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Research Article

# Nanodefects of membranes cause destruction of packed red blood cells during long-term storage

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ABSTRACT

Packed red blood cells (PRBC) are used for blood transfusion. PRBC were stored for 30 days under 4 °C in hermetic blood bags with CPD anticoagulant-preservative solution. Hematocrit was 50–55%. The distortions of PRBC membranes nanostructure and cells morphology during storage were studied by atomic force microscopy. Basic measurements were performed at the day 2, 6, 9, 16, 23 and 30 of storage and additionally 2–3 days after it.

Topological defects occurred on RBC membranes by day 9. They appeared as domains with grain-like structures ("grains") sized up to 200 nm. These domains were appeared in almost all cells. Later these domains merged and formed large defects on cells. It was the formation of domains with the "grains" which was onset process leading eventually to destruction of PRBC. Possible mechanisms of transformation of PRBC and their membrane are related to the alterations of spectrin cytoskeleton. During this storage period potassium ions and lactat concentrations increased, pH decreased, intracellular concentration of reduced glutathione diminished in the preservative solution. Changes of PRBC morphology were detected within the entire period of PRBC storage. Discocytes predominated at the days 1 and 2. By day 30 PRBC transformed into irreversible echinocytes and spheroechinocytes. Study of defects of membranes nanostructure may form the basis of assessing the quality of the stored PRBC. This method may allow to work out the best recommendations for blood transfusion.

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## 1. Introduction

Blood transfusion in acute and chronic anemia, in massive blood loss, in leukemia and other diseases implies utilization of packed red blood cells (PRBC). According to WHO guidelines [1] PRBC can be stored under 4 °C within 30–40 days. These PRBC are supposed to be acceptable for blood transfusion. But the storage environment of PRBC differs significantly from physiological. PRBC are stored in hemopreservative solution in hermetic bags. After blood transfusion at least 25% of transfused red blood cells (RBC) are eliminated from circulation during one day [2]. Preservation of the stability of PRBC is one of the key problems of transfusion medicine [3]. The final protocol including long-term storage of PRBC and the efficient blood transfusion is still under discussion [4].

Alterations of PRBC structure during storage are conventionally split into reversible and irreversible. The reversibility of the cell shape is inversely proportional to the storage time [4]. The quality of PRBC is mainly determined by the PRBC shape and the structure of their membranes [5]. Changes of PRBC shapes and alterations of their membrane structure [6] may lead to a decrease of RBC membrane deformability, deterioration of blood rheology and decrease of gas transporting function.

Changes of RBC morphology and their membrane structure may be caused by oxidative processes which develop in solution volume during prolonged storage and also by activation of free-radical processes in RBC themselves [7,8].

One of the effective methods of RBC morphology and their membrane investigation is atomic force microscopy (AFM). This method requires no preliminary modification of the object, and its resolution is less than 1 nm, which provides us with a possibility to study the cell membrane structure in detail.

*The aim* of the investigation was to study with AFM the alterations of membrane nanostructure of PRBC during long-term storage.

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## 2. Materials and methods

### 2.1. Packed red blood cells

PRBC in hermetic blood bag (400 ml) with CPD preservative solution were obtained from 3 independent blood transfusion centers (Moscow, Russian Federation). In total 12 blood bag were used in study (blood groups O(I), A(II), B(III), AB (IV), three bags of each group). Blood at these stations was routinely screened for infections, centrifuged and filtered according to the National guidelines. Packed RBC hematocrit was 50–55%.

PRBC were preserved for 30 days under 4 °C in accordance with WHO guidelines [1]. 15 ml of blood were withdrawn from each bag without breaching of hermetic sealing and diluted with phosphate buffer (pH=7.4) up to normal hematocrit (34–40%). Basic measurements were done at day 2,6,7,16,23 and 30th of storage and additionally 2–3 days after it. Smears preparations, AFM scanning in the current research were performed under the temperature of 19–20 °C without glutaraldehyde fixation of cells.

### 2.2. Analysis methods

Complete blood count was done by the blood analyzer «ADVIA 60» (Germany), biochemichal anlysis – by the «Miura One» (I.S.E. Group, Italy). Blood acid–base balance was done by the ionometric device «I-510» (Russian Federation). Electrodes ES-10603 with a reference electrode ESr-10103 were used to measure pH. Potassium ions concentration was measured by potassium-selective electrode ХС-К-001.

