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RESEARCH ARTICLE

Erythrocyte Changes in Death after General Over Cooling

Revo Z. Alekseev[1], Aitalina S. Golderova[2], Victoria A. Platonova[3], Ariana A. Kuzmina[4]

1. *Medicine Department of Adaptation Mechanisms Study Yakut Scientific Centre of Complex Medical Problems 4 Sergelyakhsky Highway, Yakutsk, Republic of Sakha (Yakutia), 677019, Russian Federation.*
2. *Medicine Department of Public Health, Healthcare, General Hygiene and Bioethics Institute of Medicine M.K. Ammosov North-Eastern Federal University 58 Belinsky Str., Yakutsk, Republic of Sakha (Yakutia), 677000, Russian Federation.*
3. *Department of General and Experimental Physics Institute of Physics and Technologies M.K. Ammosov North-Eastern Federal University 58 Belinsky Str., Yakutsk, Republic of Sakha (Yakutia), 677000, Russian Federation.*
4. *Department of Pharmacology and Pharmacy Institute of Medicine M.K. Ammosov North-Eastern Federal University 58 Belinsky Str., Yakutsk, Republic of Sakha (Yakutia), 677000, Russian Federation.*

Abstract

In medicine, the problems of hypothermia and body 'revitalization' remain open. Insufficient knowledge of the mechanisms of dying at ultralow temperatures gives grounds to supplement the idea of the cellular-molecular mechanisms of deadly hypothermia. The aim of this study was to assess the morphology of red blood cells during fatal hypothermia at extremely low temperatures (i.e. below -40°C) using atomic force and scanning electron microscopy, high resolution JSM-7800F. The subject of the study was blood smears of people who died from hypothermia and from a gunshot wound. In a man who died of hypothermia at extremely low temperatures (i.e. -42°C), whose smear was taken within 1 day, red blood cells were characterized by minimal changes than when dying from a gunshot wound. The discocytic form of erythrocytes with a flat surface was preserved; however, we found an increase in the depth of the central concavity of red blood cells, which may be associated with an increase in the volume of red blood cells. It was found that even a slight difference in the temperature of the body regions (i.e. Δ 2.4°C) at the time of blood sampling affects the ratio of different forms of red blood cells after complete thawing. The lower the temperature, the more degenerative forms of red blood cells appear. After complete thawing of the corpse on the 4th day, all red blood cells (100%) had a different degree of dysmorphism, a more flattened surface, an increase in diameter and heterogeneity and roughness of the plasma, indicating signs of hemolysis.

Keywords: *Red blood cells; Death from hypothermia; Atomic microscopy; Scanning electron microscopy.*

Introduction

In modern conditions of Arctic exploration, the problem of hypothermia in humans and animals takes on a fundamental and applied character. Despite the progress made over the past few decades in the field of forensic pathology, the possibilities for posthumous diagnosis of hypothermia remain relatively limited [1].

The world has not yet fully studied the issues of death from general cooling in ultra-low temperatures (i.e. below -40°C). In real conditions, persons who died from hypothermia (according to external signs) without resuscitation were taken to the morgue. An important point is that during the first day (a very rare pulse and low blood pressure), the victims are in a state of cold suspended animation and the restoration of the body's vital activity is not excluded [2].

The results showed that deaths due to hypothermia are characterized by elevated levels of ketones in the blood and other body

fluids, elevated urine adrenaline concentrations, elevated serum cortisol levels after death from femoral blood, and elevated urinary free cortisol levels [3]. A study of the red blood cells state in critical, terminal and post-resuscitation conditions allows us to identify how the cells responsible primarily for gas exchange in the body react to strong metabolic changes that occur during critical conditions, and how their functional, structural and biochemical properties change.

