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Original paper

Opposite effects of electroporation of red blood cell membranes under the influence of zinc ions

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The goal of the study was to investigate the effects of zinc ions of various concentrations on the nanostructure of membrane of red blood cells in in vitro experiment. The suspension of red blood cells extracted from whole human blood was used.

The calibrated electroporation and the atomic force microscopy (AFM) were used to analyse damage to membrane nanostructure. We studied the haemolysis after the electroporation at different zinc concentrations. A low concentration of zinc (0.15–0.5 mM) increased significantly the rate of haemolysis and reduced the residual level of non-haemolyzed cells. At high concentrations of zinc ions (0.5–10 mM), the rate constant was sharply reduced, at the same time the residual level increased. The relationship between haemoglobin coagulants and the zinc concentration was examined. High concentration of zinc caused haemoglobin aggregation. It was shown by AFM that the membrane nanostructure was essentially changed. It was experimentally established that there existed a special point of zinc concentration $C = 0.5 \pm 0.1$ mM at which the course of the conjugate processes on the membranes of red blood cells was changed.

Key words: *red blood cells, membrane, electroporation, zinc, AFM, competitive processes*

1. Introduction

All living creatures are composite dynamic systems, in which several processes take place simultaneously. The processes can have different kinetics and directions, and the result of their action is very difficult to predict. In this article, we studied the effect of only one factor, i.e., zinc ions, on the erythrocyte membrane. Different concentrations of zinc cause simultaneous competitive processes, which can change significantly the result of the action, up to an opposite result.

Zinc is one of the essential metals in biological systems as it plays the role of active centers of enzymes and supports the structure of the globular proteins. Zinc binds with several centers of protein and can induce changes in the conformation of the secondary structures [1] and their aggregation. In the body, zinc can have antioxidative effect [2], [3]. On the other hand, an excess of zinc ions in the blood may be responsible for anemia and the haemolysis of red blood cells [4]–[6].

Zinc is used in model studies to examine the aggregation and clustering of proteins. Its ions can interact with the internal domain of band 3, which participates in the connection between spectrin and cell membrane and induces their clustering [7]. The cytoplasmic domain of band 3 can interact with haemoglobin [8]. Zinc-modified haemoglobin can be coagulated [9].

The processes of the clustering of band 3 proteins in the membrane, aggregation of haemoglobin in the cell, formation of the membrane-attached haemoglobin (Hbm) can proceed simultaneously and with different kinetics. Therefore the resulting changes of the

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structure and properties of membranes can be various and essentially dependent on zinc ion concentration.

The results of experimental research can provide us with the possibility of establishing biophysical mechanisms of zinc ion effect on the membranes of biological cells. This will be important for understanding the cell toxicity in human organism exposed to metal ions, for example, under adverse environmental conditions or while working at industrial plants.

The effective methods of studying the effect of ions on erythrocyte membranes are the calibrated electroporation [10] and the atomic force microscopy (AFM). These methods allow us to record the surface of erythrocyte membranes in the nanoscale and to analyze the disturbance of the kinetics of the membrane functioning, to assess quantitatively damages and to consider their statistical distributions.

The goal of the study was to investigate the effects of zinc ion action of various concentrations on membrane nanostructure of red blood cells in in vitro experiment.

2. Materials and methods

The experiments were conducted with the suspension of red blood cells extracted from the whole human blood of four healthy males aged between 27 and 32. Blood sampling was performed voluntarily during preventive examination. Institutional Ethics Committee approval was obtained. Blood was placed in test tubes with K3 EDTA coagulant. A suspension of blood was prepared in a buffer solution, i.e., “Diahim-Buffer-G”. This phosphate whose pH ranges from 6.8 to 7.2 is used in haematology. Then the suspension was thermostated at 19 °C during 60 min.

2.1. The calibrated electroporation of erythrocyte membranes

Electroporation of cells is the process of a thorough formation of non-reversible pores in the membranes by an externally applied pulsed electric field. It occurs when the directed transmembrane potential $\Delta\varphi_m$ exceeds some critical value of the membrane breakdown potential $\Delta\varphi_{cr}$:

$$\Delta\varphi_m > \Delta\varphi_{cr} . \quad (1)$$

The value $\Delta\varphi_{cr}$ is determined based on the properties of the membrane, in particular, based on an average number of “active centers” (pores, lesions, heterogeneousness and other defects), which are present in its normal state. The external factors, i.e., drugs, chemicals, ionizing radiations, can break the structure of membranes due to an increase in the number of “active centers”. In this case, the value $\Delta\varphi_{cr}$ is decreasing. If an external factor heals defects, the value $\Delta\varphi_{cr}$ increases. The value $\Delta\varphi_m$ is determined based on the strength of external electrical field E:

$$\Delta\varphi_m = 1.5rE\cos\Theta ,$$

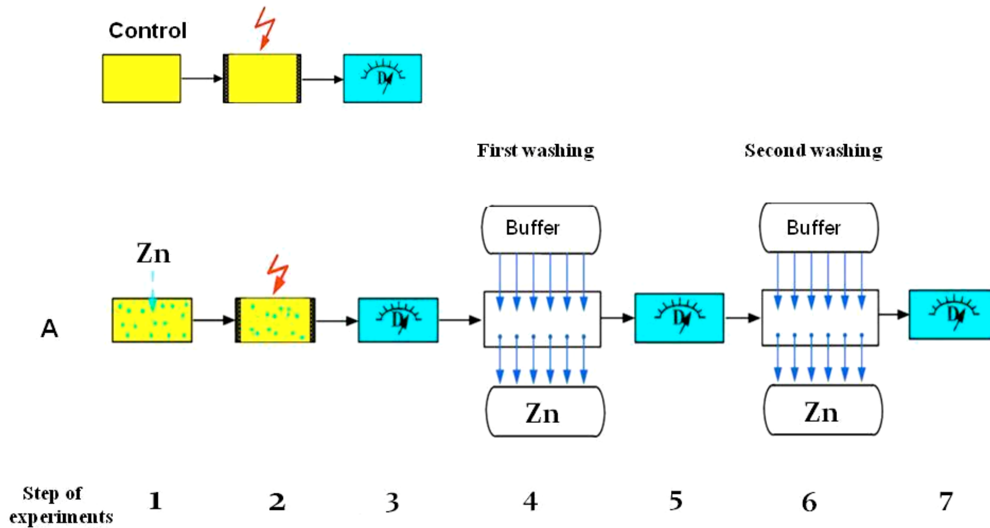

Fig. 1. Diagrams showing the experiment steps: *step 1* – preparation of control suspension (the control group) and suspension with zinc (A series), *step 2* – electroporation, *step 3* – measurement of optical density $D(t)$, *step 4* – the first zinc wash-out: centrifugation, collection of supernatant fluid, replacing it with a buffer solution, *step 5* – measurement of optical density $D(t)$, *step 6* – secondary zinc wash-out: centrifugation, collection of supernatant fluid, replacing it with a buffer solution, *step 7* – measurement of optical density $D(t)$; control – the control suspension, arrow –electrical impulse

where:

r is the radius of cell,

Θ is the angle between the radius-vector and the vector of electrical field strength $\vec{E}$.

This method was employed as follows. The clinical defibrillator «Lifepak-7» (USA) as a source of pulsed electric field was used. The electric impulse was applied by means of the titanium electrodes placed in quartz cuvette being filled with 2.4 ml of erythrocyte suspension. The distance between power electrodes was 1.7 cm. The electrodes covered the opposite sides of cuvette providing the uniformity of electric field in the solution (figure 1, step 2). The resistance of suspension was 100 ± 5 Ohm, the impulse duration – 10 ms, and energy – 200 J which corresponded to the field strength in the solution (1100 V/cm) [10], [11].

2.2.The kinetics of haemolysis

If the condition (1) in suspension is fulfilled, the osmotic haemolysis of red blood cells occurs. A decrease in a number of erythrocytes $n(t)$ due to haemolysis results in a decrease in the optical density of the suspension $D(t)$. At small concentration of the solution a linear dependence is observed:

$$D = knl,$$

where:

k is the coefficient of attenuation,

n is the concentration of erythrocytes,

l is the thickness of the layer of suspension.

The dependence $n(t)$ after the electroporation is the exponential function [11]:

$$n(t) = (n_0 - n_{\text{res}})e^{-\beta t} + n_{\text{res}},$$

where:

n_0 is the initial number of erythrocytes,

n_{res} is the total number of non-haemolyzed erythrocytes (residual level),

$(n_0 - n_{\text{res}})$ is the total number of haemolyzed erythrocytes,

β stands for the rate constant of a decrease in erythrocyte number due to haemolysis.

The plot $D(t)$ is called the kinetic curve of haemolysis.

The dependence $D(t)$ was registered by the Apel PD-303 spectrophotometer (Japan). The concentration of the suspension was adjusted so that it had an initial optical density $D = 1.0$ at the wavelength $\lambda = 760$ nm.

2.3. Capturing and analysing images by AFM

The monolayer of red blood cells was formed at room temperature on microscope slides by the «DiffSpin-2» centrifuge (USA). This specialized centrifuge is intended for the formation of blood cell monolayers. Samples were air-dried. The images of the surface membrane were obtained by using the atomic force microscope (AFM), «Femtoscan» (RF), in the mode of the continuous scanning with the mathematical software of the microscope. The force during scanning ranged from 0.1 to 5 nN. The number of scan points was 512, and the scanning fields were as follows 10 × 10 µm, 1500 × 1500 nm, 800 × 800 nm and 150 × 150 nm.

Of an initial surface we selected the surfaces of three orders to obtain informative characteristics of the processes investigated [10]. For this purpose we used the spatial Fourier transform of the original surface with three spectral windows. The first order of surface conformed to the spatial spectral window with L_1 period in the range of 1000–600 nm. The second order: L_2 – 600–80 nm and the third order L_3 – 80–10 nm. The initial profile and the image of the original surface were reinstated by the reverse addition of profiles and the surfaces of the first, second and third orders [11].

In our articles [11], [12] and in the articles of other authors [13], it is shown that the sizes of nanostructures of the cell membranes are the natural parameters of the membranes. They contain information, which is characteristics of the membrane, and information about changes of its characteristics after endo- and exogenous influence on blood cells.

2.4. The diagrams of experiments

Further the concentration of $ZnSO_4$ is marked with $C = i$ (mM) in view of the fact that these are the final values of salt concentrations in solution. Zn^{2+} ions in the further text are simply marked with Zn.

2.4.1. Diagram 1. The haemolysis due to electroporation at different Zn concentrations (figure 1, steps 1–3)

$ZnSO_4$ was added to the suspension in such a way that the salt concentration in solution was: $C = 0$ (control); 0.15, 0.3, 0.4, 0.5, 1.0, 2.0, 6.0, 10.0 (mM) (*step 1*).

For this purpose we prepared the basic solution with a high concentration of $ZnSO_4$ in the 0.9% saline solution. 3 ml of the erythrocyte suspension with a known haematocrite were poured into each cuvette. The basic solution with zinc was added to each cuvette to obtain the corresponding concentrations of Zn ions. In order to obtain the control curve of haemolysis, the erythrocyte suspension without zinc was used $C = 0$ (figure 1).

