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Hemorheological disorders in patients with peripheral occlusive arterial disease and ways to correct them

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Abstract
In vascular pathology, the reserve of vascular dilatation decreases, and the rheological properties of blood can either compensate for this negative modification or worsen tissue perfusion in a given vascular region. In view of the above, the purpose of this study was to investigate the hemorheological profile in patients with peripheral occlusive arterial disease (POAD). In 26 patients with POAD blood viscosity (BV1 and BV2) at high and low shear stresses, plasma viscosity (PV) and red blood cell (RBC) suspension viscosity (SV), hematocrit (Hct), red blood cell deformability (RBCD) and their aggregation (RBCA) were recorded and compared with the blood rheology of healthy individuals. In *in vitro* experiments, RBC microrheological responses to biologically active compounds – signaling molecules that have a positive effect on RBC microrheology, were analyzed. It was found that most of the hemorheological characteristics were abnormally changed in patients compared with healthy individuals. To a somewhat greater extent, this concerned the RBC microrheological parameters. The RBC incubation with pentoxifylline and vinpocetine revealed a positive microrheological effect. To analyze possible molecular cellular targets for the above compounds, experiments were performed with the incubation of erythrocytes with stimulators of adenylate cyclase (Forskolin), guanylate cyclase (nitric oxide), dB-cAMP (dibutyryl – cAMP), and sodium nitroprusside (SNP). Statistically significant positive RBC microrheological responses to these compounds were obtained. Taken together, it can be concluded that in POAD, along with vascular pathology, there is a negative change in the hemorheological profile. However, the data obtained in in vitro experiments indicate the possibility of correcting the existing changes in the RBC microrheology of patients.

Keywords:hemorheological profile, red blood cells, microrheology, POAD, signaling molecules, phosphodiesterase (PDE) inhibitors

1. Introduction

Numerous studies have shown that blood rheological properties are abnormally changed in vascular disorders [1-5]. It was reported that blood viscosity is increased and the oxygen transport capacity is reduced in patients with peripheral atherosclerotic vascular disease. It was combined with the increase of red blood cell (RBC) aggregation and the RBC rigidity too [6, 7]. In microcirculation, where cells must deform to pass through the narrow capillaries, RBC deformability is a major determinant of resistance to flow [8, 9]. Another red cell microrheological characteristic – red blood cell aggregation (RBCA) is known to be the main cause of increased blood viscosity under low-shear conditions [10, 11]. Increased aggregation is expected to increase the energy cost for the breakdown of aggregates as the blood approaches the microcirculation thereby causing an increase in flow resistance [12]. Besides the higher RBC aggregation may result in increased vascular resistance due to an inhibition of NO release by

the endothelium [13]. Therefore, high red cell aggregation and reduced cellular deformability may play a critical role in tissue perfusion, increasing the resistance to flow in the microcirculation in patients with peripheral occlusive arterial disease (POAD). Although the above-mentioned disease is linked to insufficient deformability and an increased aggregability of RBCs [14], there are only a few drugs that can modulate these microrheological properties of the red blood cells, including pentoxifylline [15, 16]. To correct RBC microrheological disorders drugs with well-known rheological effects can be used. However, it is difficult to estimate their direct effect on the microrheological red blood cell properties by investigating their aggregation and deformability changes after drug therapy alone. For this purpose, the investigation protocol could be supplemented with the estimation of the direct drug effect on microrheological RBC properties and the definition of the molecular targets for these drugs [17]. This study was designed to evaluate the hemorheological alterations after pentoxifylline treatment in patients with cerebral and peripheral atherosclerosis and to study the molecular change mechanism.

2.1. Subjects

Twenty-six patients (men, aged 57.6±5.8 years) with peripheral occlusive arterial disease (POAD) were enrolled in this prospective, open-label study. In order to reveal the differences between the normal hemorheological variables and those in POAD patients, the blood rheology parameters of healthy volunteers were also registered. It was a control group (aged-matched men, n=24). The use of human blood was in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The study was approved by the local ethical committee of the university (protocol No. 1 dated February 16, 2023) and informed consent was obtained from all study participants. Blood samples were obtained by venipuncture into EDTA vacutainers. RBCs were separated from plasma by centrifugation (15 min, 3000 rpm), washed three times in isotonic NaCl solution, and resuspended in Ringer's solution.