Therefore, the restoration or preservation of functional red blood cells can solve not only the problems associated with gas exchange, but also restore the metabolism disturbed in critical conditions [4]. Considering that during winter months (i.e. below -40°C) about 200 people die from hypothermia every year in the Republic of Sakha (Yakutia), as well as insufficient knowledge of the features of dying mechanisms at extremely low temperatures, this study is of interest and is relevant. The results of this study can complement the understanding of the cellular-molecular mechanisms of deadly hypothermia process. The aim of this study was to assess the morphology of red blood cells during deadly hypothermia at extremely low temperatures (i.e. below -40°C) using atomic force and scanning electron microscopy.

Materials and Methods

The object study was blood smear samples made according to the generally accepted technique without fixing and staining during autopsy of two middle-aged men. Blood smears of the dead from hypothermia and a gunshot wound were made immediately after the delivery of corpses (Day 1) to the Bureau of Forensic Medical Examination of the Ministry of Health of the Republic of Sakha (Yakutia). Additionally, with mortal hypothermia, blood samples were taken on the 4th day after the complete thawing of the corpse. Table 1 show the temperature of body areas where the blood smear was prepared from.

Table 1: Temperature body areas of the deceased from hypothermia

Body area	Temperature, °C	
	Day 1	Day 4
Brain	- 0,1°C	+17,2°C
Anterolateral surface of the neck	- 2,5°C	+17,2°C

The study of blood smears was carried out using an atomic force microscope called 'NT-MDT' (made in Zelenograd, Russia), model was SolverNext. Processing and calculation of data was performed using the 'Nova' program. The scans were performed in a semi-contact mode, the scanning area was 50x50 μm (512 points), the scanning speed was 0.5 Hz, and the NSG 10 cantilever with a radius of curvature of no more than 10 nm was used for all measurements. To assess the unevenness of red blood cells relief and thickness, the S1 line was used. Also in this study, a high-resolution scanning electron microscope (SEM) JSM-7800F ('Japanese Electron Optics Laboratory' - 'JEOL', Japan) was used. The SEM under consideration, having a magnification range of 25 - 1 000 000, allows you to explore the object with an accelerating voltage of 0.1-30 kV. In this work, a lower detector of secondary electrons was used.

Results and Discussion

In order to identify the morphology of red blood cells during mortal hypothermia for Day 1, we analyzed blood smears of two middle-aged men who died from hypothermia (at an ambient temperature of 42°C) and a gunshot wound. Analysis of the two presented AFM images of red blood cells indicates their significant differences (Figure 1). In the field of view at death from a gunshot wound, pronounced poikilocytosis is visualized, with a predominance of echinocytes and the formation of conglomerates (Figure 1a). It is known that an echinocyte resembles the shape of a sea urchin, has spikes of the same size, located evenly on the surface of the red blood cell. In some cases, the formation of an echinocyte is accompanied by the release of hemoglobin and part of the internal contents of the red blood cell through small sections of the destruction of the cell wall. The functional and physiological significance of echinocytosis has not yet been adequately studied.

One of the reasons for the formation of echinocytes is considered to be the increased permeability of erythrocyte membranes for K

+ and Na + ions [5]. Echinocytosis does not prevent the formation of aggregates, but also is accompanied by the formation of aggregates of increased strength [6]. When super cooling in an AFM image (Figure 1b), erythrocytes having a biconcave shape are visualized - discocytes with a flat surface and their conglomerates (Figure 1b).

