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Carbonylation of Plasma Proteins and Blood Hemolysis Induced by the Action of External Electrostatic Field *In Vitro*

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Abstract

The external electrostatic field (ESF) leads to an increase of oxidative modification of proteins and lipids in blood and in other tissues, as well as to changes in activity of pro- and anti-oxidative enzymes. The mechanism of such a response to the exposure of this physical factor is not well understood. In this paper the data on the influence of external ESF of 200 kV/m on carbonylation of plasma proteins and hemolysis of blood *in vitro* are presented. In order to clarify the role of blood cells in the development of oxidative processes in the plasma, ESF exposures were applied both to the whole blood and to the prior obtained plasma. In addition, samples were placed in closed and in open tubes, which allow to determine the role of air oxygen and air ions in the development of expected processes. It was shown that under the influence of external ESF plasma proteins' carbonylation increased only when the whole blood was exposed to ESF and not the plasma alone. This indicates that the pro-oxidative processes, observed in plasma after ESF exposure, are mediated by the blood cells. Further, lysis and deformation of red blood cells were observed. Moreover, the deformation of red blood cells was most pronounced around monocytes and polymorph nuclear leukocytes, indicating the importance of these cells in the observed processes. The free air exchange had no significant effect on the results of experiments. Thus, *in vitro* experiments have shown that the effects of an ESF to oxidative processes in blood have been likely mediated by the activation of phagocytes independently from air ionization.

Keywords: Electrostatic field; Protein carbonylation; Red blood cells; Hemolysis; Polymorphnuclear leukocytes

Introduction

Studys on the biological effects of external electrostatic fields (ESF) are performed at different levels and in different directions. The importance of such research is dictated by the fact that the external ESF is one of the several environmental factors under constant influence of which all biological objects exist and develop. ESF of natural origin is stipulated by the charge redistribution between the upper layers of the atmosphere and the Earth surface. The tension of natural ESF near the Earth surface in a fair weather is about 150 V/m [1]. Nowadays, the background level of the external ESF is increased due to the widespread use of electrical appliances (both at home and in industry), high-voltage DC transmission lines and production of synthetic fabrics by the textile industry. In certain cases the technogenic background of the external ESF can reach up to 500 kV/m value [2].

The elucidation of the nature of ESF interactions with and mechanisms of action on biological systems is important not only in terms of purely scientific interest, but may also have applied value. In particular, the electrostatic shower or franklisation has been accepted as a therapeutic method, in which the patient has been exposed to a constant electric field generated by a high voltage source (50-150 kV). Therapeutic effect of franklisation apparently is based on polarization of the molecules of dielectric in biological tissues, electrification of conductive structures with the advent of microcurrents, which leads to a redistribution of ions in the area of action and development of irritation effects. An additional effect is the inhalation of negative ions, which are generated near the electrode of negative polarity [3]. At the same time, the "flip side" - namely the negative effects of the influence of external ESF of high tension, the nature and mechanisms of changes in biological tissues in response to the ESF exposure are the problems which require detailed studies. Recently, it was observed an increase of cases of such pathologies as Alzheimer's disease (AD), rheumatoid arthritis, diabetes, sepsis, chronic renal failure, and respiratory distress syndrome [4]. Oxidative stress – the imbalance in pro-/antioxidative processes towards prevailing of prooxidant reactions, is considered as one of the etiopathogenic factors of these diseases [4]. There is increased experimental evidence, that externally applied ESF exerts various effects on oxidative processes and antioxidative defense systems. As it was reported previously [5], the external ESF (E=500 V/m) leads to the increase of intracellular reactive oxygen species (ROS) concentration. It was shown strong ESF caused transient inhibition of antioxidant enzymes activity in red blood cells (RBC) with subsequent adaptive stimulation of this activity after the end of exposure cycle [6]. Data provided in literature show an enhancement of the activity of superoxide dismutase (SOD) and increase of malonic dialdehid (MDA) content in plasma, liver, lung, and kidney tissues of guinea pigs after exposure to 0,8-1,8 kV/m ESF [7-8]. In our previous studies SOD activity increased with simultaneous decrease of catalase (CAT) activity in blood plasma and hemolisate of rats exposed to ESF (E=200 kV/m). Such a ratio of SOD and CAT activities might bring to H_2O_2 accumulation followed with enhanced oxidative damage of lipids and proteins in blood [1]. Subsequently, an enhancement of protein carbonylation and modification of proteome in blood plasma and serum was found at rats after ESF exposure [9-12]. It was also shown enhanced activity of phospholipase A_2 in erythrocyte membrane at rats exposed to ESF (E=200 kV/m) which might indicate the activation of lipid peroxidation scavenging system [13]. All investigations of biological

