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Rheological properties of erythrocytes in patients infected with *Clostridium difficile*

Właściwości reologiczne erytrocytów u pacjentów zakażonych *Clostridium difficile*

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C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
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Summary

Introduction: *Clostridium difficile* infection (CDI) is a bacterial infection of the digestive tract. Acute infections are accompanied by increased risk for venous thromboembolism (VTE). To date, there have been no studies of the rheological properties of blood during the course of digestive tract infections. The aim of our study was to examine the effects of CDI on red blood cell (RBC) rheology, specifically RBC deformability, RBC aggregation, and plasma viscosity. In addition, the activity of glucose 6 phosphate dehydrogenase (G6PD) and acetylcholinesterase (AChE) in RBC was studied.

Material and Methods: Our study group included 20 patients with CDI, 20 healthy persons comprised the control group. We examined the effects of CDI on the rheology of RBCs, their deformability and aggregation, using a Laser–assisted Optical Rotational Cell Analyzer (LORCA). Plasma viscosity was determined using a capillary tube plasma viscosymeter. Moreover, we estimated the activity of AChE and G6PD in RBC using spectrophotometric method.

Results: A statistically significant increase was found in the aggregation index, viscosity and activity of G6PD whereas the amount of time to reach half of maximum aggregation (t½) and the amplitude of aggregation (AMP) both showed statistically significantly decreases among patients with CDI compared to the control group. We also observed that the Elongation Index (EI) was decreased when shear stress values were low, between 0.3 Pa and 0.58 Pa, whereas EI was increased for shear stress in the range of 1.13 - 59.97 Pa. These observations were statistically significant.

Conclusions: We report for the first time that acute infection of the gastrointestinal tract with *Clostridium difficile* is associated with abnormalities in rheological properties of blood, increased serum viscosity as well as increased aggregation of RBCs, which correlated with severity of inflammation. These abnormalities may be an additional mechanism causing increased incidence of VTE in CDI.

Keywords: **aggregation • *Clostridium difficile* • erythrocyte • plasma viscosity • rheology**

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Abbreviations: **AChE** - acetylcholinesterase; **AI -** Aggregation Index; **AMP** - amplitude of aggregation; **CDI** - *Clostridium difficile* infection; **CRP** - C reactive protein; **EI** - Elongation Index; **G6PD -** glucose 6 phosphate dehydrogenase; **Isc** - intensity of scattered light; **LP -** lipopolysaccharides; **LORCA -** The Laser-assisted Optical Rotational Cell Analyzer; **MCH** - mean corpuscular hemoglobin; **MCHC** - mean corpuscular hemoglobin concentration; **MCV -** mean corpuscular volume; **NO** - nitric oxide; **PCT** - procalcitonin; **PVP -** polivinylopyrrolidone; **RBC** - red blood cells; **SA -** sialic acid; **t½ -** the time it takes to reach half of maximum aggregation; **VTE -** venous thromboembolism; **WBC** - white blood cells.

Introduction

Clostridium difficile is a Gram-positive, anaerobic, spore--forming bacillus. The frequency and severity of *Clostridium difficile* infection (CDI) has been increasing over the last ten years in Europe and North America, and have become an increasingly more common source of nosocomial infections. Transmission of *Clostridium difficile* may be endogenous or exogenous. The major risk factors of CDI include: antibiotic therapy, hospitalization, and advanced age. Patients over 65 years of age have a five- to ten-fold greater risk for infection than people less than 65 years of age [1]. The clinical picture of CDI is diverse and ranges from asymptomatic carrier status to various degrees of diarrhea which can be severe, even life threatening. The most typical symptom is watery, sometimes intense diarrhea, abdominal pain, fever, nausea, vomiting and fatigue. The most serious complications of CDI are toxic megacolon, large intestine perforation, paralytic ileus, renal insufficiency. CDI is characterized by increased inflammatory markers; laboratory tests can reveal white blood cells (WBC) counts reaching 20,000/mm^3 and C reactive protein (CRP) concentration five-to-ten times the upper limit of normal [1].