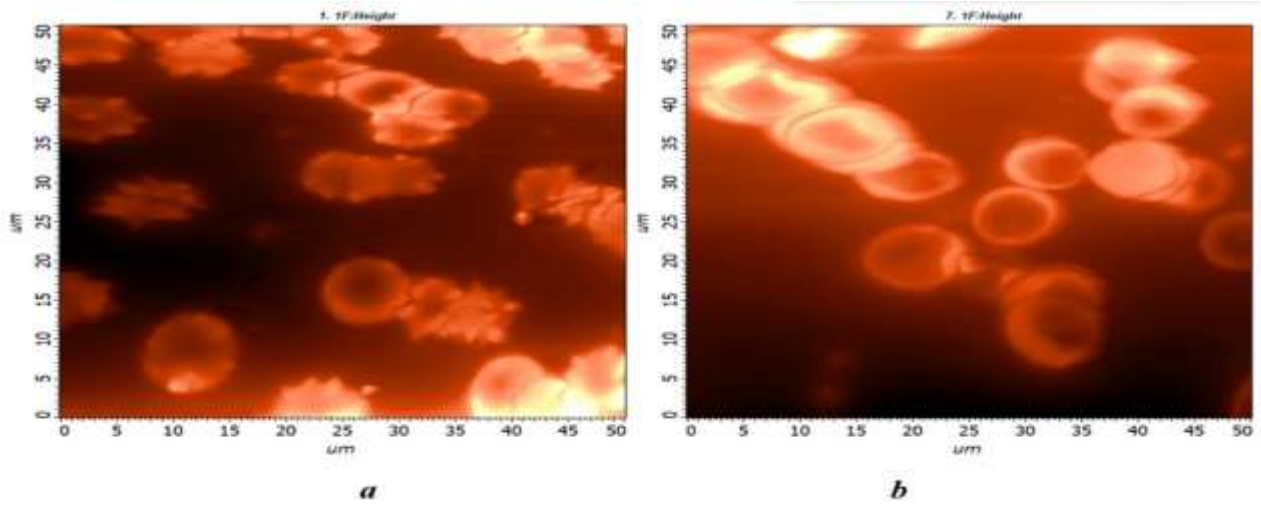


Figure 1: Two-dimensional AFM images of red blood cells on the 1st day after death: *a* – from a gunshot wound; *b* – from hypothermia

In order to assess the quantitative characteristics of red blood cells for various reasons. An AFM scan of a red blood cell during a gunshot wound indicates the presence of outgrowths on its surface (Figure 2a), as well as a significant decrease in the depth of the cavity, i.e. the acquisition of a flattened surface.

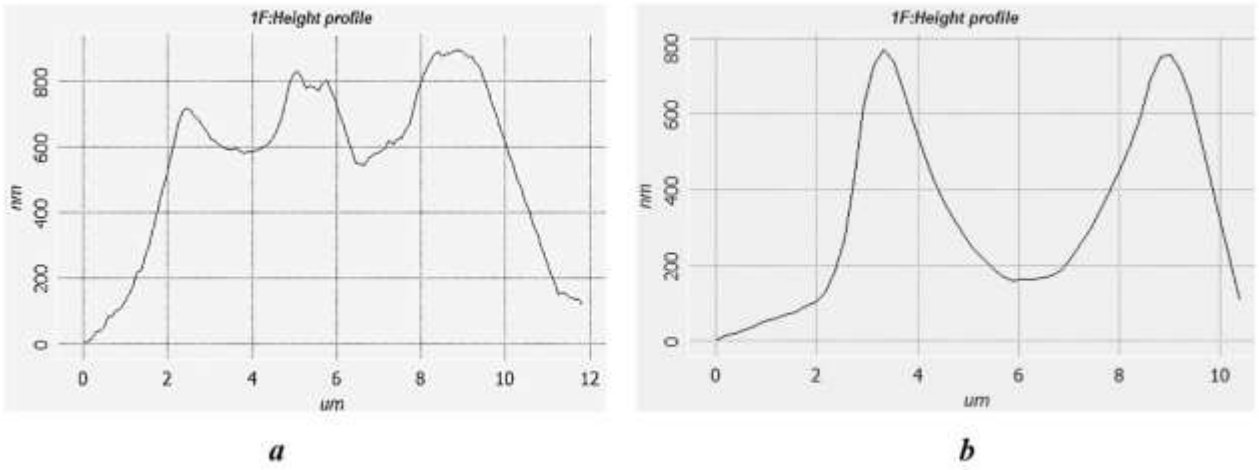


Figure 2: The horizontal section of the red blood cell from the AFM scan for Day 1 after death: *a* – a gunshot wound; *b* –sub cooling

