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Full Length Research Paper

Biological Activity of External Electrostatic Field Exceeding the Natural Background: Erythrocyte Plasma Membrane Target

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Abstract. It was hypothesized that the biological activity of external electrostatic fields is a result of field-induced physical processes on the cell membrane. Membrane is a dynamic lipoprotein structure and even the marginal changes of its physical parameters will be reflected on the structural and functional state of bilayer. For revealing the possible changes, the lipid-protein interactions in rat erythrocyte membranes after the *in vivo* chronic influence (6 days, 6 hours per day) of 200 kV/m electrostatic field were investigated. The biophysical parameters of erythrocyte ghosts were investigated by spectrofluorimetric method using the fluorescent probes 1-anilinonaftalene-8-sulfonate and pyrene. Oxidative modifications of membrane components were investigated also. The data obtained established that the field influence leads to the redistribution of membrane surface charge. It increases the immersion degree of the peripheral proteins in lipid bilayer and, in all probability, induces the conformational alterations of membrane integral proteins. These changes can be induced by the direct influence of field on the membranes and/or they can be a result of the field-generated changes in metabolic cascades. Our results emphasize the importance of membrane proteins in realization of the effect of electrostatic field.

Keywords: electrostatic field, erythrocyte membrane, lipid-protein interactions, surface charge

1. INTRODUCTION

The biological activity of external electrostatic fields (EESF) is among the disputed scientific issues. An analysis of the energy of these fields leads to the conclusion that no biologic effects are possible for these fields, except for extremely high EESFs, because an insufficient amount of energy is coupled with the quasistatic biologic system (Grandolfo et al., 1985). It is assumed that static electric fields do not penetrate the body, so they cannot have health effects, but at the same time they are used in physiotherapy (Naito et al., 2013). Different limited values of electrostatic fields for human health were set in many countries (ACGIH, 1994; NRPB, 1993).

On the other hand, there are a lot of studies about the changes observed in the living matter parameters at different levels due to the influence of EESFs. In accordance with them, EESF influence brings to the aversive behavior of rats (Creim et al., 1993), increases the electroencephalography activity and decreases the posterior hypothalamic activity (Lott et al. 1973), changes the serum protein fractions (Harutyunyan et al., 2013; Marino et al., 1974) and hematological-morphological state of blood (Antipov et al., 1983), altered the rate of glycolysis (Huang et al., 1997), decreases the hemolysis in the stored blood preparation (Nishiyama et al., 2005). The level of the above changes appears to be directly related to the EESF strength. There are numerous studies concerning the changes in the pro/antioxidant system due to the field influence (Cieślar et al., 2012). Thereby, the biological activity of EESF can be considered as a proven fact.

Since due to the scientific and technological progress more and more numbers of people appear under the impact of EESF exceeding the natural background, the identification of EESF initial mechanisms acquires the great theoretical and practical importance. This makes the occupational-pathological and ecological-hygienic assessment of the EESF biological activities urgent, which is impossible without identifying its primary mechanisms. Identification of mechanisms underlying EESF biological action in the purposeful and scientifically based use of these fields in physiotherapy is of no less importance.

We have hypothesized that the biological activity of external electrostatic fields is a result of physical processes at the boundary of the mediums with

different conductivity. The "external environment-membrane-internal environment" system can be regarded as a conductor-dielectric-conductor physical system. On the other hand, the membrane itself is a system of structures with different dielectric conductivities.

Membrane is a lipoprotein structure and even the marginal molecular rearrangement can cause the changes in the functional state of bilayer. In accordance with literature data and our own studies the influence of EESF changes the lipid matrix of artificial and natural membranes (Artsruni et al., 2013; Change, 1993; Hans et al., 2002; Lecuyer et al., 2006). The revealed changes can be a result of the metabolic displacements in lipid component of membranes. Undoubtedly, these changes, in their turn, should bring to the alterations of the structural and functional state of lipid-bound membrane proteins. On the other hand, the changes in membrane components can be induced by the alterations of the physical parameters of membrane surface layer due to the EESF direct influence.

Previously we reported about the redistribution and changes of the surface charge of rat erythrocyte membranes due to the *in vitro* and *in vivo* influences of 200 kV/m EESF (Artsruni et al., 2013; Pogosyan et al., 2007).

The aim of presented study was to investigate lipid-protein interactions in rat erythrocyte membranes after the *in vivo* influence of 200 kV/m EESF and reveal the possible molecular rearrangements in natural membranes due to the field imposition.

2. MATERIALS AND METHODS

2.1 Animals and experimental design

The main scheme of presented study has been described in our previous studies (Artsruni et al., 2013; Poghosyan et al., 2007). The object of investigation made erythrocyte ghosts of white outbreed 12-15-week aged female rats (body weight 130-150 g) preliminary exposed to 200 kV/m EESF during 6 days for 6 hours per days. All animal experiments were conducted in compliance with "The Legal Aspects of Research Ethics and Science in European Community: Directive 2001/20/EC". The same nutritional, light and thermal conditions were provided for both experimental and control animals.

