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Effect of acute immobilization on the hematological, red-ox and rheological parameters of erythrocytes in rats

Artemy V. Novozhilov[1], Tat'yana V. Tavrovskaya[1], Michael I. Kalinski[2], Lev N. Katyukhin[1], Vadim A. Ivanov[1] and Vladimir I. Morozov[1]*

[1]Sechenov Institute of Evolutionary Physiology and Biochemistry of the Russian Academy of Sciences, St. Petersburg, Russia and [2]Kent State University, Kent, OH, USA

Abstract: *Objectives:* Immobilization effects on antioxidant enzymes activities, red-ox variables and rheological parameters of erythrocytes were studied. *Background:* It is not clear to which extent immobilization-induced oxidative stress may affect erythrocyte rheology. *Method:* Hematological and red-ox variables, erythrocyte antioxidant enzymes activities, and rheologic parameters of erythrocytes were analyzed. *Result:* Immobilization stimulated release of stored erythrocytes and reticulocytes, and decrease in rheology parameters of erythrocytes. There were no significant effects of the immobilization on antioxidant enzymes activities and red-ox variables. *Conclusion:* Immobilization-induced appearance of stored erythrocytes and reticulocytes in circulation is likely to have contributed to the alterations of erythrocyte rheology than red-ox state of blood.

Keywords: immobilization, erythrocyte rheology, oxidative stress

Introduction

Immobilization stress induces distinct changes in concentration of erythrocytes, formation of ROS and is followed by lipid peroxidation in blood in rats [1-3]. The erythrocytes act as scavengers for reactive oxygen species (ROS) produced in plasma and antioxidant enzymes in erythrocytes are very important for preventing the ROS-induced lipid peroxidation [3]. However, it is not clear to which extent immobilization-induced oxidative stress may affect rheologic parameters of erythrocytes by immobilization. This study was aimed to investigate the dynamics of hematological, red-ox and rheological parameters of erythrocytes after different durations of immobilization of rats.

Material and Methods

This study was performed in accordance with Helsinki Recommendations and ICMR guidelines on human and animal research [4]. 48 male Wistar rats 3 months of age were used in experiments. Rats housed in standard vivarium conditions and had free access to water and food. Depending on the duration of immobilization, animals were randomized in four groups: control, 0.5 h, 1 h and 3 h of immobilization. Rats were immobilized by forcing them to lie on their backs. Immediately after immobilization animals were decapitated and blood samples were collected into heparinized (10 IU/ml) tubes. White/red blood cells (WBC/RBC) parame-ters were measured with hematological analy-zer *Medonic CA620* (Medonic AB, Sweden). Leukocyte types were analyzed in blood smears stained by Giemsa solution (Azur B/Methylene Blue/Eosin 1:1:1). To analyze the reticulocytes (Ret) blood smears were prepared after supravital staining of blood samples by Brilliant Cresil Blue. Four fractions of Ret of different maturity (I, II, III and IV) were visualized. Blood samples were centrifuged (10 min, 3000 rpm) to separate cellular and plasma fractions. Erythrocytes were washed three times by cold saline, aliquoted, as plasma, and stored at -80ºC until analysis. Activities of antioxidant enzymes superoxide dismutase (SOD) [5], catalase (CAT) [6] and glutathione peroxidase (GP) [7] were measured in erythrocytes. Reduced glutathione (GSH) was determined in whole blood [8]. Malondialdehyde (MDA) was assessed in blood plasma [9].

Gradient ektacytometry was used to determine DI, erythrocyte deformability index, O_{isot}, osmolality of medium solution in which erythrocytes have maximal deformability (parameter estimating the degree of hemoglobin dehydration or internal viscosity), and O_{min}, parameter is a precise measure of the S/V (surface/volume) ratio estimating the shape of erythrocytes [10]. Reversible aggregation of erythrocytes was studied by piezodynamic method in a microchamber (a cylinder glass tube with a diameter of 16 mm and 25 µm in height) filled with whole heparinized blood [11]. RBC aggregation was described by the following parameters: T_{min} and T_{max} (characteristic times of initiation and completion of destruction of RBC aggregates); $\dot{\gamma}$ is the rate of RBC spontaneous aggregation (it was evaluated as a half-period reduction of the photometric transient signal through completely disaggregated blood after power-down of piezo crystal connected with surface of the microchamber); Ia, aggregability index (it was estimated as $T_{max} \times \dot{\gamma}$). All measurements were performed at constant temperature (25±0.5º C). The content of the stored red blood cells (RBC_{sto}), released from depot, were calculated as follows: $RBC_{sto}=[(RBC_{res}–Ret_{res})–(RBC_{con}–Ret_{con})]\times 100/RBC_{res}$, where RBC_{res}, RBC_{con}, Ret_{res}, Ret_{con} are erythrocytes or reticulocytes concentra-tions in immobilization or in the control ($\times 10^{12}$/L), respectively.