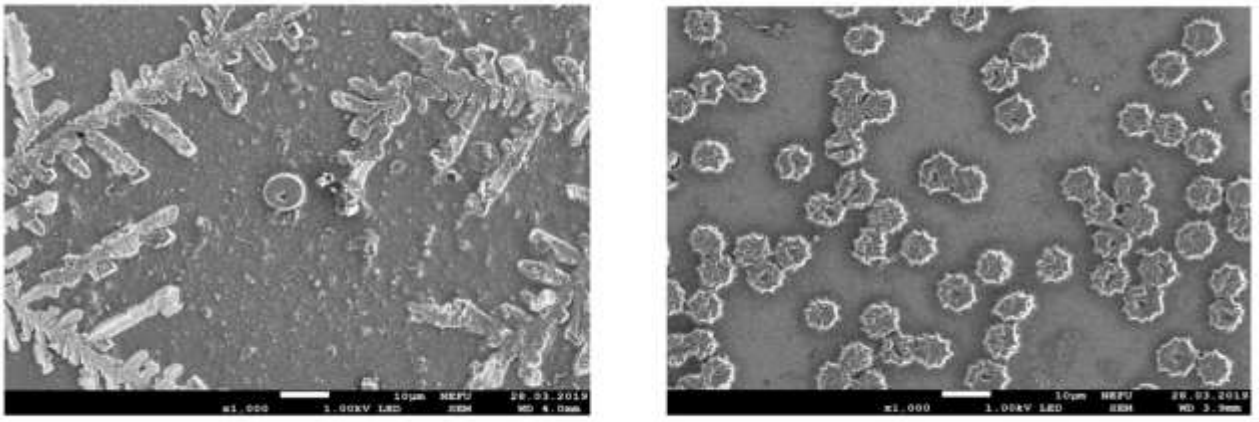
There is an even surface relief observed in the AFM scan of the horizontal cross-section of an erythrocyte on the 1st day after death from hypothermia, as well as an increase in the depth of the depression (~ 0.645 µm), which exceeds the norm (~ 0.200 µm) by 3 times (Figure 2b), i.e. the degree of red blood cells concavity becomes pronounced. The next stage of our work was the assessment of changes in the morphology of red blood cells depending on the time of corpse thawing, i.e. on Day 1 and Day 4. Most of the membrane proteins are located on the inner (cytoplasmic) side of the membrane and form a network of filaments (actin and intermediate), which serves to maintain the normal form of the red blood cell. The erythrocyte membrane elasticity is provided by the interaction of cytoskeletal proteins. Aggregation activity and deformability largely depend on the structural organization of erythrocyte cell membranes; these indicators determine the ability of cells to micro circulates. In red blood cells, ATP is formed during anaerobic glycolysis. When ATP is inhibited, erythrocyte deformability decreases the shape of the cell changes, and the membrane permeability to ions increases

[4]. During echinocytic transformation, the charge of the erythrocyte surface membrane also changes significantly, which is a necessary condition for maintaining the stability of the erythrocyte suspension, i.e. plasma. Echinocytosis entails the aggregation of red blood cells [7]. Echinocytic transformation is due to the formation of cross-links between spectrin and hemoglobin. Membrane material is lost by microvesiculation [8].

Thus, AFM data indicate fundamental differences in the morphology of erythrocytes depending on the cause of death, in particular, in the first hours after death, with deadly hypothermia, erythrocytes have a flat (without outgrowths) surface and with an increased central concavity.

In blood smears in the first hours of death from a gunshot wound, red blood cells take an echinocytic form, which is characterized by a decrease in the depth of the cavity. Red blood cell conglomeration is observed in both cases, i.e. with a gunshot wound and hypothermia. To determine the effect of ambient temperature on red blood cells, in our case, when the corpse was completely thawed (Day 4) after fatal hypothermia, we compared SEM images of blood samples on Day 1 and Day 4.