Red blood cells present in the solutions of the known concentrations of zinc (Series A and control) were subjected to electroporation (*step 2*). We recorded the kinetic curves representing the erythrocyte haemolysis, and then we calculated the rate constants (*step 3*). According to diagram 1 we carried out 3 tests with the blood of each of the four donors – a total of 12 experiments. In each experiment, we obtained nine correlations $D(t)$ for various concentrations of zinc. 108 kinetic curves of haemolysis were registered.

2.4.2. Diagram 2. The haemolysis after electroporation and Zn washing-out (figure 1, steps 1–7)

In series A (figure 1), the concentration $C = 6.0$ mM was used, at which the haemolysis stopped even after the electroporation in the experiments according to figure 1. The experiments were performed in the following steps.

Zinc at the concentration $C = 6$ mM was added into the original erythrocyte suspension (*step 1*). Then, the solution was subjected to the electroporation (*step 2*), the kinetic curves of $D(t)$ representing haemolysis of red blood cells were registered (*step 3*). In *step 4*, the solution was centrifuged in a «HettichMikro 220R» (Germany) – 1200 g, 5 minutes. Thereafter we removed the supernatant fluid with the dissolved Zn and replaced it with the buffer solution of the same amount. Then we registered the kinetic curves $D(t)$ of haemolysis (*step 5*). Afterwards, we centrifuged and conducted the secondary selection of the supernatant fluid with Zn and replaced it with buffer solution (*step 6*). Then, we eluted gradually zinc ions from the solution and after the secondary selection of zinc ions we registered the optical density of solution (*step 7*). According to diagram 2, we conducted three experiments of series A, one control experiment on blood from four donors – in total 12 experiments.

Two experiments with the blood of each donor taken for analysing the nanostructure of the erythrocyte membranes exposed to different Zn concentrations were conducted. We scanned 2–3 cell images in the field of 10 × 10 μm from the samples of each experiment with AFM. Then, we scanned three fragments in the field of 1500 × 1500 nm for each cell, and selected the fields of 1000 × 1000 nm and smaller than these fragments. 126 images were obtained and analyzed. Thus, we analyzed the effect of different Zn concentrations on the nanostructure of erythrocyte membranes and the effects of the washing of zinc ions and compared the surfaces of the corresponding orders of the control samples and the membranes after these procedures.

The samples and their quantity for each series of experiments were arranged according to statistical representativeness. The results were statistically processed by using the «Origin» program, errors for ensembles were calculated and the significances of the differences between the results of all stages of the experiment were estimated.

3. Results

3.1. Electroporation

Figure 2 shows the results of experiments on diagram 1 – the calibrated electrocorporation of erythrocyte suspension at different concentrations of zinc solution. We used the following concentrations: $C = 0$ (control), $C = 0.15, 0.3, 0.4, 0.5, 1.0, 2.0, 6.0, 10.0$ (mM). The kinetic curves are presented for blood donor number 2.

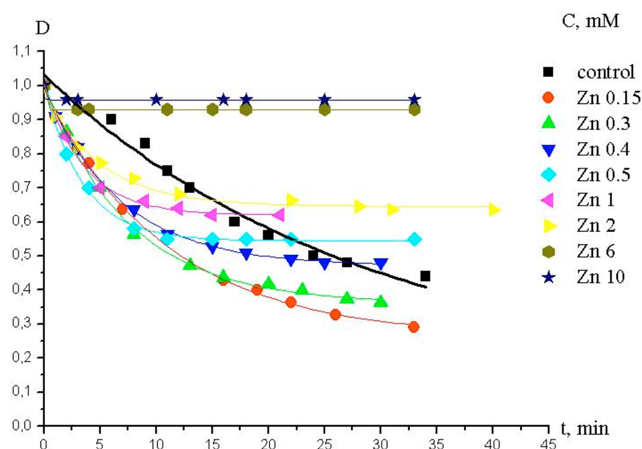

Fig. 2. Kinetic curves $D(t)$ of erythrocyte haemolysis after electroporation at different concentrations of zinc ions. D – optical density, t – time after electroporation. At zinc ion concentration of 0.5 mM haemolysis was faster than that of control. At zinc concentration higher than 0.5 mM haemolysis slowed down, being slower than that in the control, and at $C = 6$ mM and higher haemolysis stopped

The optical density of the control suspension was reduced to the level of 0.7 for 13 minutes. If the Zn concentration increased from 0.15 to 0.5 mM, the haemolysis accelerated and the rate constants increased. The kinetic curve for the concentration of 0.5 mM reached the point D = 0.7 faster than another curves. After that, when the zinc concentration increased, the haemolysis was slow; at the concentration of 6 mM and higher, the haemolysis stopped.

The kinetic curves reached the residual level of n_{res}, which remained constant during the next two hours. The residual level n_{res} increased with an increase in concentration from 0.32 (at the concentration C = 0.15 mM) to 0.7 (at the concentration C = 2 mM) and to 0.9 (at the concentration C = 6 and 10 mM).

The dependence of the rate constants β_n of haemolysis normalized to the control on the Zn concentration is shown in figure 3a. The rate constant β of haemolysis increased at the concentration C = 0.5 mM. At this point, the value β_n was maximal and approximately seven times higher than that of the control. Then it dropped sharply at C = 4 mM. When C reached 6 mM, the rate constant remained invariable, close to zero. If the concentration C was higher than 6 mM, we determined β for decreasing the optical density during 40 minutes.