The EESF is created using the condenser type device with controlling parameters of the field. The schematic diagram of ESF setup is provided in Figure 1. As a source of high voltage the step-up transformer and a rectifier from X-ray apparatus "RUM-17" (former USSR) was used. Output step-up transformer is connected to the rectifier, assembled by voltage multiplication scheme, which allows straightening and repeatedly raising the voltage obtained from the transformer. Leakage current between the electrodes was controlled using micro-ammeter and was immeasurably small due to the large distance between the electrodes. This allowed straightening the voltage with minimal pulsation, which was additionally, smoothed by capacitances of the electrodes themselves, and by parallel switched capacitor of 0.5 μF. Application of this scheme let to supply to the electrodes the voltage up to 200 kV. The ability to adjust the distance between the electrodes and the voltage across the electrodes enabled to create a strictly calibrated ESF in a wide range of tensions. Smooth regulation of the voltage on the electrodes was carried out by means of the regulating transformer (variator). As a voltage indicator on electrodes a magnetoelectric device with almost uniform scale (graduated in kilovolts) was used. This device (kilovoltmeter) is powered by a special step-down transformer through a germanium rectifier. Actually voltage across the electrodes depends on the mains voltage and on the voltage in setup itself. Voltage drop in the network reflected on testimony of kilovoltmeter. The voltage drop in the setup itself is taken into account by specially applied compensation scheme. Voltage across the electrodes was additionally monitored by static kilovoltmeter C-96 type that was connected directly to the electrodes.

Upon setup turning off the high-voltage capacitors are discharged automatically. Discharging device consists of two series-connected resistance 1.8 mega ohm and 2.7 mega ohm by 20 watts each, which at the setup time off grounding the capacitor plates by means of a magnetic contact. Entrance to the room where the setup operates blocked by entrance voltage so that at the time of opening the door automatically turns off high voltage and the capacitor discharged. Remote control of the setup and the testing equipment were located in an adjacent room. These precautions ensure complete safety of the experimenter.

Experimental chambers (plastic cages with rats inside) were placed between two round aluminum electrodes; to avoid edge effects, the diameter of the electrodes exceeds the size of the chambers and composes 150 cm. The electrode surfaces are carefully polished to provide a uniform charge distribution on the electrode surface. The upper electrode is movable, which enables to change the gap between the electrodes and thereby adjust the tension of ESF at a fixed voltage on the electrodes. The lower electrode simultaneously with its function acts as a stand for the experimental chambers. Dimensions of the chambers were: length - 40 cm, width - 30 cm, height - 15cm. Cages were located side by side on the

lower electrode in the center. Rats were located in two cages by 5 in every one.

We used ESF of 200 kV/m strength. In order to obtain this strength, on the upper electrode the constant negative potential of 60 kV was applied, while the lower electrode was grounded. Electrodes were fixed on the distance of 30 cm from each other.

2.2. Blood sampling and processing

Blood samples were obtained by the method of cardiac puncture (Weis et al., 1971). Two groups of animals, 60 rats in each, were investigated. The blood was drawn from the intact animals of both groups. It is well known that this blood obtaining method brings to the activation in release of immature and young red cells to the blood circulation (Morton et al. 1993). In 7 days after the recovery of above mentioned processes, the first group was exposed to EESF during 6 days (EESF-group). Immediately after the influence, the blood of experimental animals obtained. For understanding and differentiation of the field and cardiac puncture induced effects, the second group was kept under the same conditions and time frame as the first one after the cardiac puncture, but without the field exposure. The blood of the second group rats was obtained in parallel with samples taken from experimental animals for revealing the possible effects of the release of young red cells to the blood after the cardiac puncture (Cardio-group).

Control animals and animals of Cardio-group were placed within the switched off exposure setup, in the same light and nutritional conditions (sham control). For the avoidance of circadian rhythms, the blood preparation was done in the same time of day.

2.3. Preparation of erythrocyte ghosts

The isolation of erythrocytes, preparation of their membranes and formation of ghosts was carried out by Dodge method in our modification (Dodge et al., 1963). Particularly, we used the solution containing 0.145 M NaCl, 0.02 M Tris/HCl (pH 7.6) for red cells isolation, which allowed us to increase the membrane outcome. For each sample the blood of 6 animals was used.

2.4. Fluorescent Investigations

The biophysical parameters of erythrocyte ghosts were investigated by spectrofluorimetric method using the fluorescent probes 1-anilinonaftalene-8-sulfonate (ANS) and pyrene. ANS is a water-soluble, non-penetrating probe with unit negative charge, which reacts in the sites of protein–lipid connections in the cell surface (McLure et al., 2011; Vladimirov et al., 1980). The bounding parameters of this probe with membranes serve as indicators for revealing the molecular reconstructions in membrane surface structure.