Cell-free hemoglobin in the preservative solution was measured by spectrophotometer «Unico-2800» (USA). To do this PRBC were centrifuged 2 times (1500 /min, 5 min), supernatant was withdrawn, its optical density was ($D$) was registered on wavelengths 500–760 nm. The exit parameters of free hemoglobin in solution were calculated by formula $D = \varepsilon Cl$, ($\varepsilon$ – molar absorptivity, $C$ – hemoglobin concentration, $l$ – layer thickness).

### 2.3. Atomic force microscopy

Cells and their membranes images were obtained by the AFM «NTEGRA Prima», (NT-MDT, Russian Federation) in semicontact mode. Monolayers of PRBC on glass slide were prepared with the aid of blood smear preparation device, the model V-Sampler (Vision, Austria) prior to AFM scanning. Cantilevers NSG01 (Switzerland) were used, force constant 5 N/m, tip radius 10 nm. The amount of scanning points was-512, 1024 within each line of image. Scanning fields: $120 \times 120\ \mu m^2$, $10 \times 10\ \mu m^2$, $3 \times 3\ \mu m^2$. Images and their profiles were analyzed in 2D and 3D formats. This method is described in detail in our works [9–11]. The typical membrane parameters of each image were quantified by the software FemtoScan Online (Advanced Technologies Center, Moscow, Russian Federation). To characterize the membrane topology of nanosurface the spatial Fourier analysis was used to decompose the image of the membrane surface into three constituents based on space periods of membrane patterns revealed by AFM. This methodological approach was proposed and described in detail in our previous studies [10,15] and also is used now the by other authors in study of membrane nanosurface [12].

### 2.4. Statistics

Analysis of cells shapes for each probe was done within 5 scans $120 \times 120\ \mu m^2$. Every day of measurement 12 probes with 4500 cells were analyzed. For 6 basic days the total statistical database was 27,000 cells. Analysis of membrane structure was done in $10 \times 10\ \mu m^2$ and $3 \times 3\ \mu m^2$ scans. For each basic days we obtained not less than 80 fragments of RBC membranes.

The data were statistically processed by Origin 6.1. (OriginLab Corporation, MA).The one-way ANOVA was used, $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. PRBC morphology

Alterations of RBC morphology were detected during the whole period of PRBC storage. Figs. 1–4 shows AFM images of cells at different days of storage. Fig. 1A shows typical 3D AFM image of RBC in 120x120 $\mu m^2$ scale. At early stages of storage due to natural poikilocytosis various cell shapes were presented on monolayer.

On days 1–2 of storage discocytes made up to 68 ± 8% of cells

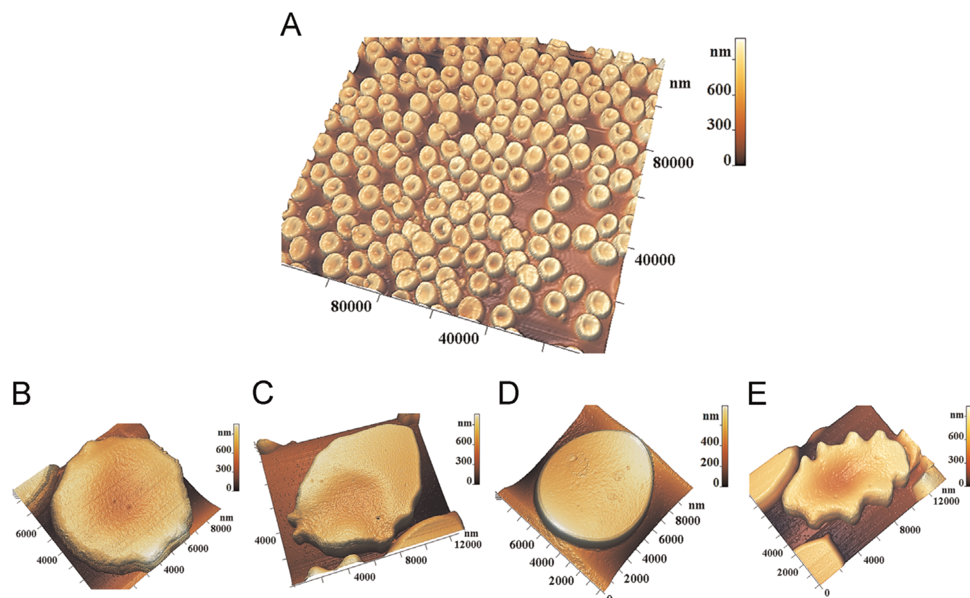


**Fig. 1.** AFM images of PRBC on days 1–2 of storage. (A) PRBC monolayer in $120{,}000 \times 120{,}000\ nm^2$ scale. (B) Discocyte, 68%. (C) Ovalocyte, 12%. (D) Planocyte, 5%. (E) Echinocyte, 15%.

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