3.2. Non-haemolyzed cells and coagulants of haemoglobin

If the haemolysis did not occur after the electroporation at zinc concentration C = 6 mM, the questions arose: What substrates did remain in suspension, which could prevent the haemolysis?

To answer this question we added the solution of Zn into 50 μl of blood and placed into 3 ml of distilled water. The final concentration of zinc in the solution with 0.7% haematocrit was 1 mM. It is obvious that the haemolysis of erythrocytes must occur in the absence of zinc ions (optical density of erythrocyte ghosts at a wavelength λ = 760 nm is not hihger than 0.03). However, for C = 1 mM the optical density D at the wavelength of 760 nm was 0.95. Consequently, the optical density 0.95 (λ = 760 nm), which was obtained in the experiment, was determined either by the light scattering by the non-haemolyzed erythrocytes, which remained in solution, or by the coagulants of haemoglobin, which appeared due to the zinc ion influence.

Non-haemolyzed cells. Is it possible to maintain the rest of non-haemolyzed cells in the suspension under the existing conditions? For this study the AFM images of corresponding smears were obtained.

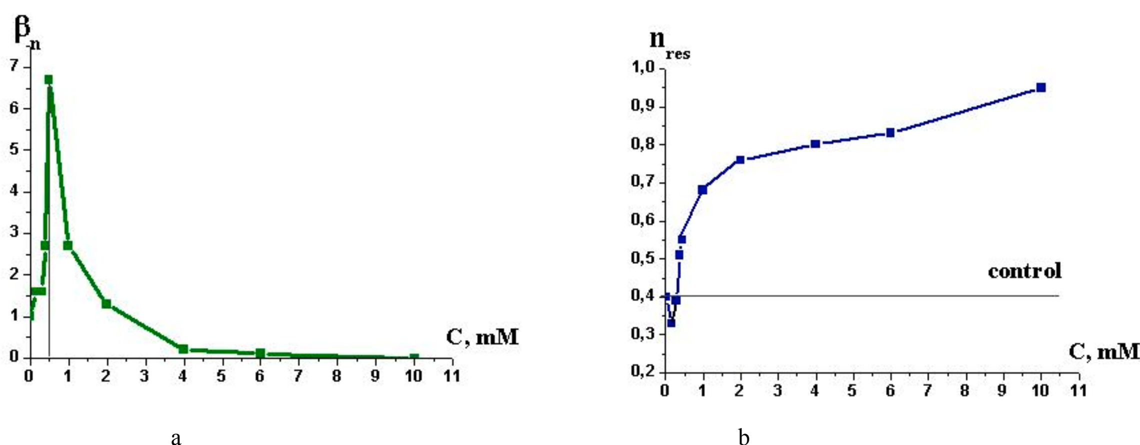

Fig. 3. The parameters of haemolysis kinetics in erythrocyte suspension after electroporation:
a) the dependence of the rate constant β_n normalized in controls on the zinc concentration C,
b) the dependence of residual level of non-haemolyzed erythrocytes n_{res} on the zinc concentration C.
Control – residual level of non-haemolyzed erythrocytes in control suspension

Figure 3b shows the dependence of the residual level of non-haemolyzed erythrocytes n_{res} on zinc concentration. Horizontal line (control) represents n_{res} for the control suspension. The residual level was lower than that of control as long as the concentration C = 0.3 mM, after reaching the control point it continued to rise steadily up to 0.9 mM.

Two of these images in 3D and their profiles are shown in figure 4. In this figure, the non-haemolized red blood cells have standard dimensions and slightly distorted form. The dimensions of each of these cells

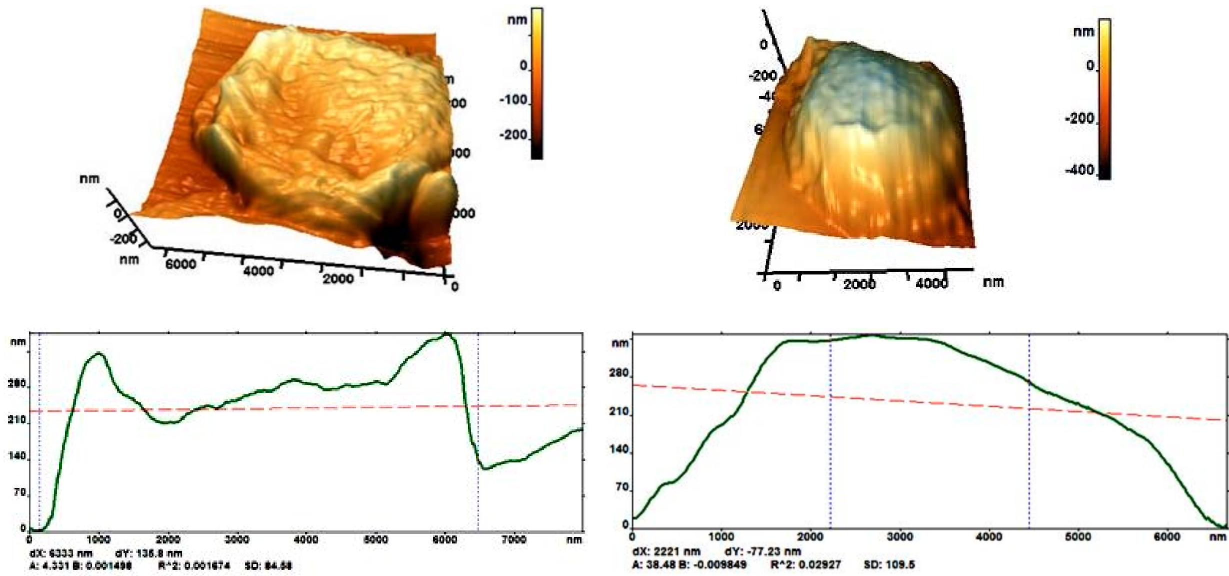

Fig. 4. Red blood cells, which were non-haemolyzed in the distilled water as the result of zinc action. Two red blood cells and their profiles, obtained by AFM, are represented in 3D. In the solution with 0.7% haematocrit, the final concentration of zinc $C = 1$ mM. Non-haemolyzed erythrocytes have standard sizes and are partially deformed. The diameters of each of them range from 6400 to 6800 nm and their heights exceed 300 nm

