

Effects of Preconditioning on RBC Deformability in Critically Ill Patients

OBJECTIVES: RBCs from critically ill patients have depressed deformability, especially in sepsis. Prolonged exposure of RBCs from healthy volunteers to physiologic shear stress (the preconditioning technique) has been associated with improved deformability, but the effect of preconditioning on RBCs from critically ill patients with or without sepsis has never been studied.

DESIGN: Prospective study.

SETTING: A 32-bed medico-surgical ICU and a university-affiliated cell biology laboratory.

SUBJECTS: RBCs from 26 healthy volunteers and 40 critically ill patients (20 with and 20 without sepsis).

INTERVENTIONS: RBC deformability was measured using the elongation index (EI) with an ektacytometer, at shear stress levels ranging from 0.3 to 50 Pa. To assess the effects of preconditioning in the three groups, we measured EI after first applying a shear stress of 5 Pa for 300 seconds. To study the potential mechanisms involved in preconditioning, we looked at deformability after incubation of an RBC solution from the healthy volunteers with glutaraldehyde, a membrane-stabilizing protein, and neuraminidase, an enzyme that releases membrane sialic acid.

MEASUREMENTS AND MAIN RESULTS: Baseline RBC deformability was significantly depressed in the septic patients compared with the volunteers at all shear stress levels greater than or equal to 4.89 Pa. Preconditioning improved deformability only in the volunteers (at shear stress levels of 0.48 and 0.76 Pa). Among the critically ill patients, preconditioning worsened RBC deformability at higher shear stress levels. After incubation (with glutaraldehyde or neuraminidase) of RBCs from five volunteers in whom preconditioning had significantly improved deformability, the positive effect of preconditioning was lost with glutaraldehyde.

CONCLUSIONS: RBC deformability is depressed in septic patients. There was a deleterious effect of preconditioning on RBC deformability in septic patients, unlike the positive effect on RBCs from healthy volunteers. The effect of preconditioning may be associated with elasticity of the cell membrane.

KEY WORDS: critically ill patient; deformability; microcirculation; preconditioning; red blood cell; shear stress

The microcirculation, where tissue oxygenation occurs, consists of vessels with a diameter less than 100 μm (capillaries, arterioles, venules). It can be modified by the action of endothelial and smooth muscle cells, as well as by various blood cell elements, including RBCs (1). Apart from their capacity to transport oxygen bound by hemoglobin, RBCs play a key role in the regulation of oxygen distribution within the microcirculation. Under hypoxic conditions, RBCs release—via intracellular signaling pathways—adenosine triphosphate (ATP) and nitric oxide (NO) which induce local vasodilatation, thereby improving tissue oxygenation (2, 3).

Anthony Moreau, MD¹

Karim Zouaoui Boudjeltia, PhD²

Patrick Biston, MD³

Michael Piagnerelli, MD, PhD^{2,3}

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One essential feature of RBCs for their microcirculatory role is their deformability. The term “erythrocyte deformability” refers to the capacity of a RBC to modify its shape in response to pressure (stress) being applied to its membrane. Because of its biconcave shape, the total surface area of the RBC membrane is larger than the area necessary to contain the cell volume (4). This feature allows an RBC to adopt different shapes while maintaining its volume, making it possible for it to pass through blood capillaries with narrower diameters. RBCs in the blood stream are exposed to different levels of shear stress, resulting in passive deformation (5–7), but active deformation, via complex intracellular signaling (8), has also been described.

Recent studies performed on RBCs from healthy volunteers have shown that a preconditioning technique, consisting of exposure of a highly dilute suspension of RBCs to a constant shear stress of between 5 and 20 Pa for a period of 300 seconds, can improve RBC deformability at certain shear stress levels (9, 10). The authors hypothesized that this preconditioning technique activates production of NO, which influences deformability (11). By contrast, the hypothesis that preconditioning can improve RBC deformability by modifying the elastic properties of the RBC membrane has never been studied.