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effects of ESF mentioned above were based on *in vivo* experiments. The goal of the current study was to clarify *in vitro* influence of the external ESF of 200 kV/m tension on plasma proteins carbonylation and blood hemolysis.

Methods

The study object and experimental design

The experimental animals were noninbred male rats weighing initially 250 to 300 g (2 months old); they were fed a standard diet (ration of food value from natural foods). Blood collection was initiated by the heart puncture using syringes washed with heparin. All manipulations with animals were done in accordance with necessary requirements of bioethics and were approved by the local Ethical Authority of Yerevan State Medical University. Blood, collected from 6 animals were combined into one. Total volume of whole blood was 16 ml. With the goal to clarify the role of cellular factor in the development of oxidative processes in the plasma, ESF exposure was applied both to the whole blood and to the plasma free of cells. Thus, blood cells were removed before (Part 1) or after (Part 2) the exposure of ESF (centrifugation, 2800 × g, 10 min). In order to elucidate the impact of the air ions in ESF-dependent modification of oxidative processes samples of the whole blood and the plasma were placed in the open tubes (free air exchange) or in the closed tubes (no air exchange). Pilot samples were subjected to the exposure of ESF (E=200 kV/m) during an hour. ESF was generated in the special capacitor type device, which was designed on the basis of X-ray apparatus "RUM-17" (former USSR) [14]. Control samples were treated in the same manner with the exception of ESF exposure. Design of the experiment is detailed in the Table 1.

Measurements

Carbonylation of proteins was measured in the samples of blood plasma using the reaction with 2, 4-dinitrophenylhydrasine (DNPH) by the method of Levine [15]. Results were expressed in terms of nmoles of carbonyl groups × mg^{-1} of protein. Total protein content in plasma was determined by biuret reaction. The protein concentration was expressed in terms of mg × ml^{-1} of plasma. CAT activity in plasma was measured spectrophotometricaly by determination of the rate of H_2O_2 specific absorbance (l=240 nm) decrease in accordance to the method of Beers and Sizer [16]. The enzyme activity was expressed by means of nmol H_2O_2 min^{-1} mg^{-1} of protein. The rate of blood hemolysis was determined spectrophotometricaly by measurement of plasma absorbance at l=550 nm [17]. Results were expressed by means of units of the optical density (OD). All spectrophotometrical measurements were done using spectrophotometer Specord M40 (Analytik Jena, Germany). The RBC content in blood was determined by visual counting of cells in the Goryaev's counting chamber using the light microscope "Micros" (Austria) at magnification 400×. Blood smears were prepared by the standard method and stained with azur-2-eosin by Romanovski-Giemsa. 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). Two independent groups were compared using Mann-Whitney U test. All data were expressed as mean ± standard error of mean (M ± m). P-value<0.05 was considered statistically significant. n=5.

