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Abstract

[1)]Tishko T. T.*, [1)]Tishko D. N.,
[1)] Titar V. P. ,[2)] Prykhodko O. O.,
[2)] Bumeister V. I.
[1)] V. N. Karazin Kharkov National University,
4, Svoboda Sq., Kharkov, Ukraine, 66077;
[2)] Sumy State University,
2, Rymskogo-Korsakova St., Sumy, Ukraine, 40007

APPLICATION OF DIGITAL HOLOGRAPHIC INTERFERENCE MICROSCOPY AND ELECTRON MICROSCOPY TO STUDY RATS' ERYTHROCYTES OWING TO THE INFLUENCE OF HEAVY METAL SALTS

We employed the combination of the digital holographic interference and electron microscopy methods to study 3D morphology of rats' blood erythrocytes o. The rats drank water with high concentration of heavy metal salts for a month. The results showed considerable morphological changes of blood erythrocytes in animals from experimental group: the number of transformed and degenerative erythrocytes increased, the average diameter of normal erythrocytes decreased and shape transformations of normal erythrocytes regarding sphericity coefficients increased.

Key words: digital holographic interference microscopy, electron microscopy, red blood cell morphology, 3D imaging, sphericity coefficient, heavy metal salt, rat.

Corresponding author: * tishko@univer.kharkov.ua

[1)]Тішко Т. Т.*,[1)] Тішко Д. Н.,
[1)] Тітар В. П.,
[2)] Приходько О. О. ,
[2)] Бумейстер В. І.
[1)]Харківський національний університет ім. В. Н. Каразіна,
площа Свободи, 4,
Харків, Україна, 66077;
[2)]Сумський державний університет,
вул. Римського-Корсакова, 2,
Суми, Україна, 40007

ВИКОРИСТАННЯ ЦИФРОВОЇ ГОЛОГРАФІЧНОЇ ІНТЕРФЕРЕНЦІЙНОЇ МІКРОСКОПІЇ ТА ЕЛЕКТРОННОЇ МІКРОСКОПІЇ ДЛЯ ВИВЧЕННЯ ЕРИТРОЦИТІВ КРИС В УМОВАХ ВПЛИВУ СОЛЕЙ ВАЖКИХ МЕТАЛІВ

Ми використовували комбінацію методів цифрової голографічної інтерференційної мікроскопії та електронної мікроскопії для створення тримірних зображень еритроцитів крові щурів, яких поїли протягом місяця водою з високим вмістом солей важких металів. Отримані результати відображали принципові морфологічні зміни еритроцитів крові у тварин експериментальних груп, збільшення кількості змінених та дегенеративних форм еритроцитів, зменшення середнього діаметру нормальних еритроцитів, трансформація форм еритроцитів обумовлювалась зростанням коефіцієнту сферичності.

Ключові слова: цифрова голографічна інтерференційна мікроскопія, електронна мікроскопія, морфологія червоних кров'яних тілець, тримірне зображення, коефіцієнт сферичності, солі важких металів, щур.

Website: http://www.ujcem.med.sumdu.edu.ua/
e-mail: jcandemr@gmail.com

Резюме

[1)]Тишко Т. Т., [1)] Тишко Д. Н., [1)] Титар В. П., [2)] Приходько О. А. , [2)] Бумейстер В. И.*

[1)]Харьковский национальный университет им. В. Н. Каразина
Площадь Свободы, 4,
Харьков, Украина, 66077;
[2)]Сумский государственный университет,
ул. Римского-Корсакова, 2,
Сумы, Украина, 40007

ПРИМЕНЕНИЕ ЦИФРОВОЙ ГОЛОГРАФИЧЕСКОЙ ИНТЕРФЕРЕН-ЦИОННОЙ МИКРОСКОПИИ И ЭКЛЕКТРОННОЙ МИКРОСКОПИИ ДЛЯ ИЗУЧЕНИЯ ЭРИТРОЦИТОВ КРЫС В УСЛОВИЯХ ВЛИЯНИЯ СОЛЕЙ ТЯЖЕЛЫХ МЕТАЛОВ

Мы использовали комбинацию методов цифровой голографической интерференционной микроскопии и электронной микроскопии для создания трехмерных изображений эритроцитов крови крыс, которых поили на протяжении месяца водой с високим содержанием солей тяжелых металов. Полученные результаты отражали принципиальные морфологические изменения эритроцитов крови у животных экспериментальных групп, увеличение количества измененных и дегеративных форм эритроцитов, уменьшение среднего диаметра нормальных эритроцитов, трансформации форм нормальных эритроцитов обусловленных увеличением коэффициента сферичности.

