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Blood Elements Activation in Hemodialysis – Animal Model Studies

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***Abstract*—**Haemodialysis (HD) is a procedure saving patient lives around the world, unfortunately it brings numerous complications. Oxidative stress is one of the major factors which lead to erythrocytes destruction during extracorporeal circulation. Repeated HD procedures destroy blood elements and the organism is not able to keep up with their production. 30 HD procedures on healthy sheep were performed to evaluate effects of such treatment. Oxidative stress study was performed together with an analysis of basic blood parameters and empirical assessment of dialyzer condition after the procedure. A reversible decline in absolute leukocyte count, during first 30 min of HD, was observed. Blood clots were formed in the area of the blood inlet and outlet of the dialyzer. Our results are consistent with outcomes presented throughout the literature specifically with respect to the effects observed in humans and will provide a basis to evaluate methods for blood protection during haemodialysis.

***Keywords*—**Animal model, blood components, haemodialysis, leukocytes, oxidative stress, sheep.

I. Introduction

HAEMODIALYSIS (HD) is a procedure that uses the extracorporeal circulation to remove waste products from blood, especially small and medium-sized molecules. This technique involves the use of semi-permeable membranes through which takes place the exchange of substances between blood and a specially prepared dialysis fluid. The treatment is performed periodically until the restoration of kidney function, until the kidney transplantation or even up to the end of patient's life. Treatments involving devices that have direct contact with the organism often associate different types of complications. This is a major problem in procedures using artificial organs [1], [2].

Blood of a haemodialysis patient is in constant contact with the material, which causes the activation of plates, leukocytes, mainly neutrophils, which release reactive oxygen species (ROS) leading to the destruction of the cellular components of blood [3], [4]. Oxidative stress is a major factor in red cell damage during extracorporeal circulation. A short interval between treatments causes inability of the body to regenerate and lagging production of new cells [5], [6].

Currently, there are many studies aimed at protecting the blood during such procedures. There are created new, more biocompatible materials, new assisting drugs. Surgical techniques with the use of artificial organs are still being developed and improved and therefore there is a need to search for new solutions that are tested in animal models. In this type of research it is very important to choose the appropriate test conditions in order to meet the expectations of modeling necessary organism functions.

In this work we have tested a suitable animal model and investigated the influence of extracorporeal circulation on the blood. We have modeled haemodialysis and studied a healthy animal response to irritants which are disposable parts of the artificial kidney in contact with the patient's blood, especially the effect of haemodialysis on blood cell counts in sheep model and compared it with literature reports regarding humans.

II. Procedure - Materials and Methods

A. Animals

Experiments were conducted on three adult, clinically healthy, male Merino sheep weighing 54-62 kg. A total of 10 HD treatments were performed on each animal. Permanent vascular access was obtained by percutaneous placement of a 14.5 French 28cm long dual-lumen haemodialysis catheter into the right jugular vein (vena jugularis) (Fig. 1). Sheep were premedicated (Xylazine 0.2mg/kg body weight iv) and with local infiltration anesthesia (2% lidocaine).



Fig. 1 Permanent vascular access

B. Haemodialysis Procedure

Experiments were performed using a Fresenius 4008B haemodialysis device on sheep standing in boxes without pharmacological sedation (Fig. 2). Four hour HD sessions

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were performed three times a week with the use of polysulphone dialyzer Fresenius F4HPS and standard blood line (AV-Set, Fresenius MC, USA). Before the procedure, 5000 IU of sodium heparin (WZF Polfa SA, Poland) dissolved in 500mL Ringer's solution (B. Braun Melsungen AG, Germany) was administered as intravenous infusion. During the experiment heparin was introduced into the blood line at a constant rate determined in preliminary studies. 36L of blood was processed within 4 hours of HD (blood pump speed: 150mL/min), total ultrafiltration was set at 200mL. Dialysate flow rate (composition: Na^+ 142.0, K^+ 3.0, Mg^{2+} 0.5, Ca^{2+} 1.5, Cl^- 110.0, HCO^{3-} 32.0mmol/L, glucose 1.0g/L) was 500 mL/min.



(a) (b)

Fig. 2 (a) Sheep standing in boxes (b) Artificial Kidney

C. Blood Collection for Research

During each HD blood samples were collected into 7 EDTA tubes and 5 citrate tubes. The first blood sample was collected before the start of haemodialysis session. It was important in order to check the general condition of patients. Two further samples were collected in the fifth minute after the start of treatment from venous and arterial lines of artificial kidney. Fourth and fifth respectively, were taken 15 and 30 minutes after the start of procedure from arterial line. The last two samples were collected just before the end of the circulation from the same points as the second and third sample. Placement and time of collection of the samples have been carefully selected on the basis of literature reports and preliminary studies, according to which the greatest changes in the blood are observed during the first 30 minutes of circulation.

