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Calculation of Intracellular Pressure of Red Blood Cells at Jaundice According to Atomic Force Microscopy Data

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The present work is devoted to the analysis of three-dimensional data of atomic force microscopy for research of the morphology of red blood cells. In this paper we built a biomechanical model of the erythrocyte, which allowed calculating the intracellular pressure of erythrocyte based on data of atomic force microscopy. As a result, we obtained the dependence intracellular pressure on the morphology of red blood cell. We have proposed a method of estimating of intracellular pressure of erythrocytes based on numerical modeling and data of atomic force microscopy of erythrocytes scan, which involves a comparison of the experimental data with the results of numerical calculation. The method is applied to the data of atomic force microscopy of erythrocytes of experimental animals - dwarf domestic pigs with different degrees of obstructive jaundice and normal. It is shown that with increasing severity of the disease and the concentration of bilirubin in the blood there is an infringement erythrocyte membranes, by an average increasing their volume and intracellular pressure.

Keywords: Atomic force microscopy, Erythrocyte membrane biomechanics, Intracellular pressure, Obstructive jaundice.

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1. INTRODUCTION

Method of atomic force microscopy (AFM) is finding wide application for biology and medicine. AFM has several advantages over optical or electron microscopes. In addition to three-dimensional imaging AFM has the capability of analyzing mechanical properties, allowing you to delve deeper into cellular processes, to investigate such important properties as elasticity, mobility of the surface layer, adhesion, molecular binding and electrostatically.

At present a new direction is formed in cytology, which defines the term "Nanomechanics cell" or "cell elastography". These concepts involve imaging of shear elastic characteristics of biological soft tissues, which complement the traditional methods of imaging and are considered promising for medical diagnosis of various pathologies, especially in the study of cancer [1-8].

The studies on biological object using AFM actively carried out in respect red blood cells. The membrane condition of erythrocytes and the ion pumps of membrane uniquely change intracellular osmotic pressure, which in turn changes the morphology of erythrocytes and volume thereof. Based on the thermodynamic principle of minimization of the free energy of the lipid bilayer membranes [1, 2] the three-dimensional shape of the erythrocyte is obtained by analytic calculation. Based on this model and the regulation of ion exchange and thus the volume of red blood cells in works [3-5] numerical calculation of the relationship of pressure and intracellular corpuscular volume was done, including taking into account biomechanical model membranes [5, 7]. These models are in good agreement with the experimental data in which the change occurs when changing the volume of red blood cell and ion exchange under the influence of various external chemical factors that alter pH of the solution.

However, to compare the calculated data with experimental data obtained by AFM, these models need to be supplemented for several reasons [7-18]. The erythrocyte shape does not change and its volume at 95-99 % filled hemoglobin [6, 7], while it was assumed that the percentage of hemoglobin is in the range of 50-80 % in the works [3-5]. Secondly, the ratio of volume-pressure will vary in thermodynamic system, in which hemoglobin affects significant on erythrocyte membrane, that is mentioned briefly in papers [3, 7]. Thus in work [3] the process of expansion of the erythrocyte was studied under internal pressure, which vary widely – from a few Pa to 5 kPa. At the same modeling of volume reduction due to dehydration or compression by forces has not been met. Finally, in the experimental study of AFM the erythrocytes are deposited on the surface, its adhesion and shape change occur under gravity and adhesion forces [10-15]. Thus, the aim of this work is the numerical calculation of the erythrocyte morphology, depending on the intracellular pressure to analyze the data by atomic force microscopy, i.e. for the case where erythrocytes stored in air on a microscope slide.

2. ERYTHROCYTE MODEL AND NUMERICAL CALCULATION OF MORPHOLOGY

There are several models which describe the processes of regulation of ion exchange and volume of erythrocytes [1-8]. The focus of these studies is given to chemical and electrochemical processes that affect the volume regulation. Ion transfer is carried out using a class of enzymes hydrolases adenosine triphosphatase (ATP), which catalyze the cleavage of adenosine triphosphate of one or two residues of phosphoric acid. In

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the process of the biochemical reaction it is freed of the energy used in the transport of substances through the membrane. High concentration ratio of potassium in extracellular and intracellular fluid (38 : 1) is supported by the action of the Na^+, K^+ -ATP, actively transporting potassium ions into the cell and sodium ions - from it in a ratio of 2 : 3 [8]. ATPase of proteins of plasma membranes performs ATP- dependent transmembrane transport of Na^+ and K^+ ions in cells, that ensures the maintenance of electrochemical and osmotic gradients of monovalent ions required for the normal functioning of cells.