Ключевые слова: цифровая голографическая интерференционная микроскопия, электронная микроскопия, морфология красных кровяных телец, трехмерное изображение, коэффициент сферичности, соли тяжелых металлов, крыса

Автор відповідальний за листування*:* * tishko@univer.kharkov.ua

Introduction

The study of 3D morphological features of blood erythrocytes has great importance for estimation their functionality especially in different pathologies and under influence of different external damaging factors. Solution of the problem of erythrocyte 3D morphology study is directly connected with the development of 3D imaging methods of transparent biological microscopic objects.

Until now electron microscopy remained the single method for 3D imaging of blood erythrocytes. The results of electron microscopy showed that, in addition to haematological diseases, the diseases of various geneses could be the reason for modifications of the 3D shape of blood erythrocytes [1; 2]. Morphological modifications were detected in toxic anaemia caused by the chronic effect of lead and chlorobenzene [3], and influence of heavy metals salts [4]. Increase of the quantity of transformed and degenerative erythrocytes was observed in the blood of the patients and rats. However, electron microscopy requires special sample preparation, and is unable to study untreated blood cells, and the observed morphological modifications can be caused by various effects related to this process. Moreover, this “destructive” method is not quantitative.

The problem of 3D imaging of untreated blood cells, for the first time, was solved by combining the holographic interferometry methods of phase microscopic object visualization with the methods of digital image processing. We developed the digital holographic interference microscopy method (DHIM) which enabled to obtain 3D images of native blood erythrocytes and measure their morphological parameters from a single recorded interferogram by a numerical process [5]. Though, DHIM method is an optical method that allows studying microscopic objects with sizes not less than the wavelength of the used coherent radiation. As the type of interferometry, DHIM allows measuring phase increments inserted by a transparent microscopic object into the wave passing through it to a fraction of the wavelength in the direction of observation. The interferogram of blood erythrocyte presents a phase profile of erythrocyte. Phase increments, inserted by the microscopic objects into passing through them wave, are the multiplication between the difference in refractive indices between the blood specimen and the surrounding medium, and the thickness of the sample. For blood erythrocytes which refractive index is constant, the thickness profile can be directly obtained from the phase profile.

The DHIM method was used to study erythrocyte morphology of patients after the course of ozone therapy; of newborns with anemia (sickle-cell anemia) and after the course of radiation therapy (gamma-radiation influence) [6]. The DHIM method enabled to detect and quantitatively estimate pathological and morphological transformations of main mass of normal blood erythrocytes, which were observed in blood smears of patients either in different disease, or under the influence of an external factor – radiation. Such parameter as sphericity coefficient was introduced by us to characterize 3D shape of red blood cells and their morphological functionality. It was detected that the pathological morphological transformations of the erythrocytes gradually increased the sphericity coefficients of separate red blood cells and the mean sphericity coefficient of the blood sample that corresponded to the decrease of surface area of red blood cells [7].

The problem of accurate 3D imaging of transparent living cells is very acute and urgent. That's why, several variants of digital holographic microscopy have been developed and applied to study blood erythrocytes since 2005 [8–10].

The article described application of the combination of DHIM and electron microscopy methods to study 3D morphology of rats' erythrocytes under influence of heavy metals salts. Environment pollution leads to accumulation of heavy metals in water, soil, air, and, as a consequence, in organisms of people and animals. Such heavy metals can damage biostructures of different organs and systems, including blood cells. The research of rats' blood cells functionality under heavy metals salts effect can be used as a model to study pathological processes in a human organism because rats' erythrocytes similar to human red blood cells in their morphological and functional properties.

Materials and methods

Cell preparation for electron microscopy study

The experiment on the rats was carried out in accordance with the European Convention for Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986).