Computer programs Statistica 6.0 was used for data processing. Means, standard errors (SE) of means, correlation coefficients (r) were calculated. The statistical evaluation of the data was carried out by applying Mann-Whitney criterion, ANOVA and *t*-test for two independent samples.

Results

Table-1: Erythrocytes and leukocytes count

Index / Group		RBC ($\times 10^{12}$/l)	Reticulocytes [1]					Lympho cytes, %	Neutroph ils, %	Eosinophi ls [2]
			% of RBC	I	II	III	IV			
Control		7.4±0.1	1.7±0.2	1.7±1.9	16.7±7.1	39.3±4.7	43.8±10.5	89.8±0.7	7.2±0.5	14.6±2.5
Immobilization, h	0.5	8.8±0.2 ***	3.1±0.2 ***	7.5±3.5 ***	18.5±2.7	34.1±2.6 **	38.2±7.2	87.9±0.8	9.0±0.7 *	13.5±2.4
	1	8.6±0.2 ***	3.0±0.3 **	7.1±4.1 ***	21.0±5.2	34.7±5.0 *	37.2±6.2	80.5±1.2 ***	12.5±1.0 ***	14.3±2.2
	3	8.7±0.3 **	5.2±0.8 ***	16.6±5.1 ***	26.0±6.4 **	28.9±6.5 ***	28.7±6.0 **	63.8±3.1 ***	29.9±2.9 ***	7.4±1.9 *

Means +/- SEM are presented; N = 12; * P < 0.05, ** P < 0.01, *** P < 0.001; Mann-Whitney test. [1] I-IV – reticulocyte types are presented as percentage of the total reticulocytes; [2] eosinophils are presented as percentage of the total granulocytes.

Blood cell counts (Table 1): The concentration of erythrocytes was increased, reached maximal value after 0.5 hour (+ 19%) of immobilization and remained elevated until the completion of observation. The RBC_{sto} count tended to decrease and was 14.1, 12.9, 11.1 %, respectively, at 0.5, 1 and 3 h of immobilization. The concentration of Ret was 3-fold higher as compared to the control at the end of immobilization. Alterations in concentration of different maturity reticulocytes were also revealed. After 3-hour immobilization Ret I and Ret II were 9.5- and 1.5-fold higher, respectively, as compared to the control level, which suggest reticulocyte release from the red bone marrow. Blood total leukocyte concentration did not change during immobilization, however, at 3 hours lymphocyte content was reduced by 29%, while neutrophil content increased by four times. Eosinophil percentage of the total granulocyte content decreased by 3 times after 3-hour immobilization.

Table-2: Red-ox variables and correlation coefficients

Group \ Index		SOD (Rel.U/g Hb)	r (SOD/MDA)	GP (µmol GSH/min × g Hb)	r (GP/MDA)	GSH (µmol/g Hb)	r (GSH/GP)	CAT (mmol H_2O_2/min× g Hb)	r (CAT/MDA)	MDA (µmol/L)
Control		5.88±0.38	*0.497*	33.1±0.7	*0.191*	11.39±0.80	*0.560*	29.3±1.1	*0.137*	2.38±0.08
Immobilization, h	0.5	5.12±0.43	*0.035*	32.4±0.8	*0.593*	10.38±0.34	*0.434*	30.4±1.0	*0.152*	2.53±0.08
	1	5.09±0.20 *	*0.524*	32.1±0.6	*0.713 **	10.58±0.63	*0.617*	26.5±0.5 *	*0.819 ***	2.55±0.06 (p=0.07)
	3	5.97±0.35	*0.002*	33.0±0.7	*0.176*	11.62±0.57	*0.735 **	33.2±1.2 *	*0.472*	2.38±0.08
r		SOD/MDA		GP/MDA		GSH/MDA	GSH/GP	CAT/MDA		
		-0.99		*-0.98*		*-0.96*	*0.90*	*-0.61*		

Means +/- SEM are presented; N = 12; * P < 0.05, ** P < 0.01, *** P < 0.001; ANOVA

Erythrocyte red-ox balance (Table 2): There were no significant effect of the immobilization on MDA concentrations (it tended to be increased at 0.5 and 1 hour immobilization). Activities of SOD and CAT were significantly decreased by 16% and 10%, respectively, after 1-hour immobilization. However, at 3 hour the CAT activity was 25% higher as compared to its value at 1 hour. Activity of GP and the concentration of GSH were practically unchanged during the period of immobilization. The correlations between erythrocyte SOD, CAT, GP activities and MDA were the highest at 1 hour of immobilization (SOD-MDA, r=0.52, p=0.15; GP-MDA, r=0.71, p<0.05; CAT-MDA, r=0.82, p<0.01). Increase in correlation between GP and MDA (r=0.59, p=0.09) was also found after 0.5 hour of immobilization. The correlation values between GP activity and GSH concentration went up during the immobilization and had the maximal value at 3 hours (r=0.74, p<0.05). The recovery of red-ox parameters in rat's blood was observed at 3 hours.