It is known that RBC rheology can change during the course of inflammatory diseases [2,20,45]. This fact is very important in the pathophysiology of many diseases. Changes in the biochemical properties of RBC can modify microcirculatory hemodynamics [46]. The relationship between intestinal inflammation and venous thromboembolism (VTE) has been previous established as patients with inflammatory bowel disease have an increased risk of VTE [8,19,35,36]. Lately, similar observations have been reported in CDI patients by Barmparas *et al.* [4].

The aim of our study was to examine the effects of CDI on RBC rheology, specifically deformability and aggregation. To date, there have been no studies of the rheological properties of blood during the course of digestive tract infections. The exact mechanisms of this abnormalities are not fully elucidated. We hypothesized that RBCs in a patient with CDI can undergo similar changes as RBCs in sepsis. We based this hypothesis on our clinical observation of patients with CDI who had large increases not only in WBC counts and CRP concentration and also procalcitonin (PCT) concentration. Barmparas *et al.* recently published a study confirming our hypothesis. Specifically they reported that CDI increases the risk for venous thromboembolism [4]. Hence it is very important to understand the mechanism of alteration of blood rheological properties seen in CDI. Moreover we chose to study two enzymes associated with RBC - acetylcholinesterase (AChE) and glucose 6 phosphate dehydrogenase (G6PD). Acetylocholine (ACh) affects the rheological properties of RBC [15,31], but similar effects for AChE have not yet been described. G6PD has important antioxidant properties, but its potential impact on the rheological properties of blood has not yet been evaluated [24].

ACh acts as a neurotransmitter inside the central and peripheral nervous systems in humans. After being released from the pre-synaptic junction ACh acts on receptors in the post-synaptic junction. It is very quickly degraded by the AChE. Recent experiments show that ACh and AChE have been found in various non neuronal tissues such as epithelium, endothelium, immune cells, and blood cells. ACh receptors are expressed in immune, lymphoid, and also myeloid cells [15,53]. G6PD is the first enzyme in the pentose phosphate pathway of glucose metabolism. It reduces NADP to NADPH, which has strong antioxidant properties. The presence of G6PD in RBC allows them to produce sufficient NADPH to protect themselves from oxidative stress. G6PD deficiency is the subject of numerous studies and the most common human enzyme deficiency worldwide, affecting over 400 million people

[24,26]. To date there have been no studies of the role of G6PD during acute infection in patients without deficiency of this enzyme.

The Laser–assisted Optical Rotational Cell Analyzer (LORCA; RR, Mechatrionics, Hoorn, Netherlands) is unique in its capacity to assess important rheological traits, specifically the deformability and aggregation of RBCs. This method allows measurement of both static and kinetic parameters of the aggregation process. The instrument is equipped with a video camera for detection of diffraction patterns. A thermostation unit and ellipse-fit computer software calculate the Elongation Index (EI) as a measure of cell deformability [23].

Material and methods

Patients

The study group included 20 patients with CDI hospitalized at the Infectious Disease Department and Gastroenterology and Hepatology Department, University Hospital in Krakow, aged from 24 to 87 years (mean age: 63±19,7 years), and 20 healthy volunteers from 24 to 56 years old (mean age 43±9,8 years). Testing of patients with CDI was performed within 48 hours of the beginning of treatment. Patients with CDI were treated with oral vancomycin 125 mg every 6 hours (n=10) or oral metronidazole 500 mg every 8 hours (n=10). Blood samples were obtained no later than 48 hours after initiating therapy, when the therapeutic effect of the antibiotic is not yet fully realized and patients still manifest clinical symptoms. The diagnosis of CDI was based on history, epidemiological data, physical examination, and laboratory tests. It was confirmed by detection of *Clostridium difficile* antigen and toxins in feces using TOX A/B Quick Check Complete (Wampole, TechLab, USA). Exclusion criteria included the presence of other acute or chronic inflammatory diseases, any hematologic disorders, the use of immunosuppressive therapy, anticoagulants, statins and/or antiplatelet agents. Blood samples for rheological examination were collected in the morning, between $7.^{00}$ and $8.^{00}$, after an overnight fast, into vacutainer-type tubes containing EDTA K2 as an anticoagulation agent. Moreover we assessed complete blood count with differential leukocyte count, electrolytes, blood urea, creatinine, alanine transaminase, prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen, and CRP. All tests were performed according to generally accepted standard methods. The study was conducted in accordance with the Declaration of Helsinki (1975) and approved by the local ethics committee.