Results and Discussion

The degree of protein carbonylation is a sensitive indicator reflecting the intensity of oxidative processes in tissues [18]. Therefore, in order to identify the effect of an external ESF on the system of pro-/antioxidant homeostasis of blood we have been mainly based on the data of plasma protein carbonylation. Blood, being a liquid tissue, contacting to almost all organs and tissues, is also a convenient object for research, since the state of the blood to some extent reflects the state of the whole organism. This was the reason for the choice of the object of study. Antioxidant protection of blood is represented by enzymes localized inside the cell (SOD, CAT, peroxidase, etc.) – specifically neutralizing ROS and compounds represented both inside the cell and in blood plasma (albumin, vitamin C) – non-specifically neutralizing the ROS excess [15,19-20]. The starting compound responsible for the formation of ROS is the oxygen (O_2), which is gradually being reduced and converted to superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), and other compounds having a high reactivity and therefore can damage the various molecular and supramolecular structures of the body [21]. Thus, in experiments *in vitro*, air oxygen might have a significant effect on oxidative processes in the blood. However, we did not find any significant differences in the carbonylation of plasma proteins between samples placed in open and closed tubes (Figure 1). This fact is apparently due to the buffer capacity of the antioxidant defense of blood. At the same time, external ESP differently reflected on the carbonylation of plasma proteins, depending on the whole blood (Part 2) or the plasma (Part 1) was exposed.

The effect of ESF on the level of plasma protein carbonylation

After ESF exposure the protein carbonylation in plasma had a tendency to decrease (-15%; P>0.05), compared with similar control samples (Figure 1). Furthermore, in the control samples (without exposure of ESF) alterations of plasma protein carbonylation also observed. So, carbonylation of the plasma proteins at the sham exposed (during an hour) plasma samples (Part 1) was higher by 33-52% (P

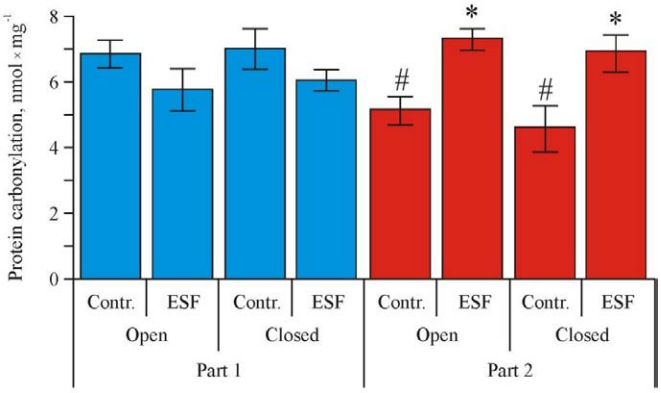

Figure 1: Effect of the ESF (E= 200kV/m, 1 hour) to the content of carbonyl groups of the plasma proteins in the experiments *in vitro*. Part 1 - The blood cells were removed prior to the exposure; Part 2 - the blood cells were removed after the exposure. *- P value versus appropriate control sample; #- P value against appropriate sample of part1. n =5.

Part 1 (blood cells were removed before ESF exposure)				**Part 2** (blood cells were removed after ESF exposure)			
Open (samples were placed in the open tubes)		**Closed** (samples were placed in the closed tubes)		**Open** (samples were placed in the open tubes)		**Closed** (samples were placed in the closed tubes)	
Control	ESF	Control	ESF	Control	ESF	Control	ESF

Table 1: Experimental design.

<0.05) compared to carbonylation of the plasma proteins at the sham exposed whole blood samples (Part 2) (Figure 1). This indicates the importance of the antioxidant system of blood cells. From this point of view it seems contradictory that after plasma exposure in ESF (Part 1) carbonylation was not increased, as it was shown in the control samples, but was decreased compared to the results obtained in similar samples in which the whole blood was exposed in ESF (Part 2). Lower values of plasma proteins' carbonylation in test samples of part 1 may be attributed to the following factors. Firstly, the ESF can lead to an activation of the antioxidant system of the plasma; and secondly, the ESF may accelerate proteolysis of oxidatively damaged proteins and thus the carbonylation of remaining proteins can be lower. To clarify the validity of the assumptions made, we have measured the total protein content in the blood plasma and the activity of CAT - as one of the major antioxidant enzymes in plasma. ESF exposure did not affect significantly (P>0.05) the total protein content in plasma at the samples of Part 1 (Figure 2). This fact indicates that proteolysis may contribute to decrease of the carbonylation of proteins. It should also be noted that the total protein content in the plasma samples placed in an open or closed tubes were almost equal. CAT activity in plasma at ESF exposed samples (Part 1) did not differ significantly from appropriate control samples (P>0.05) (Figure 3).