Pyrene is a hydrophobic, membrane-penetrating probe. The usage of this probe allows determining intra-membrane changes, particularly the immersion degree of membrane proteins in lipids, membrane viscosity and microviscosity (Isuev, 2009; Nandy et al., 1983).

To get the whole picture, fluorescence parameters of ANS and pyrene were measured in the same ghost samples. At the same time, for revealing the role of non-structured proteins in realization of EESF effect, the isolation of erythrocytes and formation of ghosts was carried out in two modes: directly from the mass of erythrocytes after the three-time washing in Tris-buffer (0.0145 M NaCl in 0.02 M Tris/HCl, pH 7.6) and from the mass of erythrocytes after the threetime washing in 0.9% NaCl solution.

ANS fluorescence of each ghost-containing sample (λext= 360 nm, $\lambda emis$ = 470 nm) was measured under the conditions of constant membrane-protein concentration (0.3 mg/ml) by titration with ANS (5-100 μM), and under the conditions of constant ANS concentration (5 μM) by titration with different protein concentrations (0.1-0.6 mg/ml). The obtained data were expressed in reversed coordinates, and the graphics made by Klotz (Vladimirov et al., 1980). The rate constant of membrane-binding reaction (Kc) and the amount of ANS-binding centers (N) were counted by the formula of Scetchard (Vladimirov et al., 1980). The concentration of proteins in samples was determined by Lowry.

The measurement of fluorescent parameters of pyrene expressing the immersion degree of membrane proteins in lipid bilayer was carried out by method described in (Tereshchenko et al., 2002), in accordance to which the isolation of erythrocytes and formation of ghosts was carried out in two samples as described above.

The fluorescence of pyrene in ghost-containing suspensions in λext= 284 nm and $\lambda emis$= 334 nm was determined for estimation of fluorescence of tryptophanyl groups. The immersion degree of membrane proteins in lipid bilayer was estimated by inductive-resonance mechanism in tryptophanyl-pyrene system. Briefly, after the measurement of tryptophanyl fluorescence, 30 μl ethanol solution of pyrene with 100 μmol/l end concentration was added to the ghost-containing suspension.

The part of fluorescence of tryptophanyl groups present in a range not more than one Fester radius was calculated by formula: $P = (F_0-F)/F_0$, where F_0 is a fluorescence of tryptophanyl groups before pyrene

adding, F is the same parameter after the probe adding.

The constant of the degree of relationship between the peripheral proteins with membranes was calculated by formula: $K= |(P_1-P_2)/P_1|$, where P_1 is the value for ghosts obtained from erythrocytes washed in Tris-buffer, P2 is the same parameter value for ghosts obtained from erythrocytes washed in NaCl solution.

Membrane microviscosity was estimated according to values of ratios I_{370}/ I_{470}, I_{390}/ I_{470} fluorescence intensities of pyrene in λ_{ext}=284nm. The increase of these parameters in erythrocyte membranes testified the increase of microviscosity or the decrease of the hydrophobic volume of the zone of protein–lipid contacts (Isuev, 2009; Nandy et al., 1983).

The values of ratios I_{370}/ I_{470}, I_{370}/ I_{470} fluorescence intensities of pyrene in λ_{ext}= 340 nm in all investigated samples were measured for estimation of viscosity of lipid bilayer.

All measurements were done in 1cm quartz cuvettes at the room temperature by the spectrometer Hitachi MPF-4 (Japan). The results were expressed in relative fluorescence units per mg of protein (RFU/mg).