Measurement of RBC deformability and aggregation

Measurements of the deformability and aggregability of RBC were carried out using a LORCA machine. A detailed description of the instrument is provided in [23].

When measuring deformability, 25 µl of blood was diluted in 5 ml of 0.14 mM PVP solution (polivinylopyrrolidone, M = 360 000, Sigma, viscosity at 37°C up to 31 mPa × s) with the addition of PBS at pH 7.4. The cell is subjected to increasing shear stress, which causes it to elongate, thus changing the dispersion of light recorded by the sensor of the apparatus. EI is obtained using the following formula: EI = (L – W)/(L + W) where L denotes length of cell and W its width. Elongation graphs were obtained that illustrated the relationship between EI and the shear stress applied [23].

For the aggregation measurement, undiluted blood was used. The blood was oxygenated for 15 minutes prior to measurement through slow rotation of the glass vessel. A sample of 2 ml of blood prepared in this way was poured into the space between the two cylinders. The apparatus analyzes the intensity of light scattered

Table 1. Results of basic laboratory tests in the patients and healthy controls

Parameter	Patients with CDI	Control group	
	mean ± SD	mean ± SD	p
WBC, $x10^9$/l	13.45 ± 3.50*	4.52 ± 0.59	0.01
RBC, $x10^{12}$/l	4.24 ± 0.37*	4.48 ± 0.37	0.07
Hb, g/dl	12.09 ± 1.35	12.11 ± 1.07	0.1
Hct, %	40.31 ± 3.59	42.56 ± 3.31	0.44
MCV, fl	87.53 ± 3.44	92.6 ± 3.45	0.41
MCH, pg	28.52 ± 1.40	31.1 ± 1.29	0.31
MCHC, g/dl	32.83 ± 1.18	32.61 ± 0.59	0.44
PLT, $x10^9$/l	306 ± 101*	188 ± 44	0.01
Fibrinogen, g/l	7.55 ± 2.45*	3.75 ± 0.44	0.01
CRP, mg/l	97 ± 48*	1.19 ± 0.58	0.01

CDI, *Clostridium difficile* infection; CRP, C reactive protein; Hb, hemoglobin; Hct, hematocrit; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PLT, platelets; RBC, red blood cells; SD, standard deviation; WBC, white blood cells; * $p<0.05$

Table 2. Elongation Index (EI) in patients and healthy controls

Shear stress, Pa	EI in patients with CDI	EI in control group	
	Mean ± SD	Mean ± SD	p
0.3	0.04 ± 0.02*	0.06 ± 0.03	< 0.001
0.58	0.07 ± 0.02*	0.08 ± 0.03	<0.001
1.13	0.16 ± 0.03*	0.09 ± 0.02	< 0.001
2.19	0.26 ± 0.03*	0.15 ± 0.04	< 0.001
4.24	0.37 ± 0.02*	0.23 ± 0.03	< 0.001
8.23	0.45 ± 0.03*	0.32 ± 0.04	< 0.001
15.96	0.51 ± 0.02*	0.40 ± 0.03	< 0.001
31.04	0.56 ± 0.02*	0.49 ± 0.06	< 0.001
59.97	0.59 ± 0.02*	0.55 ± 0.06	< 0.001

CDI, *Clostridium difficile* infection; EI elongation index; SD, standard deviation; * p<0.05