D. Performed Assays

1. Morphology

Several assays were performed in order to investigate the behavior of white blood cell (WBC) count, examine their activation and observe oxidative stress that occurs during haemodialysis. Red blood cell (RBC) count, haematocrit (Ht), haemoglobin concentration (Hb), haematological indices (mean cell volume, mean cell haemoglobin and mean cell haemoglobin concentration) and total leukocyte count were performed using an automatic blood cell counter (scil Vet abc, Horiba Ltd., Kyoto, Japan). Differential WBC count was determined by examining blood smears stained with Hemacolor® staining kit. Blood smears were examined by evaluator blinded to the experimental conditions.

2. Oxidative Stress

Lipid peroxidation is a widely used oxidative stress indicator of cell membranes. This method is based on malondialdehyde (MDA) production during the oxidation of polyunsaturated fatty acids. The reaction between MDA and thiobarbituric acid (TBA) yields a reddish color, which peaks at 535 nm.

Briefly, equal amounts of blood, 0.37% trichlorobarbituric acid (TBA) in 0.25 M HCl (w/v) and 15 % trichloroacetic acid (TCA) in 0.25 M HCl (w/v) (reagents from Chempur, Poland) were mixed and heated at 100ºC for 15min. Reaction was stopped by transferring tubes on ice. After centrifugation of the reaction mixture at 2500 · g for 20min, absorbance of the colored supernatant was measured at 535nm and corrected for unspecific turbidity by subtracting the value of the control sample which comprised of blood sample, TCA and distilled water instead of TBA. The peroxidation of blood lipids was expressed as micromoles of thiobarbituric acid-reactive substances (TBARs) per g of haemoglobin using extinction coefficient of $155mM^{-1}cm^{-1}$ [7].

E. Statistics

Morphology results were calculated as mean and standard deviation with Student's t-distribution at 95% confidence level ($p<0.05$)

Single WBC count measurement uncertainty was estimated according to declared device measurement error (±3% or $\pm 0.2 \cdot 10^3$ cells per micro liter, whichever is greater)

Statistical analyses were performed using STATISTICA rel.8 (Statsoft 2008). Nonparametric paired Wilcoxon test was used to compare the TBARs concentration in arterial and venous lines. Friedman ANOVA test and Kendall's coefficient of concordance were used to compare TBARs changes during haemodialysis. Differences with a *P* value less than 0.05 were considered statistically significant.

III. Results

No significant differences in RBC, Ht, Hb and haematological indices during consecutive experiments were observed. There were also no significant intradialytic changes in RBC count and morphology.

A. The Total Number of Leukocytes WBC:

Total WBC count and differential WBC count changed during each haemodialysis session in a specific manner.

In the first 15 minutes of HD the total number of leukocytes decreases (average 20%), and after 30 minutes, begins to return to normal level. After 4 hours, of the procedure an increase in the number of leukocytes is observed. In samples

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taken on input and output of the dialyzer after dialysis no significant changes were noticed (Fig. 3)

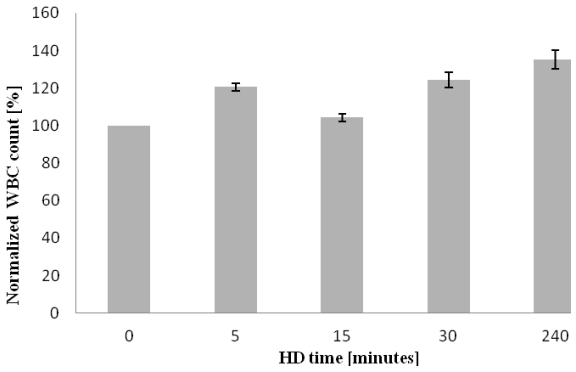


Fig. 3 Normalized mean WBC count with respect to sample collected at the beginning of haemodialysis (0 minutes) acquired during 4 hours HD

Additional studies were carried out to exactly determine at what time does the effect, seen clearly in the 15th minute, start and how long does it last. The results are shown in the graph (Fig. 4). It was observed that the decrease in the number of leukocytes starts after five minutes and lasts for 15 minutes, then begins to occur increase of WBC count and stabilization on the initial level in the 30 minute.

