Comparative analysis of cats’ lymphocytes structural features with and without retroviral infection using atomic force microscopy

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Abstract. The results of the comparative analysis of morphometric and elastic parameters of the fixed lymphocytes from intact and infected with viral immunodeficiency and leukemia cats using atomic force microscopy are presented. It is found that the adhesive properties and the roughness of the cell surface of cats’ lymphocytes with FeLV and FeLV - FIV infection are reduced by 27 – 45 % and 19 – 32 %, respectively. These indicators in the lymphocytes of FIV infected cats did not significantly differ from the intact cats’ lymphocytes. An increase in the Young’s modulus of lymphocytes in cats with retroviral infection of 9 - 16 % compared to the control has been shown. It is found that lymphocytes from cats with retroviral infection are characterized by a decrease in volume at 36 - 77 % compared to the control cats’ lymphocytes. Intact animals’ lymphocytes have a domed shape, whereas infected cats’ cells have a greater perimeter and length with reduced height. Peculiarities of blood lymphocytes in FIV, FeLV and FeLV - FIV infected cats can be important in the study of immunological mechanisms in the retrovirus pathogenesis.

1. Introduction.
Viral immunodeficiency (FIV) and leukemia (FeLV) are chronic incurable retroviral cats’ infections, accompanied by alteration of immunocompetent cells, in particular lymphocytes [1, 2]. Replication and viremia can cause structural damage to the cell membrane, which is manifested in the change of the morphometric and biophysical properties of peripheral blood lymphocytes.

A key role in ensuring cellular metabolism, intra- and extracellular homeostasis belong to biological membranes. Some elastic properties, stiffness and roughness of a cell membrane are the most important. They determine lymphocyte adhesion abilities and cell migration through the vessels and extracellular matrix [3, 4, 5].

Structural disorganization of lymphocytes cytoplasmic membranes while retroviral infection creates preconditions for humoral and cellular immunity violations. Studying of membrane biophysical properties and lymphocytes morphometric parameters both in human and animals was possible by atomic force microscopy (AFM) using [6, 7]. Several structural and functional properties of some cells in norm and in pathology have already been studied by AFM [8, 9, 10].
The purpose of this study was a comparative analysis of the functional state and some structural features of the intact and retrovirus-infected cats’ lymphocytes cytoskeleton using atomic force microscopy.

2. Materials and methods
The material used for the study were blood lymphocytes of cats with viral immunodeficiency (FIV) (n=27), leukemia (FeLV) (n=24) and retroviral co-infection (FeLV-FIV) (n=31). As a control, cells of 12 uninfected cats were used.

The diagnosis of “viral immunodeficiency” and “leukemia” was based on the clinical and laboratory examinations, using the PCR kits “VIC” and “LAKES” (InterLabServis, Russia) on the equipment Rotor-Gene™ 6000 (Corbett Research, Australia) and IHA tests (VetExpert Antigen, Korea). These studies were performed in the Educational-research Centre “Veterinary hospital” of the Saratov State Vavilov Agrarian University.

The blood taking was made from saphenous vein (V. cephalica). The blood was mixed with sterile Hanks’ solution in ratio 1:1.

Blood lymphocytes were isolated by centrifugation in ficoll - verografin density gradient (ρ = 1.077 g/cm³) according to the Recalde method 9. The lymphocytes suspension (20 µl) was adhered on clean fat-free glass slides (1x2.5 cm) at 37 °C for 60 minutes in a humidified incubator. Anhydrous methanol (5 min) was used as a fixer. Additionally, the Romanovsky - Gimz staining technique was applicable to assess the cells localization.

AFM scanning was implemented in the Laboratory of scanning probe microscopy of the Scientific-research technological Institute, Ulyanovsk State University. For the lymphocytes surface analysis, a scanning probe microscope Solver P47-PRO (NT-MDT, Zelenograd) was used. Over 15 scans of fixed lymphocytes of each test group were performed in noncontact mode in air. Scanning of blood lymphocytes was performed using silicon probes series NSG10 (NT-MDT) with a stiffness of 5.5 N/m, resonance frequency 150 kHz, the radius of curvature of 10 nm (figure 1).

