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Studies of the Structure and Functions of Blood Cells in Senile Patients with Pneumonia on the Biological Model of Hypoxia by Scanning Probe Microscopy

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Biological model of hypoxia can be used for the diagnosis of functional changes in human erythrocytes under the effect of the hypoxic factor. The use of this model together with modern methods of scanning probe microscopy for evaluation of the severity of pulmonological disease in senile patients will help to predict treatment efficiency and outcome.

Key Words: *scanning probe microscopy (SPM); Young's modulus; pneumonia*

Physiological changes in human tissues during aging manifest in impaired adaptation capacity to metabolic stress and general deterioration of the health status. The older the individual, the higher is the risk of disease development, of which pulmonological diseases, including acute community-acquired pneumonia (called pneumonia in the text below) are most hazardous [1]. Respiratory diseases often cause a potent unfavorable effect, oxygen deficit in tissues, and therefore, studies of tissue hypoxia is the main problem of pulmonology and physiology [2]. Studies of the mechanisms of cell response in hypoxia, evaluation of regularities of disease development, and the search for prognostic and diagnostic criteria are expected to improve evaluation of the severity and progress of various diseases, including pneumonia [8]. Scanning probe microscopy (SPM) is an informative method for studies of the physiological status in health and disease, specifically, for studies of blood cell parameters.

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Extrapolation of experimental data on animal blood obtained on a biological model to the data obtained in studies of hypoxia in patients is expected to show the trend of changes in blood cell structure and functions, indicating the effect of the hypoxic stress factor on the organism.

The strong impact of infection is essential in pneumonia, which can modulate the cell parameters, specifically the erythrocyte values [1]. Use of the biological model will help differentiate the hypoxic effects on changes in human blood erythrocyte characteristics from the bacteriological factor. If the trend of changes in the rat red blood cells is similar to changes in patients with pneumonia, presumably, it is the hypoxic, but not the bacterial factor, that is mainly responsible for changes in human erythrocytes.

We used the biological model for SPM studies of the blood cell structure and functions in senile patients with pneumonia.

MATERIALS AND METHODS

The study was carried out at Research Laboratory "Physiology of Adaptation Processes", Belgorod Sta-

te National Research University, and included two stages: animal studies to detect the common trend of changes in blood cell parameters under conditions of experimental hypoxia and studies of blood cells from senile patients with pneumonia. Stage 1 was aimed at detection of changes in the parameters of animal blood cell under conditions of hypoxic exposure. The aims of stage 2 are to study the same blood cell parameters in senile patients with pneumonia and to exclude the effect of the bacterial factor; to compare the results of animal experiment with the results of studies of patient blood; to identify changes in blood cell morphology and functions caused by the negative effect of hypoxia.

Studies on animal blood. Peripheral blood erythrocytes and neutrophils from 60 outbred male laboratory gray rats (*Rattus norvegicus*) were studied. The animals were handled in accordance with the Helsinki Declaration Philosophy of Humane Handling of Animals, presented in the EEC Directive (86/609/2008) and the Ethic Regulations of Biomedical Studies [6]. The rats were kept in standard cages under controlled environmental conditions at 19-21°C and 30-70% air humidity at 12:12 h day:night regimen, with 11 air changes per hour. The animals received water and standard fodder. The rats were adapted to experimental conditions for 10 days before experiment in groups of 5 animals per cage, their status evaluated visually. After adaptation, the animals were distributed at random into experimental, control, and intact groups (20 animals per group); the main criterion of distribution was body weight differences <10%. No infections were detected in animals throughout the experiment.

Acute hypoxia was induced by placing the animals in a closed 750-cm^3 vessel for 25 min. Controls were kept in ventilated vessels for 25 min under standard environmental conditions. In order to rule out the effects of stress and hypodynamia on blood cell reactions under conditions of oxygen deficit, an intact group was formed, kept in cages under normal environmental conditions. The blood was collected after decapitation of narcotized animals (inhalation narcosis with ethyl ester). Heparin was used as the anticoagulant (10 U/ml).