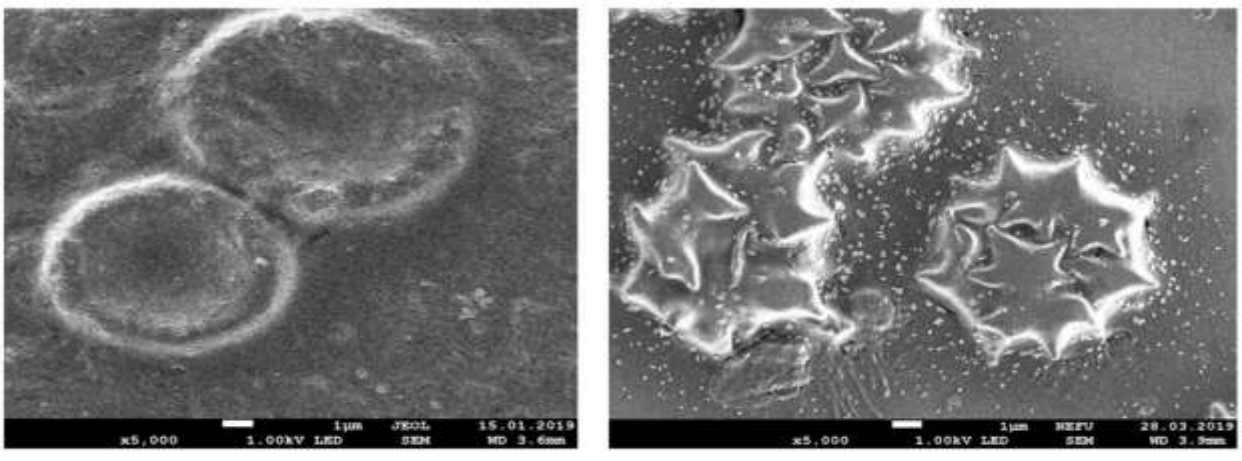
SEM images (1x1000) show a smear of blood mixture with cerebrospinal fluid on Day 1 after death taken from the intracranial area; the crystallization process is visualized in Figure 3a, because 90% of the cerebrospinal fluid is water.



a *b*

Figure 3: SEM images (1x1000) of the brain area in death from hypothermia: *a* - for 1 day; *b*-for 4 days

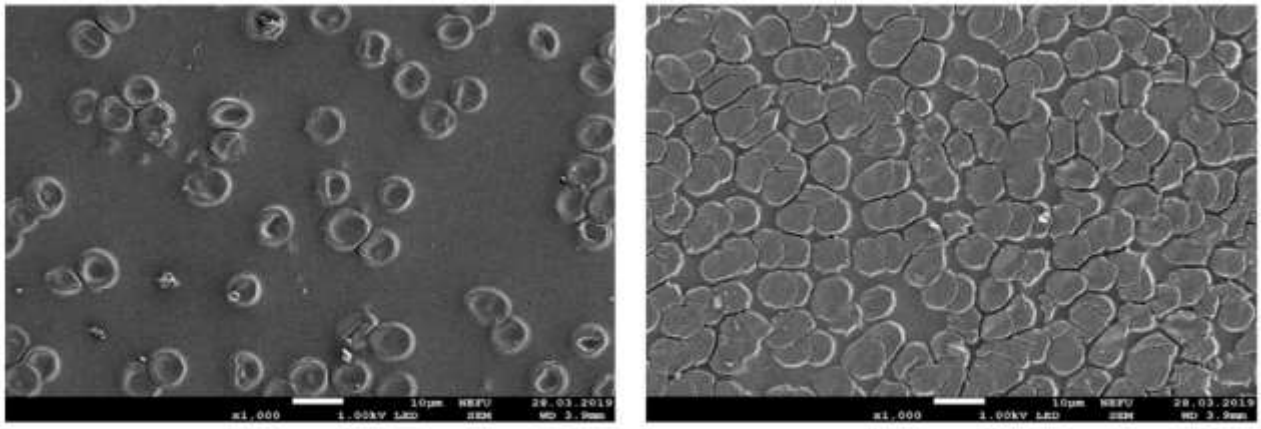
Despite crystallization, surviving discocytes (normal red blood cells) with preserved concavity (Figure 3a and Figure 4a) can be observed. In the intercellular fluid on Day 1, many small particles are visualized (Figure 3a), which disappear after complete thawing of the corpse (Day 4), but with a large increase (1x5000), smaller particles are visualized located around the dysmorphic red blood cells (Figure 4b).