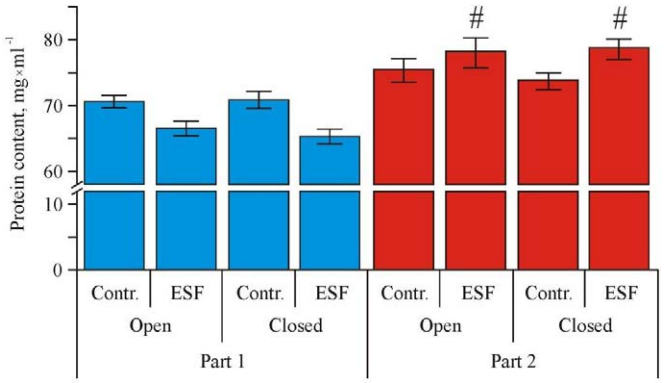

Figure 2: Effect of the ESF (*E*= 200kV/m, 1 hour) to the total protein content in blood plasma in the experiments *in vitro*. Part 1 - The blood cells were removed prior to the exposure; Part 2 - the blood cells were removed after the exposure. *- P value versus appropriate control sample; #- P value against appropriate sample of part 1. n =5.

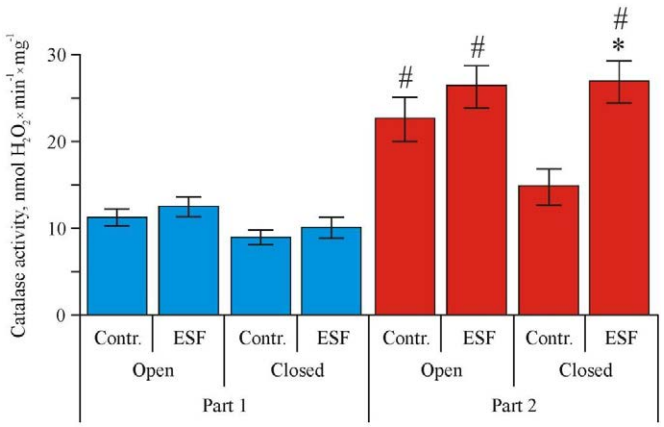

Figure 3: Effect of the ESF (*E*=200kV/m, 1 hour) to the CAT activity in blood plasma in the experiments *in vitro*. Part 1 - the blood cells were removed prior to the exposure; Part 2 - the blood cells were removed after the exposure. *- P value versus appropriate control sample; #- P value against appropriate sample of part1. n =5.

In contrast to the data obtained after the plasma exposure in ESF, the whole blood exposure in ESF during an hour (Part 2) was reflected with significantly enhanced carbonylation of plasma proteins (+42-50%; P <0.05) compared to similar control samples (Figure 1). Furthermore, there was a slight increase of the content of total protein - by 4-6% (P> 0.05) (Figure 2), and the increase of CAT activity - by 16% (P> 0.05) - in samples placed in open test tubes; and by 77% (P <0.05) – in samples placed in closed tubes (Figure 3). This fact indicates that the air oxygen makes some changes in the specific pro-/anti-oxidant system. In our studies exposure to air expressed only on the values of CAT activity in plasma and only in the control samples of the part 2 (whole blood exposure). ESF also leads to an increase in CAT activity in plasma, placed in both open and closed tubes - thus overlapping the effect of air oxygen. In other words the impact of ESF on the investigated parameters apparently was independent of the air ions.