2.2. Study design

One week prior to entering this study, all drug therapy was discontinued, and at the end of this washout period, baseline clinical and hemorheological measurements were obtained for each subject. The clinical assessments included complete medical history, 12 lead electrocardiograms, blood pressure, heart rate, body mass and complete physical examination.

In vitro research session the microrheological characteristics of red blood cells (RBCs) were measured in patients prior to and after cell incubation (at 37^0C, 30 min) with:

1) pentoxifylline (10 μM), nonselective PDE inhibitor;
2) isobutyl-methyl-xanthine (IBMX, 100 μM), nonselective PDE inhibitor;
3) forskolin (10 μM), adenylate cyclase stimulator;
4) dB-cAMP, Dibutyryl cyclic-AMP (dB-cAMP, 10 μM),
5) sodium nitroprusside (100 μM), soluble guanylate cyclase stimulator, nitrogen oxide (NO) donor.

In each experiment, a suspension of RBCs incubated in Ringer's solution was used as a control, without the addition of the above drugs. Stock solutions of the compounds were prepared in Dimethyl sulfoxide (DMSO) or water. All analyses were completed within 4 h after the blood collection. Chemical compounds were purchased from Sigma-Aldrich.

2.3. Hemorheological measurements

Hemorheological profile parameters were recorded: blood viscosity (BV) and RBC suspension viscosity (SV) with hematocrit (Hct) 40% at five shear stress levels (0.36, 0.72, 1.08, 1.44 and 1.80 N/m^2), together with plasma viscosity (PV) and hematocrit measurement. Viscosities were measured using a capillary viscometer at room temperature. The hematocrit index was determined on an Elmi SM-70 hematocrit centrifuge (Latvia). The hemorheological efficiency of oxygen transport was assessed by the ratio of hematocrit/viscosity (Hct/BV) [18]. Red blood cell aggregation was assessed by the Myrenne

Aggregometer. The resulting index, termed «M1» by the manufacturer and «RBCA» herein, increased with enhanced RBC aggregation. To assess red blood cell deformability (RBCD), the cell elongation index (EI) was recorded in a flow microchamber [19].

2.4. Statistics and data presentation

Statistical processing included obtaining the mean value (M) and standard deviation (SD). The sampling distribution was tested using the Shapiro–Wilk test. Nonparametric statistics of the program Statistica 10.0 (StatSoft Inc., USA) was used. When conducting paired comparisons of indicators within groups during repeated measurements, the Wilcoxon test was used. Differences at $p < 0.05$ and $p < 0.01$ were taken as statistically significant. The data correlation hypothesis was tested using Pearson's correlation coefficients.

3. Results

3.1. Hemorheological characteristics of blood in patients with POAD

Analysis of the complex of hemorheological parameters showed that the blood viscosity under relatively higher and lower shear stresses was significantly higher in POAD patients as compared with control data (Table 1). This was combined with plasma viscosity and RBCA increase and also with RBCD decrease ($p<0.05$). The efficiency index of oxygen transport of blood in patients was 23% ($p<0.05$) and it was lower than in the group of healthy individuals (Table 1).

Plasma viscosity in patients correlated better with whole BV ($r=0.66$ – patients and $r= 0.42$ – healthy individuals).

