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Research Article

MORPHOFUNCTIONAL INDICES OF ERYTHROCYTES AND POLYMORPHONUCLEAR LEUKOCYTES *RANA RIDIBUNDAPALL* UNDER THE INFLUENCE OF TEMPERATURE FACTOR

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Abstract

Using force spectroscopy, a quantitative analysis was performed for morphometric parameters and functional features of the plasma membrane of red blood cells and polymorphonuclear leukocytes in frogs *Rana ridibunda* Pall. It has been established that an increase of the temperature of incubation of hemocytes (up to 40° C) as compared with the room temperature (20 ° C) can reduce the area and height of polymorphonuclear leukocytes and erythrocytes, while decrease of temperature of incubation (5°C) reduces the above-mentioned parameters only of white blood cells. By decreasing the incubation temperature to 5°C and increasing it to 37 ° C, the value of the modulus of elasticity and adhesion of plasma membrane of eritrotcytes and polymorphonuclear leukocytes raises, and incubation of hemocyte at temperature40°C reduces these indices.

Keywords: Red blood cells, Polymorphonuclear leukocytes, Frog, Adhesion, Elasticity, Morphometric parameters

Introduction

An important task of modern cellular biology is the study of morphometric characteristics of biological objects, since it is the size and form that largely determine the principle of their functioning. It is known that all physical and chemical processes that support vital processes and provide functional activity of the cells depend on the temperature [1-3].

An intensive development of scanning probe microscopy in the last decade has led to the emergence of new methods of research parameters and morphological characters of plasmalemma. These methods include atomic force microscopy (AFM), which allows to measure the morphometric parameters of cells and study their elastic properties (Young's modulus). In turn, the measurement elasticity of membranes of blood cells allows to assess their

deformability [4, 5], which depends on a number of cell parameters, in particular elastic membrane, viscosity of cytoplasm, form (area of surface) of the cell [6]. By means of AFM we have revealed morphometric characteristics of erythrocytes and leukocytes in mammals and man when exposed to various environmental factors, including temperature [7-10]. The morphometric parameters of blood cells of other vertebrates, in particular the representatives of Amphibia, are insufficiently studied. Even less studied is the question of the effect of temperature on the hemocytes of frogs.

The objective of this study was to investigate an effect of temperature on the morphofunctional characteristics of red blood cells and polymorphonucleocytes *Rana ridibunda*Pall by the method of atomic force microscopy.

Material and Methods

The studies were performed on peripheral blood cells of frogs *Ranaridibunda* Pall (30 individuals), captured from the Vezelka River within the city of Belgorod. The objects of study were erythrocytes and polymorphonuclear leukocytes (PMNL). Blood sampling was effected in the animals anesthetized with ether from the heart. The anticoagulant heparin was used in an amount of 10 u / ml. The obtained blood was centrifuged for 4 min at 400g. One collected the lower part of the plasma, that is leukocytes-rich, and leukocytal ring, erythrocyte suspension was left in tubes. Blood films were prepared on the defatted glass substrates in a conventional manner.

On the basis of the freshly prepared blood smears by AFM method, the morphometric characteristics of the cells, their adhesive elastic properties using scanning probe microscope Vita Integra NT-MDT were studied. The images of blood cells were obtained in the mode of semicontact scanning using silicon probes of NSG03 series (NT-MDT) of rigidity 1.1 N / m with a radius of curvature of 10 nm and frequency of scanning of order 0.6-0.8 Hz [9]. The native cells under conditions preventing their drying at room (20°C) temperature and the native cells incubated for 120 minutes at low (5°C) and elevated (37°C, 40°C) temperatures *in vitro* were scanned. AFM measurements were performed on 25 cells in each series sample processing.The obtained scans demonstrated area (mcm^2), volume (mcm^3), short and long axes (m) height (m) of red blood cells, and - area (mcm^2), volume (mm^3), diameter (m) and height (m) of white blood cells. These data with the help of the software «Nova» (NT MDT, Zelenograd, 2009) were used to build the profile curves of the scanned cells. The adhesion (nN) of cells was assessed on the basis of the obtained curves. The elastic properties of erythrocytes and PMNL were studied using the method of force spectroscopy, and recording the topography and the map of vertical deflection probe point of AFM upon application of load to the cell surface in 36 local sites [9]. Quantification of membrane elasticity was performed by calculating

the Young's modulus (kPa). To calculate the value of the modulus on the force curves, the program ImageAnalysis 3.5 was used.