Thus, data obtained in samples of part 2 demonstrated the importance of the cellular factor in modification of the oxidative processes in plasma. In this particular case we are talking about intensification of prooxidant processes in blood in response to the action of an external ESF. As it is known, the main ROS-generating enzymes in the blood cells are NAD(P)H oxidases [22]. Earlier, in experiments *in vivo*, we have shown the increase of the activity of NADPH oxidase in polymorphonuclear leukocytes (PMNL) [23] and the increase of the activity of NADH oxidase in RBC of the peripheral blood of rats [9]. It is natural to assume that similar processes may also take place at the influence of ESF on isolated blood samples - in the experiments *in vitro*. It was found that the generation of ROS by leukocytes, especially PMNL, increased significantly at their activation [24]. Generated ROS can damage different supramolecular structures. In particular plasma membrane damage can lead to cell death. In our experiments this was manifested by an increase of CAT activity in plasma after the initial whole blood exposure in the ESF. It is well known that CAT is an intracellular enzyme [25]. Therefore, increased CAT activity in plasma can be interpreted as a consequence of enhanced permeability of plasma membrane or cell lysis/death - in particular RBC. We did not observe any statistically significant difference between samples placed in open and closed tubes which mean negligible effect of the air oxygen to mentioned parameters. Thus, based on the data obtained in the part 2, it can be assumed that the ESF apparently leads to activation of leukocytes, and consequently intensifying the generation of ROS. This causes an increase of carbonylation of plasma proteins and the blood hemolysis, which was manifested in CAT activity increase in plasma. In order to verify the assumptions made, we investigated parameters characterizing blood hemolysis: the content of RBC in blood and hemoglobin in the plasma. Also we made an analysis of stained blood smears to identify damaged RBCs.

The effect of ESF on blood hemolysis

RBC count in blood samples exposed in ESF for an hour did not differ significantly from that in the control samples (Рис. 4). Only a tendency to decrease of RBC count in blood was noted (-3%; P>0.05). The high inaccuracy of the data neither allows to state the fact of ESF induced hemolysis, nor determines the exact number of lysed RBCs. It is supposed that the number of lysed RBCs in blood samples exposed to ESF has the order of 10^8 cells × ml^{-1} (Figure 4). Data on CAT activity in plasma also indicate that the hemolysis occurred in blood samples exposed to ESF. Hemoglobin content in plasma was measured to clarify the degree of hemolysis as the backup "reverse" test.

Measurement of the optical density of plasma at l=550 nm showed a significant level of hemolysis induced by an external ESF. Thus, the optical density of the plasma in ESF exposed samples exceeded that

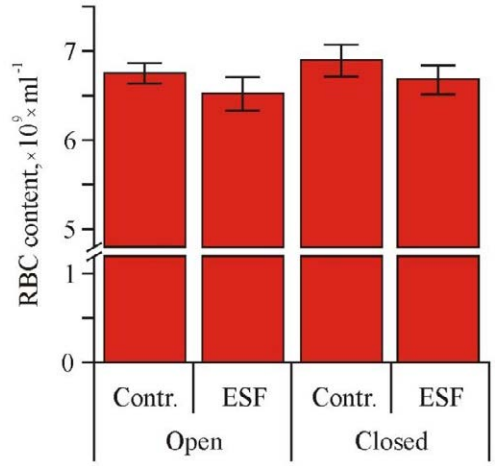

Figure 4: Effect of the ESF (*E*= 200kV/m, 1 hour) to the RBC content in blood in the experiments *in vitro*. n = 5.