a *b*

Figure 4: SEM images of a smear (1x5000) taken from the area of the brain: *a* - for 1 day; *b* - for 4 days

On Day 4 a large number of red blood cells with a pronounced degree of dysmorphism can be observed. Visualization of red blood cells at a large increase (1x5000) (Figure 3b) allowed us to attribute all red blood cells (100%) to degenerative (irreversible) pathological forms, i.e. acanthocytes (cells with denticles of unequal size) associated with a structural defect in the membrane. For quantitative characteristics in dynamics, we compared SEM images of red blood cells from the neck of a man who died from hypothermia on Day 1 and Day 4. Day 1 SEM images are characterized by moderate anisocytosis of discocyte red blood cells with pronounced concavity (Figure 5a).



a *b*

Figure 5: SEM erythrocytes images (1x1000) of a deceased from hypothermia taken from the cervical area: *a* - for 1 day; *b* - for 4 days

Further, the erythrocytes were differentiated by their forms, which showed that normocytes prevail on Day 1 and in a blood smear - 68%; spherocytes - 5%; target cells - 5% and ovalocytes - (22%) (Figure 6).

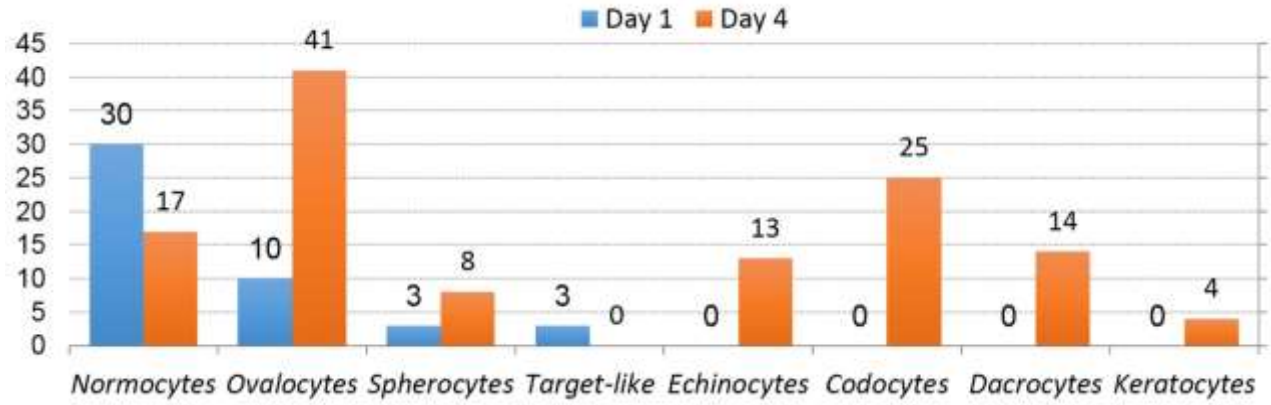


Figure 6: Erythrocyte forms distribution from the neck area in deadly hypothermia on Day 1 and Day 4

