

Study of Morphological Characteristics of Pigeon's Erythrocytes and Hemoglobin Properties under the Influence of Ions Ca^{2+}

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Abstract

By the methods of laser-interference microscopy and Raman spectroscopy has shown the changing of erythrocytes morphological characteristics, hemoglobin's condition and distribution depends on the content of free ions Ca^{2+} extracellular (outside the cells) and intracellular (inside the cells). High concentrations of Ca^{2+} changes distribution and conformation of Hemoglobin. It is assumed that the observed changes in the morphology of the erythrocytes caused under the action of ions Ca^{2+} , and it is one of the mechanisms for maintaining a major function of erythrocytes - transportation of oxygen by the action of extreme impacts.

Keywords: Pigeon erythrocytes; Calcium ions; Laser-interference microscopy; Hemoglobin

Introduction

It is known, that ions Ca^{2+} are universal physiological agents influencing on the many properties of erythrocytes. Ion's actions Ca^{2+} are manifesting on the level of the whole cell [1-4], on the condition of cell membrane bilayer [5], on the level of individual membrane and enzyme systems [6-8]. There are some findings about the influence of Ca^{2+} on the structure of erythrocyte and red blood cell [9].

However, up to the present moment there is no information about the impact of high concentrations ions Ca^{2+} and the duration of their impact on the morphological characteristics of erythrocytes, the distribution of HB and oxygen transport properties.

Taking into account the above data was of interest to establish a correlation between morphological characteristics of red blood cells, oxygen transport and distribution functions of HB under the influence of high concentrations and prolonged exposure of ions Ca^{2+} .

Methods and Materials

The objects of the research were erythrocytes of peripheral blood of a pigeon. Instead of anticoagulant we used heparin (5000 U/ml). Erythrocytes obtained by centrifugation of the whole blood over 1500 g during 15 minutes. Then the supernatant was removed, which contained plasma and leukocytes, and erythrocytes were washed three times with cooled solution 0.9% NaCl. After that erythrocytes were diluted 1:1 (v/v) with saline solution and with a solution of $CaCl_2$, to the effect that the final concentration Ca^{2+} would be 3.5 mM. Incubation is carried out with a temperature of 40°C. Sampling was performed during 0, 10, 20, 30 minutes.

For research of intracellular concentration Ca^{2+} we used the dye Fura Red (Invitrogen, USA). For this purpose, erythrocyte suspension were incubated in the presence of 0.5 mM Fura Red with 37°C during 45 minutes. After incubation period Ca^{2+} containing solution was added to 3 ml in a quartz cuvette for fluorescence measurement. Fluorescence spectra were recorded using a spectrofluorimeter RF-5301PC (Shimadzu, Japan) with a xenon lamp with a power 150 W. Intracellular concentration Ca^{2+} ($[Ca^{2+}]_i$) determined with the help of standard form [10]:

$$[Ca^{2+}]_i = K_d \times \frac{R - R_{min}}{R_{max} - R} \quad (1)$$

Where the K_d – Dissociation constant Fura red;

R – the ratio of fluorescence intensities measured at 420 Nm and 460 Nm;

R_{min} – fluorescence intensity without calcium medium;

R_{max} – fluorescence intensity with a presence of calcium.

Morphological changes in erythrocytes were studied with the help of the method of LIM on an automated interference microscope MII-4M (RUSSIA). Using this method we can research the dynamics of changes in cell shape and structure, as well as functional condition [11,12]. In this work was used the laser with wavelength 650 Nm, the lens with magnification x32 and a numerical aperture 0.65. Researching samples which are diluted 10 times was faced to a special microscope slide with a smooth surface and placed under the microscope lens. Processing of the interference image was performed with the program fiji-win32.

The principle of operation is based on the Linnik interferometer optical system. The device allows you to record the optical distance difference F (ODD).

Researching of transport oxygen properties of Hb erythrocytes was accomplished by the method of Raman spectroscopy (RS). Spectra of RS was obtained with the microscope InVia Raman Microscope (Renishaw, United Kingdom), with the laser wavelength – 532 Nm and with a peak power– 50 mW. As a data logger was used CCD detector with a Peltier cooling. We used the diffraction grid with 1800 lines per millimeter. The width of the slit of the spectrograph was 65 microns. Analysis of spectral data was made by the programme WIRE 3.3 (UK). For researching of changes in conformation Hb we analyzed characteristic frequency in the Raman spectrum Hb [13].