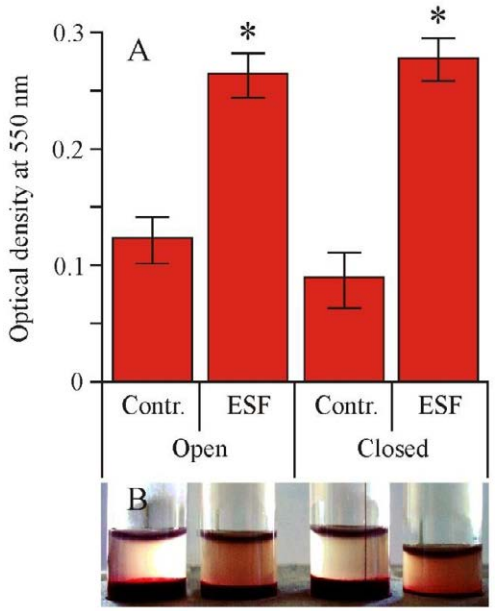

Figure 5: Effect of theESF (*E*= 200kV/m, 1 hour) to the hemolysis of blood in the experiments *in vitro*. A - graphical representation of data; B - the picture of the appropriately treated samples. *-P value versus appropriate control sample. n=5.

parameter in the similar control samples by 115% (P<0.05) - in the open tubes; and by 215% (P<0.05) - in the closed tubes (Figure 5). Smaller difference in the samples placed in the open tubes was stipulated by the fact that a certain degree of hemolysis occurred also in the appropriate control samples. The optical density of the plasma at l=550 nm allowed us to calculate the number of lysed RBCs by the following equation:

$$C_{RBC}=((\Delta OD/e) \times M)/b$$

Where CRBC - the concentration of lysed RBCs ($\times 10^8 \times ml^{-1}$); ΔOD - the difference in absorbance between the control and test samples (0.139 and 0.189 – for the samples in open and closed tubes respectively); *e* - molar extinction coefficient of oxyhemoglobin (HbO_2) at l=550 nm ($14 \times 10^6 \times mol^{-1} ml^{-1} cm^{-1}$) [26]. M - molecular weight of hemoglobin (6.45×10^{16} pg mol^{-1}); b - the average content of hemoglobin in one RBC (20 pg). After substitution of the appropriate values of the variables the following results were obtained: 0.32×10^8 lysed RBCs $\times ml^{-1}$ - for the ESF exposed samples placed in the open tubes; 0.44×10^8 lysed RBCs $\times ml^{-1}$ - for the ESF exposed samples placed in the closed tubes. The calculated data agree well with the data obtained by direct counting of RBCs in the blood and determining the activity of CAT in the plasma.

Lysis of RBCs in the samples exposed to ESF apparently is stipulated by the damage of their plasma membranes which may be caused by the exposure of ROS. Furthermore, ROS may be generated by both RBCs and other blood cells. It should be noted that hemolysis was the extreme consequence of the impact of ROS to the plasma membrane of RBCs. The intermediate stage of RBCs' damage appears evident

