Series on Biomechanics, Vol.31, No.3 (2017), 16-24

Evaluation of the elastic properties and topography of leukocytes' surface in patients with type 2 diabetes mellitus using atomic force microscope

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Abstract

The aim of study was to examine some morphometrical parameters (height, diameter) of the leukocytes (white blood cells – WBCs), their specific surface morphology (globular prominences and depression in WBCs) as well as their local elastic properties (Young's modulus) in healthy persons and in patients with type 2 diabetes mellitus (T2DM) by means of the atomic force microscopy (AFM).

Morphological and morphometrical parameters of human leukocytes were evaluated by AFM in tapped mode. A twenty subjects were investigated divided into 2 groups: 1st group – 10 patients with T2DM; 2nd group – 10 healthy controls. In each of the groups, 10 leukocytes were examined. The samples for scanning were prepared by the methods of M. Yu. Skorkina et al. (2009, 2011).The elastic properties of leukocytes were characterized in the mode of force spectroscopy.

The results show that in the patients with T2DM the diameter of leukocytes was increased, but the cell height was significantly decreased, in comparison with the corresponding parameters in healthy donors. It was observed an significantly increase in the Young's modulus in the T2DM. A significant increase in the number of globular prominences and depressions on the WBCs in T2DM patients was found, in comparison with leukocytes of healthy individuals.

Keywords: Atomic force microscopy (AFM), leukocytes (WBCs), type 2 diabetes mellitus (T2DM)

1. Introduction

Type 2 diabetes mellitus is associated with abnormal (increased) blood glucose concentration, injured endothelial cell functions, defective leukocyte-endothelial interactions [1], disturbances in the hemorheological parameters, such as: increased haematocrit, increased erythrocyte aggregation, decreased erythrocyte deformability [2], increased both plasma and whole blood viscosity (WBV) [3, 4, 5], increased platelet aggregation and adhesion [6].

These disturbances are significantly responsible for appearance of severe complications in the patients with diabetes mellitus type 2 (T2DM), such as cardiovascular, neurovascular- and peripheral vascular pathological states [7], endothelial dysfunction [7], increased risk of atherosclerosis, retino-, nephro-, neuropaties, etc. [8]. In the patients with T2DM the increased blood viscosity values were associated with impaired cerebrovascular and peripheral vascular responses [4]. Reduced cutaneous microvascular responses to local thermal stimulation in the plantar sides of the toes of both T1DM and T2DM patients with polyneuropathy were established [9]. The obtained results reflect autoregulatory neurovascular and endothelial disorders which might further the ischemic microcirculatory manifestations [9].

1.1. Biomechanical properties of the WBCs in cases of T2DM

The biomechanical properties of the leukocytes (white blood cells - WBCs), influenced microcirculation in different vascular diseases [10].

The WBCs and erythrocytes (red blood cells - RBCs) circulate through the capillaries with a diameter smaller than their own size. The WBCs migrate through the endothelium, "rolling" along the endothelium (through the process of leukocyte adhesion) and penetrating (through the process of leukocyte transmigration) [11]. All these processes depend on the leukocyte deformability [12, 13].

Blood cell deformability could be influenced by three biofactors: (i) the morphological feature of blood cells such as diameter, concave depth, surface area, and volume; (ii) the viscous coefficient of cells, and (iii) the elastic properties of blood cell membranes [5, 10, 14, 15]. For describing of the elastic properties of the cells could used the Young's modulus (the elastic modulus) [16].

The viscous coefficient of WBCs is several orders of magnitude higher than that of erythrocytes: leukocytes are stiffer than the RBCs and thus not only erythrocytes, could block microcirculation and blood flow (so-called leukocyte plugging) [17].

Additionally, the defective WBCs in diabetes might interrupt capillary flow in the retina, kidney, etc. (A. Barnes, 1981) [18]. The vascular complications of T2DM could be also associated with the presence of less deformable lymphocytes in non-obese diabetic (NOD-) mouse model (C. Perrault et al., 2004) [19]. In patients with T2DM the deformability of the RBC membranes was decreased, but the stiffness increased in comparison with healthy persons (E. Drozd et al., 2010) [20].

Therefore, we supposed that the evaluation of the morphological and elastic properties (Young's modulus) of the leukocyte surface from patients with T2DM, could show changes in the biomechanical characteristics of WBCs and their cell membranes, which is reason for the appearance of vascular complications in DM patients.

