752. Electronic-microscopic estimation of changes in erythrocytes ultrastructure under influence of low-frequency ultrasound

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Abstract. The paper presents the estimation of changes of erythrocytes ultra-structure under influence of low-frequency ultrasound using various types of waveguides. It was demonstrated that application of ultrasound contributes to change of erythrocyte form and size, which depend on intensity, duration and type of the waveguide.

Keywords: US thrombolysis, wave guide, intensity, erythrocyte.

1. Introduction

One of the most perspective methods of treatment of an arterial thrombosis is ultrasonic (US) thrombolysis. High efficiency of the given method was demonstrated during experiments in vitro [1-4], on animals [5, 6] and in clinic [7].

The main result of the experimental studies in recent years in the area of US-recanalization is the establishment of influence of waveguide head form in achieving significant US-thrombodestructive effect [8]. The most preferable is to use the waveguide with the spherical head in terms of destruction efficiency of blood clots and atherosclerotic plaques. The waveguide with the flat head also provides an effective restoration of affected vessel permeability, however at its application the frequency of a vessel damage increases in comparison with the waveguide having spherical head [8]. It was proposed to move the waveguide on an intravascular conductor, which is installed through an aperture in the waveguide head aiming to exclude vascular wall perforation [7].

Despite the obtained results, there are negative effects of US-influence on various parts of a hemostasis. In particular, it was established that US causes hemolysis of erythrocytes, changes the physical condition of a membrane lipid bilayer thus breaking their functional condition [7, 9, 10].

At the same time it is known about the role of erythrocytes in formation and progressing of occlusing vessel defeat [12-14].

It is possible to assume that the type of the waveguide head can influence functional erythrocytes condition, which is necessary to take into account when selecting the optimum waveguide type.

In this connection the aim of this research work is to study the influence of a waveguide head form on morphofunctional changes of erythrocytes in vitro at influence of low-frequency high-intensity US with various parameters. As membrane cell system defines its reaction on any influence so there were studied the diameter and the form of erythrocytes to estimate morphofunctional erythrocytes changes.

2. Materials and Methods

The studies in vitro were performed using the stabilized 3,8 % solution of natrium citrate of fresh donor blood obtained from the Hematology and Hemotransfusion Institution.

For US-influence there was used at work the plant of acousto-induced thrombolysis (the Republican Research Center "Cardiology", the Technopark of the Belarusian Technical University "Metolit") consisting of US-generator, piezoelectric converter and a waveguide. Target power of the generator is 80 W. The waveguides were made from steel 12X18H10, 23, 5 cm by length with spherical and flat head shapes without apertures and with apertures on a distal end.

2 series of experiments were conducted to study the influence of various US parameters on erythrocytes diameter: set 1 - depending on time of US influence (n = 1000); set 2 - depending on US intensity (n = 1000). US parameters: time (t) – 15, 30, 60, 90, 120 and 180 s; intensity (I) – 4.2, 8.1, 16.2, 25.1, 46.2 W/cm^2; duty of cycle (S) – 5 %.

The sonicated blood samples were placed in 1 % glutaric aldehyde on phosphatic buffer for fixing, then they were washed in the phosphatic buffer (pH = 7.4) and distilled water, after there were prepared erythrocytes smears. Ultrastructural erythrocytes changes were studied using scanning electronic microscopy ("Cam Scan", England) and the software for image processing and analysis "Autoscan" (the Scientific Research Institute named after A. N. Sevchenko, Minsk). The calculation of erythrocyte diameter was provided by photos on samplings including not less than 200 cells and expressed in percentage.

Control samples for microscopy were prepared from non-sonicated blood.

The final processing of the results was carried out using the software package for statistical research "Statistica 6.0". The data are submitted as $M \pm m$. The reliability of distinctions of average values was defined by Student pair t-criterion. The distinctions were considered to be authentic at $p < 0.05$.

3. Results and Discussion

In control blood samples the erythrocytes diameter made up from 4 up to 8.6 microns. Erythrocytes were divided into 2 groups: group 1 included cells in diameter 4-5.8 microns, group 2 included cells in diameter 5.81-8.6 microns.