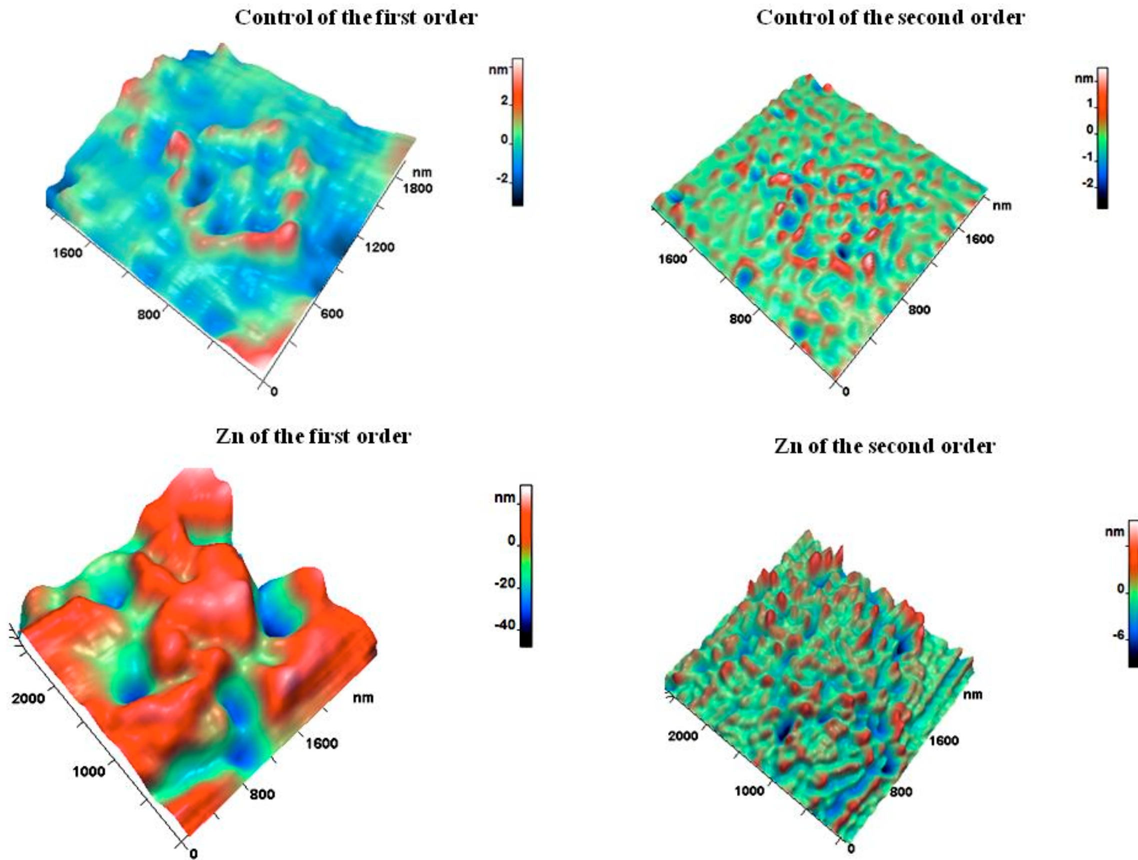

Fig. 5. AFM images of membrane nanosurfaces in 3D format for the 1st and 2nd orders for control ($C = 0$) and for zinc concentration of 0.5 mM. Scales of heights and scales of scanning are given

ranged from 6400 to 6800 nm, the height was over 300 nm. The first of them assumed the shape of echinocyte, and the second, inflated, the shape of spherocyte. It is noteworthy that both were the undamaged non-haemolyzed cells. Thus, in the suspension tested, the individual undamaged cells were detected, which

did not undergo the complete destruction as a result of haemolysis.

We separated the plasma from blood, washed the erythrocytes with the buffer solution and haemolized the cells in 3 ml of distilled water to determine the possible contribution of the haemoglobin coagulants to $D(t)$. We removed the ghosts by centrifuging. Then, we added C = 1 mM. The optical density of the solution was 0.3 – this was the contribution of the haemoglobin coagulants to the optical density of the solution. Consequently, the rest of the contribution to the optical density of the solution could only give non-haemolized erythrocytes, as is shown in figure 4.

We wanted to find whether or not the nanostructure of erythrocyte membranes changed after the action of zinc ions at different concentration. For this purpose the red blood cells were separated from plasma by using a buffer solution. The suspension of erythrocytes in the buffer solution with 50% haematocrit was prepared. The corresponding quantity of the basic solution of $ZnSO_4$ was added in order to obtain the corresponding concentrations of zinc ions. Then the monolayers of erythrocytes were formed on microscope slides. On the surface of each cell membrane we scanned 1 × 1 μm fragment in the field of AFM and decomposed the membrane surface with the use of the spatial Fourier transform into three orders.

characteristic heights h_i in all orders with an increase in the Zn concentrations indicates that zinc causes the total damage to membrane structures: an increase in the flickering-amplitude [14] (the first order), the violations of the spectrin matrix and the aggregation of membrane proteins (the second and third orders).