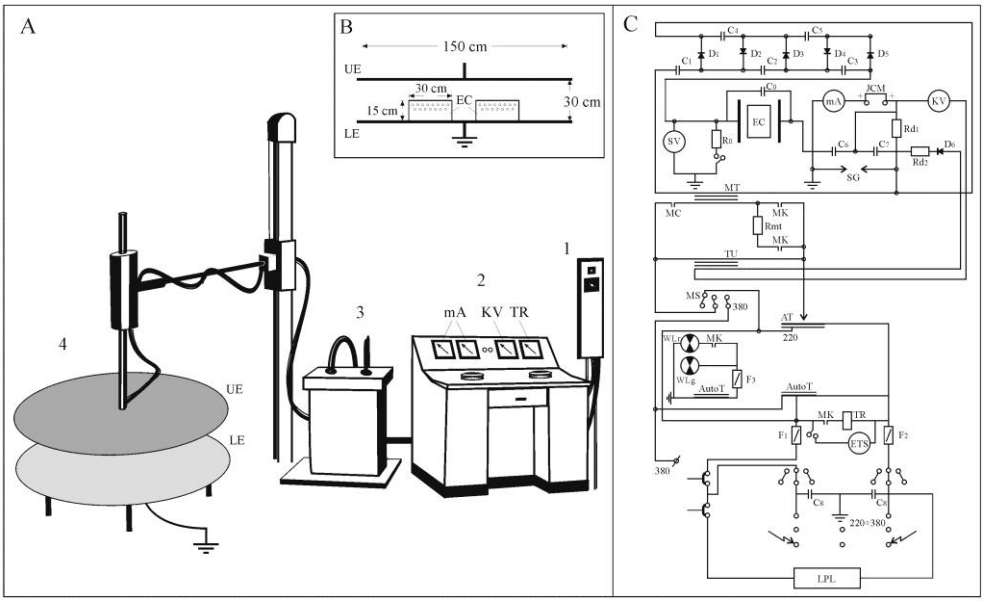

Fig. 1: Electrostatic field experimental setup: drawing of setup (A); dimensions of the electrodes and the experimental chambers (B); the schematic diagram of setup (C). 1 – Toggle; 2 – Remote control; 3 – a high voltage generator; 4 – electrodes; UE – upper electrode; LE – lower electrode; C1, C2, C3, C4, C5 – capacitor voltage multiplier; D1, D2, D3, D4, D5 – diode voltage multiplier; C0 – smoothing capacitor; C6, C7, C8 – capacitors; D6 – rectifier diode device; JCM – jack control milliammeter; mA – milliammeter; KV – kilovoltmeter; SV – static voltmeter; EC – experimental chamber; Rmt – the resistance of the main transformer; R0 – resistance of the arrester; Rd1, Rd2 – resistance devices; SG – spark gap; MT – main transformer; TU – transformer unit; AT – adjusting transformer; AutoT – autotransformer; MK – main contactor; MS – main switch; WLr – warning light red; WLg – warning light green; F1, F2, F3 – fuses; TR – time relay; ETS – electric time switch; LPL - light panel lock.

2.5. Protein Carbonyls

The protein carbonyl content was measured by the method of Levine (Levine et al., 1990). The carbonyl content was expressed in terms of nmol/mg of protein.

2.6. Lipid Peroxidation

Lipid peroxidation in the ghosts was determined by measuring the amount of malondialdehyde (MDA) produced by the thiobarbituric acid (TBA) reaction as described by Uchiyama and Mihara (Uchiyama et al., 1978). The results were expressed as MDA nmol/mg of protein.

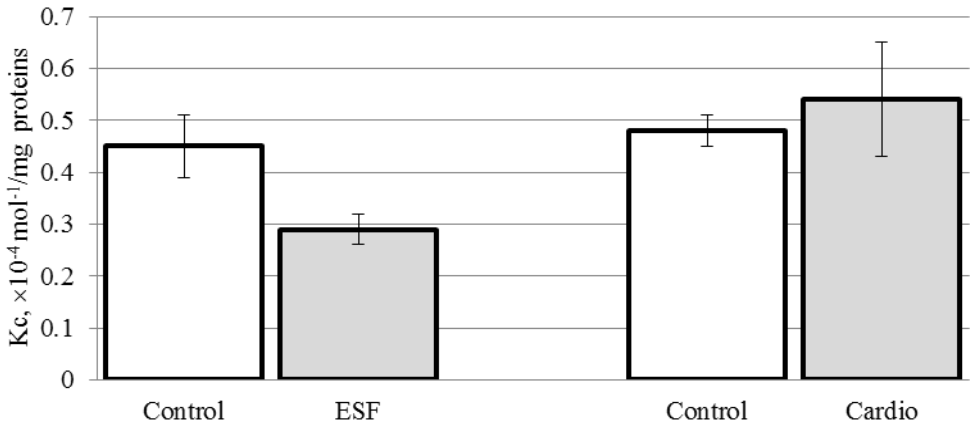

Fig. 2: The rate constant (Kc) of ANS binding reaction with the ghosts prepared from the mass of Tris-buffer (0.0145 M NaCl in 0.02 M Tris/HCl, pH 7.6) washed erythrocytes in EESF- group, Cardio-group and in appropriate control samples, *×10^{-4} mol^{-1}/mg proteins*