The results obtained were processed using the methods of variation statistics with applied software on a personal computer. Significance of differences was determined by Student's *t*-test ($p \leq 0.05$).

Findings of Investigation

The AFM images of erythrocytes obtained by semicontact scanning under various incubation temperature are shown in Figures 1a-g. As seen in Figures 1a and 1b, after incubation at temperatures of 5° C and 37° C, the cell surface is strongly convex at the center (in the core zone), it is concave closer to the edge, well defined small creases are on the very edge.

At the room temperature (20° C) (see Figure 1b) red blood cells have an elliptical shape, a convex surface in the vicinity of the nucleus and weakly creasy plasma membrane. The degree of convexity of erythrocytes at the temperature of uncubation of 40° C is weaker as compared with temperatures of 5°C and 37° C, however, the roughness of plasma membrane increases (see Figure 1d).

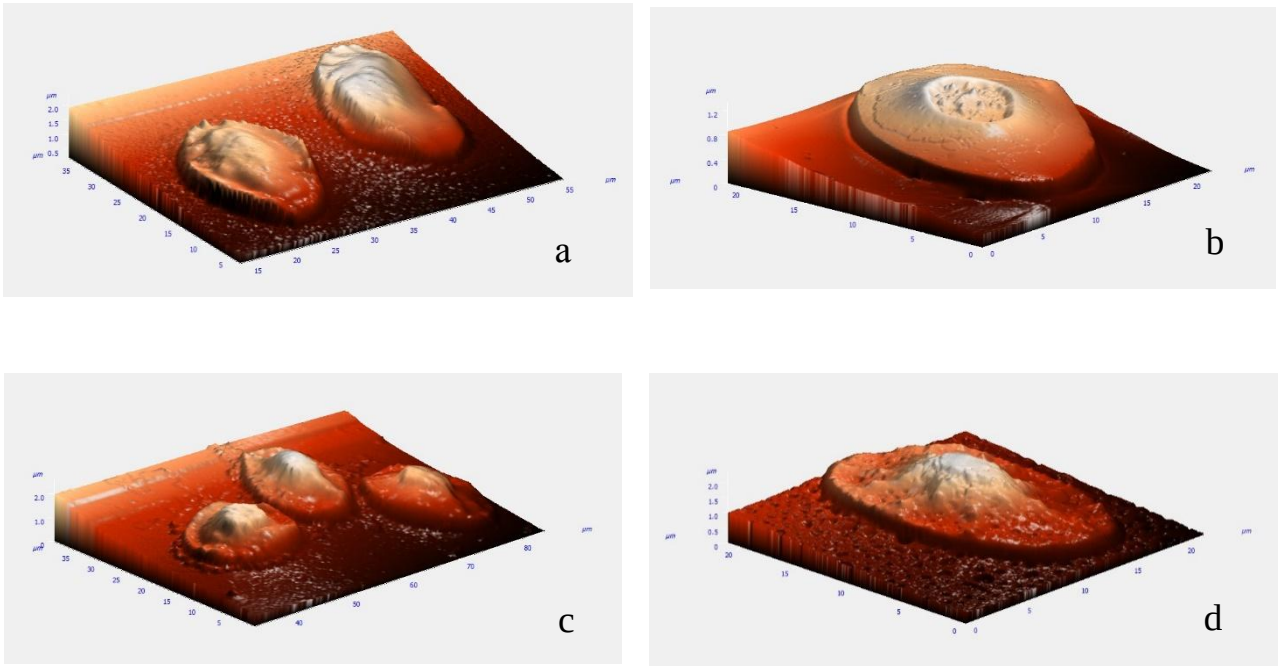

Fig. 1. AFM images of erythrocytes of frog at different incubation temperatures: a –5°C; b – 20°C; c – 37°C; d – 40°C

Fig. 2 shows AFM-scans of polymorphonuclear leukocytes of the frog *Ranaridibunda*, obtained at different incubation temperatures.