Plotting was made by the programme Origin Pro 8.1.

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Discussion and Results

It is known, that physiological concentration of ions Ca^{2+} in erythrocytes is varied and depending on the methods for determining it is from 8 to 160 nM. With increasing the concentration Ca^{2+} in the incubation medium the content of ions Ca^{2+} erythrocytes also increases [10].

At the first series of experiments we recorded the change of the content Ca^{2+} in intact erythrocytes during 30 minutes in physiological solution. It is found that in case of incubation of erythrocytes there will be decreasing of intracellular concentration of ions Ca^{2+} . The most intensive ionic yield Ca^{2+} occurs during 10 minutes. In this case the losses Ca^{2+} is 46.03% from the initial value and it is continuing in the following 20 and 30 minutes (Figure 1).

In the next series of experiments erythrocytes incubated at the same solution but with adding 3.5 mM Ca^{2+} solution. The experiment results showed that in the first 10 minutes of erythrocytes incubation medium, containing Ca^{2+} is also has losses in intracellular Ca^{2+} . However, ion yield Ca^{2+} in this variant 16.03% less then with the same time interval in the first experiment (Figure 1).

At the longer-term period of erythrocytes incubation medium in Ca^{2+} observed the increasing in the intracellular content of the ions. During 30 minutes the amount of intracellular Ca^{2+} exceeds the reference level of 200%.

It may be noted that observed changes Ca^{2+} flows into the cell or from the cell depending on concentration of extracellular Ca^{2+} . And one of the mechanisms responsible for entry or ion yield Ca^{2+} is Na-Ca-exchanger and K^{+} -channels, is activated by hyperpolarization.

At the next stage of our work, we researched the influence of ions Ca^{2+} on the morphological characteristics of erythrocytes. We found that in normal conditions the area of erythrocytes is equal to $316.17 \pm 6.64 \mu\text{m}^2$ (Figure 2). With incubation of erythrocytes medium Ca^{2+} in concentration 3.5 mM area of erythrocytes does not change during 20 minutes of our experiment.

Increasing of exposure time Ca^{2+} on the erythrocytes to 30 minutes leads to decreasing of red blood cells area on 9.6%. Long-term effect of researched ions during 60 minutes virtually didn't change from the control values.

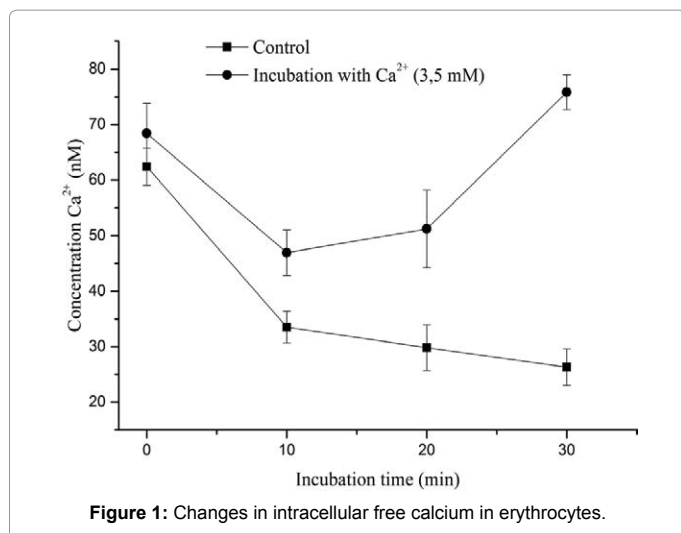


Figure 1: Changes in intracellular free calcium in erythrocytes.

In parallel with the measurement area erythrocytes we recorded the phase height of erythrocytes (Figure 3).

It found that after 10 minutes of ion's exposure Ca^{2+} occurs a decreasing of physical height of erythrocytes. At the incubation time of erythrocytes medium observed further decreasing of ODD (Figure 3). In this case, increasing of ODD reached 23 and more percent from the control ratio. Therefore, the ion's influence Ca^{2+} leads to a change in the physical height of erythrocytes.