1.2. Atomic Force Microscopy for investigations of blood cells

The recent AFM-techniques contribute elucidation of blood cell biomechanics:
- three-dimensional visualization topography of the living blood cells;
- morphological integration of the image on the cell surface with the local biomechanical properties of the cells (cell membrane deformability, viscoelasticity) [21, 22, 23].

Atomic Force Spectroscopy (AFS) is a dynamic analytical technique in the atomic force microscopy, which allows the evaluation of the mechanical properties of blood cells – elasticity and adhesion. With atomic force spectroscopy (single point) or force mapping (multiple points), the tip of the cantilever approaches and indents the sample, then retracts at each point, generating a force - distance curve at a specific point on the cell surface [24, 25, 26].

2. Materials and methods

2.1. Preparing the blood samples

Blood samples were prepared and collected (by venepuncture), by special medical staff in the Clinical-Diagnostic Laboratory of the Hematological Department Belgorod Regional Hospital of St. Ioasaf, Belgorod, Russia. The experimental part of the work was made at the Department of Biology of the Belgorod State National Research University, Belgorod, Russia.

The total and differential analysis was performed by participation laboratory assistant in the clinic laboratory of the Belgorod Regional Hospital. It was carried out counting the number of formed elements by automatic hematology analyzer Beckman Coulter LH500 (France, 2010).

Experiments were carried out on peripheral blood samples from 10 healthy donors (6 men and 4 women, mean age 47,6 ± 4,5 years) and 10 patients with T2DM (3 men and 7 women, mean age 52,1 ± 3,4 years). The leukocytes were isolated in two times centrifugation of the whole blood (at 5000 rpm for 10 min). The upper layer of the plasma was removed and the lower leukocyte-rich portion and the leukocyte ring, were collected and then re-suspended in the culture medium RPMI-1640. Drops of cell's

suspension were placed on a clean degreased slide.

2.2. Procedure for the studying of WBC topography by AFM

The topography of blood cells were studied using an Integra Vita NT-MDT scanning probe microscope (configuration on the basis of Olympus IX-71 inverted light microscope), in tapped mode (scan frequency of 0.6-0.8 Hz), using cantilever NSG03 (NT-MDT) with tip radius curvature of 10 nm and spring constant of 1.1 N/m. The samples for scanning were prepared by the method "The pathway of investigation of native cells", developed by M. Skorkina et al. (2009) [27]. AFM measurement were performed on a least 10 cells per each blood sample. The obtained scans with Nova software (NT-MDT, Zelenograd, Russia 2009), were used for construction of:

- 3×3 μm surface profiles for evaluation of morphological structures (the globular prominences – number, height, width and the depression in the WBCs – number, depth, width);
- 30×30 μm surface profiles for evaluation of morphometrical parameters (height and diameter of the leukocytes) [28, 29].

2.3. Procedure for the studying of leukocyte elastic properties by AFS

Elastic properties of the leukocytes were measured in the mode of AFS. The samples for scanning were prepared by the method "The pathway of determine of the elastic properties of blood cells" developed by M. Skorkina et al. (2011) [30]. AFS is based on the two procedures:

- 10 cells from each sample were scanned in tapping mode;
- in 25 local points of each blood cell surface, was applied force (by contact mode).

The obtained AFM curves with Nova software (NT-MDT, Zelenograd, Russia 2009), were used for calculation of Young's modulus (basing on Sneddon's modification of the Hertz model for the elastic indentation of a flat soft sample by a stiff cone).

The interaction force for the system: sample - tip of the cantilever, was calculated by the Hooke's law (Eq. 1):

$$F = k.x \tag{1}$$

where F is the force, k is the spring constant of the cantilever and x is the cantilever deflection.

The Young's modulus for the sample - tip system was calculated by the formula (Eq. 2):

$$F = \frac{4\sqrt{R}}{3} E\delta^{3/2} \tag{2}$$

where F is the force acting on the sample, R is tip radius, δ is depth of probe indentation into the sample, and E is Young's modulus [21, 31, 32].

2.4. Statistical analysis

Data analysis was performed using the software packages Sigma Plot 11.0. All data are presented as means ± SD (standard deviation). Significance of differences was evaluated using t-test and Mann-Whitney Rank Sum Test. Differences were accepted as significant at $p<0,05$.