Studies on patient blood. Erythrocytes and neutrophils from senile patients with pneumonia (11 patients aged 80-84 years; Table 1) were studied. All patients presented with signs typical of an infectious disease: the leukocytic formula shifted to the left, high erythrocyte sedimentation rate (ESR=24±2 mm/h). Blood cells from 10 age-matched normal subjects served as control. The blood (3 ml) was collected from the vein no later than 40 min after admission in patients with manifest signs of disease exacerbation hospitalized at Pulmonological Department of Municipal Clinical Hospital No. 1, (Belgorod).

Methods for cell separation, sample processing, and scanning of formed elements of the blood were identical for animals and humans. Leukocytes and erythrocytes were separated by centrifugation (10 min, 1500 rpm). The leukocyte layer was collected, in which the erythrocyte admixture was destroyed by 0.83% ammonium chloride solution. Leukocyte and erythrocyte suspensions were washed twice in isotonic buffer (Dulbecco's solution; pH 7.4). ESR was assayed by the Westergren method, blood pH was measured using a pH 827 lab pH-meter (Metrohm). Structural and functional characteristics of blood cells were studied under a NTEGRA Vita atomic force microscope (NT-MDT, configuration based on Olympus IX-71 inverted stage microscope). Scanning was carried out after processing of blood cell specimens from each sample in a semicontact mode using NSG, CSG cantilevers (Nanoworld) with rigidity <0.5 N/m. The preparations for scanning mounted on clean degreased glass were placed into a humid box to preserved native characteristics of cells [4]. A total of 30 neutrophils and 50 erythrocytes were scanned in each experimental series

TABLE 1. Patients Characteristics

Disease	Number of patients	Age, years
Community-acquired left lower lobe pneumonia (severe course)	3	82, 84, 84
Community-acquired left upper lobe pneumonia (severe course)	2	82, 82
Community-acquired left focal lower lobe pneumonia (medium severe)	2	80, 82
Community-acquired bilateral polysegmented pneumonia (severe course)	1	80
Community-acquired right upper lobe focal pneumonia concomitant with chronic obstructive pulmonary disease (severe course)	1	80
Community-acquired bilateral lower lobe pneumonia (severe course)	1	83
Community-acquired left lower lobe pneumonia (medium severe)	1	82

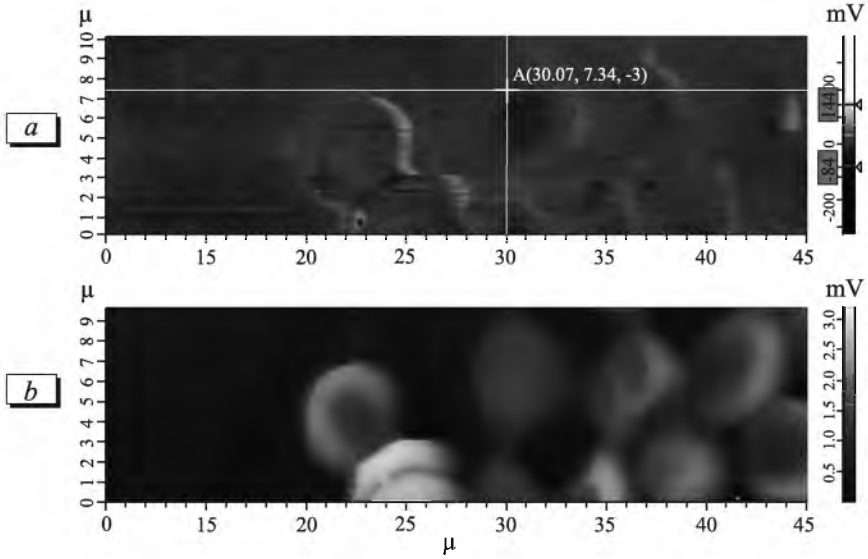

Fig. 1. Distribution of potential (*a*) on the surface of group of cells (*b*).

in the semicontact mode by the method for studies of native blood cells [5].