[Figure 1. Scan of lymphocytes obtained using AFM contactless mode, three-dimensional image.]

The AFM scans were processed in the program Nova 1.0.26.1443.

Further, the data processing was performed using the software ImageJ, Excel, Statistica 8. For authenticity, the differences between the experimental groups at a significance level of 95% (p < 0.05) were accepted.
3. Results
To study the biophysical parameters of cats’ lymphocytes cell membranes with and without retroviral infection, the module of isometric contraction of the membrane (Young’s modulus) was evaluated. Young’s modulus characterizes the ability of the cells to the strains arising from the interaction of the membrane with the apex of the AFM probe, and the greater its value, the smaller the elastic deformation of the cell. Young’s modulus (Pa) of lymphocytes cells membranes was measured by atomic force spectroscopy.

To calculate the Young’s modulus, the Hertz model was used [6].

![Figure 2. AFM scan, power curves to calculate the Young’s modulus.](image)

The scan of lymphocytes with a grid of points the impact force on the surface is presented in the figure 2 on the left (1A). The derived power curves are shown in the figure 2 to the right. The position of the probe at a height of inlet and outlet is presented on the horizontal axis. And the ordinate axis is the level of the power signal DFL is shown, which is transferred from the display format in amperes in the display format in newtons using the integrated script DFL_to_Force.

From these series of force curves (figure 2) Young’s modulus was calculated in accordance with the Hertz’ model for a hemispherical tip.

The morphometric parameters of each scanned lymphocyte (length, width, height, perimeter, diameter, etc.) were automatically calculated by using the software, and the area and volume of the cells were calculated too (figure 3).

Then, using these AFM data, we calculated the area of contact of the cells with the substrate (S), volume (V) and coefficient of flatness (Kv). The square of the cell-substrate contact was calculated using the formula for the spherical segment [Zubareva et al. 2011], and the volume of cells was determined by the formula for the volume of the spherical segment [9]. The flatness of lymphocytes was calculated as the area of contact of the cells with the substrate (μm²) and the average height of the cell (μm) ratio.

To characterize the surface roughness of the samples an arithmetic average roughness (Sa) was used. The adhesion strength of the probe to the membrane of lymphocytes was determined by the portion of the force curve of the outlet corresponding to the negative margin of cantilever bending before the separation from the cell surface (figure 4).
Figure 3. Mode «Roughness Analysis», image of the lymphocyte surface (the maximum and minimum height of the lymphocyte, the average surface roughness, as well as other parameters characterizing the surface of the lymphocyte are shown).

The adhesion strength of the probe to the membrane of lymphocytes was determined by the portion of the force curve of the outlet corresponding to the negative margin of cantilever bending before the separation from the cell surface (figure 4).

Figure 4. Determination of the adhesion of lymphocytes (x and y are the coordinates of the point of adhesion determination, on the axis z – value of adhesion strength at nN).

The graphic depiction of software indicated adhesion magnitude is shown in figure 4 on the left. The map of distribution of adhesion forces is shown in figure 4 to the right. The color intensity corresponds to the magnitude of the adhesion force.
The study of morphometric and biophysical features of the lymphocytes from intact and infected with viral immunodeficiency and leukemia cats using AFM revealed that the healthy cats’ lymphocytes authentically have higher actual values of the calculated parameters (Table 1).

**Table 1. Indicators of morphometric and biophysical parameters of lymphocytes from intact and retrovirus-infected cats.**