Table 1

Characteristics of the hemorheological profile in healthy individuals and in patients with peripheral occlusive arterial disease (POAD) (M±σ)

Parameters	Control (n=24)	POAD (n=26)
BV1, mPa.s	4.94±0.32	6.06±0.76**
BV2, mPa.s	16.57±1.12	22.96±0.88**
PV, mPa.s	2.08±0.08	2.51±0.12
Hct, %	45.08±3.10	46.31±5.88
SV, mPa.s	4.12±0.32	5.22±0.24
Hct/BV1	9.91±2.42	7.59±2.12
RBCA, units	10.08±1.12	14.78±1.49
RBCD (EI), units	2.02±0.04	1.85±0.05

Note: BV_1 –blood viscosity at shear stress 1.80 N/m^2; BV2 – at 0.36 N/m^2; PV – plasma viscosity; SV – RBC suspension viscosity (Hct=40%), at high shear stress (1.80 N/m^2); Hct/BV_1 ratio – an index of blood O_2-transport efficiency; RBCD – red blood cell deformability; EI – red blood cell elongation index, as an index of RBC deformability; RBCA – red blood cell aggregation; POAD – peripheral occlusive arterial disease. *p<0.05, *vs.* group 1; ** p<0.01, *vs.* control group.

It is important to note that the RBC microrheological characteristics - their deformability and aggregation were not only significantly and negatively changed in comparison with healthy individuals (Table 1), but also more pronouncedly correlated with blood fluidity, as a value of inverse viscosity (Table 2). A more pronounced correlation of blood fluidity and its transport potential with individual rheological characteristics and, especially, with indicators of RBC microrheology, points to ways to

correct the negatively altered hemorheological profile of patients with POAD. To solve this problem, it is possible to study the microrheological responses of erythrocytes to several biologically active compounds for which molecular targets in erythrocytes are described.

Table 2

Coefficients of correlation in healthy individuals and patients with POAD

Correlations	Control (n=22)	POAD (n=26)
$1/BV_1$ – PV	–0.38	–0.64**
$1/BV_2$ – PV	–0.24	–0.46*
$1/BV_1$ – RBCD	0.24	0.52*
$1/BV_1$– Hct	–0.35	–0.56**
1/BV1–SV	– 0.26	– 0.43*
$1/BV_2$–SV	–0.44*	–0.60**
$1/BV_2$–RBCA	–0.24	–0.34
Hct/BV_1 – RBCD	0.27	0.39
Hct/BV_1 – RBCA	–0.22	– 0.40*
Hct/BV_1 – SV	–0.28	–0.52*

Notes: * p<0.05; **p<0.01;
$1/BV_1$ – blood fluidity at higher shear rate; $1/BV_2$ – blood fluidity at lower shear rate; other designations as in Table 1.

A more pronounced correlation of blood fluidity and its transport potential with individual rheological characteristics and, especially, with indicators of RBC microrheology, points to ways to correct the negatively altered hemorheological profile of patients with POAD. To solve this problem, it is possible to study the microrheological responses of erythrocytes to several biologically active compounds for which molecular targets in erythrocytes are described.