As one can see from the pictures, PMNL have a circular form. At all temperatures of incubation the surface of white blood cells of frogs is rough, and at the temperature of incubation of 5° C and 37° C, it is concave, and at the temperature of 20° C and 40° C – convex.

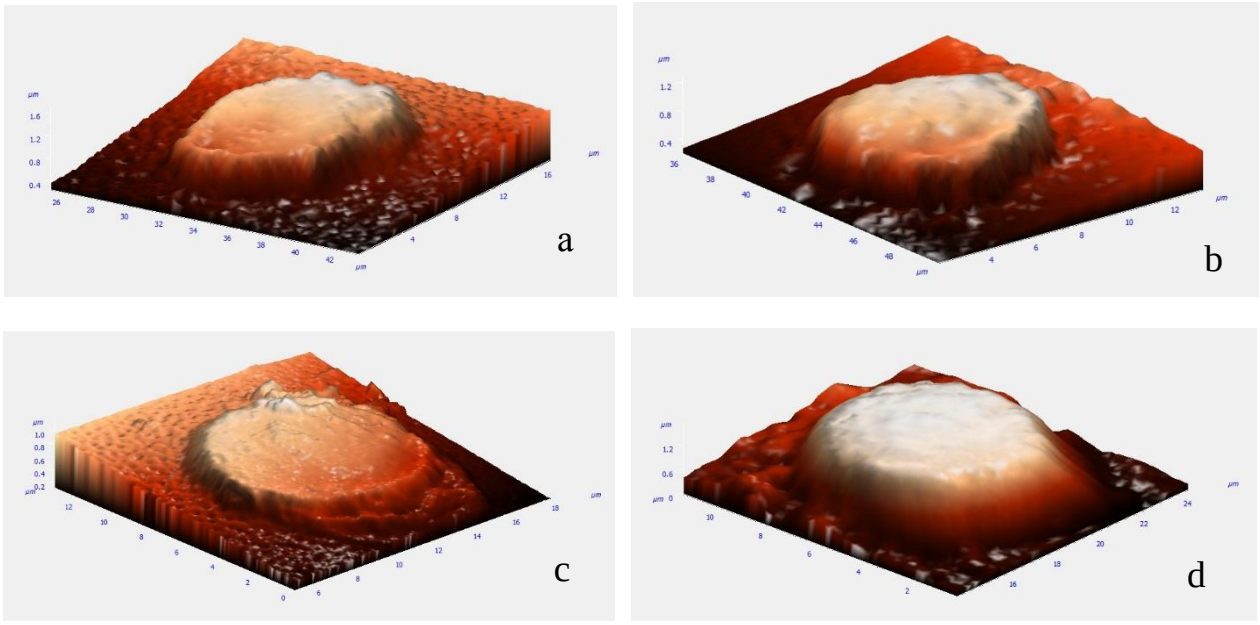

Fig. 2. AFM images of polymorphonuclear leukocytes of a frog at different incubation temperatures: a – 5°C; b – 20°C; c – 37°C; d – 40°C

Morphometric parameters of frog blood cells obtained by the method of the AFM are shown in Tables 1-2.

Table 1: The morphometric parameters of erythrocytes *Rana ridibunda* Pall.

Morphometric parameters	Incubation temperature, °C			
	5	20	37	40
Area, mcm^2	252.1±41.8	257.37±17.53	225.92±25.14 *	217.05±25.31 *
Volume, mcm^3	275.06±77.61	303.73±28.85	261.77±42.82 *	253.85±28.13 *
Short axis, mcm	17.83±1.37	18.06±0.59	17.00±0.97 *	16.60±0.95 *
Long axis, mcm	26.21±1.86	27.00±1.21	25.39±1.52 *	24.87±1.35 *
Height, mcm	9.59±0.97	9.50±0.31	8.91±0.56 *	8.67±0.52 *

Note: Here and in Table 2 * - significance of differences compared with the temperature at 20 ° C according to Student's *t*-test (P <0.05)

It has been found that reducing the incubation temperature does not cause change of morphometric parameters of erythrocytes as compared with incubation at the room temperature, whereas at the elevated incubation temperature these parameters are significantly reduced (see Table 1). Thus, incubation of the red blood cells at temperatures of 37°C and 40°C reduces the magnitude of the volume by 13.9% ($p < 0.05$) and 12.5% ($p < 0.05$), the area - by 16.5% ($p < 0.05$) and 15.6% ($p < 0.05$), respectively, as compared with the room temperature.