3. Results

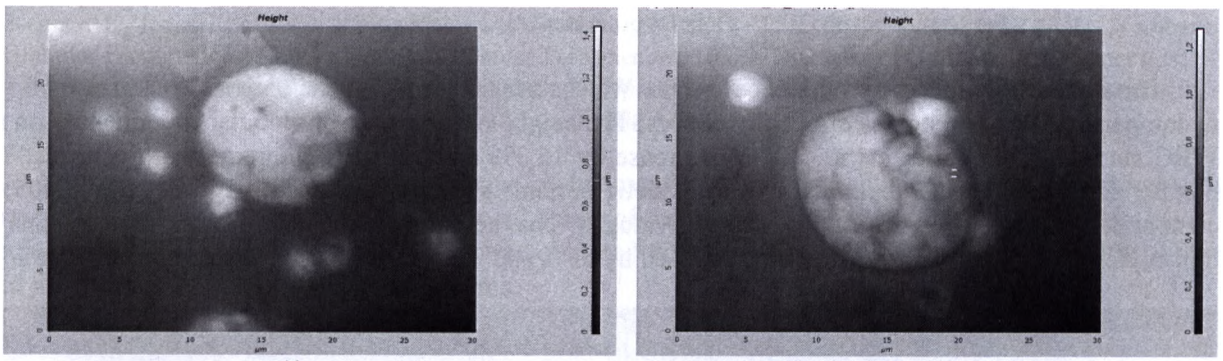
The hematological/hematometrical parameters from healthy donors and patients with T2DM was established by the total and differential analysis of the blood samples. The value of the glucose level of the patients with T2DM significantly increased by 95 % ($p<0,001$), in comparison with glucose level of the healthy donors. The hematological/hematometrical parameter - red blood cell distribution width (RDW) increased by 23%, compared to the control (Table 1).

Table 1
Mean values and standard deviations of glucose level and hematological/hematometrical parameters from healthy donors and patients with T2DM.

Hematological hematometrical parameters	**Healthy donors n=10**	**Patients with T2DM n=10**
Hb, g/l	145,1 ± 4,6	132 ± 6,2
RBC, 10^2 /l	4,8 ± 0,2	4,7 ± 0,2
Ht, %	0,45 ± 0,01	0,4 ± 0,02
MCV, fl	93,4 ± 1,3	86,5 ± 3,7$^{\#}$
MCH, pg	29,9 ± 0,6	28,4 ± 1,7
MCHC, g/l	322,4 ± 4,8	325,7 ± 12,7
RDW, %	**13,1 ± 0,2**	**16,1 ± 1,4**
PLT, 10^9/l	288,1 ± 17,7	235,4 ± 36
MPV, fl	10,1 ± 0,5	10,9 ± 0,6
WBC, 10^9/l	7,5 ± 0,7	6,3 ± 0,5
Glucose, mmol/l	**4,4 ±0,2**	**8,6 ± 0,7$^{\#\#\#}$**

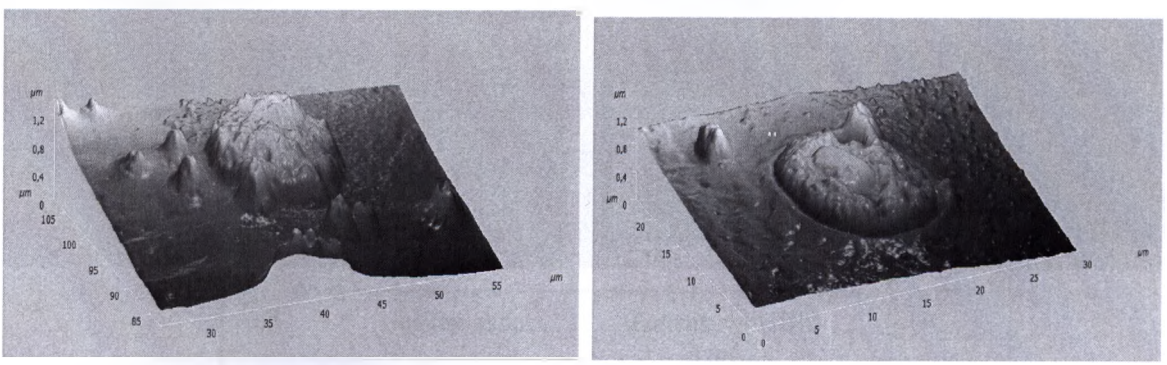
Hb - hemoglobin, RBC - number of red blood cells, Ht - hematocrit, MCV - mean red blood cell volume, MCH - mean corpuscular hemoglobin concentration, RDW - red blood cell distribution width, PLT - number of platelets (thrombocytes), MPV - mean platelet volume, WBC - number of leukocytes.