3.2. Microrheological responses of erythrocytes to pentoxifylline and vinpocetine

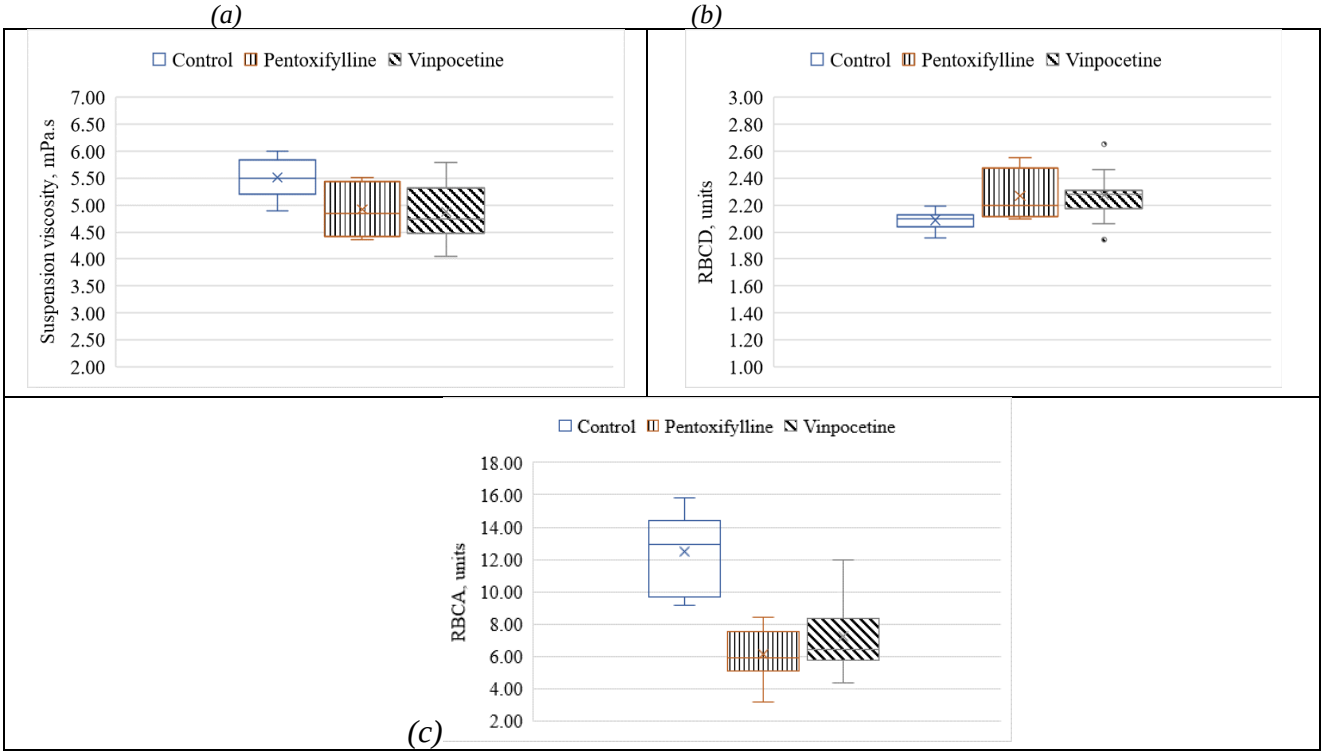

Fig. 1. Alteration of RBC suspension viscosity (*a*) RBC deformability (*b*) and RBC aggregation (*c*) after cell incubation with pentoxifylline and vinpocetine: vs. control (red blood cell suspension without above substances).
Note: Data are presented as median (Me) [Q25:Q75].

When erythrocytes were incubated with drugs such as pentoxifylline and vinpocetine, a significant decrease in SV by 10 and 12%, respectively, was observed (Fig. 1*a*).

Approximately the same amount (by 9 and 11%, $p<0.01$, fig. 1*b*) showed an increase in the RBC elongation index as an indicator of their deformability (RBCD). In response to these drugs, RBC aggregation decreased significantly, by 55 and 62%, pentoxifylline and vinpocetine, respectively ($p<0.01$, Fig. 1*c*).

3.3. Microrheological responses of RBCs to forskolin, IBMX, dB-cAMP and sodium nitroprusside

The above compounds inhibit phosphodiesterase (PDE) activity in cells [17] and increase the concentration of cAMP [20]. These data are confirmed in experiments with the RBC incubation with a non-selective PDE inhibitor, IBMX. Under these conditions SV decreased by 12% ($p<0.01$), and RBCA by 44% ($p<0.01$, Fig. 2a, 2b). The increase in RBCD was 13% (from 2.03±0.02 to 2.35±0.03 units, $p<0.01$, fig. 2*c*). Since PDE inhibition is accompanied by an increase in the concentration of cAMP in cells, it is important to check how this will affect the RBC microrheological responses.

Indeed, incubation of RBCs with the adenylate cyclase (AC) stimulator forskolin and the permeant cAMP, dibutyryl - cAMP (dB-cAMP), resulted in positive changes in RBC microrheological characteristics (Figs. 2a, b, c). In addition, one can add that, the magnitude of these changes was significant ($p<0.01$) and comparable with the shifts of these values in response to incubation with IBMX. In addition to stimulation of RBC AC, one can expect their positive microrheological responses at guanylate cyclase (GC) activation [19].