Sepsis is a syndrome characterized by organ dysfunction and caused by a dysregulated immune response to an infection (12). Several studies have shown that RBC deformability is reduced in patients with sepsis (13) and when persistent is associated with higher mortality (14). Several factors, including inter alia the products of glycolysis (2,3 diphosphoglycerate and ATP), NO, and membrane components (carbohydrates like sialic acids and lipids), may be associated with decreased RBC deformability in sepsis (15).

We compared RBC deformability in healthy volunteers, nonseptic patients, and septic patients. We then assessed the effects of preconditioning on RBC deformability in these three groups and studied the potential mechanisms involved.

MATERIALS AND METHODS

This prospective study was approved by a central and a local ethic committee (Erasmus University Hospital and Intercommunale de Santé Publique du pays de Charleroi Ethical committees; P2018/088) on March 29, 2018. Procedures were followed in accordance

with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975. A consent form was signed by each participant.

1. Selection of the Participating Subjects

Healthy volunteers had to be at least 18 years old and with no known pathology, in particular no active infection. Patients were included over a period of 6 months. The ICU patients were grouped into two categories: those with sepsis (including patients in septic shock) diagnosed according to international criteria (12) and those without sepsis. Exclusion criteria were as follows: age less than 18 years old, had received a blood transfusion within 3 days before the study, were receiving treatment with iron and/or erythropoietin, active hemorrhage, hematological pathology including pathologies affecting RBCs or leukocytes, leukopenia unrelated to sepsis, active cancer or chemotherapy within the past 3 months, and pregnancy.

2. Preparation of the Suspended Solution of RBCs

A blood sample was taken from each participant using blood collection tubes with an EDTA anticoagulant. The sample was taken within the 24 hours preceding the experiment. Five microliters of whole blood were diluted in 1 mL of polyvinylpyrrolidone, at a viscosity determined by the manufacturer (29.3 mPa/s at 37°C); 600 µL of the RBC-polyvinylpyrrolidone mixture were then placed in an ektacytometry system (LORCA MaxSis, Mechatronics, Hoorn, the Netherlands).

3. The Laser-Assisted Optical Rotational Cell Analyzer

See **Supplemental Digital Content** (<http://links.lww.com/CCX/B64>).

4. Measurement of Deformability and Effects of RBC Preconditioning

We applied each suspended RBC solution to shear stress levels ranging from 0.3 to 50 Pa (0.3, 0.48, 0.76, 1.21, 1.93, 3.07, 4.89, 7.78, 12.39, 19.72, 31.4, and 50 Pa) and measured the EI at each level. Measurement errors existed for each shear stress, with variation coefficients

of 51%, 8.2%, 3.9%, 2.4%, 1.7%, 1.6%, 1.1%, 1.3%, 1.7%, 1.2%, 1.4%, and 1.2%, respectively (14).

To assess the effect of preconditioning on the RBCs, we then applied a constant shear stress of 5 Pa to a suspended RBC solution over a period of 300 seconds (protocol applied in the studies of Meram et al [9] and Simmonds et al [10]), immediately followed by evaluation of the deformability of these preconditioned RBCs using the method described above.

5. Study of Potential Mechanisms Involved in Preconditioning

To study the role of the membrane explaining the effects of preconditioning of RBCs, we used two molecules. First, we incubated RBCs with glutaraldehyde (C₅H₈O₂, used for protein fixation) to increase membrane rigidity. Second, we incubated RBCs with neuraminidase to decrease carbohydrate membrane content, as has been observed in RBCs from septic patients, and is known to modify RBC sphericity (16).

Using the blood samples from volunteers, we first mixed 1 mL of blood with 14 mL of “Roswell Park Memorial Institute” (RPMI; a cell culture medium) (step 1). The resulting mixture was then centrifuged for 10 minutes at 1,700 relative centrifugal force (step 2), after which the supernatant was aspirated and removed (step 3). Steps 1, 2, and 3 were then repeated on the RBC sediment. The resultant solution of “washed” RBCs was then mixed with RPMI to obtain a 1 mL mixture. This solution was then divided into three “aliquots.” The first “aliquot” served as the control sample. To the second “aliquot,” we added a 0.005% solution of glutaraldehyde, and to the third “aliquot,” we added a solution of neuraminidase at a concentration of 0.5 U/mL.