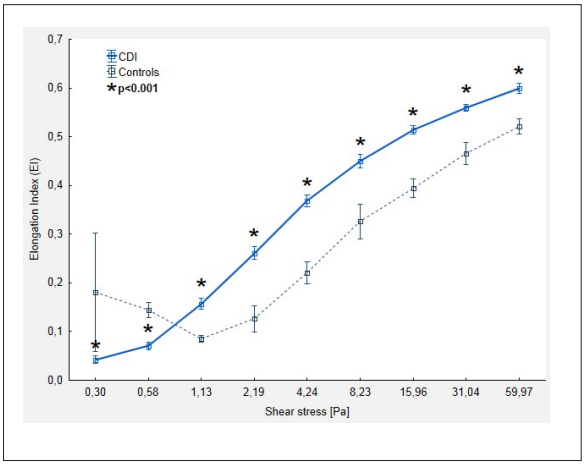

Fig. 1. Comparison of Elongation Index (EI) in patients and healthy controls
The graph shows significant difference in Elongation Index (EI) between study groups. RBC deformability was monitored using a Laser-assisted Optical Rotational Cell Analyzer (LORCA, RR, Mechatrionics, Hoorn, Netherlands) as previously described [23]. RBCs are subjected to increasing shear stress. The resulting laser diffraction pattern, changing from circular (rest) to elliptical (high shear) are expressed by EI, calculated from the major (L) and minor (W) ellipse axis as (L-W)/(L+W). More deformable RBCs are more elongated and produce a higher EI. In our study RBC deformability was lower in CDI patients when there was less pressure on RBC (shear stress 0.3 Pa and 0.58 Pa). When there was increased pressure on RBC, (sheer stress from 1.13 to 59.97 Pa) RBC deformability in CDI patients increased when compared to the control group.
CDI, *Clostridium difficile* infection; EI, Elongation Index; LORCA, Laser-assisted Optical Rotational Cell Analyzer; * p<0,05

by the blood sample. The measurement is then represented in the form of a syllectogram, a curve illustrating the change in the intensity of scattered light (Isc), expressed in arbitrary units (au) during 120 seconds corresponding to the course of aggregation. The first stage of aggregation measurement is an initial disaggregation of blood cells. It is obtained by subjecting the sample to 120 seconds of shear stress at 400 s-1. Then the motor driving the cylinder is stopped abruptly, the cells lose their elongation and return to a round shape, causing an upstroke of the Isc value. This value may be deemed to be the measure of elasticity of RBC in whole blood. Next, aggregation of RBC occurs and is accompanied by a drop in the Isc value [23].

Measurement of plasma viscosity

Plasma viscosity was assessed using a capillary tube plasma viscosymeter (Myrenne GmbH – Germany). Viscosymeter comprises a capillary tube 0,38 mm in internal diameter and 200 mm long through which a plasma sample of 0,5 ml is forced at a constant positive pressure of 17,2 kPa. Plasma viscosity is proportional to its flow time through the capillary. Readings are made at ambient temperature and corrected to a viscosity value at 37°C [14].

Measurement of the activity of AChE, G6PD

AChE and G6PD RBC activity was estimated using spectrophotometric method after Beutler. The activity measurement was made by monitoring the increase in absorption at 412 nm for AChE and 340 nm for at 37°C [9].

Statistical Analysis

Results were subjected to statistical analysis. The normality of the distribution was tested using the Shapiro-Wilk test. We used the student's t-test for analysis of independent variables and the U Mann-Whitney test for analysis of dependent variables. Correlation between selected variables was evaluated using a Pearson correlation coefficient; in the absence of normal distribution, Spearman's rank correlation was used. All calculations were performed using STATISTICA 10 PL software (StatSoft, Inc., USA) licensed to Jagiellonian University. Only results which had a p value <0.05 were considered statistically significant.