A result of work ion channels in cell an asymmetry in the ion concentrations occurs inside and outside the cell, which ensures the maintenance of its constant volume. Produced by metabolic processes in the cell osmotic pressure causes deformation of the erythrocyte membranes. Thus morphology of erythrocyte is set by the lipid layer, which is determined by the internal forces arising due to construction of proteins layer (phospholipids) by nonpolar ends inside layer. Arising due to ionic interaction of the mechanical energy of the lipid bilayer takes a minimum value, and forming erythrocyte morphology [1, 2, 9].

To calculate the impact of ion pumps on erythrocyte volume regulation constitute a system of equations [3-8], which includes streams of ions and water flow. It is known [7] that the main contribution to the regulation of human erythrocyte volume contribute sodium and potassium cations, as well as chlorine and anions HCO_3. Kinetics of intracellular potassium and sodium concentrations is described by equations that take into account the active transport of these cations Na^+, K^+ ATP and passive flow through the membrane on the concentration gradient. Considering the electroneutrality condition for intracellular content of erythrocyte we can get known equation of dependencies of volume from osmotic pressure in erythrocyte:

$$\frac{d}{dt}\left(\frac{V}{V_0}\right) = J_{\mathrm{H_2O}} = \frac{SP_f}{V_0}\upsilon_{\mathrm{H_2O}}\Delta C = \frac{SP_f}{V_0}\upsilon_{\mathrm{H_2O}}\frac{\Delta P}{RT} \quad (1)$$

where V and V_0 – current and physiologically normal erythrocyte volume, and flow of water J_{H2O} is proportional to the concentration difference ΔC of osmotically active ions inside and outside of the erythrocyte membrane, which is expressed through osmotic pressure ΔP and thermal potential RT, S – membrane surface, ϑ_{H2O} – molar volume of water and P_f – osmotic membrane permeability to water.

In works [3, 7] it was performed numerical calculation of equations (1) taking into account the impact of the elastic shell on metabolism and change of erythrocyte volume. Due to ability to resist deformation, the erythrocyte shell has an impact on the process of osmosis in the form of reactive pressure ΔP_r, which is applied to its volume. When this in calculations of equation (1) difference $\Delta P - \Delta P_r$. was instead of pressure ΔP. The model showed good agreement with the experimental data with increased volume of erythrocyte in the process of changing of ion channels in the membrane after treatment with amphotericin B and the consequent increase of permeability for ions, as well as due to the reduction of osmosis of environment [3].

In the case of measurements using atomic force microscopy it were determined erythrocyte parameters most often on blood smear on the glass, where erythrocytes are located by thin layer in the air. As shown in the works [11-18] it occurs on air that water exits from erythrocyte and, accordingly, its volume is reduced in 4-6 times. Despite this in works [11-15] it was demonstrated experimentally that the shape of the erythrocyte membrane and its properties are maintained for 2-3 weeks, depending on the method of their storage. Further drying the erythrocytes causes a change the elastic properties of with a sharp increase in the Young's modulus from 1-2 kPa to 50 MPa [12-14], and the shape of the erythrocyte is stored. Because of drying of a sample with erythrocytes during AFM measurements the water environment disappears, the large gradient ΔP occurs and water exits from erythrocyte and that's why it loses mass and volume in accordance with the formula (1). Thus form of erythrocyte remains unchanged and is determined by the state of its membrane [11-18], i.e. reactive pressure ΔP_r.

On the one hand the reactive pressure ΔP_r of erythrocyte membrane will depend on the state of the membrane, on the other hand the erythrocyte morphology will depend on the biomechanics of its membrane [1, 2, 15-18]. Thus to determine of intracellular pressure from AFM data we need carry out numerical calculation, in which the initial reactive pressure ΔP_r is set and we will get morphology of erythrocyte.

For the model of numerical calculation we take known reference data and biomechanical model of a homogeneous body covered with a membrane consisting of lipid bilayer, elastic properties of which are described in [1, 2] in the form of asymmetrical tensor that forms the erythrocyte morphology To solve the system of equations we used PARDISO software product (PARallel DIrect SOlver), in which the subroutine library is used for solutions to systems of linear equations with rarefied matrix by Gaussian methods of super node exceptions as well as decomposition of Holesskij. To conduct numerical calculation by method of finite elements we used software package Comsol multiphysics (license 1029477) and module optimization of MATLAB software package (license 512916) [15-18].