The WBC diameter was evaluated by the method of AFM – state of 2D profile of the leukocyte surface (30×30 μm). It was found that the diameter of leukocytes (n=100) from patients with T2DM was increased by 7,5% in comparison with diameter of leukocytes (n=100) from healthy donors (Fig. 1, Table 2).



A) B)

Fig. 1. 2D profile of the leukocyte (polymorphonuclear granulocyte) surfaces: A) from healthy donors; B) from patient with T2DM.

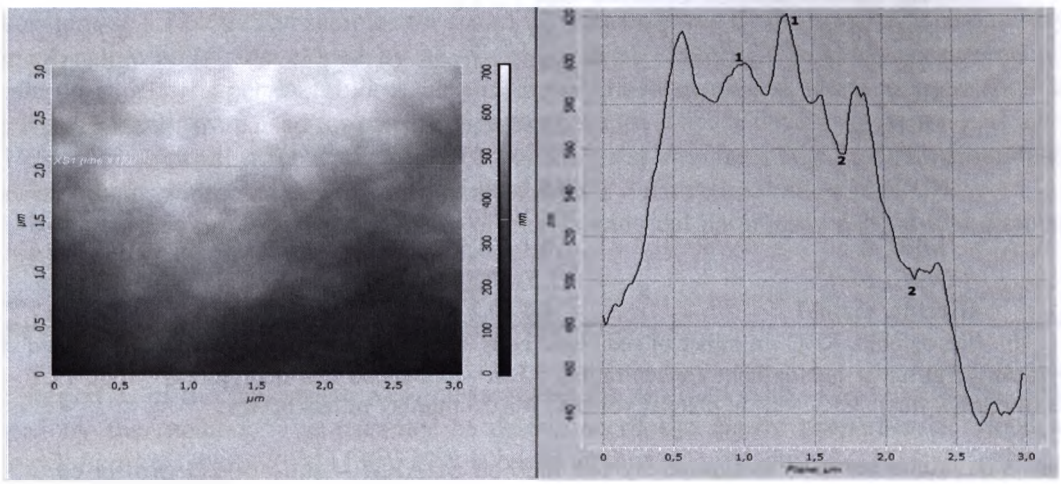


A) B)

Fig. 2. 3D profile of the leukocyte (polymorphonuclear granulocyte) surfaces: A) from healthy donors; B) from patient with T2DM.

The leukocyte height was evaluated by 3D profile of the leukocyte surface (30×30 μm). It was found that the height of leukocytes (n=100) from patients with DM was significantly decreased by 13,1% ($p<0,005$), in comparison with height of leukocytes (n=100) from healthy donors (Fig. 2, Table 2).

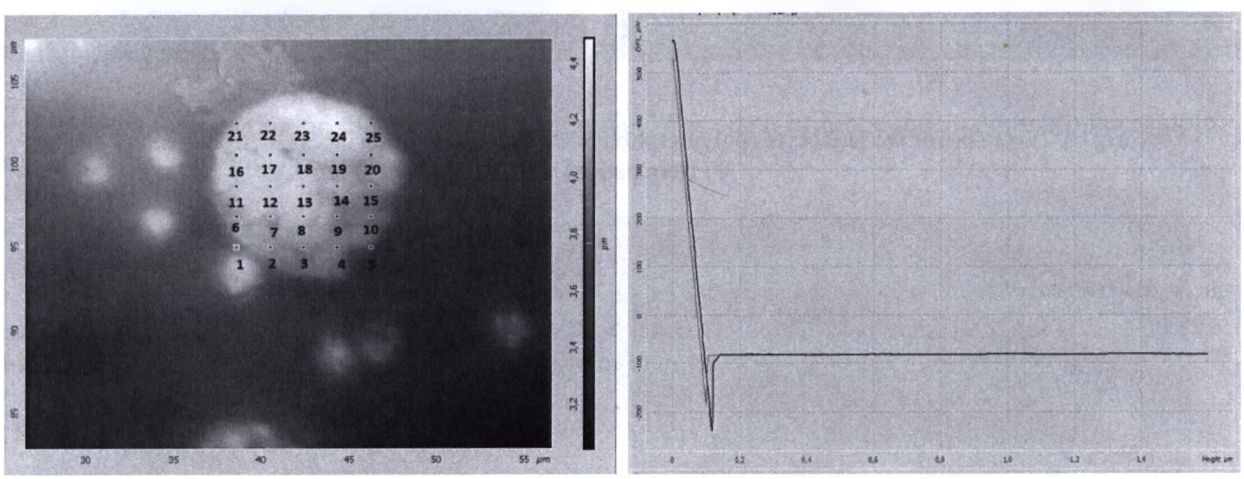
For evaluation of different morphological structures of the WBC surface - presented as globular prominences and/or depressions on the WBCs, the 3×3 μm surface profile curves of leukocytes from healthy donors and T2DM patients, were found (Fig. 3).