It is our belief that these changes connected with ions Ca^{2+} inside erythrocytes. It is known that with increasing concentration of outside Ca^{2+} activated Ca^{2+} -sensitive K-channels, that leads to membrane with hyperpolarization losses ions K^{+} , and that's why the cell will have a different volume [14].

On the next stage of our work we studied a distribution structures in the cytoplasm and the transverse profile of erythrocytes. In control sample erythrocytes has an oval form and intracellular structures uniformly distributed. Erythrocyte membrane has a smooth surface. Control sample cell profile has two limbs and a single peak which is peculiar to nucleus (Figure 4a, c).

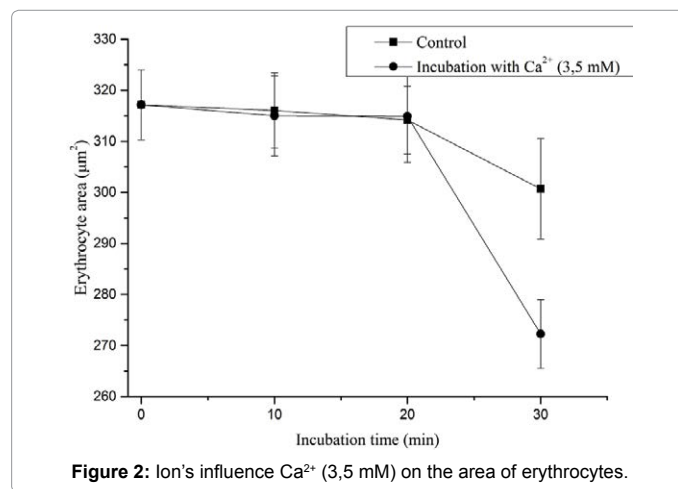


Figure 2: Ion's influence Ca^{2+} (3,5 mM) on the area of erythrocytes.

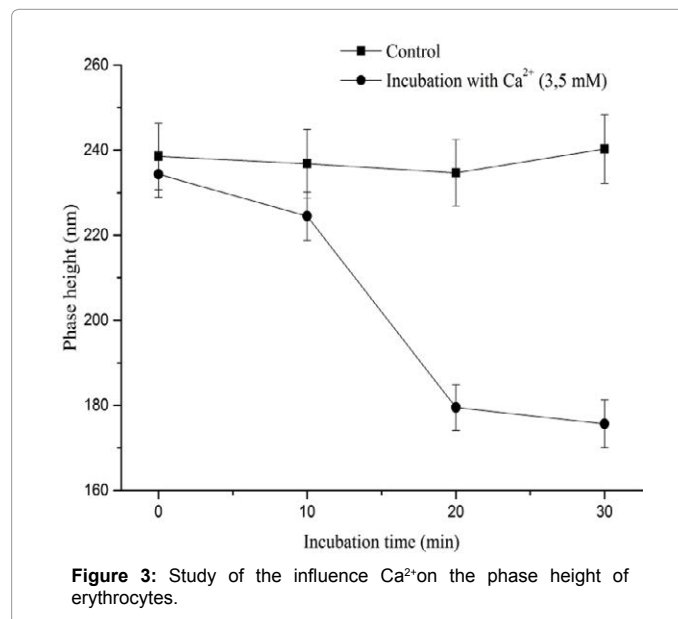


Figure 3: Study of the influence Ca^{2+} on the phase height of erythrocytes.

With concentration increasing Ca^{2+} in the medium of incubation, the form of erythrocyte will change (Figure 4b). Equal distribution of the cytoplasm cell structure will be disturbed. Erythrocyte membrane of the prototype has heterogeneous phase height on the cell's perimeter. Graph of the cross-section erythrocyte has 3 distinct peaks, two of which are characteristically to the membrane of erythrocyte, as for the big one – central peak – it is characteristically to nucleus. By comparison of erythrocytes profiles we can see that the erythrocyte phase height is lower than the reference value. Width of erythrocyte I also have changed. In the sample incubated medium with a high content of Ca^{2+} decreases the erythrocyte width ten the reference value.

Therefore, ions Ca^{2+} can change morphological characteristics of erythrocytes. It is also known that the structure of erythrocyte can change with membrane hyperpolarization caused under the influence of ions Ca^{2+} [15]. In these conditions ions Ca^{2+} cause a change in condition of the lipid bilayer (Figures 4 and 3D).