White adult male rats were used in our experiment. The rats (8 months old) were divided into two groups: experimental and control. The rats in the experimental group (10 rats) took drinking water with high concentrations (that in 10 times exceed the concentrations in ordinary water) of zinc, copper, iron, manganese and lead salts: $ZnCl_2$ – 5 mg/l; $CuSO_4 \times 5H_2O$ – 5 mg/l; $FeSO_4$ – 10 mg/l; $MnCl_2 \times 4H_2O$ – 1 mg/l; $Pb(NO_3)_2$ – 3 mg/l. The rats in the control group took normal drinking bottle water. The experiment lasted for one month. Blood erythrocytes were the material for the study. Blood was sampled from the tail vein after the end of the experiment.

The samples were prepared following the procedure: separation in the centrifuge at 1 000 rpm for 15 minutes; fixation in 1% glutaric dialdehyde prepared in phosphate buffer; separation from the fixing solution by centrifugation with further fixation in 1 % osmium solution; washing in the phosphate buffer three times and dehydration with ethanol of elevated concentrations (60, 70, 80, 90, 100 %) [1]. Then erythrocyte samples were placed on a graphite tables, and were dried on air. The samples were deposited by carbon using the "VUP 5".

Cell preparation for DHIM study

The dry untreated blood smears on glass substrates were prepared for DHIM study by the standard procedure. It was necessary to have fragments of blood samples where cells were placed one layer.

Electron microscopy study

Study of erythrocyte's morphology was carried out using a scanning electron microscope REM 106" (Ukraine, Sumy, "Electron") with a low vacuum chamber; photos were taken with the magnification from ×2000 to ×40000. The taken electron photos of erythrocytes were analyzed using a computer analyzing system. Percentage of different morphological types of erythrocytes was calculated from a thousand of the cells ($n = 1000$). Calculations were performed on the sample fragments where erythrocytes were placed in one layer. Morphometry and measurement of mean diameter of the erythrocytes were made for 100 cells from each blood sample using the systems of computer image analyses "Video Test 5.0" and "Video size 5.0". Study results were processed using the Student's-test. Statistically significance was $p < 0.05$.

DHIM study

DHIM setup

The DHIM can combine all techniques of holographic microinterferometry in one device: the interference-contrast, phase-contrast and polarization contrast methods in real time or by double exposition [11; 12]. W employed the real-time interference-contrast method (interferometry

in fringes of finite width). The DHIM consists of three main units: holographic interference microscope, digital video camera and computer. Real-time interferograms of the specimens are digitally processed to obtain their 3D images of erythrocytes and measure their morphological parameters. In Fig. 1 you can see the optical lay-out of the DHIM.

A He-Ne laser with the wavelength 0.63μm is used as a source of coherent light. The hologram 12, which was previously recorded on a holographic plate using the reference wave formed by the collimator 9 in the absence of the specimen, is used as an optical element of the DHIM. This hologram produce "empty" object wave.