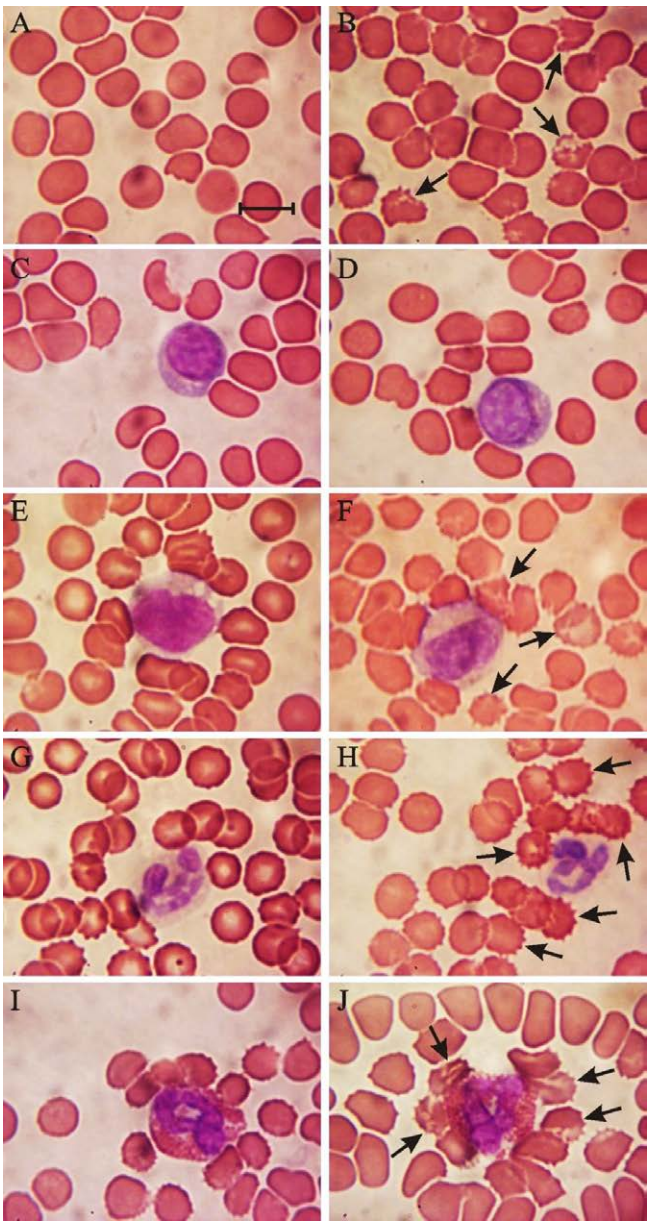

Figure 6: Effect of the ESF (*E*=200kV/m, 1 hour) to the blood cells morphology in the experiments *in vitro*. A,B - RBCs; C,D - RBCs+lymphocytes; E,F - RBCs+monocytes; G,H - RBCs + neutrophils; I,J - RBCs+eosinophils. A,C,E,G,I - control samples of blood; B,D,F,H,J - blood samples exposed to the ESF. Blood smears were stained with Romanovsky-Giemsa. Light microscopy with magnification 2250×. Bar - 10 nm. The arrows indicate the RBCs that have abnormal shape.

in their deformation. Thus, oxidative damage of membranes leads to various forms of abnormal RBCs - elliptocytes, echinocytes, knizocytes, codocytes etc [27]. In order to identify the RBCs' deformation we analyzed the blood smears. Deformed RBCs - mainly echinocytes with a plurality of evenly sharp edges really were detected in the samples exposed to ESF. Clusters of echinocytes were mainly observed around monocytes, neutrophils and eosinophils, i.e. the cells capable of phagocytosis (Figure 6). The deformation of red blood cells was most pronounced around the neutrophils of blood samples exposed to ESF. Slight deformation of red blood cells was also observed around the neutrophils of the control blood samples. Around lymphocytes the deformed RBCs were not observed. The data provided above suggest the assumption that the external ESF (E=200 kV/m) leads to the activation of phagocytes, which in turn leads to increased generation of ROS and all subsequent pro-oxidant processes in the blood. It has been described above - in the experiments *in vitro*, and in the experiments *in vivo* observed earlier.

Conclusion

A significant increase of the carbonylation degree of plasma proteins was shown after exposure of whole blood of rats in the ESF (E=200 kV/m, 1 h) to the *in vitro* experiments. At the same time no significant effect was detected if only the plasma was exposed to the ESF. Similar results were observed for CAT activity in the plasma, indicating a key role of the blood cells in ESF-induced modification of oxidative processes in the plasma. Contact of the samples with the air had no significant influence on the effects induced by an external ESF. Nevertheless, some fluctuations of data were observed in the control samples which led to a certain distortion of results of investigations. Exposure of whole blood in the ESF also causes a deformation and the lysis of RBCs. The deformation of RBCs was most pronounced around the phagocytic leukocytes (neutrophils, monocytes, eosinophils), which indirectly indicated the ESF-induced activation of blood leukocytes. The last was manifested in the damaging of the neighboring RBCs, which likely was a result of increased generation of ROS.

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