A) B)

Fig. 3. AFM image and graphic presentation of part of WBC surface: A) profile of leukocyte surface along the scanning path; B) 1 - globular prominences; 2 - depressions in the WBCs.

The number of globular prominences on the WBC surfaces from diabetic patients increases by 6,1%, in comparison with leukocytes of healthy donors. The height and diameter of globular prominences on the WBC surfaces from patients with T2DM decrease by 15,3% and 5,3%, as compared with controls. The number of depressions in leukocytes from diabetic patients significantly increases (66,1%, $p<0,005$) in comparison with leukocytes of healthy individuals. The height and diameter of the depressions in leukocytes from patients with T2DM are reduced by 53% and 22,7%, comparing with control (Table 2).



A) B)

Fig. 4. A) Points of nanoindentation on the surface of WBC from diabetic patient (see the same leukocyte on Fig. 1B); B) Force-displacement curve of the one point of nanoindentation.

Table 2
Mean values of morphometrical parameters and morphological structures of the leukocyte surface from healthy donors and T2DM patients.

Healthy subjects №	**1 F**	**2 F**	**3 F**	**4 F**	**5 M**	**6 M**	**7 M**	**8 M**	**9 M**	**10 M**	**Mean values±SD**
WBCD (μm)	7,42	8,3	7,43	7,02	7,29	9,82	7,78	6,19	7,03	7,47	**7,58 ± 0,35**
WBCH (μm)	1,93	1,43	1,69	2,06	1,76	1,06	2,38	1,83	1,7	1,65	**1,75 ± 0,11**
NGP	6,2	3,2	1,2	1,6	2,3	9,3	4,9	0,4	4,1	3,1	**3,63 ± 0,88**
HGP (nm)	45,7	27,62	26,7	12,35	44,2	15,18	12,87	27,9	34,08	57,15	**30,38 ± 10,32**
DGP (nm)	177,7	142,8	93,35	49,73	116,4	92,84	47,6	90,5	67	110,5	**98,82 ± 23,63**
ND	4	2,6	0,9	1,5	2,1	7,6	4,6	1,2	2,3	2,7	**2,95 ± 0,85**
DepthD (nm)	13,51	47,39	34,08	62,82	89,6	10,82	51,12	7,12	39,91	22,78	**37,92 ± 19,65**
DD (nm)	94,96	146,7	120,6	105,5	137,6	84,92	99,96	37,25	79,21	60,36	**96,71 ± 23,92**
E (μPa)	55,56	57,15	15,45	15,28	16,14	24,53	12,43	18,08	18	16,28	**24,99 ± 0,04**
T2DM patients №	**1 F**	**2 F**	**3 F**	**4 F**	**5 F**	**6 F**	**7 F**	**8 M**	**9 M**	**10 M**	**Mean values±SD**
WBCD (μm)	12,02	10,5	6,35	7,08	7,55	7,81	7,02	8,18	7,71	7,27	**8,15 ± 0,43**
WBCH (μm)	1,21	1,12	1,15	1,92	1,9	1,87	1,35	1,65	1,66	1,37	**1,52 ± 0,11*****
NGP	8	3,7	4,8	2,9	2,2	2,4	1,7	3	3,4	6,4	**3,85 ± 0,84**
HGP (nm)	19,95	19,61	42,49	36,15	53,88	12,43	34,03	12,84	7,58	18,34	**25,73 ± 7,71**
DGP (nm)	140,4	114,9	116,4	109,5	160,3	47,02	64,74	65,9	47,31	69,14	**93,56 ± 17,99**
ND	7,2	6,2	4,9	2,8	2,6	4,5	3,1	4,4	5,2	8,1	**4,90 ± 0,99**$^{\#\#\#}$
DepthD (nm)	16,52	10,86	13,84	13,34	30,22	13,68	26,87	28,58	8,6	12,77	**17,53 ± 3,83**
DD (nm)	79,24	62,26	82,1	64,1	87,53	48,66	74,86	105,5	73,67	69,88	**74,78 ± 11,57**
E (μPa)	93,83	71,53	8,12	16,21	18,56	33,85	16,78	15,12	19,55	15,42	**30,9 ± 0,05**$^{\#\#\#}$