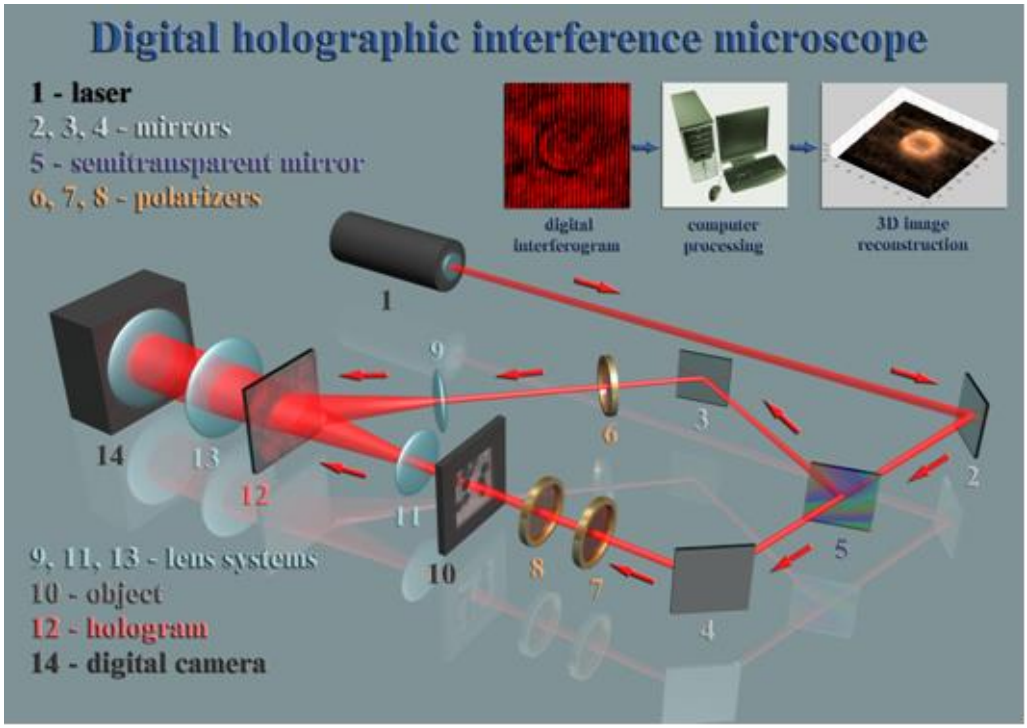

Fig. 1. An Optical lay-out of the digital holographic interference microscopy (DHIM). A He-Ne laser produces coherent light (λ = 0.63 μm), which is divided by beam splitter *5*. The specimen*10* is illuminated by one beam. The transmitted wave is collected by microscope objective *11* and form the object wave. The second beam passes through collimator 9 and forms the reference beam. Hologram *12* is illuminated by the reference beam and produces the "empty" object wave. Two object waves interfere to produce the interferogram recorded by the digital camera *14*

The hologram in the microscope creates the ideal conditions for interferometry because the real-time interferogram of the studied specimen is produced by interference of two identical object waves passing the same optical path (through objective 11 and eyepiece 13), but in different instants of time (the real wave transmitted by the specimen and the reconstructed "empty" wave). These waves differ only in phase shifts inserted by the specimens. Due to identity of the waves such interferogram is a system of straight interference fringes, which are deviated in the image of an erythrocyte representing its phase profile. Period of interference fringes is determined by the angle between the interfering object waves, which can be controlled by shifting the hologram12. Polarizes *6*, *7* and *8* are used to control intensities and polarizations of the interacting waves to reach maximum contrast and quality of the interferogram. 40 x 0.65 objective and 10X eyepiece are used in the microscope. The hologram is recorded on PFG-03 plate. The real-time interferograms of the specimen are recorded by the digital camera 14. In Fig. 2 one can see the typical interferogram of individual rat's erythrocyte. The digital interferograms are computer processed using the mathematical algorithms that can reconstruct the 3D images of blood specimens and measure morphological parameters of erythrocytes.

3D reconstruction procedure

The phase shifts manifest themselves in deviations of the interference fringes in its interferogram (Fig. 2). The interferogram is formed by interference of two identical waves, so its interpretation is simple and clear. Phase shift depends on multiplication of refractive indices between the blood specimen and the surrounding medium, on the one hand, and the thickness of the

sample, on the other hand. Rat's erythrocyte has homogeneous refractive-index distribution. So, it is possible to calculate its thickness *t(x, y)* in any point by measuring the deviation of the interference fringe in the corresponding point in its interferogram and the period of interference fringes [9]:

$$t(x,y) = \frac{\lambda h(x',y')}{T\Delta n}, \quad (1)$$

where $h(x', y')$ is the deviation of the interference fringe in the corresponding point of the interferogram; T is the period of reference interference fringes, λ is the wavelength of the radiation being used, Δn is the difference of refractive indices of the erythrocyte and the ambient medium.

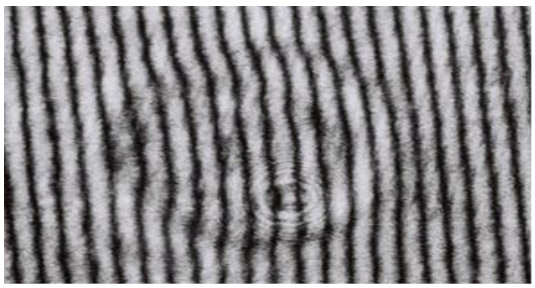

Fig. 2. Interferogram of rat's erythrocyte

As it follows from the formula (1) the thickness is a ration of two values measured in one reference system. So, it is not needed any calibration of the DHIM. Distances (deviations of the interference fringes and the period) are the values directly measured in the method. So, resolution of the method is determined by the minimal measured deviation. It depends on several factors: the accuracy of a used measuring device and quality of the interferogram. Coherent noise and speckle structure of the image, which occur using coherent light, determine the physical limit of the method accuracy. The methods can measure thickness regarding to a fraction of the wavelength of the used coherent light wave.