In PMNL at low incubation temperature the area and the height are reduced by 8.6% ($p < 0.05$) and by 16.1% ($p < 0.05$), respectively, compared with the room temperature (see Table 2). Increasing the incubation temperature also initiates reduction of cells. The increase in the incubation temperature up to 37°C reduces the diameter and the height of cells by 10.9 ($p < 0.05$) and by 14.7% ($p < 0.05$), respectively, but still does not cause a change in their area or volume. Increasing the incubation temperature up to 40°C causes a significant decrease in white blood cells: the value of the surface area is reduced by 24.3% ($p < 0.05$), the adiametr and the height by 22.6 ($p < 0.05$) and 21.3% ($p < 0.05$), respectively.

Table 2: Morphometric parameters of polymorphonuclear leukocytes *Ranaridibunda*Pall.

Morphometric parameters	Incubation temperature, ^{o}C			
	5	20	37	40
Area, mcm^2	79.76±8.35*	87.24±5.96	88.28±9.67	66.04±11.12*
Volume, mcm^3	97.03±4.79	113.18±22.48	118.29±39.86	100.94±15.13
Diameter, mcm	11.19±0.55	11.85±1.12	10.56±0.57 *	9.17±0.85 *
Height, mcm	5.01±0.06 *	5.97±0.14	5.09±0.73 *	4.70±0.18*

Application of atomic force microscopy also allowed to estimate the change of the elastic membrane indices and the adhesion of bloods cells *Ranaridibunda*Pall to nanoprobe after incubation of the red blood cells and PMNL at different temperatures (Table 3).

Table 3: Changes of elastic modulus and adhesion of blood cells *Ranaridibunda*Pall.

Incubation temperature, ^{o}C	Types of cells	modulus of elasticity, kPa	Adhesion to the nanoprobes, nN
5	E	25.27±3.24*	8.10±1.03*
	PMNL	27.62±3.29*	6.91±1.97*
20	E	18.05±1.92	6.98±0.76
	PMNL	22.61±2.32	6.34±0.72
37	E	27.73±3.71*$^{\&}$	7.46±1.70*$^{\&}$
	PMNL	23.75±1.12*$^{\&}$	7.51±1.02*$^{\&}$
40	E	18.34±3.04$^{\&\#}$	5.88±1.21*$^{\&\#}$
	PMNL	17.31±1.52*$^{\&\#}$	5.24±1.32*$^{\&\#}$

Notes: E – erythrocytes; PMNL – polymorphonuclear leucocytes; significance of differences compared to the temperature: * –20^{o}C; $^{\&}$ –5^{o}C; $^{\#}$ –37^{o}C according to Student's *t*-criteria ($p<0.05$)

As can be seen from Table 3, the value of Young's modulus (kPa), which characterizes the elasticity of the cell surface of the red blood cells and PMNL, changes under the action of different incubation temperatures. Thus, by reducing the incubation temperature to 5°C, elastic modulus of nucleated red cells and PMNL increases by 28.6% ($p < 0.05$) and 18.1% ($p < 0.05$), compared with the room temperature. By increasing the incubation temperature to 37°C the elasticity of red blood cells and PMNL also increases by 34.9% ($p < 0.05$) and 4.8% ($p < 0.05$), respectively, compared with the room temperature. After incubation of PMNL at 40°C as compared with the temperature of 20°C elasticity cell membranes decreases by 23.4% ($p < 0.05$), whereas incubation of the nucleated red cells at a similar temperature does not cause the change of the indicator. Compared with the temperature of 20^o adhesion to nanoprobes of the membrane of nucleated red cells increases after incubation at temperatures of 5 ° C and 37° C 13.8% ($p < 0.05$) and 6.4% ($p < 0.05$) and decreases by 15.8% ($p < 0.05$) after incubation at 40°C. A similar trend is also observed in PMNL. Reducing the adhesion to nanoprobes of the membrane in PMNL occurs only at their incubation at 40°C compared to the room temperature.