Using the control “aliquot,” we studied RBC deformability without and after preconditioning at 5 PA for 300 seconds. Using the other two “aliquots,” we then studied RBC deformability after preconditioning at 5 PA for 300 seconds in RBCs incubated with glutaraldehyde for 30 minutes or with neuraminidase for 4 hours. The concentrations of glutaraldehyde and neuraminidase and the incubation durations used in our study were based on results from previous studies conducted by Piagnerelli et al (16) and by Baskurt et al (17), in which these concentrations and incubation times had the greatest effect on deformability.

6. Statistical Analysis

We used the software SigmaPlot Version 12.5 (Systat Software, Chicago, Illinois, United State) for Microsoft Windows. Data are shown as mean \pm SD or as median with 25–75 percentiles. The demographic data were compared with nonparametric Kruskal-Wallis (with a correction using the Dunn test) and Mann-Whitney tests. Baseline deformability in the healthy volunteers and nonseptic and septic patients was compared using the one-way analysis of variance (ANOVA) test. The effect of preconditioning was assessed using the parametric Student test. The effect of glutaraldehyde and neuraminidase was assessed using the one-way ANOVA test. A value of p less than 0.05 was considered statistically significant. The SDs are not shown in the figures included in the text so as not to impair readability.

RESULTS

During the 6 months of the study, we included 26 healthy volunteers and 40 patients, 20 with sepsis and 20 without.

Demographic Data

The demographic characteristics of the healthy volunteers and critically ill patients are shown in **Table 1**. The diagnoses at ICU admission in the septic and nonseptic patients are shown in **eTables S1** and **S2** (Supplemental Digital Content, <http://links.lww.com/CCX/B64>). The patients with sepsis were older than the nonseptic patients and the healthy volunteers ($p < 0.001$). As expected, the Acute Physiology and Chronic Health Evaluation II score was significantly higher in the septic than in the nonseptic patients. The septic patients were more anemic than the healthy volunteers ($p < 0.001$) and had a more marked inflammatory syndrome (higher C-reactive protein concentration) than the healthy volunteers and the nonseptic patients. There was no statistically significant difference in the mean cell volume of the RBCs among the three groups.

Study of Deformability at Baseline

RBC deformability at baseline was significantly depressed in the septic patients compared with the healthy volunteers at all shear stress levels greater than or equal to 4.89 Pa (**Fig. 1**). The EI_{max} = elongation index max was significantly lower in septic patients (0.633 ± 0.048) than in healthy volunteers