Coagulants of haemoglobin. Figure 6 shows the images of the fragments of haemoglobin coagulants, which were obtained by AFM. We showed three fragments at the concentration of C = 6 mM, which were obtained consistently 30 minutes (a), 60 minutes (b) and 90 min (c) after their exposure.

The fragments of haemoglobin coagulants comprise individual grains (figure 6a), whose sizes range from 20 to 70 nm. These individual grains began to unite two by two, four or more. After 30 minutes (figure 6b) grains created large coagulants and their size increased. These coagulants developed large formations which after 90 min were several hundred nanometers long, and longer (figure 6c). At the same time in the solution, small structures like the separate clusters of grains (figure 6b, c) were preserved. With time the optical density of the solution (λ = 760 nm) increased: from 0.16 to 0.3 at C = 1 mM and from 0.26 to 0.48 at C = 6 mM. This indicated an increase in the conglomerate size with the time.

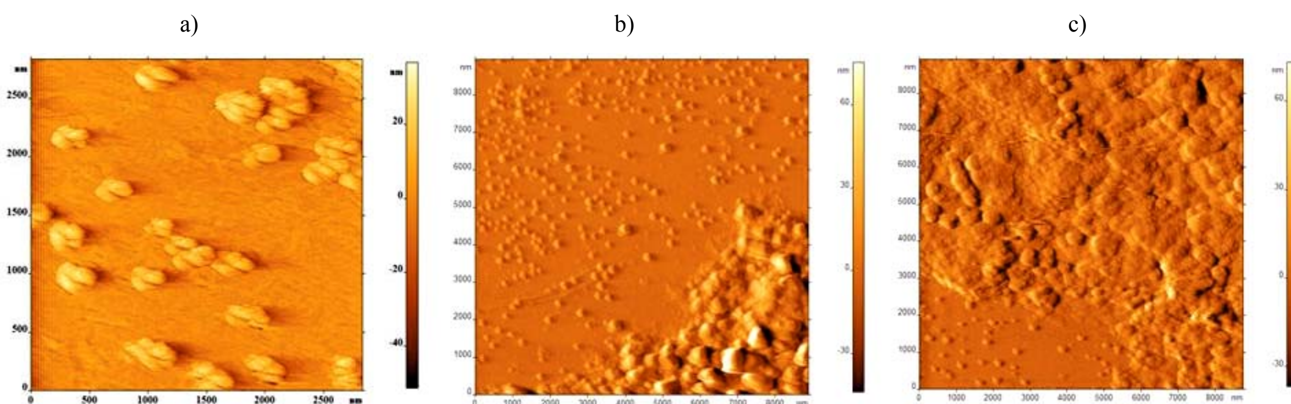

Fig. 6. AFM images of haemoglobin coagulants at Zn concentration C = 6 mM:
a) 30 min, b) 60 min, c) 90 min after adding Zn to haemoglobin solution

The example of the changes in the texture of nanosurface of membranes for the first and second orders are given in figure 5. These images are shown in 3D format for the control membranes (C = 0) and the membranes exposed to the zinc concentration C = 0.5 mM. At this concentration there is observed an essential development of nanostructure damages. Figure 5 shows the scale of scanning and the separate scales of heights for comparing the parameters of nanostructures. An increase in the

Is the effect of zinc on erythrocyte membranes reversible? Figure 1 (series A) shows the diagram of the next experiment. Initially we added C = 6 mM into the erythrocyte suspension, then thermostated it at 19 °C for 30 minutes and electroporated. The initial optical density was 1.0. The control suspension reached the point D = 0.7 after 14 min. The maximum value of rate constant equalled 0.045 s^{-1}, and the residual level n_{res} to 100 minutes was 0.32.

The solution with zinc was centrifuged (1200 g for 5 min), then we collected the supernatant fluid and replaced it with the buffer solution of the same volume. After an initial washing of zinc ions, the optical density of the solution dropped abruptly to 0.87 and the process of haemolysis began. The rate constant was 0.03 s^{-1}. Then the procedure was repeated. The optical density dropped abruptly to 0.44 and the haemolysis continued. Its rate constant increased to 0.04 s^{-1}. Thus, after the first and second flushing, the rate of haemolysis increased simultaneously with a decrease in the zinc ion concentration in suspension. The haemolysis developed almost as well as in the control in the time range from 20 to 100 minutes.

As a result of all our experiments we showed that there is a special point of zinc concentration (C = 0.5±0.1 mM) at which the course of the conjugate processes on the membranes of red blood cells was changed. The results obtained allow us to establish mechanisms and to suggest the model of zinc ion influence on the membranes of red blood cells.

4. Discussion

The results of the research are the following. Two ranges of the Zn concentration typical of the processes examined were set at 0.15–0.5 mM and 0.5–10 mM. These ranges were established based on the electroporation, the atomic force microscopy, the osmotic resistance, and the optical microscopy.

- At zinc ion concentrations of 0.15–0.5 mM, the residual level of the kinetic curves was below that of the control, and the rate constant β increased sharply.
- At zinc ion concentrations of 4–10 mM, the residual level of the kinetic curves was close to 1, the rate constant β was close to 0. The haemolysis of red blood cells caused by the electroporation stopped.

The concentration C = 0.5±0.1 mM was the point of inflection on a curve representing the residual level and the point of the maximum for the rate constant. At this concentration some essential changes in membrane nanostructure arose. The typical ranges of concentration, in which it occurred, were similar in different donors.