The algorithm for the digital 3D imaging is very simple and consists of interpreting of the array of values of the deviations as an array of values of the thicknesses of the erythrocyte in accordance with equation (1). Dry blood smears on glass slides were used in our experiment, thus Δn was the refractive-index difference of the erythrocytes and air. The refractive index of blood was measured by means of an Abbe refractometer using a He-Ne laser. The value of blood refractive index equal to 1.352 was used in digital processing of interferograms.

Sphericity coefficient

Volume is one of the most important morphological parameter of erythrocytes. Erythrocyte can form different shapes and, consequently, can have different surface areas under the given volume. We detected in blood of people and rats three main morphological types of erythrocytes in our previous DHIM experiments. They were biconcave disk, flat disk and spherocytes. We used the sphericity coefficient *k* as a ratio of the erythrocyte thickness at the center t_c to the thickness at half of its radius t_r (Fig.3) to characterize the morphological type of erythrocytes:

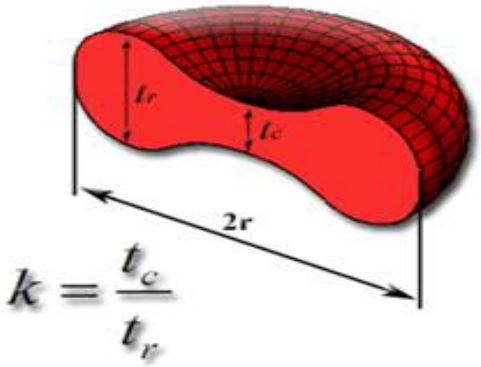

Fig. 3. The sphericity coefficient *k* determination. t_c is the thickness in the center; t_r is the thickness at a half of the radius *r*

In Fig.4 you can see the main morphological types of erythrocytes and their sphercity coefficients.

The sphericity coefficient was measured upon the computer processing of the erythrocytes' interferograms. The sphericity coefficient was less than unity, about unity, and greater than unity, respectively for the biconcave erythrocytes, flat disks and spherocytes. The increase of the sphericity coefficient (transformation of the biconcave erythrocyte to the spherocyte) corresponded to the decrease of its surface area [7]. The sphere had the minimum surface area under the given volume. Functionality of spherocytes was minimal. So, sphericity coefficient can be the parameter of erythrocyte morphological functionality. In this study the sphericity coefficient was calculated for every erythrocyte, and the average value for a blood smear.

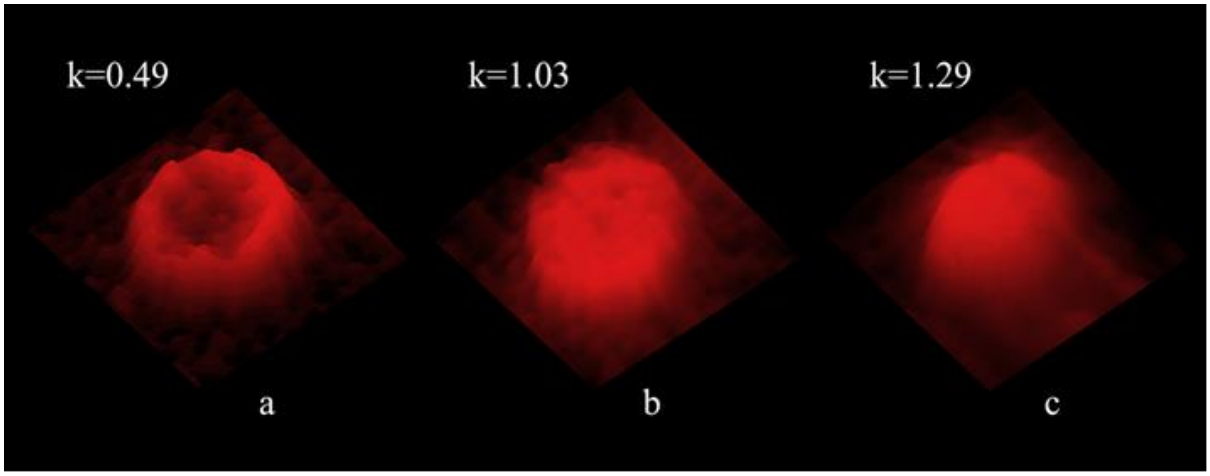

Fig. 4. Three main morphological types of blood erythrocytes and their sphericity coefficients. From left to right: biconcave disk, flat disk, spherocyte

