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THE CHANGES OF ERYTHROCYTE MEMBRANES OF WHITE OUTBREED RATS EXPOSED TO ELECTROSTATIC FIELD WITH THE TENSION EXCEEDING THE NATURAL BACKGROUND

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The in vivo influence of 1-hour 200 kV/m electrostatic field (ESF) on the biophysical parameters of rat erythrocyte ghosts is investigated. Studies have shown that ESF influence changes the physicochemical state of ghosts. The field influence results to the lipid-protein intermolecular reorganization in the superficial layer of membrane, increases the strength of association of peripheral proteins with integral proteins and lipid bilayer, and increases the membrane viscosity. Comparison of literature data with the results obtained, allows us to suggest that the investigated factor leads to the redistribution of membrane surface charge, which can be the both a reason or/and a result of revealed changes.

Electrostatic field – erythrocyte ghosts – surface charge

Հետազոտվել է 200 կՎ/մ լարվածությամբ էլեկտրաստատիկ դաշտի (ԷՍԴ) 1-ժամյա ազդեցությունը սպիտակ ոչ ցեղային առնետների էրիթրոցիտների ստվերների կենսաֆիզիկական չափանիշների վրա: Ցույց է տրվել, որ ԷՍԴ-ի ազդեցությունը բերում է ստվերների ֆիզիկա-քիմիական վիճակի փոփոխության: Դաշտի ազդեցության հետևանքով մեմբրանի ներքին շերտում տեղի են ունենում սպիտակուց-լիպիդային մոլեկուլային վերադասավորություններ, մեծանում է ծայրամասային սպիտակուցների և լիպիդային երկշերտի միջև առկա կապի ուժը, աճում է թաղանթի մածուցիկությունը: Գրականության տվյալների և հետազոտության արդյունքների համադրումը թույլ է տալիս եզրակացնել, որ հետազոտվող գործոնը բերում է թաղանթի մակերևութային լիցքի վերաբաշխման, ինչը կարող է լինել ինչպես բացահայտված փոփոխությունների պատճառ, այնպես էլ հետևանք:

Էլեկտրաստատիկ դաշտ – էրիթրոցիտների ստվերներ – մակերևութային լիցք

Исследовано *in vivo* 1-часовое воздействие электростатического поля (ЭСП) напряженностью 200 кВ/м на биофизические параметры теней эритроцитов белых беспородных крыс. Показано, что воздействие ЭСП приводит к изменениям физико-химического состояния теней. В результате воздействия поля происходят липид-белковые молекулярные перестройки на наружном слое мембраны, повышается сила связи периферических белков с липидным бислоем и вязкость мембраны. Сопоставление литературных данных с полученными результатами, позволяют предположить, что исследуемый фактор приводит к перераспределению поверхностного заряда мембраны, что может являться как причиной, так и результатом выявленных изменений.

Электростатическое поле – тени эритроцитов – поверхностный заряд

The analyses of literature data and the results of our previous studies allow us to suggest that the electrostatic fields (ESF) with tensions exceeding the natural background are directly effect the physical and chemical properties of biological macromolecules and

their membrane-like ordered structures [4, 5, 9, 12]. We have advanced a hypothesis, according to which the initial mechanisms of the biological activity of ESF are conditioned by the physical processes on the border of the layers with different electrical conductivity [3]. The cell membrane is a system with obviously expressed borders of components with different electrical conductivity.

In available literature we did not find works testifying the concrete structural reconstructions in native membranes due to the ESF influence, when even the marginal changes of physical parameters of membranes can lead to the extremely considerable changes of their structural-and-functional state.

The presented study tries to make up for this deficiency. This paper introduces the results of analyses of physical parameters of erythrocyte ghosts of rats exposed to one-hour 200 kV/m ESF.