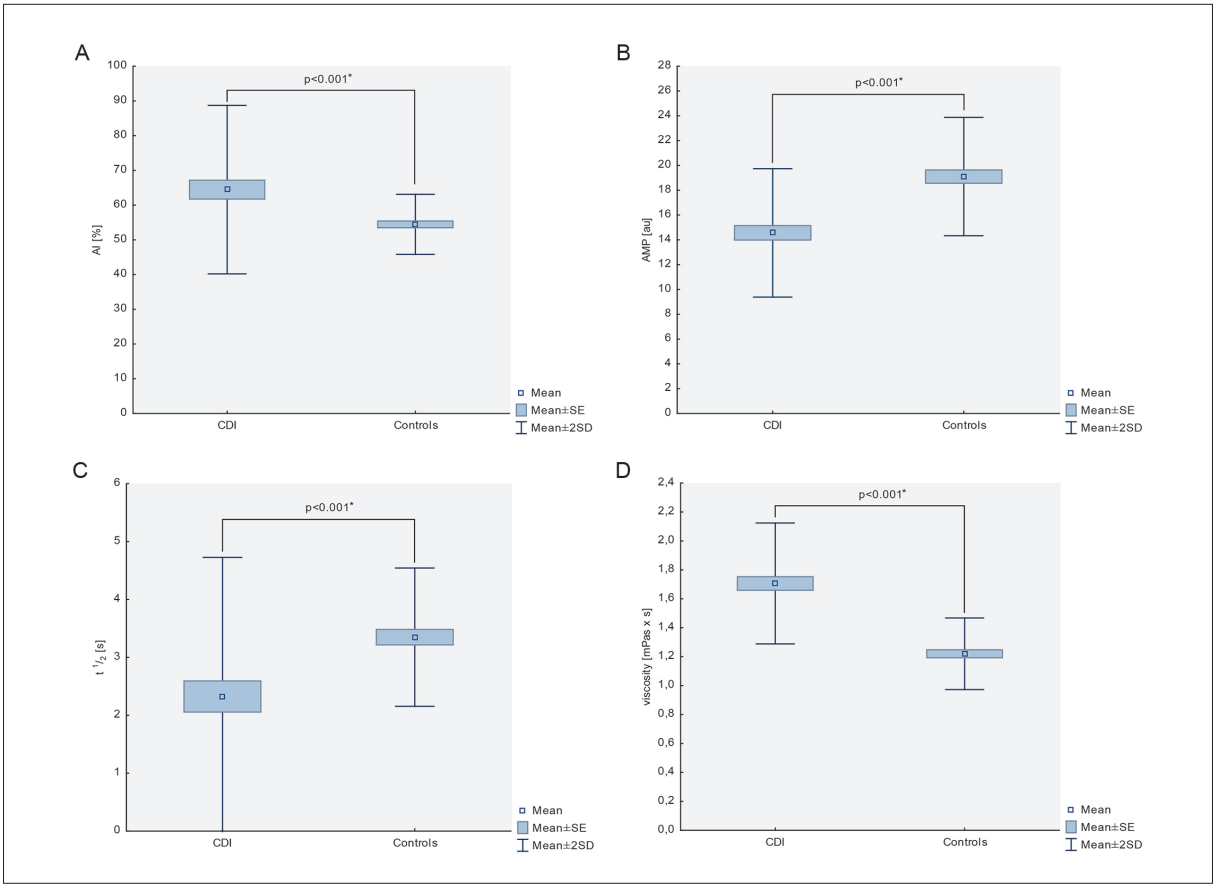

Fig. 2. Comparison of aggregation parameters and plasma viscosity in study groups

The figure shows significant differences in RBC aggregation parameters and plasma viscosity in study groups. An increase in AI (Fig. 2A), plasma viscosity (Fig. 2D) and decrease in AMP (Fig. 2B) and t½ (Fig. 2C) was found in CDI patients when compared to the control group.

AI, Aggregation Index; AMP, amplitude of aggregation; CDI, *Clostridium difficile* infection; t½, the time it takes to reach half of maximum aggregation

Table 3. Comparison of aggregation parameters and AChE/G6PD activity in RBC in the study groups

Parameter	Patients with CDI	Control group	
	mean ± SD	mean ± SD	p
AI, %	64.46 ± 12.13*	54.45 ± 4.33	< 0.001
AMP, au	14.56 ± 2.59*	19.30 ± 2.39	< 0.001
t½, s	2.32 ± 1.20*	3.3 ± 0.60	< 0.001
Viscosity, mPas x s	1.71 ± 0.21*	1.22 ± 0.12	< 0.001
AChE, U/g Hb	20.24 ± 5,79	17.16 ± 3.01	0.1
G6PD, U/g Hb	2.22 ± 0.98*	1.75 ± 0.55	0.04

AChE, acetylcholinesterase; AI, Aggregation Index; AMP, amplitude of aggregation (the difference between minimum and maximum values of intensity of scattered light); CDI, *Clostridium difficile* infection; G6PD, Glucose 6 Phosphate Dehydrogenase ; t½, time to reach one half of maximum aggregation; * p<0.05

Results

Effect of Clostridium difficile on laboratory tests

Patients with CDI were characterized by statistically significant increases in WBC, platelets, fibrinogen and CRP when compared with the control group. The average WBC count was three fold higher while the average plasma fibrinogen level was two times higher in patients with CDI when compared to the control group. We did not observe any changes in RBC counts which allowed us to effectively compare RBC deformability and aggregation between our test groups as the RBC count and hemoglobin levels have an effect on rheological characteristics of blood [43]. No significant

differences were found with respect to number of RBC, hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration MCHC (Table 1). Additionally, though the data are not shown, no fluctuation in the concentration of electrolytes, urea, creatinine, alanine transaminase, PT or APTT were observed.