Take account of this data we have assumed that the changes in the morphological characteristics of erythrocytes can influence on the physical, chemical and functional parameters and primarily on the

function of oxygen transport of Hb associated with the structure of packed red cells.

That's why on the next stage of our work we researched Hb oxygen transport function of erythrocytes by recording Raman spectrum under similar conditions. Figure 5 shows a Raman spectrum of the erythrocytes, which reflects the form of Hb located in the blood (Figure 5).

For conformation analysis and O_2 -binding properties of Hb we used the following lines of Raman spectroscopy of blood spectra: 1172, 1355, 1375, 1540-1552, 1580-1588 cm^{-1} . Line 1172 cm^{-1} appeared resulting from asymmetric vibrations of the Hb pyrrole rings. Proportion I_{1375} / I_{1355} contains information about the severity of symmetric and asymmetric vibrations of the pyrrole rings, and its variation may be due to conformational changes of pyrroles. Lines 1355 and 1375 cm^{-1} connected with symmetric vibrations of pyrrole rings in the d-Hb and Hb, connected with ligands. Since the amount of O_2 in blood exceeds the content of NO and CO, so line intensity 1375 cm^{-1} in general determined by the content of, o-Hb. Thus, the intensity ratio $I_{1375} / (I_{1355} + I_{1375})$ is a characteristic of the relative number of Hb-blood or erythrocyte suspension.

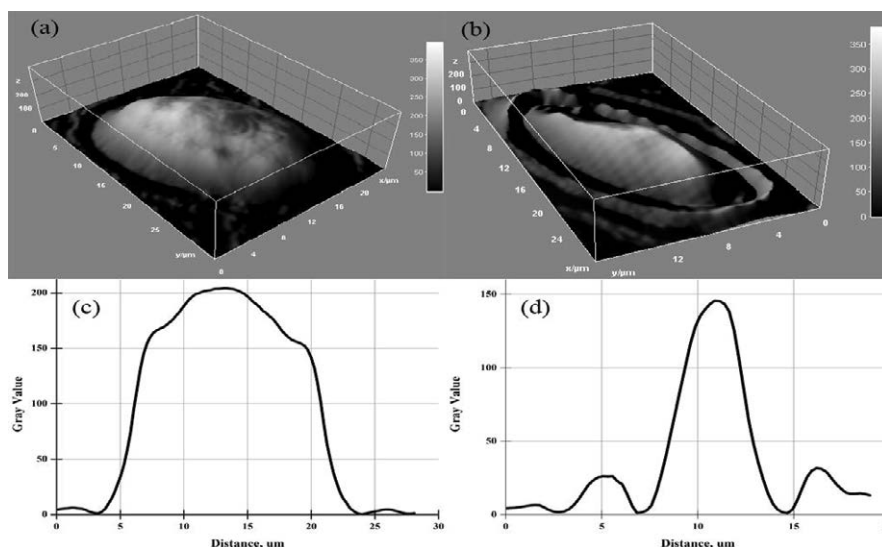


Figure 4: 3D models and profiles of erythrocytes: a, c – 3D erythrocyte model and profile of prototype; b, d – 3D model and profile of erythrocyte incubated with a high content of calcium.

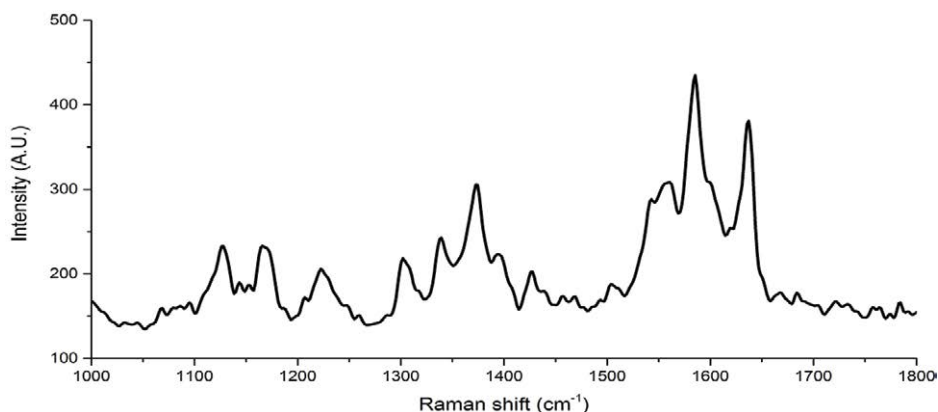


Figure 5: The Raman spectrum of pigeon erythrocytes.