Materials and methods. The erythrocyte ghosts of 150-200 g male outbreed rats, which are preliminary exposed in 200 kV/m ESF during an hour, are served as an object of investtigations. The ESF is created using the condenser type device with controlling parameters of the field [2]. Briefly, the chamber, where experimental animals are placed consists of three millimeter organic glass-made cylinder with 1 m diameter and 0.25 m height. The holes on the side walls and on the cover of the chamber ensure the ventilation and water supply. Each chamber is divided into four equal compartments. In each of them 5 rats are kept. The caging is made through a removable cover. In each compartment there is a feeding. The chamber is placed between two round aluminum electrodes of 1.5 m diameter with polished and curling edges to maximize uniform distribution of charges on the electrodes. The lower electrode is fixed and, simultaneously, with its primary function serves as support for an experimental chamber. The upper electrode is movable, which makes possible to adjust the distance between the electrodes and the voltage across the electrodes. In our case, to obtain the tension of 200 kV/m, the constant negative potential of 60 kV was applied on the upper electrode, and the lower electrode was grounded. Electrode spacing was 0.3 m.

Blood of intact animals is prepared by the method of cardio puncture [20]. Within the 7 days, after recovery of hematopoiesis, the same animals are exposed to ESF. Immediately after the influence, the blood is prepared by the above mentioned method. For the avoidance of circadian rhythms, the blood preparation is done in the same time of day.

The isolation of erythrocytes, preparation of their membranes and formation of ghosts was carried out by Dodge method in our modification [7]. Particularly, we have used the solution containing 0.145 M NaCl, 0.02 M Tris/HCl (pH 7.6) for red cell isolation, which allows us to increase the membrane outcome.

The biophysical parameters of erythrocyte ghosts are investigated by spectrophotometric 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 [10, 14, 19]. The bounding parameters of this probe with membrane are served 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 intramembrane changes, particularly the immersion degree of membrane proteins in lipids, polarity in bilayer, membrane viscosity and microviscosity [11, 15, 16].

For getting the whole picture, fluorescence parameters of ANS and pyrene have measured in the same ghost samples. At the same time, for revealing the role of non-structured proteins in realization of ESF effect, the isolation of erythrocytes and formation of ghosts have carried out in two samples: directly from the mass of erythrocytes after the tree-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 tree-time washing in 0.9 %-NaCl solution.

ANS fluorescence of each ghost-containing simple has 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 have expressed in reversed coordinates, and the

graphics have made by Klotz [19]. The rate constant of reaction (K_c) and the amount of ANS-bounding centers (N) have counted by formula of Scetchard [19]. The concentration of proteins in samples is determined by Lowry [13].

The measurement of fluorescent parameters of pyrene expressing the immersion degree of membrane proteins in lipid bilayer was carried out by method described in [18], in accordance to which the isolation of erythrocytes and formation of ghosts have carried out in two samples as described above.

The fluorescence of ghost-containing suspension in λ_{ext}= 284 nm and λ_{emis}= 334 nm is determined for estimation of fluorescence of tryptophanil groups.

The immersion degree of membrane proteins in lipid bilayer is estimated by inductive-resonance mechanism in triptophanil-pyrene system. Briefly, after the measurement of triptophanil fluorescence, 30 μl ethanol solution of pyrene with 100 μmol/l end concentration is added to the ghost-containing suspension.

The part of fluorescence of triptophanil groups arranging at a range not more than one Fester radius calculated by formula: $P = (F_0-F)/F_0$, where F_0 is a fluorescence of triptophanil groups before pyrene is added, F is the same parameter after the probe adding expressed in conventional units of fluorescence (CU).

The constant of the degree of relationship between the peripheral proteins with membranes 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, P_2 is the same parameter value for ghosts obtained from erythrocytes washed in NaCl solution.

Membrane microviscosity is estimated according to values of ratios I370/ I470, I390/ I470 fluorescence intensities of pyrene in λ_{ext}=284 nm. The increase of these parameters in erythrocyte membranes testifies the increase of microviscosity or the decrease of the hydrophobic volume of the zone of protein–lipid contacts [11,15, 16].

The values of ratios I370/ I470, I370/ I470 fluorescence intensities of pyrene in λ_{ext}= 340 nm in all investigated samples are measured for estimation of viscosity of lipid bilayer.

The polarity in lipid bilayer is estimated by the I_{370}/ I_{390} ratio of pyrene in λ_{ext}= 340 nm.

All measurements have done in 1cm quartz cuvettes at the room temperature by the spectrometer Hitachi MPF-4 (Japan). The results are expressed in conventional units (CU) of fluorescence.

For each point of measurement the ghosts of erythrocytes isolated from 6 animals are used and each considering point is taken as an average of 10 measurements.

Statistical processing of results is done according to the Student's t-parameter.