The diagram shows (Figure 6) that on Day 4, along with normocytes, a large number of different degenerative forms of red blood cells appear (normocytes - 12%; spherocytes - 9%; echinocytes - 5%; ovalocytes - 40%; dacrocytes-11%; codocytes - 20% and keratocytes - 3%), as well as the surface flattening of red blood cells (i.e. a decrease in concavity). It is likely that such a different ratio of erythrocyte forms in the brain area and the front neck surface is explained by the temperature difference (Δ 2.4°C) in different body areas. At the time of taking blood smears, in our case, the lower the temperature (Table 1), the greater the degree of red blood cell changes after a corpse thawing. Analysis of 3-D AFM images of erythrocytes from the neck during hypothermia on Day 1 revealed anisocytosis, poikilocytosis, and aggregation of dysmorphic red blood cells, as well as a relatively even and smooth plasma profile (Figure 7a). The AFM image on Day 4 is characterized by a flattened erythrocyte shape with a tendency to increase the diameter of the cells, as well as a roughness of the plasma with many small depressions, probably from membrane fragments of destroyed red blood cells (Figure 7b).

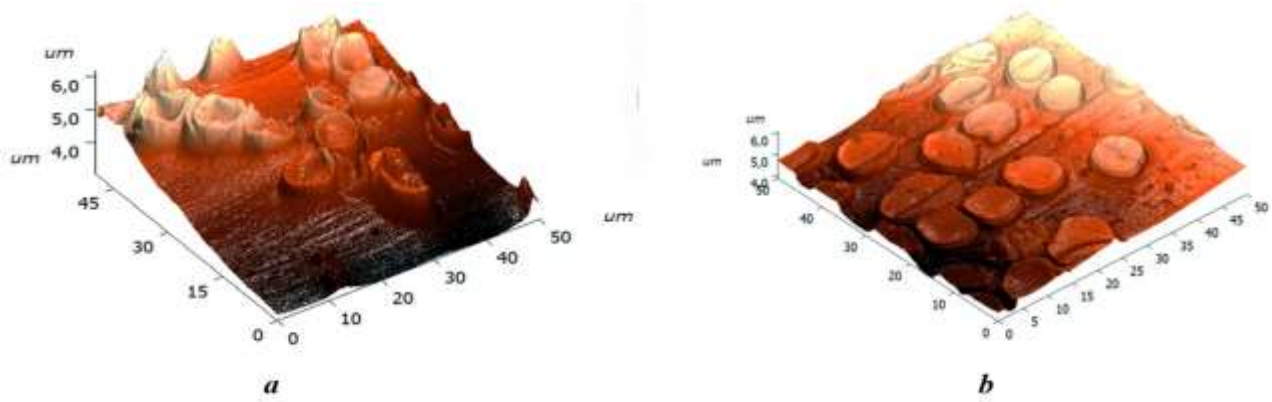


Figure 6: Three-dimensional AFM images of erythrocytes in fatal hypothermia: *a* –Day 1; *b* - Day 4

To clarify the quantitative characteristics, we measured graphical images from a scan, which revealed that erythrocytes, during Day 1 after death from hypothermia, they have indicators close to normal ranges, in particular, the average value of the depression depth was ~ 0.513 µm, the diameter of red blood cells was ~ 7 µm , the height of red blood cells ~ 0.620 microns. The average values of similar parameters of red blood cells on day 4 changed significantly. The depressions depth and the height of the red blood cells decreased (~ 0.3 µm and ~ 0.38 µm, respectively), and the diameter of the cells increased (~ 10.6 µm).

The formula was used to calculate the volume (fl) of red blood cells [9]:

$$V = k\left[\frac{l_1+l_2}{2}(R^2 + Rr + r^2) - \frac{n_1+n_2}{2}r^2\right], \quad (1)$$

where l_1 and l_2 – the height of the red blood cell walls on the left and right, respectively (i.e. microns); n_1 and n_2 are the depression depths on the left and right, respectively (µm), and R and r are the radii of the base and top of the red blood cell (µm); coefficient k is a fitting parameter that includes all constants and takes into account the deviation of the real erythrocyte volume from the 'ideal' model.