TABLE 1.
Demographic Data

Variables	Healthy Volunteers (n = 26)	Nonseptic Patients (n = 20)	Septic Patients (n = 20)	p
Age (yr)	51 (31–57)	63 (36–71)	69 (63–76) ^{ab}	< 0.001
Sex (male/female)	16/10	13/7	12/8	1
Hemoglobin (g/dL)	14.6 (13.2–15.6)	13.0 (11.3–14.8)	11.2 (10.1–12.7) ^a	< 0.001
Hematocrit (%)	43.8 (40.8–46.6)	39.8 (33.9–43.5) ^a	34.6 (30.0–40.0) ^a	< 0.001
RBC count ($\times 10^3/\text{mm}^3$)	4,840 (4,300–5,110)	4,125 (3,690–5,280)	3,630 (2,985–4,268) ^a	0.001
Mean corpuscular volume (fl)	91.0 (89.5–93.9)	91.5 (87.9–92.6)	91.2 (86.8–97.2)	0.9
WBC ($\times 10^3/\text{mm}^3$)	6.8 (6.1–7.8)	12.5 (10.5–16.2) ^a	12.9 (8.6–16.1) ^a	< 0.001
Platelets ($\times 10^3/\text{mm}^3$)	242 (212–274)	234 (203–302)	247 (184–326)	1
Blood urea nitrogen (mg/dL)	35 (30–38)	35 (28–46)	61 (47–83) ^{ab}	< 0.001
Creatinine (mg/dL)	0.82 (0.78–1.04)	0.82 (0.68–0.94)	0.93 (0.75–1.30)	0.51
Bilirubin (mg/dL)	0.51 (0.34–0.57)	0.8 (0.59–1.25) ^a	0.7 (0.55–0.96) ^a	0.001
Glucose (mg/dL)	77 (62–97)	133 (112–159) ^a	143 (114–181) ^a	0.001
C-reactive protein (mg/L)	1 (1)	41 (10–68) ^a	228 (171–267) ^{ab}	< 0.001
Lactate (mmol/L)	–	0.9 (0.7–1.4)	1.4 (1.1–2.3) ^b	0.02
Acute Physiology and Chronic Health Evaluation II score	–	10 (6–18)	22 (15–24) ^b	0.01
Pao ₂ /Fio ₂	–	329 (220–388)	203 (151–290)	0.05
Nonsurvivors (%)	–	4 (20)	7 (35)	0.3

^avs healthy volunteers.

^bvs nonseptic patients.

Dashes indicate no value available.

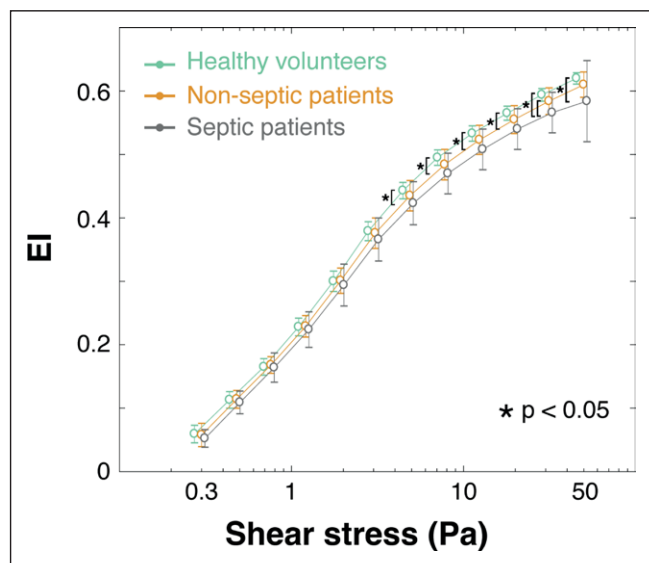


Figure 1. Baseline RBC deformability in the healthy volunteers/nonseptic/septic patients as assessed by the elongation index (EI) at different shear stress values.

(0.656 ± 0.019) ($p = 0.04$). There were no statistically significant differences in the Shear Stress 1/2 (SS1/2) values or the SS1/2/EI_{max} ratio across the three groups (eTable S3,

Supplemental Digital Content, <http://links.lww.com/CCX/B64>).

Effects of Preconditioning

After preconditioning, we observed a significant overall improvement in RBC deformability from healthy volunteers only at low shear stress levels (shear stress 0.48 Pa: EI ranged from 0.113 ± 0.013 to 0.118 ± 0.011 after preconditioning, $p = 0.01$; shear stress 0.76 Pa: EI ranged from 0.165 ± 0.013 to 0.171 ± 0.013 after preconditioning, $p = 0.02$) (Fig. 2). However, responses to preconditioning in healthy volunteers were heterogeneous: preconditioning improved RBC deformability for several volunteers, whereas among others it deteriorated (Fig. 3). There was a significant decrease in deformability in patients with and without sepsis at shear stresses of greater than or equal to 7.78 Pa. There were no statistically significant differences in the SS1/2 values or the SS1/2/EI_{max} ratio across the three groups (eTable S4, Supplemental Digital Content, <http://links.lww.com/CCX/B64>).