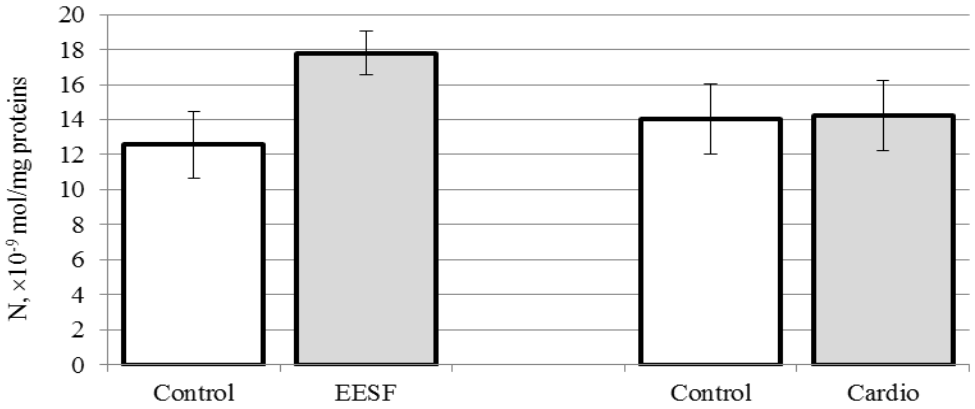

Fig. 3: The amount of ANS-bound centers N for the ghosts prepared from the mass of Tris-buffer (0.0145 M NaCl in 0.02 M Tris/HCl, pH 7.6) washed erythrocytes in EESF- group, Cardio-group and in appropriate control samples, *×10^{-9} mol^{-1}/mg proteins*

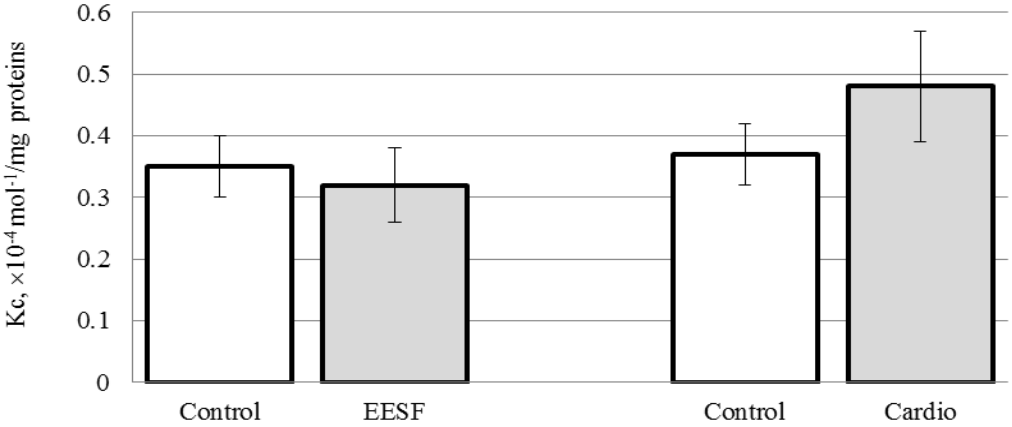

Fig. 4: The rate constant (K_c) of ANS binding reaction with the ghosts prepared from the mass of 0.9% NaCl-washed erythrocytes in EESF-group and rats Cardio-group and in appropriate control samples, *×10^{-4}mol^{-1}/mg proteins*

2.7. Assay of Aromatic Amino Acids Oxidation

Dityrosine production and tryptophan oxidation was measured in ghost samples. Tryptophan destruction and dityrosine production were measured with a "Hitachi-MPF-4" (Japan) spectrofluorometer. Dityrosine production was assessed at λ_{ext}=325 nm and λ_{em}=415 nm (Giulivi et al., 1994). Tryptophan oxidation was monitored by loss of protein

fluorescence at λ_{ext}=295 nm and λ_{em}=340 nm (Teale, 1960). Results are expressed RFU/mg.

The oxidative modifications of membrane proteins and lipids were assayed for ghosts prepared from the Tris-buffer washed erythrocytes for control and field-exposed rats.

For each point of measurements the ghosts of erythrocytes isolated from 6 animals were used and each point was taken as an average of 10 measurements.

2.8 Statistical Analysis

Statistical analysis of the results was done using the statistical functions of the GrafPad InStat software (GraphPad Software, Inc., San Diego California USA, www.graphpad.com). P-value of <0.05, <0.01, and <0.001 was considered respectively as statistically significant (*), very significant (**), and critically significant (***). All data were expressed as mean ± standard error of mean (SEM).

Table 1: Changes of I_{370}/ I_{470} and I_{390}/ I_{470} ratios of pyrene fluorescence in the excitation wavelengths λex=285 nm and λex=340 nm after the chronic (6 days, 6 hours per day) *in vivo* influence of 200 kV/m EESF in the ghosts-containing solutions prepared from the mass of erythrocytes after the washing in Tris-buffer (0.0145 M NaCl in 0.02 M Tris/HCl, pH 7.6) and from the mass of erythrocytes after the washing in 0.9% NaCl