The 1st series of experiments were dedicated to studying the influence of the waveguide type on morphofunctional erythrocytes condition depending on duration of US processing. At US absence at use of all types of the waveguides there were absent the changes in amount of erythrocytes of both groups. At the same time, increase of US influence time, the number of group 2 erythrocytes increased. As apparent in Fig. 1, application of the waveguide with a spherical form in 15 seconds induces the increase of the number of group 2 cells from 38,35±11,77 up to 55,43±5,58 % ($p < 0.05$) in comparison with the control group. Increase of US influence duration up to 60 seconds does not change the number of cells of the given group and does not differ from those for t = 15 s. While at t = 90 s the number reached the maximal value – 71,36±5,84 % ($p < 0.05$ in comparison with t = 15 s). Further, during US influence of 120 and 180 seconds the amount of erythrocytes of a big diameter decreased with sonication time increase (67,55±17,71 % and 54,66±4,82 % accordingly ($p < 0.05$ in comparison with a reference value)).

Use of the waveguide with flat head resulted in a similar tendency: in 15 seconds after influence the quantity of group 2 cells constituted 98,54 (96,18; 98,87) % ($p < 0.05$ in comparison with the control one). With increase of US influence time the number of cells was not changed in comparison with t = 15 s, remaining at previous high figures (see Fig. 1).

At use of the waveguide with spherical form aperture in 15 s of US influence, the number of large cells amounted to 57,39±4,97 % ($p < 0.05$ in comparison with the control one), in 30 s –

70,41±3,29 %, and in 60 s it reached the greatest value – 81,42±9,97 %. US influence during 120 and 180 s resulted in reduction of erythrocytes quantity in group 2 with increase in sonication time (56,55±17,99 % and 47,36±5,35 % accordingly ($p < 0.05$ in comparison with a reference value)).

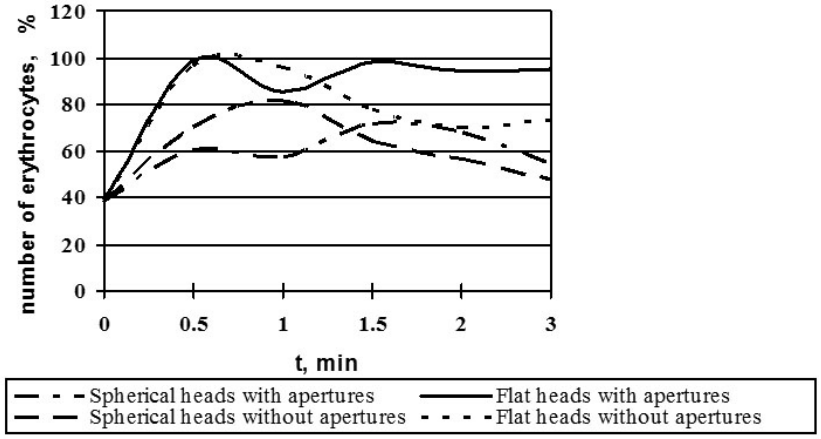

Fig. 1. Dynamics of group 2 erythrocytes amount change depending on US-influence time (I = 4,2 W/cm^2, S = 5 %) on whole blood using various types of waveguides

Use of the waveguide with flat form aperture also caused an increase in diameter of the erythrocytes. The greatest quantity of large erythrocytes is marked in 15 seconds from US influence and made up 97,74 (94,18; 97,76) % ($p < 0.05$ in comparison with the control one). With increase of US influence time up to 60 seconds the number of cells did not change in comparison with t = 15 s (see Fig. 1). At sonication during 90-180 s the number of the given cells was reduced in comparison with t = 15 s up to 73,52±22,27 % ($p < 0.05$). The decrease of group 2 erythrocytes quantity probably is associated with their hemolysis at long US influence.

The 2nd set of experiments was performed for studying the influence of the waveguide on erythrocytes diameter depending on US intensity. As Fig. 2 indicates, US intensity increase was caused by increase in a number of large erythrocytes at use of all types of the waveguides. When using the waveguide with spherical head US influence of I = 4,2 W/cm^2 resulted in increase of cells amount of group 2 from 38,35±11,77 up to 55,43±5,58 % ($p < 0.05$). At I = 8,1 W/cm^2 the quantity was 61,28±2,74 % ($p < 0.05$). At influence from I = 16,2 W/cm^2 it reached the greatest value of 71,31±4,53 % ($p < 0.05$ in comparison with I = 8,1 W/cm^2) in comparison with the control group. In turn, US blood processing from I = 25,1 and 46,2 W/cm^2, resulted in reduction of number of cells of group 2 by 14,63 and 20,74 % accordingly in comparison with I = 16,2 W/cm^2.