Elastic characteristics (Young's modulus, YM) were measured in the force spectroscopy mode based on evaluation of the sample surface deformation during its interactions with the probe [3]. The YM was recorded using a modified cantilever based on polymeric microspheres, attached to CSG 11 tipless as described previously [5]. The electric characteristics of blood cell surface were studied in Kelvin's mode using a probe with current conducting titanium coating, lot NSG03/Tin. The surface potential (SP) was evaluated by its gradient distribution in SPM scan (Fig. 1). Erythrocyte smears were prepared on a special degreased metal sublayer for SP measurements. The cell morphology (surface area, volume) was evaluated in the resultant scans using NOVA and Gwyddion softwares.

The results were statistically processed by methods of variational statistical using Microsoft Excel 14.0 and Statistica 6.0 softwares, statistical processing was carried out by Student's *t* test.

RESULTS

Analysis of morphometric parameters of erythrocytes of rats exposed to acute hypoxia showed a decrease in the surface area in experimental and control groups in comparison with intact group (by 15 and 13%, respectively; $p<0.05$; Fig. 2). The surface areas in intact and control groups differed just negligibly. Erythrocyte volumes were virtually the same in all groups (35.5±5.6 μ^3).

The erythrocyte YM of experimental animals differed significantly from the parameter in intact and control groups: by 23 and 24% ($p<0.05$), respectively. The parameter in intact and control groups virtually did not differ. The blood erythrocyte YM in rats without exposure was 1.96±0.16 μPa (Fig. 3). The neutrophil YM was 1.70±0.24 μPa in all groups.

The erythrocyte surface charge was the minimum in experimental group, differing from the parameter in intact and control groups by 56 and 44%, respectively. The YM of intact and control animals virtually did not differ (Fig. 3).

Hence, exposure of animals to stress and hypodynamia in a ventilated reservoir virtually does not tell on the erythrocyte parameters. Hypoxia as a stress factor causes just negligible changes in erythrocyte morphology and significant changes in the mechanical and electric parameters of cell surface, such as YP and SP (an increase and decrease, respectively, after exposure). It is known that the neutrophil cell membrane rigidity decreases significantly in the presence of large inflammatory foci. This shift is due to neutrophil activation and is essential for their subsequent migration from vessels to tissues [10,11]. In our study, the neutrophil YM in rats does not change, which attests to the absence of the infection impact in experimental group. In sepsis, the erythrocytes acquire a spherical shape [7,9]. In our study, rat erythrocytes had common (biconcave) shape in all groups. The rat ESR (4.0±0.3 mm/h) also indicates the absence of infections. Importantly, the blood pH in hypoxia reduces (acidosis). The mean pH of the blood in rats exposed to acute hypoxia is 7.36±0.03 *vs.* 7.41±0.02 in control rats.

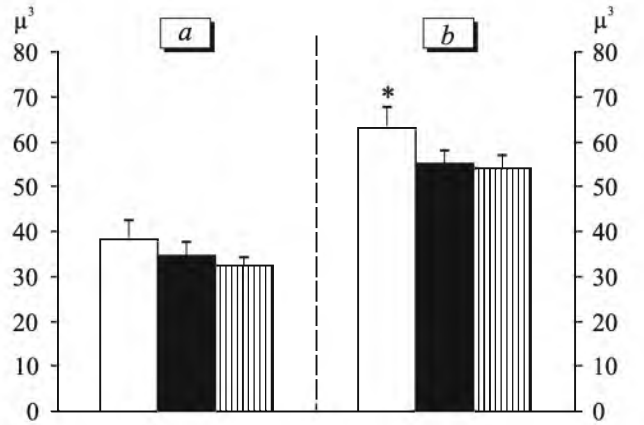

Fig. 2. Volume (*a*) and area (*b*) of erythrocytes in animals. *$p<0.05$ in comparison with the control group. Here and in Fig. 3: light bars: intact group; dark bars: control group; vertically hatched bars: experimental group.