Effect of Clostridium difficile on RBC elongation, RBC aggregation, and plasma viscosity

Elongation Index (EI) in the group of patients with CDI was lower than the control group at shear stress levels of 0.3 Pa and 0.58 Pa. For sheer stress levels from 1.13 to 59.97 Pa, patients with CDI had higher EI when compared to controls. The observed values were all statistically significant (Table 2, Fig. 1).

A statistically significant increase was found in Aggregation Index (AI) (Fig. 2A) and viscosity (Fig. 2D) among patients with CDI compared to the control group, however, AMP, the amplitude of aggregation (Fig. 2B), and t½, the time it takes to reach half of maximum aggregation (Fig. 2C), values showed a statistically significant decrease in those patients. A statistically significant increase was found in G6PD activity among patients with CDI compared to the control group. AChE activity was higher in patients with CDI as compared controls, however this variation was not statistically significant (Table 3).

Additionally, we performed a correlation analysis of our test parameters which showed that during CDI infection there is an indirect correlation between AI and t½ (r=-0,89, p<0,001), direct correlation between AI and WBC count (r=0,49, p=0,047), as well as a direct correlation between plasma viscosity and CRP levels in the blood (r=0.49, p=0.03). Other correlation analysis did not yield any statistically significant results (Table 4).

Table 4. Correlation analysis of studied parameters in the CDI group

Compaired parameters	n	r	p
AI	20	-0,89	<0,001*
t½			
AI	20	0,49	0,047*
WBC			
Viscosity	20	0,49	0,03*
CRP			

AI, Aggregation index; CDI, Clostridium difficile infection; CRP, C reactive protein; t½, time to reach one half of maximum aggregation; WBC, white blood cells; * p<0,05

Discussion

This study demonstrated that acute bacterial infections of the gastrointestinal tract may cause worsening of rheological properties of blood. The capacity of RBCs to adapt their shape to the dynamic flow conditions, is essential for their proper functioning, i.e. flow through the microcirculatory bed. RBC deformability is a major determinant of RBC survival, as deduced from the association between abnormal RBC shape, anemia, and splenic sequestration [16,33,38].

Rheological changes in inflammatory conditions have been assessed in numerous studies [2,20,45], but such changes in acute infections are not as widely studied. Such changes have almost exclusively been studied in sepsis [5,6,10,34]. Sepsis is a very serious infection both in course and prognosis, characterized by strong inflammation often ending in death. To date there is only one report of rheological changes during milder infections, even though the incidence of such infections exceeds the incidence of sepsis many times over. Biesiada *et al.* found markedly increased aggregation of RBC and decreased $t_{½}$ during erysipelas, while changes in EI were only observed at low levels of sheer stress, and RBC deformability displayed statistically significant increases [10]. In the present study, we selected patients with CDI for evaluation of their rheological parameters. We hypothesized that CDI has a negative effect on the entire body by changing the rheological properties of blood. The toxins produced by *Clostridium difficile* damage intestinal mucosa causing neutrophilic infiltration, with interleukin 8 acting as key chemotactic mediator [50]. It has been reported that this cytokine increases the risk for venous thromboembolism [51].

To date there have not been studies of the rheological properties of blood during the course of gastrointestinal tract infection, including *Clostridium difficile*. In order to assess the impact of CDI on the rheological properties of blood, we applied strict exclusion criteria to ensure the validity of results. Specifically, we excluded patients with other inflammatory diseases, hematological diseases, as well as patients treated with immunosuppressive drugs, statins, or anticoagulants.