US influence through the waveguide with flat head from I = 4,2 W/cm^2 resulted in increase of a number of group 2 erythrocytes from 38,35±11,77 % up to 98,54 (96,18; 98,87) % ($p < 0.05$) in comparison with the control group. With increase in US intensity the quantity of cells of the given group decreased: at I = 25,1 and 46,2 W/cm^2 made up 84,76 (83,9; 86,11) % and 74,97±16,08 % accordingly.

US influence from I = 4,2 W/cm^2 of the waveguide with spherical head caused increase in quantity of group 2 cells from 38,35±11,77 % up to 57,39±4,97 % ($p < 0,05$ in comparison with the control). At I = 8,1 W/cm^2 their number reached the greatest value – 95,81±2,05 % ($p < 0.05$ in comparison with I = 4,2). US influence from I = 16,2 and 25,1 W/cm^2 did not lead to statistically significant changes of group 2 cells in comparison with I = 8,1 W/cm^2. At I = 46,2 W/cm^2 the quantity of group 2 erythrocytes decreased in comparison with I = 25,1 W/cm^2 and

amounted to 85,46±6,1 % (see Fig. 2). Use of the waveguide with a flat head resulted in formation of the largest quantity of large erythrocytes already at I = 4,2 W/cm^2 ($p < 0.05$ in comparison with the control). With intensity increase the quantity of the given cells decreased. At I = 8,1 W/cm^2 there were 87,49 (84,03; 87,62) %, at I = 16,2 W/cm^2 – 60,01 (58,6; 60,87) %, at I = 25,1 W/cm^2 – 48,86±9,92 %, and at I = 46,2 W/cm^2 – 36,5±15,74 %.

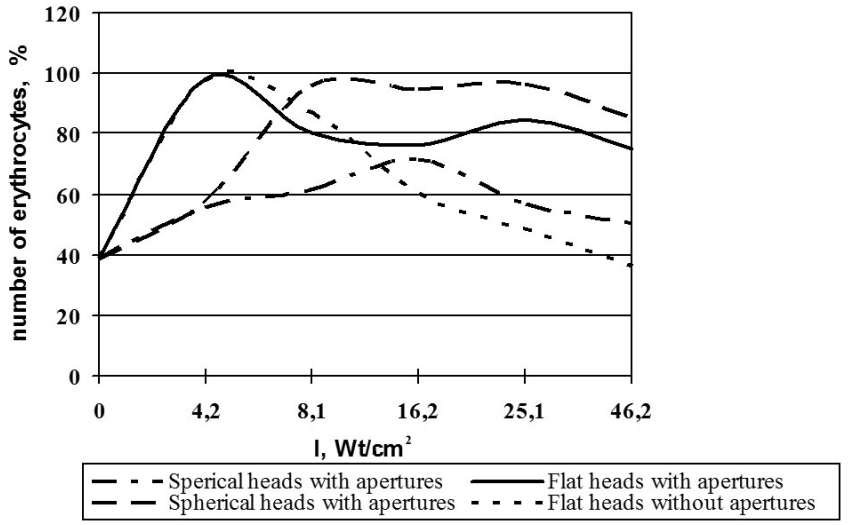

Fig. 2. Dynamics of group 2 erythrocytes amount change depending on US influence intensity (t = 15 s, S = 5 %) on whole blood at use of various types of waveguides

The analysis of erythrocytes ultra-structure allowed establishing the changes in studied blood corpuscles structure. As apparent in Fig. 3, after US processing of erythrocytes from dyscocytes they will be transformed into spherocytes.

In numerous studies the attention mainly focuses on studying of impact of various US parameters on functional condition of erythrocytes [7, 9, 10]. We did not find any studies in the available literature on the influence of waveguides type on ultra-structural erythrocytes changes.

As it is known under US influence the erythrocytes hemolysis occurs, their functional condition [7] changes. We can assume that mechanical erythrocytes destruction is at the heart of hemolysis due to cavitation bulbs impact [7, 9, 15]. Thus there is a mechanical damage of erythrocytes membranes with its subsequent hemolysis. Functional erythrocytes changes also can be connected with the secondary cavitation effects of physical and chemical character.

T. Tun et al. established that the waveguide head type defines the form and direction of cavitation stream and also its size that underlies at the heart of thrombodestructive effect various by its force [8]. The presence of an aperture in distal part of the waveguide can promote the formation of a big number of cavitation bulbs and also increase the size, direction and the area of cavitation stream. It can assist to more expressed US-influence on erythrocytes.