Table-3: Aggregability and deformability variables of erythrocytes

Group \ Index		T_{min}	T_{max}	$\dot{\gamma}$	Ia	DI	O_{min}	O_{isot}
		s		s^{-1}	Rel.U.		mosmol/kg H_2O	
Control		15.8±3.1	95.0±8.5	0.047±0.008	5.0±1.2	769±9	159±2	303±3
Immobilization, h	0.5	19.9±3.3	112.1±12.3	0.047±0.009	5.4±1.3	745±9 **	164±3	298±3
	1	12.0±1.8	88.4±10.7	0.032±0.006 **	3.3±0.8 *	759±11	156±2	299±2
	3	11.3±1.2	88.4±8.2	0.034±0.006 *	3.3±0.7 *	753±7 *	154±3 *	289±5 *

Means +/- SEM are presented; N = 8-9; * P < 0.05, ** P < 0.01; Student´s *t*-test.

Erythrocyte rheology parameters (Table 3): Erythrocyte aggregability variables (T_{min}, T_{max}) tended to increase at 0.5 hour. After 1 and 3 hours of immobilization the erythrocyte Ia was significantly decreased. Erythrocyte DI was significantly reduced at 0.5 hour and remained at a reduced level after 1 and 3 hours of immobilization. Erythrocytes shape was more spherical at 0.5 hour (O_{min}– ratio of surface area to volume of erythrocyte) compared with its control value, whereas it became more toroidal, returning to the control value after 1 and 3 hours of immobilization. Index O_{isot} was significantly decreased after 3-h immobilization.

Discussion

Cell dynamic: We observed that immobilization induced the typical stress reaction. Erythrocytes concentration increased quickly most likely due to the release of deposited old erythrocytes mainly from the spleen and of reticulocytes from the red bone marrow. Immobilization-induced release of stored erythrocytes from the spleen depot was demonstrated earlier [12]. Reticulocyte count was increased in blood and the shift of reticulogram to the left was especially pronounced after 3 hours of immobilization. The reduction of lymphocyte and eosinophil counts and an increase of neutrophil count in blood represent the typical development of the stress reaction. The alterations in red-ox and rheology variables of erythrocytes are related to the changes in erythrocyte populations in the bloodstream (reticulocytes/mature erythrocytes/ old erythrocytes) and amplification of oxidative stress. Furthermore, the erythrocytes released from depot (the stored erythrocytes) have reduced function, activities of antioxidant enzymes, GSH and elevated MDA contents as compared to young erythrocytes [13, 14]. The mature erythrocytes occupy the intermediate position on values of these variables [13].

Red-ox: In erythrocytes, the ROS species ('O_2,O_2^- , H_2O_2, OH^-) may be generated by oxidases or spontaneously and antioxidant enzymes (SOD, CAT, GP) eliminate them [1]. Correlations between erythrocyte SOD, CAT activities and MDA were stronger at the onset of immobilization (1 hour) and weaker at 3 hours. In contrast, the correlation between GP activity and GSH concentration was stronger at 3 hours of immobilization. This may be due to the increase in erythrocyte glutathione reductase activity. It suggests that SOD and CAT are more important in countering oxidative stress at the onset of immobilization, followed by an increased importance of GP at 3 hours of immobilization. It seems that changed composition of oxidative stress products during 3-hour immobilization, led to an adjustment of correlations between (SOD, CAT, GP)–(MDA), and (GP) – (GSH).

Rheology: Decrease in variables depicting deformability (DI, O_{min}, O_{isot}) and aggregability (T_{min}, T_{max}, γ, Ia) of erythrocytes was found in dynamics of immobilization. Reduction in cell volume (O_{min}) and elevation of cytoplasm viscosity of erythrocyte (O_{isot}) may be linked with contraction of cells and echinocytes formation due to ions homeostasis disturbance [15]. There were no association between rheology parameters and red-ox status of erythrocytes that may be indicative of insignificant contribution of oxidative stress to the disturbance of erythrocyte rheology parameters by relatively short immobilization. However, a contribution of lipid peroxidation increase in alterations in membrane properties and rheological behaviour of erythrocytes was found, for example, in old animals and by hypercholesterolemia [13, 16]. Therefore, it may be assumed that alterations in rheological parameters of erythrocytes induced by relatively short immobilization are, likely, the result of the release of stored erythrocytes and reticulocytes from the bone marrow, and also of disturbance of erythrocyte ion homeostasis. These changes in rheologic parameters of erythrocytes may increase blood viscosity and affect the microcirculation.

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*All correspondences to: Dr. Vladimir I. Morozov, Sechenov Institute of Evolutionary Physiology and Biochemistry of the Russian Academy of Sciences, Ave Torez 44, 194223 St-Petersburg, Russia. E-mail: vmorozov.g@gmail.com