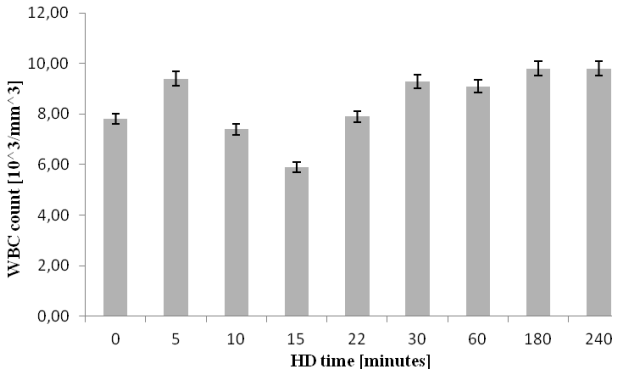


Fig. 4 Changes in WBC count measured during 4 hours of HD

B. Blood Film

Differential WBC counts also changed during HD. In the first 15 minutes of the extracorporeal circulation statistically significant ($p \leq 0.05$) decrease in the total number of neutrophils was observed in all animals. After 30 minutes neutrophil count returned to the level from the beginning of the extracorporeal circulation. Four hours of HD resulted in a significant increase in neutrophil count ($p \leq 0.05$). There were no significant differences in the neutrophil count between venous and arterial lines at the beginning and after 4 hours of HD (Fig. 5).

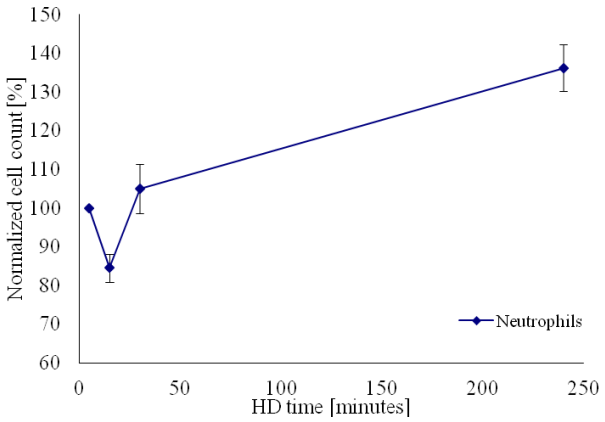


Fig. 5 Normalized mean neutrophils count with respect to sample collected at 5th minute of haemodialysis acquired during 4 hours HD

Lymphocyte count did not change significantly during the first 30 minutes of HD. Significant increase in lymphocyte count was observed after 4 hours of HD (Fig. 6). There were no significant changes in eosinophil and monocyte counts during the experiments.

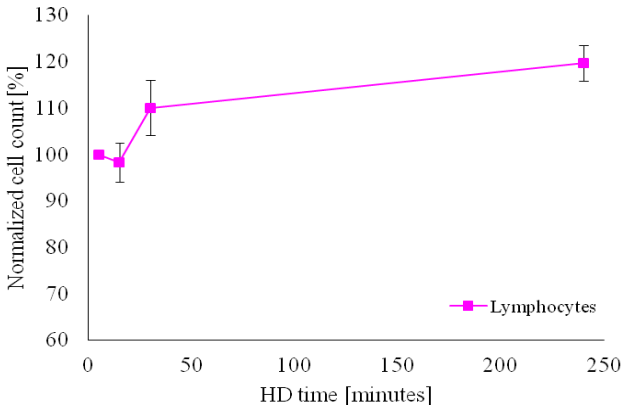


Fig. 6 Normalized mean lymphocytes count with respect to sample collected at 5th minute of haemodialysis acquired during 4 hours HD

C. Lipid Peroxidation

Haemodialysis caused the production of TBARs. The highest level of TBARs was observed in venous line after 5 minutes of HD (Fig. 7). Oxidative stress measured after 4 hours of HD in the arterial and venous lines shows no statistically significant changes (Fig. 8). The effects of haemodialysis in arterial line on the amount of substances reacting with thiobarbituric acid are presented in Fig. 9.

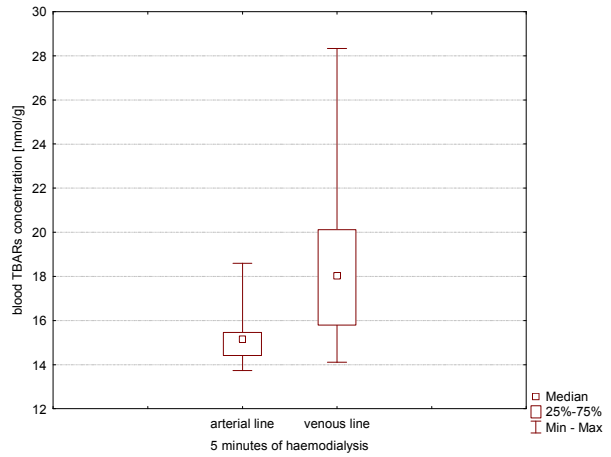


Fig. 7 Oxidative stress level after 5 minutes of haemodialysis expressed as blood TBARs concentration in arterial and venous line. Results are significantly different at a p-value of 0.05 (nonparametric paired Wilcoxon test)