Besides, we demonstrated that erythrocytes from dyscocytes will be transformed into spherocytes. The appearance of spherocytes testifies to permeability disorder of a cellular membrane. As Na-K-ATF-ase is responsible for adjustment of cells volume due to osmotic effects it is possible to assume that because of membrane microviscosity disorder the work of the given carrier is inhibited, there is water inflow to a cell, which can cause increase in erythrocyte diameter and, possibly, its subsequent rupture.

Thus, the waveguides with an aperture assist in formation of a big number of large erythrocytes, which are hemolyzed in due course.

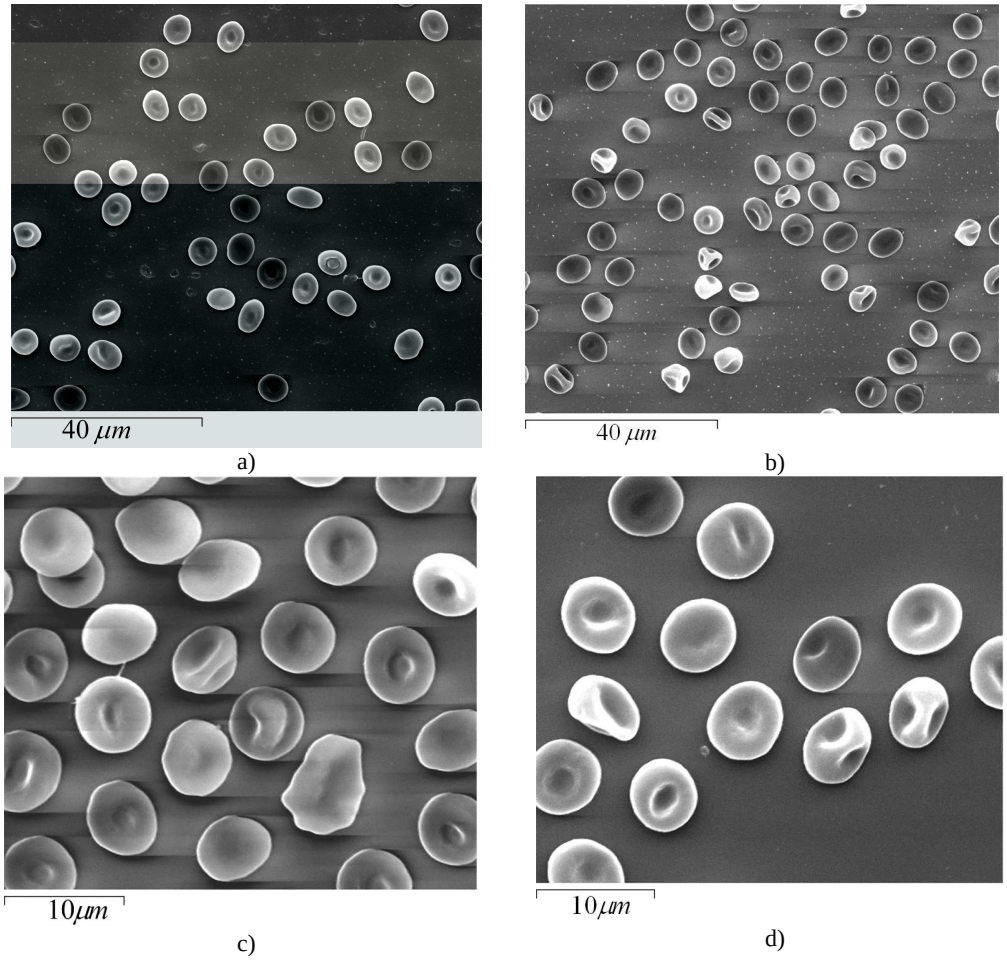

Fig. 3. Human erythrocytes: a) erythrocytes before US influence, ×1000; b) erythrocytes after US influence, ×1000; c), d) sonicated erythrocytes, ×2500

4. Conclusions

1. Low-frequency high-intensity US causes morphofunctional changes of erythrocytes: alters their form and increases their diameter.
2. The extent of morphofunctional changes depends on the waveguide type, intensity and duration of US influence.
3. The revealed morphofunctional changes may be one of the mechanisms responsible for US-induced erythrocytes hemolysis.
4. The demonstrated negative US effects must be taken into account when selecting a method of ultrasound-assisted thrombolysis.

Acknowledgments

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