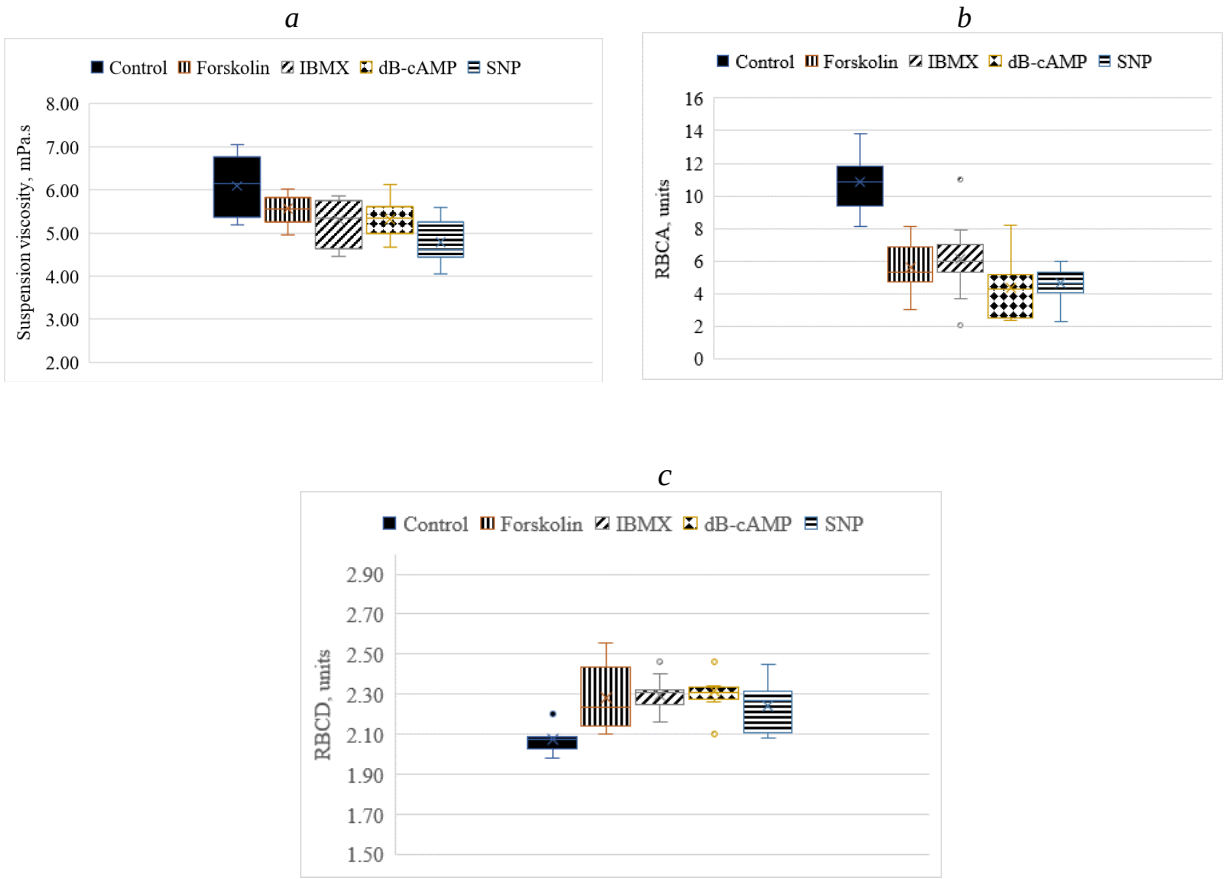

Fig. 2. Alteration of RBC suspension viscosity (*a*) RBC aggregation (*b*) and RBC deformability (*c*) after cell incubation with 5 biologically active compounds: vs. control (red blood cell suspension without of above chemicals).

Note: Data are presented as median (Me) [Q25:Q75].

This can be seen in the RBC model of microrheological responses to cell incubation with sodium nitroprusside (SNP). Under the influence of SNP, SV decreased by 16% ($p<0.01$), and RBCA – by 52% ($p<0.01$, Fig. 3-5) and this was associated with a 10% increase in RBCD ($p<0.01$).