4.1. Low Zn concentrations (C = 0.15–0.5 mM)

The low Zn concentrations were responsible for damage to the erythrocyte membranes and thus created the additional active centers of electroporation. At the concentrations of up to 0.15 mM, the number of non-haemolized erythrocytes essentially decreased in comparison with the control. At these concentrations the residual level n_{res} was the lowest. The rate constant β of haemolysis increased sharply. It was about 7 times higher than that of the control (figure 3). The residual level returns to the control level at the concentration of 0.3–0.4 mM.

Thus, severe multiple damage to the membrane appeared due to the action of zinc ions. These defects were so prominent that electroporation caused the haemolysis of a vast number of the cells in a short time. The reduction of the residual level n_{res} smaller than that of the control showed that the haemolysis occurred even in those cells, which, in the absence of zinc ions, remained undamaged.

The changes of the nanostructure of erythrocyte membrane have the same dynamics (figure 5). To the concentration C = 0.5 mM the nanostructure was significantly changed. There was a rapid change of the flickering-amplitude and spectrin matrix, which was accompanied by the process of the accelerated haemolytic destruction of red blood cells. In other words, the changes of the membrane nanostructure increase the haemolysis of red blood cells due to electroporation.

4.2. High Zn concentrations (C = 0.5–10 mM)

The rate constant β decreased sharply (7 times) at the concentration C = 0.5 mM, the heights h_i of the orders continued to grow. At the same time, the residual level n_{res} increased, and at the concentration C = 6 mM, the haemolysis nearly stopped. That is, the higher the zinc ion concentration, the smaller the number of cells were haemolyzed by the electroporation. We can assume that some substrates remained in the suspension, which prevented the osmotic haemolysis. To test this the erythrocytes were haemolyzed in distilled water with zinc ions concentration of 1 mM. The experiment showed that cells and haemoglobin conglomerates, and the ghosts remained in the suspension. And it was noteworthy that completely non-haemolized cells remained. It was clearly shown by AFM (figure 4).

Though the cells had distorted shapes, they kept their initial sizes and the typical structures. The presence of these cells increased the residual level n_{res} to 0.9, and the rate constant tended to 0. Although in our earlier studies we prove that n_{res} is not an informative indicator after ionizing radiation [15], [16], in this study, the rate constant β and the residual level n_{res} are

independent parameters. Each of them describes its own process. The residual level n_{res} shows the degree of non-haemolyzed cells. In our experiments, this process begins at the concentration of 0.5 mM and stops at 10 mM.

The electroporation at C = 6 mM before washing did not change the optical density of the suspension, which remained 1.0. After the first washing, the haemolysis was restored and then continued after the second washing. At the same time, the rate constant β was almost unchanged and nearly equal to the rate constant for the control curve in the range from 20 to 100 minutes.

4.3. Mathematical model. Competitive processes in the presence of the increased concentration of Zn ions

Zinc ions at low concentrations caused the membrane damage, which manifested itself as an increase in β and as a decrease in the residual level n_{res}. At the concentration of C = 0.5 mM, the process shifted to the other side: the rate constant decreased sharply and the residual level increased to unity. At C = 0.5, we dealt with the process directed to the reduction or with a complete cessation of the haemolysis. This process started to dominate with an increase in the concentration of zinc ions. At C = 6 mM, the haemolysis stopped sharply.

The irreversible cell electroporation is responsible for the formation of the non-reversible pores in the membranes, which causes the osmotic haemolysis. Zinc ions cause the appearance of active centers of haemolysis, which leads to an increase in the number of pores N in membranes due to electroporation. This process can be represented by the following equation:

$$\frac{dN}{dC} = \gamma C^2 , \qquad (2)$$

where γ is the coefficient of proportionality.

Due to electrocorporation, zinc ions with solution were introduced into the cell and caused the formation of the haemoglobin coagulant, whose size was comparable with the pore size of membranes or even greater. We showed (figure 6) that the haemoglobin coagulants could change their size within a wide range: from 20–70 nm to several hundred nanometers after coagulation. The sizes of the pores in the membrane after the electroporation were evaluated under experimental conditions, they ranged from 10–20 nm to 100–150 nm [10].

On the other hand, zinc ions could cause the clustering of membrane proteins of band 3. The protein of band 3 plays an important structural role within the red cell. It takes part in the connection between spectrin and the membrane. The cytoplasmic domain of band 3 could bind with haemoglobin and form the haemoglobin m (Hbm), which is associated with the inner surface of the erythrocyte membrane [17]. Chemicals, for example, $CaCl_2$, increase the number of Hbm. The growth of Hbm is observed at the $CaCl_2$ concentration of 0.5 mM [17]. This is the range, where there is a sharp drop of the rate constant β and the growth of the residual level n_{res} in our experiments.

The haemoglobin aggregation inside the cells and the protein of band 3 clustering in the membrane increase the probability of the formation of Hbm after the exposure to zinc ions. In this case, the haemoglobin flow out of the cell may be reduced or even stopped. This process depends on the zinc ion concentration: this concentration should be sufficient for the formation of the haemoglobin coagulants and the Hbm. This was observed at C = 6 mM and higher. The reduction in the number of pores during the Hbm formation is proportional to C^2:

$$\frac{dN}{dC} = -\lambda N C^2 , \qquad (3)$$

where λ is the coefficient of proportionality.

The clustering of proteins and the haemoglobin coagulation lead to slowing down the cell haemolysis (3), and the oxidative damage to the membrane leads to its acceleration (2):

$$\frac{dN}{dC} = \gamma C^2 - \lambda N C^2 . \qquad (4)$$

At $C = 0$ we have $N = N_0$.

Different signs in final equation (4) indicate the opposite effects of the zinc ion concentration on the result of the electroporation.

