidative burst (Ward & McLeish, 1995). This is inconsistent with earlier results that reported a reduced phagocytic activity in uremic patients (Vanholder *et al.,* 1991; Vanholder *et al.,* 1994). Recent studies suggested that neutrophils killing capability is reduced in HD patients, while the number of neutrophils that show phagocytic activity and produces ROS remains unchanged (Anding *et al.,* 2003). The mechanisms responsible for altered phagocytic activity of uremic neutrophils are unclear and had been related to malnutrition, iron overload, uremic toxins, elevated intracellular calcium, zinc deficiency, diabetes, duration of dialysis or, membrane biocompatibility (Waterlot *et al.,* 1985; Vanholder *et al.,* 1993; Cohen *et al.,* 1997; Banche *et al.,* 2006).

A significant increase in the number of oxidized cells as well as MFI after stimulation by *E. coli* was observed in the post-dialysis samples compared to pre-dialysis as previously reported (Gastaldello *et al.,* 2000). It is possible that uremic toxins which accumulate in the serum pre-dialysis and which are cleared by dialysis are responsible for this effect. Accordingly, a previous study suggested that uremic toxins played an essential role in PMN apoptosis and that dialysis might 'correct' or normalize apoptosis rates by clearing these toxins (Sardenberg *et al.,* 2006). In agreement with the role of uremia, PMN from chronic kidney disease patients showed increased apoptosis rates, whereas HD patients displayed PMN apoptosis rates similar to controls (Sardenberg *et al.,* 2006). Another explanation was that cytokines and, other soluble uremic toxins might not be eliminated completely by HD. This caused functional disturbances that increased the risk of infection by interfering with essential functions of the non-specific immune response (such as chemotaxis, phagocytosis, and oxidative metabolism) (Cohen *et al.,* 1998). A Recent *in vivo* study showed that uremic neutrophils with low intracellular pH (pHi) values had enhanced phagocytosis and increased oxidative burst reactions in comparison to uremic neutrophils having normal or high pHi values. In addition, *in vitro* studies on uremic neutrophils incubated in the acidic pH range demonstrated that pHi inversely correlated with phagocytosis and oxidative burst reactions (Wann *et al.,* 2007).

Role of PMNs dysfunction in liver disease had been the focus of many previous studies. Functional alteration in PMN cells was manifested in HCV infection, which may represent an additional factor in the development of liver lesions (Toro *et al.,* 1998). In the present study, *E. coli* induced oxidative burst function was significantly depressed in HCV-positive patients before dialysis however, this difference was not found in post-dialysis samples. In accordance with our result, the oxidative burst in *E. coli* phagocytizing neutrophils generated fewer amounts of active oxygen compounds in liver cirrhosis patients (Panasiuk *et al.,* 2005). According to our study, *in vitro* stimulation with the strong stimulant PMA did not differ between HCV-positive and HCV-negative patients. In contrast, PMA-stimulated neutrophils produced less reactive oxidants in liver cirrhosis patients than in healthy subjects (Panasiuk *et al.,* 2005). In our patients, severity of liver disease was not assessed, and the duration of HCV infection was around 10 years, which is a relatively short period to develop cirrhosis and advanced liver disease. Future studies on larger population with liver biopsies are needed.

We did not find any correlation between oxidative burst function and liver functions, neutrophils count, and HB levels. Previous reports showed conflicting results; a negative correlation was observed between oxidative burst MFI of PMA-stimulated neutrophils and ALT and AST levels in cirrhotic patients (Panasiuk *et al.,* 2005). In contrast, an earlier study reported a positive correlation between H_2O_2 production by PMA-stimulated cells and serum levels of ALT and AST in the HCV-infected patients (Toro *et al.,* 1998).

The role of the membrane cannot be addressed from this study since all our HD patients used the same membrane. However, according to one study, the enhanced H_2O_2 production observed during HD was unrelated to membrane type and, might be related to platelet activation factor (PAF) production (Gastaldello *et al.,* 2000).

The current study had a few limitations. First, the groups were not age-and sex-matched. Interestingly, Rao and coworkers (2004) found no correlations between PMN function and age and gender. Second, study population was relatively small hence; future studies on larger population are needed. Third, all patients used the same type of dialysis membrane, thus assessment of its role cannot be concluded from this study. In conclusion, our study supports the previous literatures that multiple factors are involved in the altered PMNs oxidative burst function in HD patients namely, uremic toxins, and dialysis procedure. However, we add that a possible role of concomitant HCV infection in this alteration is highly suggested.

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