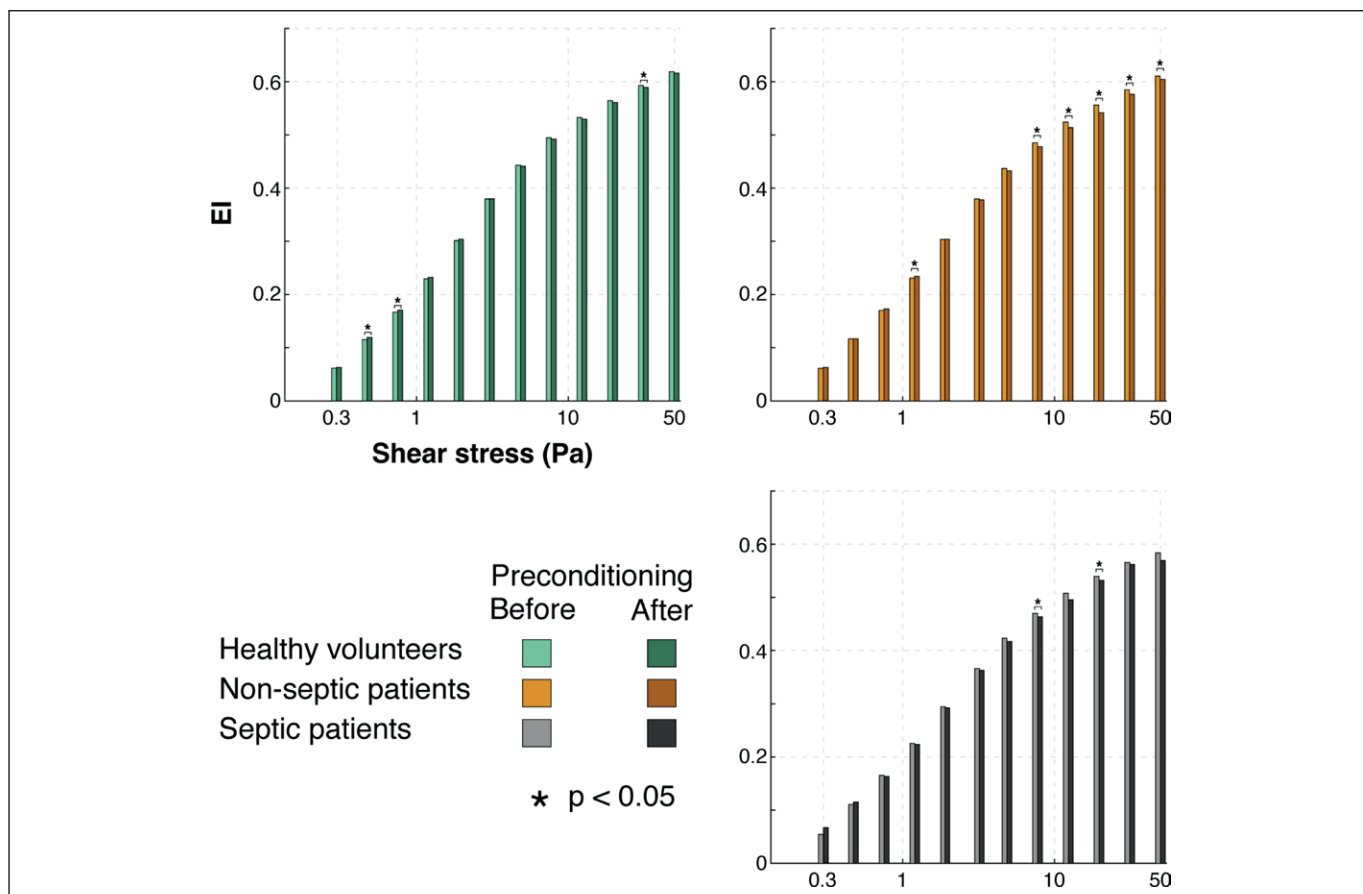


Figure 2. RBC deformability, as assessed by the elongation index (EI) at different shear stress values, before and after preconditioning in healthy volunteers (A), nonseptic patients (B), and septic patients (C).