t-test *p<0,05; **p<0,01; ***p<0,005; Mann-Whitney Rank Sum Test #p<0,05; ##p<0,01; ### p<0,005.
M - male, F - female; WBCD – White Blood Cell Diameter; WBCH – White Blood Cell Height; NGP - Number of Globular Prominences; HGP - Height of Globular Prominences; DGP - Diameter of Globular Prominences; ND - Number of Depression in WBCs; DepthD - Depth of Depressions in WBCs; DD - Diameter of Depressions in WBCs; E - Young's modulus.

For evaluation of the elastic properties of leukocytes and their cell membranes respectively, the force-displacement curves for each of 25 local points of nanoindentation on the leukocyte surface, have been analyzed (Fig. 4). The number of curves obtained were n=2325 for leukocytes from healthy donors and n=2500 for WBCs from diabetic patients.

It was found that the Young's modulus (E) of the leukocytes from diabetic patients significantly increased (by 23,7%, p<0,001), compared to the controls (Fig. 5, Table 2).

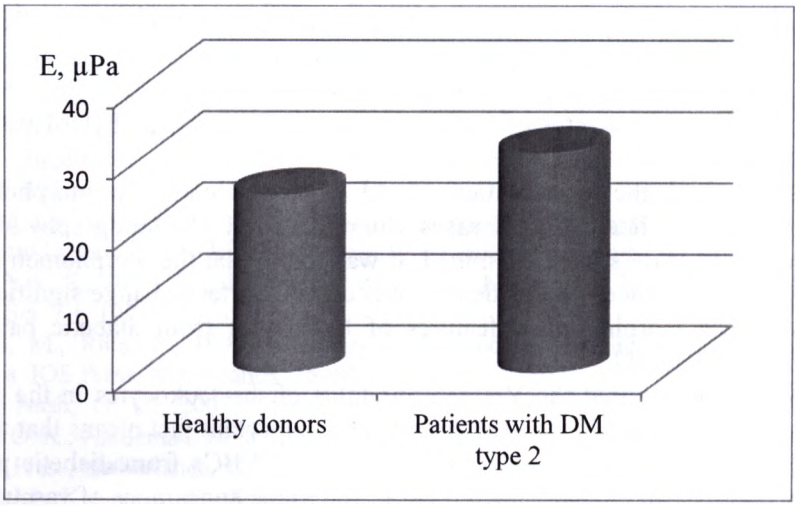


Fig. 5. Young's modulus (E, μPa) of the leukocytes from healthy donors and diabetic patients.

On the basis of the results obtained, the map of local elasticity of the WBC surface (by 25 nanoindentation points), was constructed (Fig. 6). Analysis of distribution of Young's modulus (E) values over the cell surface demonstrated no differences in the elasticity of leukocytes in the peripheral and central parts of WBCs. The peripheral parts correspond to the localization of cytoplasm and cytoskeleton structures (nanoindentation points 1, 2, 3, 4, 5, 10, 15, 20, 25, 24, 23, 22, 21, 16, 11, 6). The central parts of the WBCs correspond to the nuclear region (nanoindentation points 7, 8, 9, 12, 13, 14, 17, 18, 19), respectively. For all nanoindentation points an increased of Young's modulus of the leukocytes from patients with T2DM (by 23,7 %), as compared with Young's modulus of the leukocyte from the controls, was evaluated (Fig. 6).