RBC rheology can be influenced by many factors including intracellular calcium changes, ATP concentration, and the effects of nitric oxide (NO) [41,44]. Calcium concentration in the RBC is important for the proper functioning of those cells, and an increase in free cytosolic Ca^{2+} induces increased permeability of Ca^{2+}-activated K^+ channels, which leads to hyper-polarization of the membrane [37]. RBC membrane changes observed during the course of sepsis may lead to reduced functioning of the pumps and consequently, disturbance of Ca^{2+} homeostasis. In the course of sepsis, lipopolysaccharides (LPS) and pro-inflammatory cytokines activate inducible NOS (iNOS), resulting in the secretion of large amounts of NO. NO has an effect on Ca^{2+}-ATPase channels which leads to an increase in intracellular Ca^{2+}, causing a reduction in RBC deformability [25]. Interestingly, RBC also have the ability to secrete NO under certain conditions, as observed during *Plasmodium falciparum* infection, when

infected RBCs may produce large amounts of NO [21]. Korbut and Gryglewski showed that RBC deformability depends on WBC concentration; in the presence of low WBC counts, deformability is increased by NO donors (sydnominine, sodium nitroprusside), and is reduced by NO synthase inhibitors (L-NAME) [28]. ATP also reacts to changes in Ca^{2+} concentration [27]. During the course of sepsis, the intracellular ATP level is reduced, resulting in a decrease in energy available for the RBC membrane Ca^{2+} pump [47]. Consequently there is an increase in intracellular Ca^{2+}. Administration of pentoxifylline improves RBC deformability by direct growth of intracellular concentrations of ATP [48]. An additional factor having an impact on the functioning of RBC is sialic acid (SA). Eichelbronner et al. demonstrated *in vitro* increased adhesion of RBC to the endothelium caused by endotoxins most likely resulting from decreased SA in RBC cell membranes [18]. Piagnerelli et al. described decreases in RBC membrane SA having a significant effect on the shape of a RBC [39]. The decrease in SA during the course of sepsis may be a direct effect of bacterial infection or as a result of an increase in the activity of sialidase, as can be observed in diabetes [12,32].

RBC aggregation is a complex process that affects plasma, especially the presence of high-molecular-weight proteins that promote aggregation. These substances increase aggregation via molecules that form cross-bridges between erythrocytes or by creating an osmotic gradient, which forces RBCs together [6]. In our study we noted significantly increased plasma levels of fibrinogen in patients with CDI, which is a marker of worsening rheological characteristics of blood. Furthermore, plasma viscosity clearly increased along with strengthening of the inflammatory response, which was demonstrated by plasma viscosity increasing in proportion with the blood level of CRP.

Though plasma factors are believed to have the most significant effect on RBC aggregation, properties of RBC also seem to be important [17,40,49]. As a result of the bacteriocidal activity of phagocytic cells, reactive forms of oxygen are produced. Used for protection against bacterial invasion, these reactive forms of oxygen can be toxic towards the host's own cells by causing oxidative stress and oxidative lesions in tissues such as inter alia – the peroxidation of biological membranes [6,45]. Beppu *et al.* reported that RBC membrane proteins damaged by reactive oxygen species are preferentially degraded by membrane-bound proteases [7]. Increased proteolysis of the RBC membrane can result in increased aggregation [43]. Baskurt *et al.* assessed aggregation and deformability in animal models and observed increased RBC aggregation during states of sepsis in animals while there was a reduction of RBC deformability in human models [5,6]. Interestingly, septic rats showed significantly decreased EI only at shear stress levels of 0,5 and 1,58 Pa, whereas above 1,58 Pa EI was significantly increased [6]. Astiz *et al.* and Lam et al. showed increased RBC aggregation and viscosity in sepsis, while the others reported decreased RBC deformalibility in sepsis [3,29,30,42]. In our study we observed similar changes in RBC aggregation and blood viscosity as in sepsis. Patients with CDI had increased blood viscosity and increased rates of aggregation of RBC. RBC aggregation increased with the severity of inflammation, as demonstrated by a direct relationship between the AI and the WBC in blood. In addition, aggregation occurred faster, which indicates a decreased $t_{½}$ in the group of patients infected with CDI. The time from the preaggregation state to maximum aggregation, as measured by AMP rate, was also decreased in the CDI group. It should be emphasized that the inverse correlation between AI and t½ we observed is unfavorable for patients. During the course of CDI, with more RBC that undergoing aggregation, we observed that the process moved along faster. While in our study the changes in human RBC deformability during the course of CDI we observed differed from results observed during sepsis, they are very similar to results obtained in one study of septic rats performed by Baskurt et al. [6]. Taking into consideration the strengthening of aggregation parameters during acute infection of gastrointestinal tract, the increased EI that was observed in our study in patients with CDI may hypothetically be an adaptive response of the organism to increased aggregation. Appropriate RBC deformability is important so the cells can adequately travel through blood vessels of the smallest caliber. The decrease in RBC deformability observed during the course of sepsis could result from severe inflammation and abnormal compensatory mechanisms to counter increased aggregation of RBC and increase blood viscosity. Less intense inflammatory states did not inhibit this compensatory mechanism, as we observed in our patients with CDI.