For numerical simulation we solve the coupled problem of solid (cell membrane) and liquid (inner content of erythrocyte). In our work the parametric task was solved, as a parameter was used value of reactive pressure ΔP_r of erythrocyte membrane. Numerical experiment examines the axisymmetric problem, rotation body axis runs through the center of erythrocyte ($r = 0$). In the calculation the model of the linear elastic material was based on the following equations:

$$-\Delta\sigma = F_V, \sigma = S \quad (2)$$

$$\mathrm{s\text{-}S}_0 = C : (\varepsilon - \varepsilon_0 - \varepsilon_{inel}) \quad (3)$$

$$\varepsilon = 0.5\left[\left(\nabla u_{solid}\right)^T + \nabla u_{solid}\right] \quad (4)$$

where $\sigma = S$ – the stress tensor, F_V – the force acting on the body, ε – deformation tensor, ε_{inel} – tensor of thermal deformation, S_0, ε_0 – the initial values of the corresponding tensors, C – elasticity tensor of fourth-order, ":" – the operation of tensor works, u_{solid} – vector of component of offset, model of elastic material is applied for calculation of membrane. While the initial offset equal to 0, on the outer edge of the solid we put the following initial and boundary conditions:

$$\sigma \cdot n = F_a, F_a = \begin{pmatrix} 0 \\ \Delta P_r \end{pmatrix}, \tag{5}$$

where n – normal to surface, F_A – vector of external influence, ΔP_r – reactive pressure, which is set as a parameter in the calculation. This condition ensures the direction of pressure vector along the vertical axis. The symmetry condition is established on the rotation axis. The bottom boundary of the erythrocyte is fixed and don't move along the z axis:

$$\omega_{solid} = 0 \tag{6}$$

The physical properties of solids are defined in accordance with known experimental data [7, 9, 18, 19]: modulus of elasticity of the shell is equal to 1200-2000 Pa depending on the coordinates of r [11, 12, 15-18], the density of the shell – 1200 kg/m^3 [1, 2, 6, 19], Poisson's coefficient of shell – 0.33 [3, 11, 18]. Deformation tensor is caused by elastic properties of the membrane: shear rigidity equal to 0.006 mN/m, and on stretching, which is equal to 450 mN/m [3, 7]. When the mathematical model describes the behavior of the hydrodynamics of the internal content of model of erythrocyte, the liquid contents can move and change position depending on the applied pressure from the side of the membrane, and it is significant difference from model of membrane. As a result inside the erythrocyte the modelling of liquid medium is carried out using the Navier-Stokes and continuity equations:

$$\begin{aligned} &\rho\left(u_{fluid} \cdot \nabla\right) u_{fluid} = \nabla \cdot \Gamma + F, \\ &\Gamma = -pI + \mu\left(\nabla u_{fluid} + \left(\nabla u_{fluid}\right)^T\right) - \\ &\quad -\frac{2}{3}\mu\left(\nabla \cdot u_{fluid}\right) I \end{aligned} \tag{7}$$

$$\nabla \cdot \left(\rho u_{fluid}\right) = 0, \tag{8}$$

where p – fluid pressure, $\boldsymbol{u_{fluid}}$ – the velocity vector, ρ – liquid density, μ – dynamic viscosity, $\boldsymbol{F}$ – vector of external influences, I – unit vector, Γ – the force acting on the border of the liquid and solid.

In the calculations it was assumed that at the initial time the speed and pressure is equal 0 in a fluid. The condition on the border of solid and liquid environments made it possible to track the influence of deformation on volume of compressed liquid and pressure inside the erythrocyte and conforms to the following equations:

$$u_{fluid} = u_w \tag{9}$$

$$u_w = \frac{\partial u_{solid}}{\partial t} \tag{10}$$

$$\sigma \cdot n = \Gamma \cdot n \tag{11}$$

where u_w – speed of deformation of solids.

When the physical properties of liquids are defined by the following values: fluid density – 1000 kg/m^3, dynamic viscosity – 4 mPa·s [1, 2, 9, 15, 19].

Fig. 1 shows the dependence of the form of the erythrocyte cutoff from pressure difference on membrane, which is set after the end of calculation. It is seen that with increasing external pressure occurs a small expansion of the erythrocyte model and a significant deflection in the center of the membrane, which corresponds to the experimental data of the geometry of erythrocyte cutoff according to the AFM [11, 12, 16 - 18]. It should be noted that with proportional increase or decrease size of the erythrocyte model the established pressure inside the cells did not change. This corresponds to the theory of ion channels, because only the ratio V/V_0 is used in equation (1).