Results

Electron microscopy

After the analysis of rats' erythrocytes (control group) applying the scanning electron microscopy we concluded that the main part of the cells was biconcave; sometimes were flat discs or spherical (Fig. 5). Besides, those main types of cells were found single reversibly and irreversibly deformed erythrocytes; degenerative erythrocytes of atypical shape, for example, cells with cupola-like protrusions, cells with sharply changed forms, with single or multiple protuberances and appendages, and other forms. Spherical, irreversibly deformed, and atypical erythrocytes showed the processes of physiological aging and destruction.

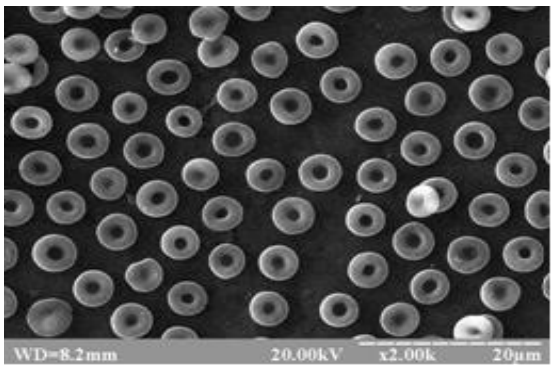

Fig. 5. Erythrocytes of a rat from the control group. The normal biconcave erythrocytes dominate

The blood of rats (experimental group) contained the same types of erythrocyte as in the control group but the correlation between them was greatly changed. We observed the decrease in the number of biconcave normocytes and the increase of deformed shapes (Fig. 6).

The number of spherical cells and atypical cells had increased significantly (Table 1). Results of erythrocyte morphometry showed the decrease of the average diameter of biconcave erythrocytes in the experimental group.

These changes in erythrocyte shapes can be considered as morphological evidence of pathological process course in under heavy metals salts effect.

Thus, application of the scanning electron microscopy showed changes in the shape of the erythrocytes. We observed the decrease in the proportion of normal erythrocytes and the great increase in the number of cells showing the stages of aging and destruction (spherocytes, atypical cells).

Table 1

Results of electron microscopy and DHIM investigations of erythrocytes in control and experimental groups of rats under heavy metal salts effect

Electron microscopy	**Control group**	**Experimental group**
Percentage of different types of RBCs		
Beconcave RBCs (%)	92.6 ± 2.1	71.4 ± 2.1%
Reversibly deformed RBCs (%)	3.5 ± 1.5	7.6 ± 0.6
Irrevesibly deformed RBCs (%)	2.6 ± 0.35	19.4 ± 0.35
Degenerative RBCs (%)	0.85 ± 0.06	1.6 ± 0.1
Diameter	4.97 ± ± 0.02 µm	4.78 ± ± 0.02 µm
DHIM		
Mean sphericity coefficient	0.41 ± 0.03	0.81± 0.03

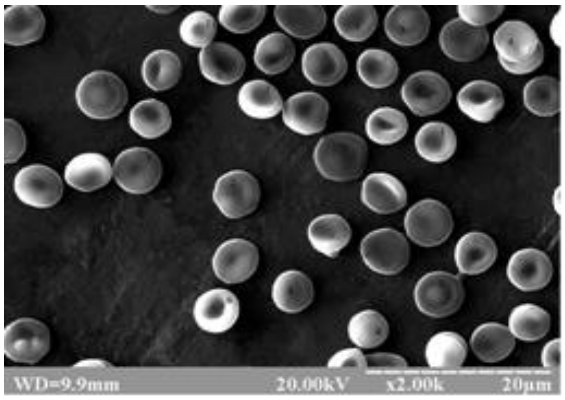

Fig. 6. Erythrocytes of a rat from the experimental group. Different shapes of atypical erythrocytes are present

DHIM

Study of erythrocytes of rats (control group) using the DHIM showed that in blood smears dominated biconcave erythrocytes (Fig. 7). The average sphericity coefficient of erythrocytes in the control group of rats was equal 0.41.