Many physiological characteristics of RBC are regulated via ACh, however the role of ACh or AChE in the normal functioning of RBC is not fully understood. It is known, however, that of all the elements of blood, RBC has the highest activity of ACh [15]. ACh is able to modulate the hematoreologic properties of RBC; it has an effect on aggregation, deformability, lipid membrane fluidity, and on the ability of RBC to transport oxygen [53]. It was shown in vitro studies that ACh decreases RBC aggregation and increases deformability at lower shear stress in blood samples from healthy donors [15,31]. It has also been shown that an enhanced activity of circulating ACh is observed in inflammatory conditions [11,22,52]. AChE rapidly hydrolyzes ACh, which terminates chemical synaptic transmission [53]. Little is known about the RBC AChE activity other than its influence on limiting ACh activity, though it has been investigated as a potential marker of cell membrane integrity. AChE is located on the outer side of RBC membrane, it is a marker of older RBC since its activity drops sharply with RBC age [15,53]. The results of our study show that during the inflammatory process AChE is increased and at the same time an increase in RBC deformability was noticed. In the control group, lower activity of AChE were accompanied by decreased EI in RBC, however the observed differences in AChE were not statistically significant.

The viscoelascity of the RBC membrane is impaired under oxidative stress, mostly due to changes of cytoskeletal proteins, transmembrane proteins, and the lipid bilayer. There are some known mechanisms which are able to protect this RBC membrane disturbance, for example thiol-compounds are able to prevent diamide-induced oxidative damage in RBCs, protecting erythrocytes against alteration of their rheological properties [13]. G6PD deficiency leads to a number of adverse consequences, particularly hemolytic anemia [26]. It has also been shown that patients with G6PD deficiency who suffer from certain infections tend to have a more severe disease course [26,54]. Taking this into consideration, and knowing that G6PD plays an important antioxidant role, its behavior in the population without enzyme deficiency may play an important role in the pathomechanism of infections. During aging of the RBC, the quantity of active G6PD decreases, and older RBC become more vulnerable to oxidative stress. Mature RBC are not able to synthesize new proteins [26]. The increased activity of G6PD in RBC of individuals infected with *Clostridium difficile* in our study may be a result of the intensified hemolysis, particularly of older RBC which are more vulnerable to oxidative stress. If this hypothesis were true, one would observe a relative increase in young mature RBC. Additional confirmation would be provided in the form of decreased total RBC count and Hb concentration in patients with CDI when compared to the control group. Although we observed such results in our study, they were not statistically significant.

In conclusion, acute gastrointestinal infection may worsen the rheological properties of blood. During the course of CDI, blood is more viscous and RBC tend to aggregate, which may be one of the factors leading to an increased risk of thromboembolic events in these patients. Increases in viscosity and RBC aggregation observed during CDI correlates directly with the severity of inflammation. The increased activity of G6PD in RBC of individuals infected with *Clostridium difficile* may be a result of the intensified hemolysis, particularly of older RBC which are more vulnerable to oxidative stress. We did not observe statistically significant differences in AChE level among study groups.

Further studies are required for elucidate the mechanisms responsible for the association of the CDI and VTE. Some relevant, preliminary data for our laboratory may serve as a starting points to develop preventing a strategies to decrease the incidence of VTE in CDI.

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