Results and Discussion. As stated above, the isolation of erythrocytes and formation of ghosts is carried out in two samples: directly from the mass of erythrocytes after the tree-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 tree-time washing in 0.9%-NaCl solution. The analyses of the results of control simples show that depending on the way of erythrocyte washing the bounding parameters of ANS with the ghosts are changed. So, in the case of washing in NaCl the amount of ANS-bound centers (N) increases by 50.13 % (fig. 1) and the rate constant of reaction (K_c) decreases by 55.26 % (fig. 2) in comparison with the same parameters in the case of Tris-buffer washing. These results allow us suggesting that the NaCl washing of erythrocytes leads to the charge redistribution on the surface of cell membrane, which, on the one hand, brings to the increase of the amount of positive charged centers, which are the targets for ANS bounding. On the other hand, in all probability, the denudation of negative charged groups also takes place, which is clarify the decrease of affinity between ANS and ghosts, consequently K_c.

After 1-hour *in vivo* influence of ESF neither the change of values, nor the change direction of N in investigated samples is revealed compared with control (fig. 1), which testifies that the field influence did not affect the amount of ANS-bound centers.

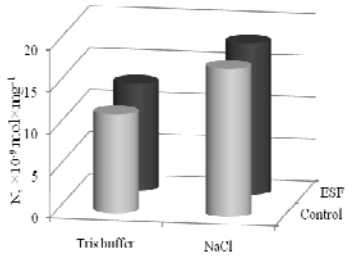

Fig. 1. The amount of ANS-bound centers N the ghosts prepared from the mass of erythrocytes after the washing in Tris-buffer (0.0145 M NaCl in 0.02 M Tris/HCl, pH 7.6) and from the mass of erythrocytes after the washing in 0.9 %-NaCl solution in control samples and after the 1-hour *in vivo* influence of 200 kV/M ESF; in mol/mg proteins

$N(Control_{Tris\text{-}buffer}) = (11.77\pm1.54)\times10^{-9}$; $N(Control_{NaCl}) = (17.67\pm4.01)\times10^{-9}$;
$N\ (ESF_{Tris\text{-}buffer}) = (12.77\pm1.71)\times10^{-9}$; $N\ (ESF_{NaCl}) = (18.04\pm2.56)\times10^{-9}$, n=10

In accordance with obtained data (fig.2), after the influence of field in Tris buffer-washed erythrocyte ghosts K_c practically does not being changed, while in NaCl-washed ghosts it increased by 223.54 %. Comparing these results and taking into account the fact that ANS is a non-penetrative probe and interacts with membrane in the protein-lipid binding sites [19], we can assume that the influence of the field leads to the intermolecular reconstructions in membrane surface layer; in particular, it changes the interactions of peripheral proteins with integral protein molecules and lipid bilayer. At the same time, since ANS is a negative charged probe and Kc directly depends on the affinity between the interacting molecules, we can suggest that due to ESF influence the membrane charge is redistributed. It can be both the result, and the reason of intermolecular reconstructions in bilayer.

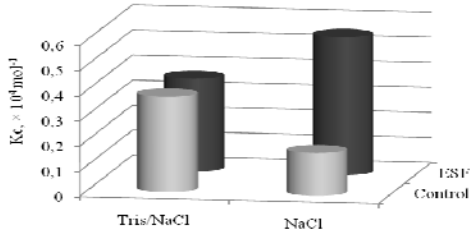

Fig. 2. The rate constant (K_c) of ANS binding reaction with the ghosts prepared from the mass of erythrocytes after the washing in Tris-buffer (0.0145 M NaCl in 0.02 M Tris/HCl, pH 7.6) and from the mass of erythrocytes after the washing in 0.9 % NaCl in control samples and after the 1-hour in vivo influence of 200 kV/M ESF,

$K_c(ControlTris\text{-}buffer)=(0.38\pm0.05)\times104$; $K_c(ControlNaCl)=(0.17\pm0.05)\times104$;
$K_c(ESFTris\text{-}buffer)=(0.37\pm0.11)\times104$;
$K_c\ (ESFNaCl)=(0.55\pm0.05)\times104$, n=10

Erythrocyte-washing in NaCl allows us to reveal the ESF-induced changes, which are not identified in the case of softer treatment of erythrocytes in Tris-buffer. These results, in our opinion, testify about the changes of the relation strength between peripheral proteins and membrane due to the *in vivo* one-hour influence of ESF.