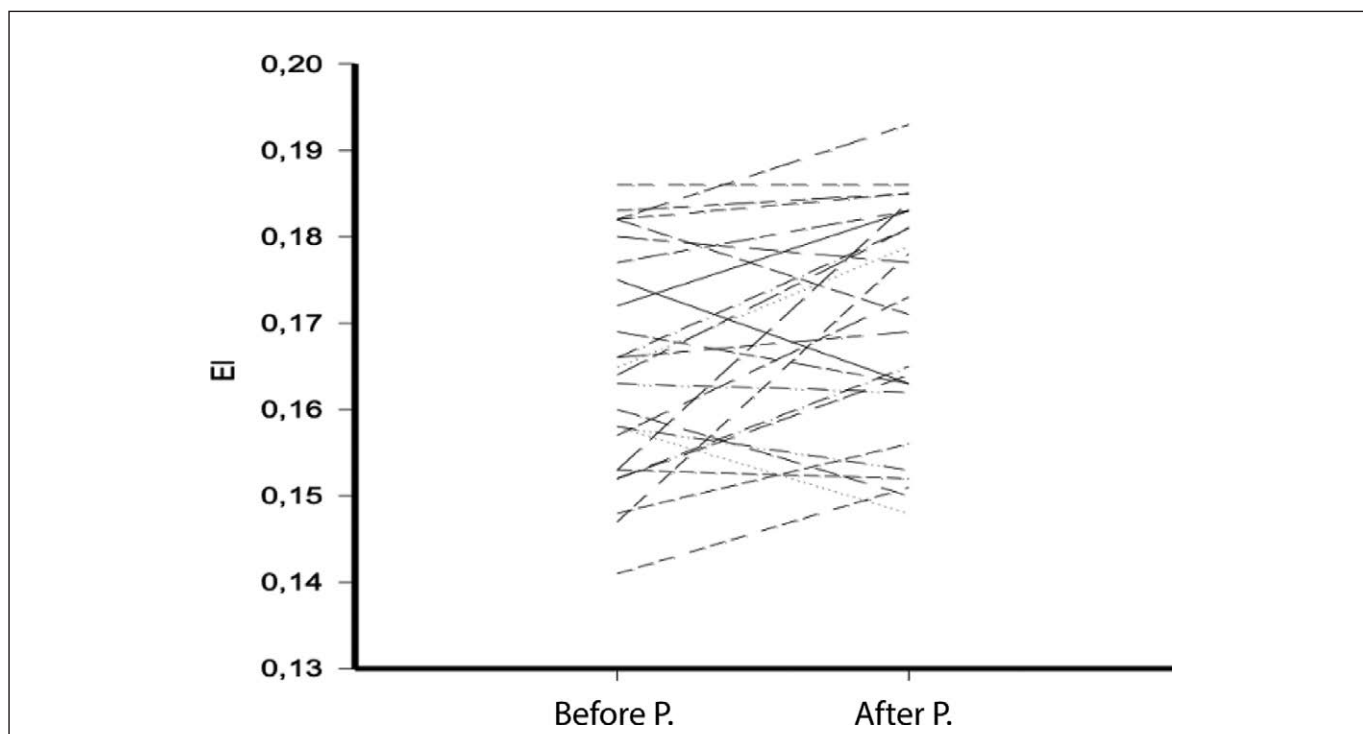


Figure 3. Effects of preconditioning (P) on RBC deformability as assessed by the elongation index (EI) at a shear stress of 0.76 Pa in healthy volunteers ($n = 26$).

Study of the Potential Mechanisms Involved in the Preconditioning

Because of the heterogeneity of the results for the healthy volunteers following preconditioning, we decided to study the effects of glutaraldehyde and neuraminidase in five volunteers whose RBC deformability was significantly improved at certain shear stress levels after preconditioning. In these RBCs, the positive effect of preconditioning was no longer present after incubation with glutaraldehyde. By contrast, there was no statistically significant change in deformability after incubation with neuraminidase (eTable S5, Supplemental Digital Content, <http://links.lww.com/CCX/B64>).