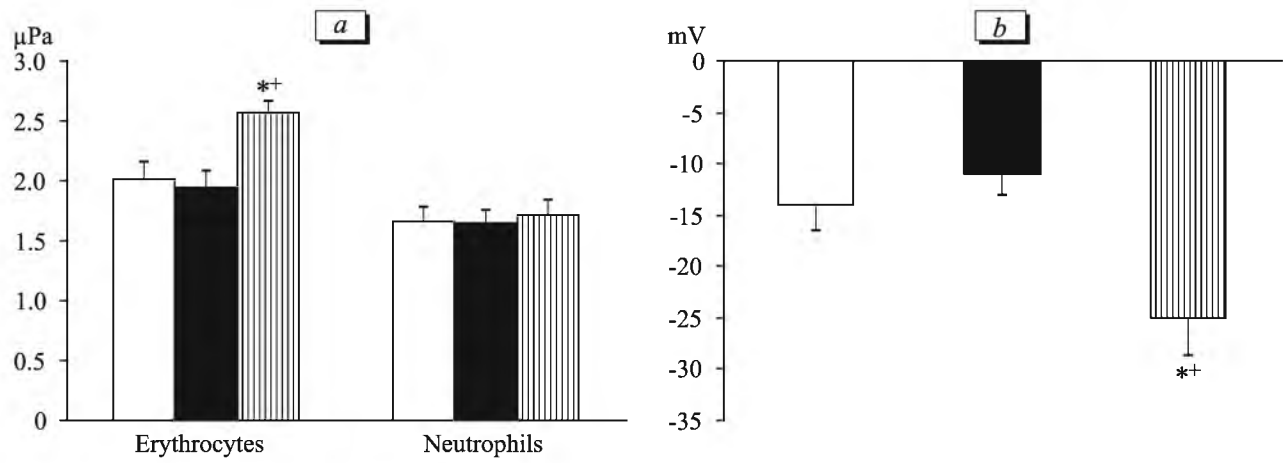

Fig. 3. Erythrocyte and neutrophil YM (*a*) and erythrocyte SP (*b*) in animals. $p<0.05$ in comparison with *control, +intact groups.

If changes in the blood cell parameters in patients with pneumonia are similar to the above "model" changes in experimental animals, we can speak about the effect of the hypoxic factor on human erythrocytes as the main factor in pneumonia. Blood cell parameters of patients according to SPM are presented in Table 2.

Erythrocyte YM in experimental group increased by 32% ($p<0.05$), SP decreased by 56% ($p<0.05$,) and blood pH shifted to the acid values in comparison with the control. It seems that the impact of infection manifested by a 30% decrease ($p<0.05$) of the neutrophil YM in patients with pneumonia [10,11]. We find no data on the bacterial effects on human erythrocyte YM and SP. Analysis of the results of stages 1 and 2 of our study showed a trend to changes in the erythrocyte YM and SP in experimental animals and humans.

Hence, the biological model of hypoxia can be used for the diagnosis of changes in the erythrocyte function under the effect of hypoxia in patients with pneumonia. The probability of a favorable outcome of the disease is higher when these accessory diagnostic instruments are used, as it is possible to correct the treatment strategy, which can be aimed at the underlying disease control and at prevention of complications, which is essential in senile patients with concomitant diseases.

TABLE 2. Human Blood Cell Parameters (*M*±*m*)

Parameter	Control	Experiment
YM, μPa		
erythrocytes	1.87±0.34	2.75±0.46*
neutrophils	1.01±0.12	0.71±0.22*
Erythrocyte SP, mV	-4±1	-9±3*
Blood pH	7.40±0.02	7.37±0.02
ESR, mm/h	14.5±1.5	24.0±2.0*

Note. *$p<0.05$ in comparison with the control group.

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