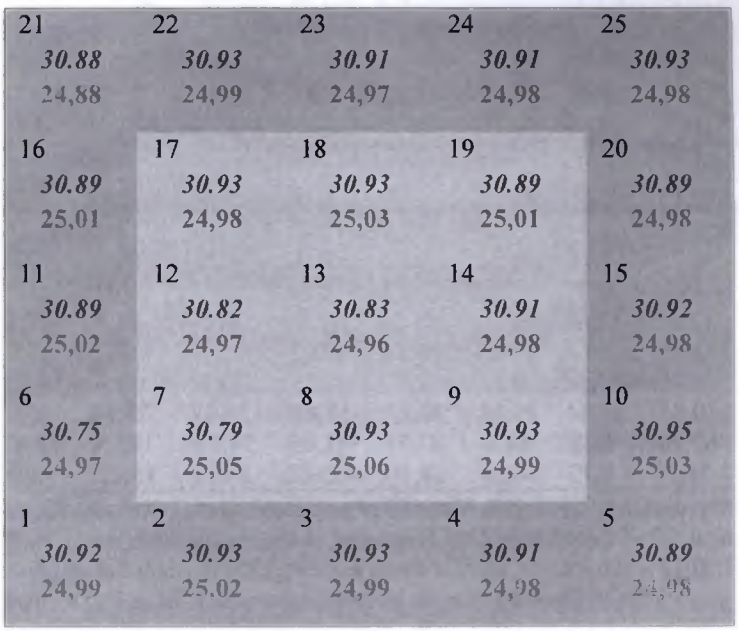


Fig. 6. A map of local elasticity of leukocyte cell surface.
Upper row: Mean values of the Young's modulus (E, μPa) of leukocytes from patients with T2DM;
Bottom row: Mean values of the Young's modulus (E, μPa) of leukocytes from healthy donors.

4. Discussion

Diabetes mellitus (DM) is a chronic, socially important disease. In 2035 year 592 million people with diabetes (10,1 % of the world's population) have been predicted [33].

In our and in previous studies [2, 34] was found that red blood cell distribution width (RDW) was higher in diabetic patients compared to healthy subjects. RDW is an independent marker of cardiovascular-,cerebrovascular - and peripheral artery diseases [35, 36]. Significant increase of Fibrinogen and WBV at shear rates of 0,0237 s^{-1} to 128,5 s^{-1} in the T2DM patients in comparison to controls was found [4].

Our results showed the possibilities of AFM to evaluate the morphological/morphometrical characteristics of WBCs in health and diseases. Simultaneously, the topography of leukocyte surface and the elastic properties of WBCs were examined. It was found that the morphometrical parameters - height of WBCs, globular prominences and/or depressions on cell surface change significantly for leukocytes in T2DM patients. These morphological features of leukocytes from diabetic patients influence on the deformability of the WBCs [5].

Using AFM was found that the Young's modulus of the leukocytes in the patients with T2DM is higher than in healthy individuals (control group). This important fact means that the deformability of the WBCs was decreased in patients with T2DM. Therefore, WBCs from diabetic patients are stiffer than leukocytes from healthy donors, which could be related to the appearance of vascular complications in the T2DM.

Additionally, not only deformability of the erythrocytes [20], but also the deformability of the leukocytes were decreased in T2DM patients, resulting in a injuring in microcirculation and leading to complications in the blood flow.

The results obtained could be used to identify earlier changes in the morphological, morphometrical and functional characteristics of leukocytes in patients with T2DM. The parameters determined pathological changes in the topography of leukocyte surfaces as well as in the WBC elastic properties. The data can serve as objective markers and tools for diagnostic, disease stratification and prognostification of T2DM.

Acknowledgements

The experiments were realized within the agreement between the Institute of Mechanics, Bulgarian Academy of Sciences and the Belgorod National Research University, Belgorod, Russia and the Yaroslavl State Pedagogical University "K. D. Ushinski", Yaroslavl, Russia to conduct dissertation-related experiments of the PhD student A. Alexandrova. The trip was supported by a personal donation of Acad. Ya. Ivanov. The authors also thank Prof. Dr. E. Zvetkova from the Bulgarian Society of Biorheology - Bulgarian Academy of Sciences, for the helpful discussions and valuable advices!

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