	Erythrocyte washing	Control	EESF	Control	Cardiac puncture
λex = 284nm	Tris-buffer	3.47 ± 1.51	2.78 ± 0.52*	3.24 ± 1.17	2.85 ± 0.72*
I_{370}/I_{470}	NaCl	3.42 ± 1.77	2.07 ± 0.24*	3.18 ± 1.07	2.37 ± 0.91*
λex = 284nm	Tris-buffer	4.23 ± 1.00	2.47 ± 0.58**	3.71 ± 1.06	2.38 ±0.37*
I_{390}/I_{470}	NaCl	4.21 ± 1.34	1.92 ± 0.23**	3.73 ± 0.91	2.26 ± 0.39*
λex = 334nm	Tris-buffer	1.17 ± 0.17	1.28 ± 0.14	1.27 ± 0.13	1.22 ± 0.13
I_{370}/I_{470}	NaCl	1.17 ± 0.17	1.20 ± 0.10	1.27 ± 0.13	1.14 ± 0.14
λex = 334nm	Tris-buffer	1.17 ± 0.17	1.28 ± 0.14	1.27 ± 0.13	1.20 ± 0.11
I_{390}/I_{470}	NaCl	1.17 ± 0.17	1.20 ± 0.10	1.27 ± 0.14	1.15 ± 0.15

Table 2: Parameters of the oxidative modifications of the proteins and lipids in rat erythrocyte membranes after the chronic (6 days, 6 hours per day) *in vivo* influence of 200 kV/m EESF

Tested parameter	Groups	
	Control	EESF
Tryptophan *(RFU/mg)*	12.90 ± 0.91	16.00 ± 1.09**
Dityrosine *(RFU/mg)*	4.54 ± 0.78	6.33 ± 0.44**
Carbonyl groups *(nmol/mg of protein)*	3.90 ± 0.60	4.4 ± 0.81
MDA *(nmol/mg of protein)*	3.42 ± 0.79	3.32 ± 0.56

3. RESULTS

The estimation of the binding parameters of ANS with erythrocyte ghosts showed that the influence of investigated field brings to the changes of Kc and N for ghosts prepared from Tris-washed erythrocytes. Thus, Kc decreases by 35.56% (Fig. 2) and the N increases by 41.64% (Fig. 3) after the influence of EESF. At the same time these parameters are not changed in ghosts of the Tris-washed erythrocytes of Cardio-group (Fig. 2 and 3).

In case of NaCl washing no significant changes of Kc are observed for ghosts of the both investigated groups in comparison with the appropriate controls, but in Cardio-group Kc has an increasing tendency by 29.73% (Fig. 4). So, we have the significant decrease of Kc in EESF-group compared to Cardio-group by 33.33% (Fig. 4).

The analysis of N for the ghosts prepared from NaCl-washed erythrocytes revealed the changes in the both investigated groups. So, in EESF-group this parameter increases by 52.30%, while in Cardio-group it decreases by 33.84% compared to appropriate controls. Thus, the value of N is greater for ghosts in EESF-group by 113.95% in comparison with the same parameter in Cardio-group (Fig. 5).

The estimation of the fluorescence parameters of pyrene showed that the chronic influence of EESF leads to the decrease of the constant of the degree of relationship between the peripheral proteins with membranes K by 62.07%, while in the Cardio-group this parameter showed a not reliable tendency to the increase by 21.15% (Fig. 6). In accordance with (Tereshchenko et al., 2002), the decrease of this parameter testifies the increase of the immersion degree of peripheral proteins in lipid bilayer and vice

versa. Thereby, we can say that the EESF influence increases the immersion degree of peripheral proteins in lipid bilayer, and the difference with respect to the effect of cardiac puncture is 65.07%.

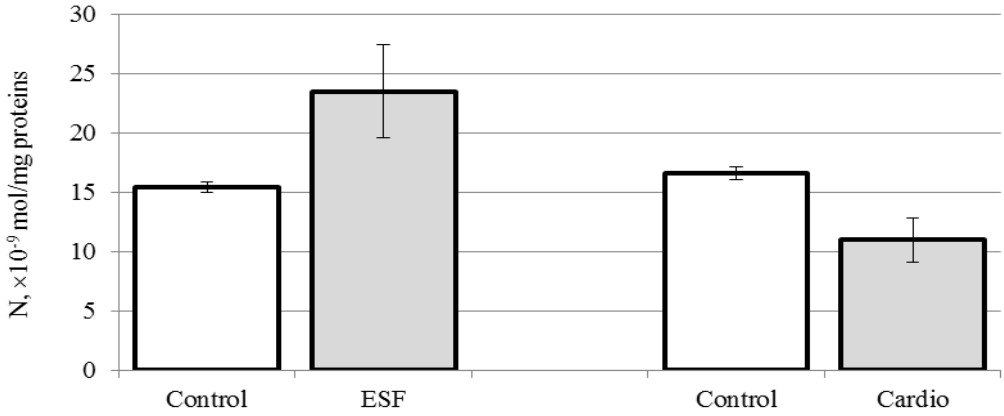

Fig. 5: The amount of ANS-bound centers N for the ghosts prepared from the mass of 0.9% NaCl- washed erythrocytes in EESF- group, Cardio-group and in appropriate control samples, *×10^{-9} mol/mg proteins*