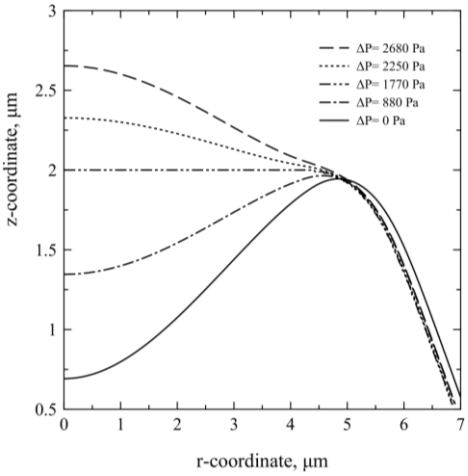

Fig. 1 – The results of calculation of erythrocyte morphology model under pressure: dependence of the shape of erythrocyte cutoff on the pressure difference on the of erythrocyte membrane

3. EXPERIMENTAL RESULTS

To calculate the intracellular pressure we carried out the experimental studies by AFM methods in semicontact mode on a microscope Solver-P4 company NT-MDT, and the samples were blood smears taken from experimental animals (minipigs - miniature pigs) with varying degrees of disease obstructive jaundice (class A, B and C) and in healthy individuals (normal). The results are shown in Fig. 2, this shows only the vertical sections of erythrocytes, which allowed to determine the degree of change in depth of the erythrocyte membrane in the center and its volume. The obtained averaged measured data for all erythrocytes on scan are shown in Table 1. Obstructive jaundice is characterized in that the concentration of bilirubin in the blood increases with increasing class of disease, bilirubin destroys membrane of erythrocytes, penetrates into erythrocytes, distributes in the body and leads to the accumulation in the liver and other organs [20]. On AFM scans of red blood cells we can see that with in-

creasing degree of disease it is a growth in both the number and size of violations in the membrane. In addition, it was found that normal red blood cells are shaped like biconcave spherocytes, with an increase of hyperbilirubinemia and bilirubin concentration in the blood the erythrocyte changes form: from spherocytes turns into an oval, of biconcave - becomes lenticular. All these data indicate the destruction of the red blood cell membrane and violation of its function, which is likely to lead to changes in its biomechanical properties.

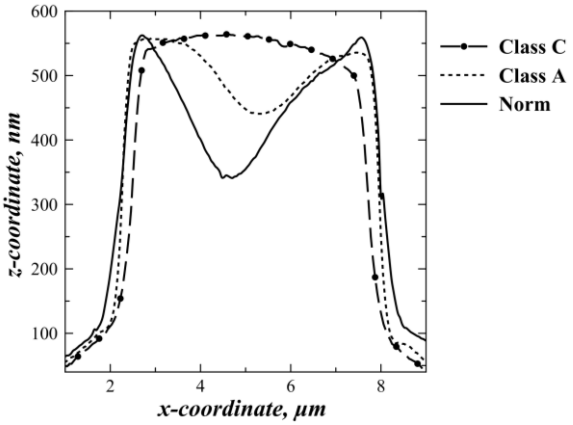

Fig. 2 – The erythrocyte morphology obtained using atomic force microscopy: cross section of a three-dimensional image of erythrocytes with different degrees of obstructive jaundice and normal

According to three-dimensional data of the AFM of the scan of blood smear the each erythrocyte was in a separate block of data for which calculated the geometric characteristics of sections of erythrocytes, and calculate its volume. Averaged erythrocyte volume and depth in the center of depression are shown in Table 1, which also shows the calculated value of the intracellular pressure. Calculated value intracellular pressure given in Table 1 for the average value of the ratio V/V_0. It is seen that with increasing degree of obstructive jaundice the growth of the average volume of erythrocytes occurs on the one hand, the corresponding change in the deformation of the membrane in the center of erythrocytes and an increase in its intracellular pressure.

Table 1 – The size of erythrocyte at different degrees of obstructive jaundice and the results of the calculation of the intracellular pressure

Degree of obstructive jaundice	Volume of erythrocytes, μm^3	The intracellular pressure, kPa
Norm	7.5 ± 2.0	0
Class A	8.8 ± 1.3	0.8
Class B	10.2 ± 1.9	1.6
Class C	12.3 ± 2.6	2.2

4. CONCLUSION

Thus, in this paper we propose a method of estimating intracellular pressure of erythrocytes based on the numerical simulation of erythrocyte morphology and data of atomic force microscopy. We calculated the intracellular pressure of erythrocytes for experimental animals at different degrees of obstructive jaundice. It is shown that with increasing concentrations of bilirubin in the blood the erythrocyte membrane is violated, an average increase in volume and a significant change in intracellular pressure. The value of intracellular pressure in the long term can be used to diagnose the state of erythrocyte membranes.

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