| Options                  | Control group | FIV - infection | FeLV - infection | FIV - FeLV - infection |
|--------------------------|---------------|-----------------|------------------|------------------------|
| Diameter, μm             | 9.53 ± 0.18   | 7.83 ± 0.17*#   | 5.40 ± 0.16*#    | 5.01 ± 0.13*#          |
| Height, μm               | 10.20 ± 0.20  | 7.40 ± 0.15*#   | 6.20 ± 0.13*#    | 5.7 ± 0.11*#           |
| S<sub>c</sub>, (μm)<sup>2</sup> | 81.81 ± 2.44  | 59.35 ± 1.77*#  | 49.72 ± 1.46*#    | 45.71 ± 1.29*#         |
| V<sub>c</sub>, (μm)<sup>3</sup> | 157.30 ± 4.71 | 99.31 ± 2.97*#  | 67.52 ± 2.02*#    | 35.31 ± 1.06*#         |
| K<sub>y</sub>, r.u.      | 34.24 ± 0.02  | 53.22 ± 0.23*#  | 45.32 ± 0.13*#    | 49.23 ± 0.22*#         |
| Adhesion, nN             | 6.26 ± 0.18   | 5.97 ± 0.15#    | 4.57 ± 0.12*#     | 3.41 ± 0.09#           |
| Young’s modulus, Pa      | 140.41 ± 7.02 | 117.63 ± 3.52*# | 145.98 ± 7.26*#   | 127.83 ± 2.98*#        |
| Roughness (Sa), nm       | 419.14 ± 11.25| 398.86 ± 10.19| 338.88 ± 9.03*#   | 284.49 ± 8.02*#        |

Note:
* - statistically significant differences between the control and experimental groups (p <0.05);
# - statistically significant differences between the experimental groups (p <0.05).

The lymphocyte diameter of infected with *FIV*, *FeLV* and *FIV-FeLV* co-infected cats reduced by 18 %, 43 % and 47 %, respectively, compared to intact cats. Furthermore, the height of the cells decreases 1.5 - 2 times. Reduction of the lymphocytes diameter and height in cats with *FIV* and *FeLV* infection is accompanied by decrease in the cell volume. The volume of lymphocytes from *FIV* – infected, *FeLV* - infected cats and cats with *FIV - FeLV* – co-infection decreases by 36 %, 57 % and 77 %, in comparison with this parameter in healthy cats. The same tendency is noted for other indicators: area of contact of the cells with the substrate and coefficient of flatness (Table 1).

According to our research data, the adhesive properties and the roughness of the cell surface of *FIV* infected cats’ lymphocytes did not significantly differ from the intact cats’ lymphocytes. However, in cats infected with *FIV*, *FeLV* and *FIV-FeLV* co-infection, the indicators of adhesion and roughness reduce by 19 % or 32 % and by 27 % or 45 %, respectively, compared to intact cats’ lymphocytes.

The Young’s modulus in peripheral blood lymphocytes of *FIV*-infected cats and cats with *FIV-FeLV* co-infection was decreased by 16 % and 9 %, respectively, compared to healthy cats’ lymphocytes. An increase in elastic properties of the lymphocytes membrane of cats with *FeLV* infection may be due to changes in the structural elements of the phospholipid basis of the cell membrane and associated with the special aspects of the virus reproduction.

Less rough surface lymphocytes were predominated in the peripheral blood of retroviruses infected cats. It is may be possibly due to decrease in the height of the various forms of globular cytolemma protrusions, which may be result of both the cell cytoskeleton and the cell membrane ultrastructure reorganization. This is evidenced by a decrease in the cats’ lymphocytes roughness, while *FIV*, *FeLV* and *FIV-FeLV* co-infection, by 14%, 19% and 32%, respectively.

4. Conclusion
The results of our studies indicate that infected with retroviruses cats’ lymphocytes have significant morphometric and biophysical aberrations. Destabilization of cells membrane structure can cause of
violation of the lymphocytes functional activity. Identified by atomic force microscopy the morphometric and biophysical characteristics of FIV and FeLV infected cats’ lymphocytes can have fundamental theoretical and significant practical importance.

First of all, the findings partially explain the stability of the infected lymphocytes. It is known that an infected cell, rather than virus can cause these infections. Increase the rigidity of the lymphocyte’s membrane can be the cause of enhancement of cell resistance to the physical (antibody binding) and chemical (lysosomal enzymes) factors during the initial infection or the diseased animal’s immune response. In addition, a significant reduction of lymphocytes roughness, especially when co-infection, can be an indicator of antigenic properties losing. It can also adversely affect the development of immune reactivity in cats.

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