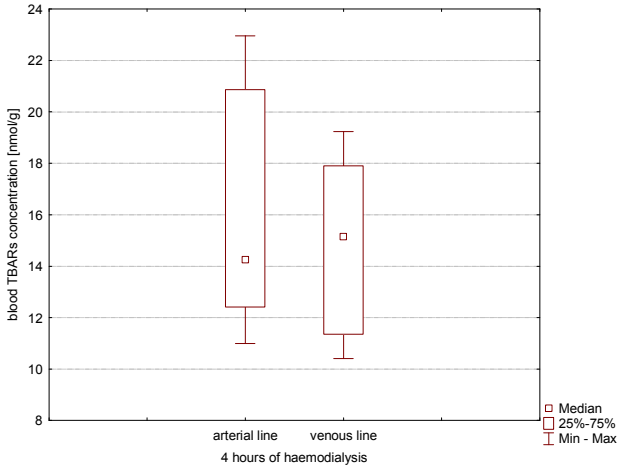


Fig. 8 Oxidative stress level after 4 hours of haemodialysis expressed as blood TBARs concentration in arterial and venous line. Results are statistically insignificant (nonparametric paired Wilcoxon test)

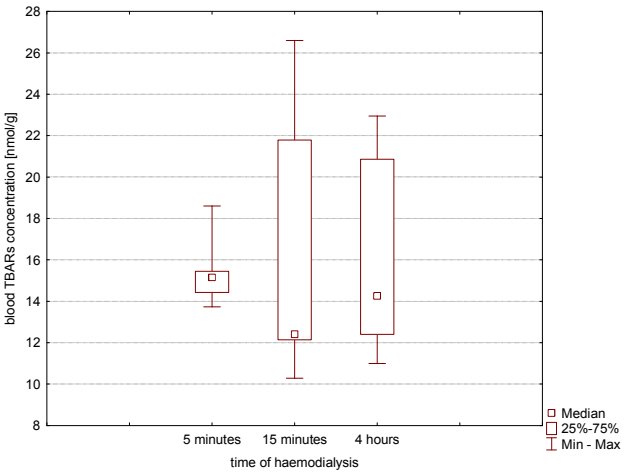


Fig. 9 Oxidative stress level during haemodialysis measured in arterial line. Results are statistically insignificant (Friedman ANOVA test & Kendall's coefficient of concordance)

IV. Conclusions

In 5th minute of HD we observed an increase in TBARs production as an indicator of oxidative stress caused by blood passage through the dialyzer. This influences the, TBARs production dependent, decrease in WBC count lasting up to 15th minute of HD that was not permanent. Then we observed a gradual increase in leukocyte number until 30th minute of HD. After that WBC count stabilizes around the number from the HD beginning. Until the end of HD there are no more signs of changes in TBARs production and WBC count. Blood clot formation was observed on the surface of a dialysis membrane, both the input and the output of the dialyzer. This effect may be associated with the deposition of activated leukocytes, neutrophils and platelets on the surface of the dialyzer membrane [8]. Significant increase in the level of lipid peroxidation in peripheral blood, at the HD beginning, indicates severe oxidative stress and may indicate a weakening of the body's antioxidant defense mechanisms. A similar effect was observed in patients treated with dialysis.

Transient leukopenia during HD is a well-known phenomenon that has been observed during HD with cellulose membranes [9]. It results from complement activation due to the contact of blood with bioincompatibile material. This causes activation of neutrophils, transmigration between endothelial cells into the surrounding tissues and the pulmonary sequestration [10]. The development of more biocompatible materials for dialyzer membrane production allows limiting the process, although it is not completely eliminated. We have shown that the contact of healthy sheep blood with the polysulphone membrane results in a temporary decrease in the total leukocyte count, which is caused by a decline in neutrophil count. Number of cells in the remaining populations of WBC did not change at the start of HD. Transient neutropenia well correlated with an increase in oxidative stress level. This suggests that oxidative stress occurring in the course of HD is largely related to the oxidative burst of phagocytes after non-specific activation by a dialyzer membrane.

Patients with chronic kidney disease (CKD) exhibit elevated levels of oxidative stress. On one hand, it may be one of the factors responsible for triggering of and progression CDK. On the other hand, it may be the result of the disease and the one of the complications related to the treatment with HD [11], [12]. It is also postulated that oxidative stress plays a role in the development of cardiovascular disease in the course of CKD [13]. This is the reason for searching for methods to reduce the oxidative stress in CKD patients. Our preliminary experiments on healthy sheep showed that HD procedure itself even when dialyzers made of modern materials are used causes the activation of leukocytes and increases the level of oxidative stress. They also provide a part of preliminary study on the methods of protection of blood components during the extracorporeal circulation.

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