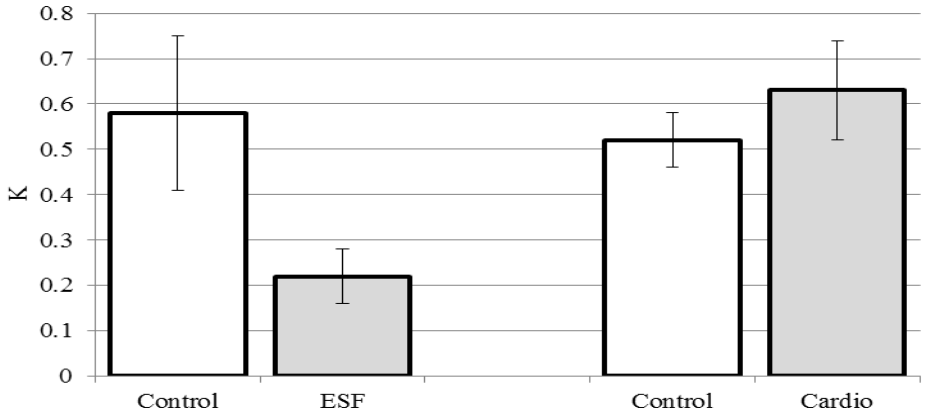

Fig. 6: Immersion degree (K) of peripheral proteins in erythrocyte membranes in EESF- group, Cardio-group and in appropriate control samples

Comparison of I_{370}/I_{470} and I_{390}/I_{470} ratios of pyrene fluorescence parameters (λ_{ext} = 284nm), which determine the membrane microviscosity, revealed changes in the both EESF and Cardio groups (Table 1). These changes have the same directions and are equivalent. It is known that the membrane microviscosity of red cells increases depending on their age (Tillmann et al., 1980).

Our findings well correlated with the literature data, in accordance to which the cardiac puncture brings to the release of young erythrocytes to the blood cycle. On the other hand, (Rollan et al., 2003) reported about the same effect due to the influence of external electrostatic fields. Thus, at this stage of our studies we cannot differentiate the effects of cardiac puncture and EESF on the ratio of the amounts of "young" and "old" erythrocytes in blood.

The I_{370}/I_{470} and I_{390}/I_{470} ratios of pyrene fluorescence parameters (λex = 334nm), which indicate membrane viscosity, were practically unchanged in both Cardio and EESF groups (Table 1). The results of the investigation on oxidative modifications of membrane lipids and proteins are presented below (Table 2). In accordance with research findings, due to the EESF influence the fluorescence of tryptophan and dityrosine increases by 24.03% and 39.43%, accordingly. At the same time no changes are revealed in protein carbonylation and amount of MDA after the field influence.

4. DISCUSSION

ANS is known to bind to polar-apolar interfaces with the chromophoric group, extending into the hydrocarbon core and the sulphonate group located in the plane of the membrane polar head groups (Slavík, 1982). So, its fluorescence is extremely sensitive to the changes in the environment. Particularly, the alterations in the lipid-protein interactions can affect the probe fluorescence. Therefore, spectral parameters

of the bound probe could provide information about the changes in its molecular environment caused by EESF.

The rate constant of membrane-binding reaction (Kc) is strongly influenced by the surface charge of membrane, and the amount of ANS-binding centers (N) depends on the number of positive-charged groups on membrane surface, which are available to interact with the negative charged ANS molecules. In accordance with our data, in case of smooth-washing of erythrocytes in TRIS-buffer, in the ghosts of Cardio-group no changes were revealed for these parameters, while in the ghosts of EESF-influenced group the decrease of Kc takes place in parallel with the increase of N. Taking into account that the Kc indicates the affinity of the ANS to the membrane, we can suggest that its decrease is caused by the increase of the negative charge or its density on the membrane surface. At the same time, the increase of N testifies that the amount of positive charged groups increases in ghost surface. It is known that the number of protein binding sites for ANS in biological membranes is considerably smaller than the number of lipid binding sites, whereas their affinity is greater (the ratio of the protein/lipid affinities to ANS is 10:1), and the probe first binds to the proteins and subsequently with lipids (Slavík, 1982). So, the comparison of data obtained allows us suggesting that the changes observed in Kc and N are the result of the lipid-protein rearrangements in the membrane surface layer due to the field influence.

No changes of Kc were revealed for the ghosts prepared from the erythrocytes washed in NaCl solution for the field influenced group. Noteworthy, this parameter has an increasing tendency for the ghosts of Cardio-group and it is significantly greater than Kc for the ghosts after the field influence. As stated above, it is the fact that after the cardiac puncture the ratio of young erythrocytes increases in blood; their membrane structure differs from the “old” ones and it can bring to the change of the ANS affinity. It means that Kc increases due to the changes in the ratio of the number of young and old erythrocytes induced by cardiac puncture. But after the field influence the affinity of membranes to the ANS decreases in comparison with the Cardio-group. So, we can assume that we have a field effect: the negative surface charge in membranes increases after the EESF influence.