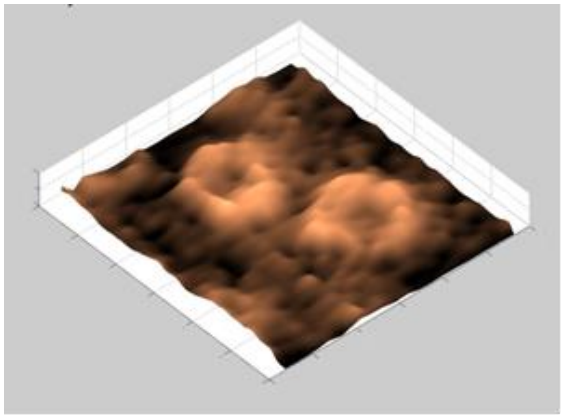

Fig. 7. Fragments of native blood smears of rats from the control group

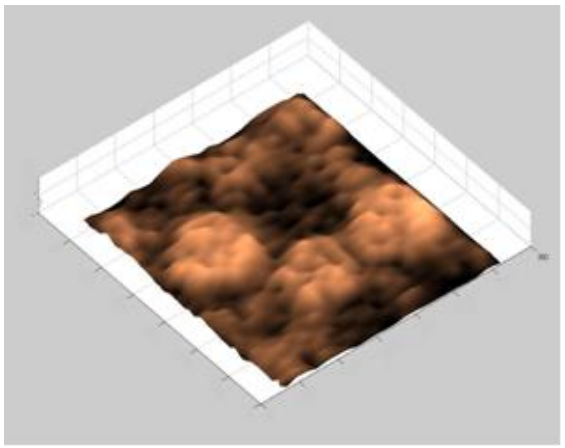

Fig. 8. Fragments of native blood smears of rats from the experimental (b) groups obtained using the DHIM

In the group of rats, which took water with high concentration of heavy metals salts, at addition to the increase of the percentage of deformed and degenerative erythrocytes, morphological changes of normal erythrocytes were observed (Fig. 8). The average sphericity coefficient of the erythrocytes in this group was equal 0.81. Thus, normal erythrocytes in the experimental group changed their shape in the direction of spericity coefficient increase; they became more spherical, their surface area decreased. Such morphological changes led to some reduction in their size. The observed morphological changes confirmed decrease of functionality of normal erythrocytes.

Discussion

Both electron microscopy and DHIM method detected morphological changes of erythrocytes in blood smears of rats which took water with high concentrations of heavy metal salts for a month.

By the electron microscopy method, we detected the decrease in the proportion of normal erythrocytes and the significant increase in the number of transformed and degenerative cells showing the stages of aging and destruction. The decrease of normocytes diameters was also observed.

DHIM method enabled to detect and quantitatively estimate fine morphological changes of normocytes that were the main part of all erythrocytes. Unfortunately, it is impossible for the electron microscopy method. These morphological modifications go in the direction of erythrocytes' sphericity coefficient increase. We used the sphericity coefficient as a quantitative parameter for description of erythrocyte shape. This is a relative parameter, it does not use absolute values, and no calibration of the system is needed. This parameter can be easily measured just from the interferograms of erythrocytes. The sphericity coefficient is inversely proportional to the surface area of an erythrocyte, and can be considered as characteristics of its morphological functionality. The obtained results allowed concluding that biological response of blood erythrocyte (both rat's or human's) during diseases and under damaging factors effect manifested itself in a similar way. That was the increase of the number of transformed and degenerative cells and transformations of shapes of biconcave normocytes in the direction of their sphericity increase. Thus, shape of an erythrocyte seems to be very sensitive to pathologies and external influences, and thus its study it is very important in diagnostics.

Combination of electron microscopy and DHIM method was very useful to study 3D morphology of blood cells. High resolution of

electron microscopy allowed visualizing and showing all morphological peculiarities of normal and atypical cells; clarifying them by their morphological type. Besides, the DHIM method allowed obtaining the 3D image of untreated native cells and estimating the morphological changes of normocytes.

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