Discussion

The studies have revealed morphological changes of erythrocytes and PMNL of the frog under the influence of incubation temperature. When changing (at decreasing and increasing) temperature of incubation, in comparison with the cells incubated at 20°C, the shape of red blood cells becomes more elongated, they have a large number of folds on the plasmalemma surface, which atypical for the red blood cells of low vertebrates [11-13]. Severe folding surface of the plasma membrane in erythrocytes of frogs under extreme environmental conditions is manifested due to the presence in cells of the pool considerable amount of membrane reserve [12, 14] and is connected with the reorganization of the actin cytoskeleton component and formation of aktin-binding domains in submembrane space, defining the formation of invaginations and protrusion in plasmalemma [15 -18].

Changing the incubation temperature has a less pronounced effect on the morphology of PMNL in comparison with erythrocytes. At temperatures of incubation of 5°C, 20°C and 37°C the white cells of frogs have circular form with small folds on the surface, which is typical of PMNL of frogs [15]. Incubation at 40^o C makes for roughness, since this temperature for the frog is more extreme [19].

Using atomic force microscopy we have established the connection between the incubation temperature and the morphometric parameters of nucleated red cells and polymorphonuclear leukocytes. Red blood cells are more resistant to low temperature of incubation, in comparison with the white cells: reducing the incubation temperature to

5° C as compared with the room temperature does not affect the morphometric characteristics of the red blood cells but causes a reduction in some of the investigated parameters in PMNL. White blood cells, including the PMNL, at a low temperature of incubation exhibit the properties being characteristic of the organism of frogs as a whole: temperature optimum for frogs amounts to 18-28^{o}C [19], cessation of the life processes goes on at 2-5^{o}C [11,19, 20]. Increasing the incubation temperature to 37°C and especially 40^{o}C as compared with the temperature of 20^{o} C makes for reduction of the studied morphometric parameters of erythrocytes and PMNL, as the temperature of 40°C is the upper limit of life activity of frogs [19].

The elastic properties of the nucleated red cells and PMNL under the action of incubation temperatures vary according to their morphometric parameters. It goes with the work by Aleksandrov V. Ya. [1], according to which protein thermostability and ambient temperature are correlated in nature. An increased elasticity of the membrane of nuclear erythrocytes and PMNL and their adhesion to the nanoprobes at the temperature away from the temperature optimum zone of life but being in the range of tolerance (reduction to 5°C and increase to 37°C) is due to changes of lability and stiffness of protein molecules [1]. Elasticity and adhesion of PMNL membrane are reduced after incubation at the temperature in prohibitive tolerance zone (40°C). It is known that there is a substantial reduction of conformational flexibility of the protein molecules [1] in cell adaptation to high temperature. It can be assumed that at the high incubation temperature in membrane, dysregulation of phase transition of lipids, microviscosity of bilipid layer and other properties of structural organization of the membrane go on [2,3, 6, 21].

Conclusion

Thus, morphofunctional indexes of red blood cells and polymorphonuclear leukocytes in frogs *Rana ridibunda*Pall under various conditions of incubation temperature have been studied by the method of atomic force microscopy. In response to increasing the incubation temperature to 37°C and especially 40°C an increased plasmolemma roughness is recorded in erythrocytes, while this reaction of plasmalemma in polymorphonuclear leukocytes is less pronounced. For all types of blood cells under study, one has established the reduction in area, height, elasticity modulus and adhesion of the plasma membrane by increasing the incubation temperature to 40 ° C, compared with the room temperature of incubation of the cells.

We have established an increase of elasticity modulus of plasma membrane and adhesion at lower incubation temperature to 5°C and higher to 37°C as compared with the temperature 20^{o}C. Incubation temperature dropping to 5°C as compared with the room temperature also leads to the reduction in area and height of the white blood cells.

The revealed changes are determined by the peculiarities of the structure of the cell membrane and also mechanisms of temperature adjustment in amphibians.

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