	$\frac{I_{1375}}{I_{1172}}$	$\frac{I_{1375}}{I_{1355}}$	$\frac{I_{1355}}{I_{1550}}$	$\frac{I_{1375}}{I_{1580}}$	$\frac{(I_{1355}/I_{1550})}{(I_{1375}/I_{1580})}$	$\frac{I_{1375}}{I_{1375} + I_{1355}}$
Control, 0 min	1.035 ± 0.048	1.077 ± 0.036	0.878 ± 0.035 *	0.698 ± 0.012	1.195 ± 0.049 *	0.493 ± 0.016 *
Control, 10 min	1.046 ± 0.035	1.084 ± 0.041	0.870 ± 0.019 *	0.709 ± 0.021	1.200 ± 0.024 *	0.511 ± 0.021
Control, 20 min	1.055 ± 0.028	1.097 ± 0.028	0.865 ± 0.025 *	0.714 ± 0.002 *	1.212 ± 0.039 *	0.523 ± 0.006 *
Control, 30 min	1.245 ± 0.023 *	1.183 ± 0.028 *	0.801 ± 0.016	0.707 ± 0.001 *	1.133 ± 0.025	0.542 ± 0.006 *
Ca ²⁺ , 3,5 mM, 0 min	1.114 ± 0.028	1.248 ± 0.043	0.716 ± 0.032 *	0.700 ± 0.034	0.964 ± 0.042 *	0.548 ± 0.012
Ca ²⁺ , 3,5 mM, 10 min	1.121 ± 0.031	1.267 ± 0.041	0.724 ± 0.028 *	0.7250 ± 0.019	0.969 ± 0.029 *	0.558 ± 0.031
Ca ²⁺ , 3,5 mM, 20 min	1.126 ± 0.014	1,288 ± 0,013	0,728 ± 0,047 *	0,740 ± 0,007 *	0,985 ± 0,055 *	0,563 ± 0,002 *
Ca ²⁺ , 3,5 mM, 30 min	1.138 ± 0.004 *	1,107 ± 0,002 *	0,873 ± 0,001	0,759 ± 0,001 *	1,149 ± 0,001	0,525 ± 0,001 *

Table 1: The ratio of the intensities of the characteristic frequencies of the HB spectrum ($p < 0.05$ on Student t-test).

Lines 1540-1552 cm⁻¹, 1580-1588 cm⁻¹ are associated with vibrations of methine bridges between Pirola molecules of Hb. In the case where the number of Hb does not change the ratio I_{1580}/I_{1548} characterizes the affinity to O₂ Hb. The intensity ratio of I_{1355}/I_{1550} reflects the relative ability of the whole Hb in the sample bind ligands, including O₂, and the ratio of I_{1375}/I_{1580} - Hb relative ability to allocate ligands. Table 1 shows the results of the calculation of these indicators.

As we can see from the data received in the erythrocytes exposed to high concentration Ca²⁺, occurs a change of conformation Hb. However, there are no significant changes Hb in ability to transport oxygen (Table 1). During 30 minutes of incubation the affinity parameters and characteristics of complexes with ligands HB did not change. This result suggests that increasing of the concentration of ions Ca²⁺ in the incubation medium did not lead to significant changes in the properties of Hb, thus retained its ability to transport oxygen. Table 1 shows the results of the calculation of these indicators.

Conclusions

Thus, from these results we can conclude that ion's influence Ca²⁺ can cause changes in the geometric characterized-acteristics erythrocytes. At that, the detected changes in the morphological characteristics of red blood cells do not affect on the oxygen transport function of hemoglobin.

The fact that the changes in the morphological characteristics of red blood cells does not lead to a breach of oxygen transport by hemoglobin possibly indicates the existence of a compensatory mechanism that allows implementation to maintain its core functions under the influence of extreme factors.

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