4. Discussion

The results of the study showed that in patients with POAD, the main complex rheological parameter of blood, its viscosity, is significantly increased compared to healthy individuals. Since blood viscosity is determined by major factors such as plasma viscosity, Hct, deformability and aggregation of red blood cells [18], then it is possible to analyze the contribution of each of the elements of the hemorheological profile (Fig. 3).

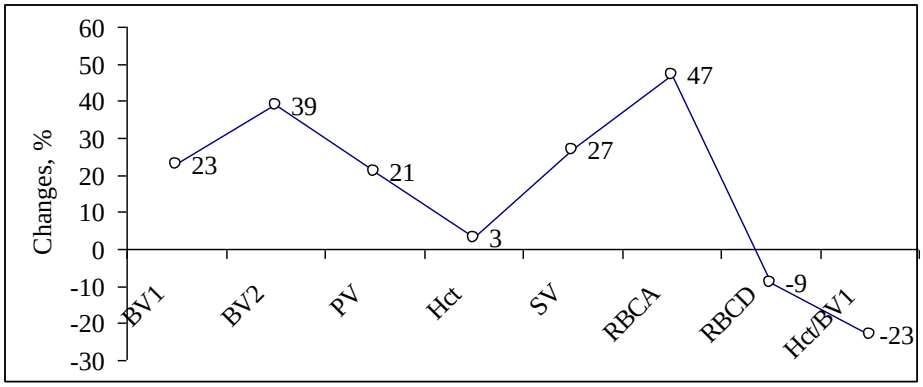

Fig. 3. Differences (in % relative to healthy individuals) of the hemorheological profile parameters in patients with POAD compared with healthy individuals (zero line)
Note: for abbreviations see Table 1.

It was found that in patients, the change in the microrheological characteristics of the profile was on average 28%, while the change in the macrorheological characteristics was 22% (Fig. 3).
For a positive correction of the hemorheological profile of patients with POAD, it is possible to influence chemical compounds that have cellular molecular targets in erythrocytes [21- 24].

It is known that drugs, based on methylxanthines, which include pentoxifylline, have phosphodiesterase (PDE) cells as a molecular cellular target, and these enzymes are well represented in human erythrocytes [25-27]. Indeed, during the incubation of erythrocytes with known selective inhibitors of phosphodiesterase activity, an increase in the deformability of erythrocytes (and a decrease in aggregation) was found. The data obtained indicate a marked increase in the deformability of erythrocytes upon stimulation of the adenylate cyclase signaling pathway.

In pathological conditions, and in vascular disorders, the aggregation of erythrocytes and their rigidity are significantly increased. One of the reasons for the change in these properties of erythrocytes may be an excess of calcium ions in the medium [28]. It has been shown that with an increase in the concentration of calcium ions in the blood serum, in several pathological conditions, there is a decrease in the deformation of erythrocytes and an increase in their aggregation [29, 30]. In model experiments with an increase in Ca^{2+} entry into erythrocytes under mechanical stress, a pronounced decrease in the filterability of erythrocytes through a 5 μm pore was recorded [31]. On the other hand, an increase in the activity of erythrocyte adenylate cyclase was combined with an increase in the fluidity of their suspensions and a significant increase in the deformation index. Therefore, stimulation of AC in erythrocytes with forskolin or dB-cAMP can not only increase the activity of protein kinase A and promote phosphorylation of membrane cytoskeleton proteins, but also block Ca^{2+} entry into the cell [32]. At the same time, phosphorylation of erythrocyte membrane proteins, and especially the 4.1 band protein and the anion transporter band 3, is combined with an increase in cell plasticity as a whole [33].

Thus, the data obtained in the study indicate that under pathological conditions, with impairment of the vascular tone of the lower extremities, there is an impairment of the parameters of the

hemorheological profile, which can be corrected with drugs, for example, pentoxifylline. In the structure of the entire hemorheological profile, only cellular elements can be targets for the drug. Therefore, it is quite logical to conclude that it is the RBC microrheology that positively changes under the action of the drug and the compounds mentioned above.

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