The calculation found that the erythrocyte volumes on Day 1 of a gunshot wound and on Day 4 during hypothermia were reduced compared with the norm (Figure 7). However, on Day 1 during hypothermia, the volume turned out to be the largest, exceeding 1.3 times the norm.

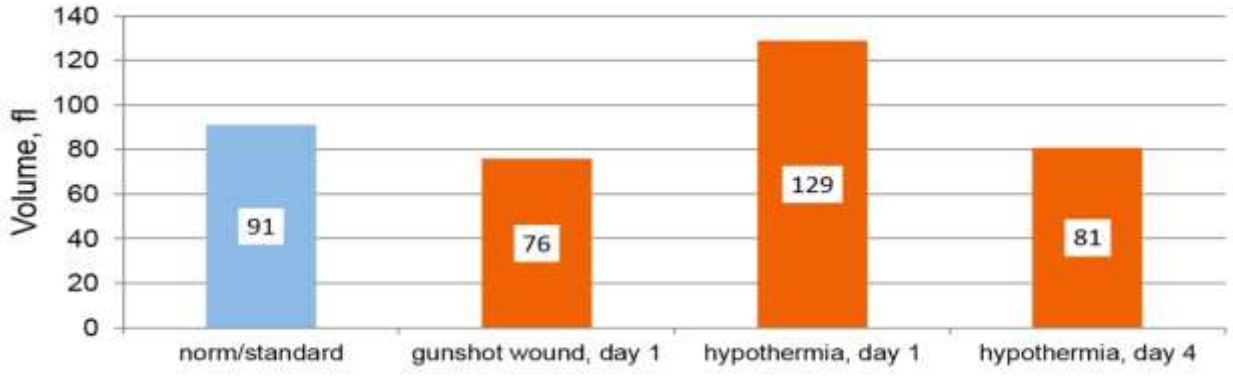


Figure 7: Red blood cells mean values

According to [10], the range of erythrocyte volume changes is small. If the volume increases by more than 1.6 times, the erythrocyte will burst. This is the maximum volume that can be enclosed in a practically inextensible cellular membrane having a normal erythrocyte surface area.

A halving of the volume is almost practically impossible, because the concentration of proteins in the cell increases so much that the hemoglobin becomes solid, and the red blood cell loses its ability to deform and pass through narrow capillaries in the spleen.

Conclusion

Thus, our data indicate significant differences in the morphology of red blood cells depending on the death cause. In particular, upon death from a gunshot wound on Day 1, most of the red blood cells take an echinocytic form, outgrowths appear, the depth of the depression (concavity) decreases and conglomerates form. On the contrary, in a man who died of hypothermia at extremely low temperatures (i.e. - 42°C), a smear of which was made within Day 1, were characterized by minimal changes.

The discocytic form of erythrocytes with a flat surface was preserved, however, conglomerate formation and an increase in the depth of the central concavity of erythrocytes were observed, which is probably due to an increase in the volume of red blood cells. It was found that even a slight difference in the temperature of body areas (i.e., Δ 2.4°C) at the time of blood collection affects the ratio of different forms of red blood cells after complete thawing. The lower the temperature, the more degenerative forms of red blood cells appear. After complete thawing of the corpse on the Day 4, all red blood cells (100%) had a different degree of dysmorphism, a more flattened surface, an increase in diameter and heterogeneity and roughness of the plasma, indicating signs of hemolysis. The use of atomic force and scanning electron microscopy allows obtaining images of red blood cells to study the morphology and topography of cells, which are associated with the physiological status of cells.

Our results indicate the need for further study of this problem to identify the possibility of cells restoration, their normal population and their functional properties, depending on the method of heating the dead from overcooling under certain temperature conditions.

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