Zinc-induced injuries to red blood cell membrane nanostructures at different zinc concentrations were studied by atomic force microscopy. In order to distinguish the intrinsic characteristics of membrane nanostructures, the membrane surfaces were represented by three orders using 3D Fourier transform. Increasing the concentrations of zinc ions modified the pattern of induced injuries: their depths and diameters and their number on the membrane surface test area increased. The injuries and their distribution for each order of membrane surface were analyzed. Albumin restored membrane nanosurface.

**Key Words:** erythrocyte; membrane; nanostructure; injuries; zinc

Zinc is one of the most important metals in biological systems. It works as an enzyme active center and supports the globular protein structures. In addition, it binds to several protein centers and can modify the secondary structures conformation [4]. Zinc binding to protein structures causes their aggregation and hence, modifies the membrane function. That is why this ion is used in model studies of membrane protein clustering [15].

The protein clusterization and coagulation effect manifests at different levels of organization by distortion of the cell shape and functioning [1]. Zinc can cause an antioxidant effect [13,14]. On the other hand, zinc ion excess in the blood can promote anemia and erythrocyte hemolysis [3,5,6].

The clusterizing effect of zinc ions can be reversible under certain conditions. Removal of zinc from the blood leads to restoration of the initial configuration of cell structures [15].

Hence, registration and studies of changes in the red blood cell molecular structures, caused by zinc ions in different concentrations, is an important experimental and clinical problem.

Atomic force microscopy (AFM) is an effective method for studies of the membrane nanostructure. This method shows the erythrocyte membrane (EM) surface in the nanoband and detects the subtle disorders in membrane structures. AFM allows quantitative evaluation of the injuries and their statistical distribution.

We studied the characteristics of disorders in EM nanostructures, resultant from exposure to zinc ions in different concentrations and demonstrated the efficiency of methods for correction of these disorders.

**MATERIALS AND METHODS**

Experiments were carried out on whole venous blood samples from 4 donors (men aged 27-32 years) who gave informed consent to use of blood samples in experimental studies. Erythrocytes were precipitated and
the plasma was removed. Blood cells were washed in Diachim-Buffer-G (Hettich Mikro 220R centrifuge, 1200g). Buffer volume identical to the volume of removed plasma was left. Hence, erythrocyte concentration in suspension was the same as in initial blood.

In experimental series I, ZnSO₄ was added to the suspension in the following concentrations: 0 (control), 0.1, 0.2, 0.5, 1.5, and 2.0 mmol/liter. The mixture was exposed for 1 h at 20°C, after which the cell monolayer was formed using DiffSpin-2 specialized centrifuge.

In experimental series II, 10% human albumin (Sigma) was added into ZnSO₄ solutions of the above concentrations and the mixtures were incubated for 1 h at the same temperature. Erythrocyte monolayer was then formed as described above.

The EM surface images were obtained by Femtoscan AFM in the constant scanning mode using mathematical support attached to this microscope. Standard cantilevers fp N10 with an angle of ≤22° and radius of ~10 nm served as the probes. Scanning force was 0.1-5.0 nN, number of scanning points 512, scanning fields: 10×10, 1500×1500, 800×800, and up to 150×150 nm.

Surfaces of three orders were distinguished from the initial surface in order to obtain informative characteristics of the studied processes [2,9]. 3D Fourier transform of the initial surface with three spectral windows was used for this purpose. Surface order I corresponded to the spatial spectral window with an L₁ period in the 1000-600 nm band, order II to L₂ in 600-80 nm band, and order III to L₃ in 80-10 nm band.

The emerging pores were statistically ranked by the heights of the respective orders – they were measured in the course of experiments. In the control, their ranges were as follows: h₁ at 5.0-2.0 nm, h₂ at 2.0-0.8 nm, and h₃ at 0.5-0.2 nm. Inverse summation of profiles of orders I, II, and III and of all three images resulted in restoration of the initial profile and initial image of the surface.

The size of cell membrane nanostructures are the membrane parameters proper [8-10]. They carry information characteristic of this membrane and changes in its characteristics in response to endo- and exogenous exposure of blood cells.

We analyzed the effects of ZnSO₄ solutions of different concentrations on the EM nanostructure and the corrective effect of albumin by comparing the surfaces of the respective orders for control samples and for Zn-treated membranes.

Twelve experiments were carried out, three with the blood of each donor. For each experiment, 3-5 cell images were scanned in AFM in a 10×10 μ field. Then 3 fragments were scanned in 1500×1500 nm field for each cell, and then fields of 1000×1000 nm and less than 500×500 fields from these fragments were scanned; 324 images were obtained and analyzed.

The membrane surface characteristics were processed by periods and heights using Origin software: histograms of surface heights and periods were plotted, errors for ensembles were calculated, and the significance of differences between all stages of experiment was tested.

RESULTS

Injuries in EM nanostructure increased quantitatively and qualitatively with increase of ZnSO₄ concentration. These injuries were presented by non-perforating pores of different depth, diameter, and shape. In the control, erythrocyte was of standard shape and 660 nm high (Fig. 1, a). A total of 10-20 small injuries (less than 160 nm in diameter) and 1-2 injuries larger than 500 nm in diameter, 1-5 nm high, were found on the entire surface of a control cell. Addition of ZnSO₄ in concentrations of 0.15 and 0.25 mmol/liter caused no appreciable changes in the membrane surface. The picture was different in the presence of higher Zn concentrations. In the presence of 0.5 mmol/liter ZnSO₄, the number of injuries of ~200 nm in diameter increased to 80-100 per cell (Fig. 1, b). This was paralleled by an increase in the number of injuries of 300-400 nm in diameter, which reached 40-50 for the surface analyzed. The height of the lesions also increased, reaching 60 nm. Addition of ZnSO₄ in a concentration of 2.0 mmol/liter led to formation of deep pores of up to 1 μ in diameter on the membrane (Fig. 1, c).

The patterns and typical configurations of disorders in EM ultrastructure changed differently in the presence of different ZnSO₄ concentrations. Six non-perforating deep (up to 40-70 nm) pores were recorded for an EM fragment (Fig. 2, a). Their diameter (dX) was measured on the profile image (Fig. 2, b) and was 872.9 nm. These were large injuries on the erythrocyte surface.

The emerging pores were statistically ranked by the heights of the respective orders and by diameters. Exposure to ZnSO₄ in a concentration of 0.5 mmol/liter resulted in emergence of pores of different size on EM (Fig. 2, c). It should be noted that the histogram was shifted towards larger lesions, though their total number was not high.

Control sample had lesions up to 220 nm in diameter, no more than 15-20 per field examined. Exposure to ZnSO₄ resulted in emergence of mainly 300-500 nm pores, maximally 800 nm in diameter (Fig. 2, d). Pores of 200 nm in diameter were the most numerous; their number on the visible surface area could surpass 100. Statistical distribution of heights and diameters of injuries were similar in all donors.