The decrease of N for the ghosts prepared from NaCl-washed erythrocytes after the cardiac puncture well correlated with the literature data testifying the membrane structural specificities of young erythrocytes. In accordance with (Tereshchenko et al., 2002), NaCl washing of erythrocytes eliminates the membrane-bound peripheral proteins, which are not inserted into the hydrophobic interior of the lipid bilayer. Instead, they are indirectly associated with membranes through protein-protein or protein-lipid interactions. These interactions frequently involve ionic bonds, which are disrupted by extreme pH or high salt. So, it is logically to suggest that the forces between the peripheral proteins and membranes of young erythrocytes are weaker and the NaCl washing of cells brings to the deeper elimination of peripheral proteins, which itself changes the amount of lipid-protein interaction sites on the membrane surface and decreases the amount of the ANS binding centers after the cardiac puncture. So, the increase of this parameter after the EESF influence as compared to the appropriate control and to the effect of cardiac puncture, allows us assuming that the field influence brings to the increase of the forces between the peripheral proteins and membranes. As a result, the amount of the positive-charged groups in membrane surface increases due to the EESF-induced structural changes in membranes.

In accordance with our results the membrane-binding parameters of ANS depend on erythrocyte washing solution indicating that the peripheral proteins have a significance role in the realization of EESF effect. The analysis of pyrene fluorescence parameters and estimation of the constant of the degree of relationship between the peripheral proteins with membranes K revealed the EESF-induced decrease of this parameter, which indicates that the immersion degree of the peripheral proteins in lipid bilayer increases; this latter means that the forces between these proteins and integral molecules (proteins and lipids) of membrane increase as well. In Cardio-group no changes of K were observed. So, we can insist that the changes of lipid-protein interactions in erythrocyte membranes occurred due to the EESF chronic influence on the rats. It is well known that electrostatic interactions play an important role in binding of protein to lipid, however the binding also depends on a very high ionic strength, which shows that additional forces, presumably hydrophobic, must also contribute significantly to the interaction of protein and the lipid (Vorobyov et al., 2011). Thus, the changes revealed in our study after the field influence can have different reasons: redistribution of the surface charge, immersion of peripheral proteins into the bilayer; conformational changes of membrane integral proteins, alterations in lipid component of membranes. Each of these suggested reasons can itself induce the other ones. So, the change of membrane surface charge can bring to the changes of the peripheral protein-lipid and peripheral protein-integral protein interactions, thus affecting the immersion degree of peripheral proteins into the bilayer.

Earlier, the enhancement of phospholipase A2 activity in erythrocyte membranes by the action of EESF was reported by Artsruni and coauthors (Artsruni et al., 1999). The changes in the activity of phospholipase A2 due to the field influence can induce the alterations in lipid component of bilayer, which itself can bring to the changes in conformation of integral proteins and immersion degree of peripheral proteins. As a result the surface charge of membranes can be redistributed.

Above it, increased activity of phospholipase A2 might explain the absence of EESF effect on MDA level in RBC membranes. This enzyme is known to play a protective role, cleaving oxidized fatty acids from the 2-position of the phospholipid glycerol backbone so that they can then be metabolized by glutathione peroxidase to the corresponding alcohols (Halliwell et al., 1989). At the same time, we revealed the reliable changes in the fluorescence of tryptophan and dityrosine. At the first view, these data manifest the changes in the processes of the oxidative modification of the protein cyclic amino acid chains, particularly, the decrease of tryptophan oxidation and the increase of tyrosine oxidation. On the other hand,fluorescence from the amino acid tryptophan has long been known to be sensitive to the polarity of its local environment (Eftink, 1991): it increases in a hydrophobic environment (Gonzalez-Horta et al., 2013; Vivian et al., 2001). Continuing to develop the assumption made above, we suggest that the field influence changes the polarity around the membrane proteins, induces the alterations in their conformational state and leads to the transition of tryptophan in a hydrophobic environment, which can increase its fluorescence. Furthermore, the increase of the tyrosine oxidation can be considered as a result of the same conformational changes, which can make this amino acid available for the oxidation.

5. CONCLUSION

The data obtained established that the *in vivo* chronic influence of 200kV/m EESF brings to the redistribution of membrane surface charge; increases the immersion degree of the peripheral proteins in lipid bilayer and, in all probability, induces the conformational alterations of membrane integral proteins. These changes can be induced by the direct influence of field on the membranes and/or they can be a result of the field-generated changes in metabolic cascades. So, if assuming that the EESF influence affected the membrane surface charge directly, as we stated above, we can allow that the changes in protein-lipid interactions are the result of the direct effect of EESF on the membranes. On the other hand, the chronic effect of field can induce the changes in lipid component of membrane, which in its turn can generate the changes in the structural and functional state of proteins, and as a result the charge on membrane surface can be redistributed. Our results emphasize the importance of membrane proteins in realization of EESF effect.

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