Divide the variables:

$$\int \frac{dN}{\gamma - \lambda N} = \int C^2 dC .$$

The general answer

$$\gamma - \lambda N = D e^{-\lambda C^3/3} .$$

We find D with the initial conditions:

$$D = \gamma - \lambda N_0 .$$

As a result:

$$N(C)=\frac{\gamma}{\lambda}+\left(N_0-\frac{\gamma}{\lambda}\right)e^{-\lambda C^3/3}. \qquad (5)$$

The haemoglobin Hbm, which is attached to the membrane, affects also the deformability of red blood cell. It is a well-known fact that Hbm and the rigidity of the erythrocyte are highly correlated: the larger the molecules of Hbm contained in a red blood cell, the more rigid the membrane and the higher the cell deformability [17]. The correlation between Hbm and the rigidity of erythrocytes allows suggestion that the change in the elasticity of dK is in proportion to the concentration C of zinc ions:

$$dK=-\theta K dC \quad \text{at} \quad C=0,\ K=K_0, \qquad (6)$$

where:

θ is the coefficient of proportionality, which depends on the membrane properties,

K_0 is the elasticity of the membrane in the absence of zinc.

We obtain:

$$K=K_0e^{-\theta C}. \qquad (7)$$

The average rate constant β of the erythrocyte haemolysis is directly proportional to the average change of the erythrocyte volume ΔV after the osmosis:

$$\beta \sim \Delta V. \qquad (8)$$

In turn the change in the volume of red blood cell is proportional to the number N of pores, which are formed on the membrane due to the electroporation, and to the average elasticity K of the membrane:

$$\Delta V \sim KN, \qquad (9)$$

$$\beta=\mu KN,$$

where μ is the coefficient of proportionality.

After the electroporation of the control cells (without Zn), the average rate constant of haemolysis depends on the average elasticity K_0 of the membrane and the average number N_0 of pores, which are formed on the membrane at the given parameters of the electric pulse:

$$\beta_n=\mu K_0N_0. \qquad (10)$$

Based on (5), (7) and (9), the rate constant of haemolysis after the electroporation can be:

$$\beta=\mu K_0e^{-\theta C}\left(\frac{\gamma}{\lambda}+\left(N_0-\frac{\gamma}{\lambda}\right)e^{-\lambda C^3/3}\right). \qquad (11)$$

The rate constant given in (10) is normalized to the reference value:

$$\beta_n=e^{-\theta C}\left(\frac{\gamma}{\lambda N_0}+\left(1-\frac{\gamma}{\lambda N_0}\right)e^{-\lambda C^3/3}\right) \qquad (12)$$

Figure 7 (a) shows dependence (12) for numeric values $\frac{\gamma}{\lambda N_0}=12$, $\lambda=50$.

Figure 7 (b) shows empiric curve representing the processes described.

A good agreement between the theoretical and the experimental correlations confirms the development of processes (2), (3), (4), (7) in the red blood cells affected by Zn ions. An observed increase in the rate constant and its subsequent decrease with

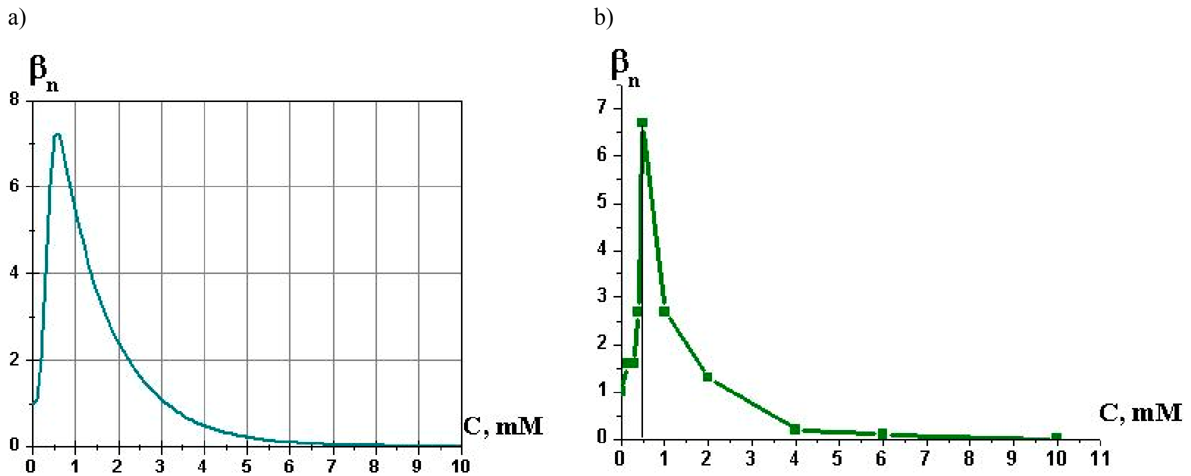

Fig. 7. The adequacy of mathematical model for the empirical data of research. The dependence β_n (C): a) mathematical model, b) empirical relationship. β_n – the rate constant of haemolysis, normalized to control, C – concentration of zinc ions

a decrease in zinc concentration shows the competitiveness of the Zn-induced processes in the system.

5. Conclusions

The concentration-dependent processes taking place in red blood cell membranes affected by zinc ions were investigated. We showed that the changes in the zinc ion concentration in the blood may unidirectionally lead to the competitive processes in the system and to the opposite results of electroporation. We experimentally established that there was a typical point of zinc concentration ($C = 0.5 \pm 0.1$ mM) at which the course of the conjugate processes on the membranes of red blood cells was changed. The results obtained allow us to suggest its mechanism and to propose the model of zinc ions influence on the membranes of red blood cells. These investigations can be useful for understanding the processes of exogenous intoxication of human organism by heavy metal ions.

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