The results of the investigations by use of the membrane-penetrating probe pyrene show that the ESF influence brings to decrease of the immersion degree of membra-

ne proteins in lipid bilayer by 62.58 % (fig.3). In accordance with [18], these results are testified about the increase of the strength of relation between peripheral proteins and membrane, which confirms our above mentioned suggestions.

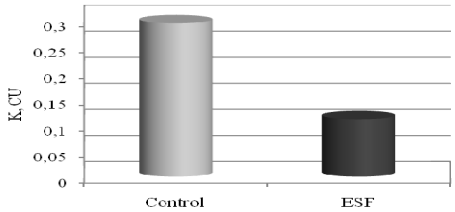

Fig. 3. Immersion degree (K) of peripheral proteins in erythrocyte membranes after the 1-hour in vivo influence of 200 kV/m ESF, CU, K(Control)=0.294±0.04; K (ESF)=0.11±0.03, n=10

The analyses of microviscosity, viscosity and polarity in the depth of membrane ghosts show that the values of investigated parameters do not depend on the way of the preparation of erythrocytes (tab. 1). As it is shown in the same table, the significance changes of microviscosity and polarity in the depth of membranes do not observed, while the viscosity increased in the both Tris buffer- and the NaCl-washed erythrocyte ghosts by 16.48 % and 14.29% simultaneously in compare with controls.

Table 1. Changes of $I_{370}/ I_{47}0$ and $I_{390}/ I470$ ratios of pyrene fluorescence in the excitation wavelength λ=285 nm and I_{370}/ I_{470}, I_{390}/ I_{470} and I_{370}/ I_{390} in the excitation wavelength λ=340 nm after the 1-hour in vivo influence of 200 kV/M ESF 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 , *p<0.05

λ	$\lambda_{emission}$	285 nm		340 nm		340 nm
	$\lambda_{extinction}$	I_{370}/I_{470}	I_{390}/I_{470}	I_{370}/I_{470}*	I_{390}/I_{470}	I_{370}/I_{390}
Control	Tris-buffer	3.69±0.49 n=10	3.25±0.43 n=10	0.91±0.01 n=10	1.19±0.18 n=10	0.79±0.07 n=10
	NaCl	3.41±0.48 n=10	3.46±0.56 n=10	0.91±0.01 n=10	1.18±0.18 n=10	0.79±0.08 n=10
ESF	Tris-buffer	3.54±0.52 n=10	3.61±0.53 n=10	1.06±0.09 n=10	1.355±0.09 n=10	0.78±0.06 n=10
	NaCl	3.99±0.71 n=10	3.78±0.44 n=10	1.04±0.01 n=10	1.215±0.14 n=10	0.86±0.08 n=10

In accordance with literature data, ESF-influence leads to the changes of lipid component and cholesterin/phospholipids ratio in liposomes and nature membranes [17, 21, 6, 8, 22]. It is also well known that the proteins adsorbed on the membrane surface scientifically acted on the structural and functional state of lipid environment of the integral proteins.

The comparison of observed changes of membrane viscosity with the literature data allows us to testify that, apparently, the lipid component of membranes is changed due to the ESF-influence, which can be both the result and the reason of the increase of immersion degree of peripheral proteins into the lipid bilayer. So, supposing that the redistribution of membrane surface charge takes place under the ESF-influence, which in its turn changes the conformation of peripheral proteins and, as a result, its immersion degree into the lipid bilayer increases and leads to the increase of membrane viscosity.

On the other hand, the field influence can change the lipid component of membranes [1], which in its turn can cause the redistribution of membrane surface charge and, as a result, the immersion of peripheral proteins can be changed.

Probably, both processes occur simultaneously, but it is impossible to differentiate the contribution of each of the processes on the base of the results of present study. But we can say with confidence that the investigated factor leads to the redistribution of erythrocyte membrane surface charge, which can be a reason or a result of changes we revealed.

The carried out investigations show that the one-hour *in vivo* influence of 200 kV/m ESF leads to the changes of physical and chemical state of rat erythrocyte ghosts. Particularly, the lipid-protein intermolecular reconstructions occur on the membrane surface, the viscosity of membrane and the strength of relation between peripheral proteins and lipid bilayer are increased.

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