DISCUSSION

The key findings of our study are as follows: first, RBC deformability was significantly depressed in patients with sepsis compared with healthy volunteers at all shear stress levels greater than or equal to 4.89 Pa; second, preconditioning improved deformability only in RBCs from the healthy volunteers; third, among critically ill patients, preconditioning worsened the deformability of RBCs at higher shear stress levels; and finally, the positive effect of preconditioning was lost after RBCs incubation with glutaraldehyde, suggesting an important role of membrane fluidity on RBC deformability.

Sepsis leads to organ dysfunction, including the microcirculation, via a dysregulated immune response following an infection (12). Among septic patients, alterations of the microcirculation are characterized inter alia by a decrease in the density of blood vessels and in their perfusion; these alterations are more marked and persistent among septic patients with poor outcomes (18). RBC deformability is also depressed during sepsis. Reggiori et al (19) showed that RBCs were less deformable in patients with sepsis admitted to the ICU than in nonseptic patients or healthy volunteers irrespective of the level of shear stress applied. In our study, RBC deformability in the patients with sepsis was significantly depressed compared with that of healthy volunteers but only at shear stress levels greater than or equal to 4.89 Pa. One possible explanation for this finding is the limited number of subjects included in our study ($n = 66$ vs $n = 216$). Furthermore, the time factor may also play a role: certain septic

patients included in our study had been hospitalized for several days prior to the measurements, in contrast to those in the study by Reggiori et al (19) which included only patients within 24 hours of admission.

Two recent studies showed that preconditioning in healthy volunteers significantly improved RBC deformability but only at low shear stress levels (9, 10). In our study, we also only showed an improvement in RBC deformability in healthy volunteers at low shear stress levels (0.48 and 0.76 Pa). However, in contrast to these two studies (9, 10), we observed a heterogeneous effect of preconditioning among healthy volunteers, with improved deformability in some subjects, but worsened deformability in others. This heterogeneity could be explained by the larger number (26 vs 5 and 10 in the studies of respectively Meram et al [9] and Simmonds et al [10]) of healthy volunteers included, given that our methodology was identical to that used in those studies.

Interestingly, the RBCs of the patients showed no improvement in deformability after preconditioning, which may even have had a negative effect at high shear stress levels. This absence of response to preconditioning could be explained by different mechanisms. Piagnerelli et al (16) showed that the activity of neuraminidase, the enzyme responsible for cleaving sialic acid (a carbohydrate found on the membrane's surface), was higher among septic patients than among volunteers and nonseptic patients. They also demonstrated that, in vitro, incubation of a solution of RBCs with neuraminidase resulted, as expected, in desialylation and induced a more spherical RBC shape and lower deformability. In their in vitro experiments, they were able to reproduce, in the RBCs of septic patients admitted in ICU, through desialylation, the changes they had observed (20). In our study, when adding neuraminidase to a solution of RBCs from volunteers in whom preconditioning had a positive effect on deformability, we did not observe any significant loss in the effectiveness of preconditioning. These results suggested that the RBC membrane content of sialic acid did not participate in the effects of preconditioning.

However, the improvement in RBC deformability following preconditioning in certain volunteers disappeared at certain shear stress levels when glutaraldehyde was added to the solution, because it rigidifies the membrane. This observation leads us to suppose that the beneficial effect of preconditioning is—at least in part—linked to the membrane's elastic properties.

The RBC membrane is made up of carbohydrates, a lipid bilayer with its many transmembrane proteins, and the cytoskeleton, a structural network of proteins. The interactions between these various components determine membrane elasticity. In sepsis, these components, their interactions, and their biochemistry are altered. Piagnerelli et al (21) observed identical membrane protein contents in septic and nonseptic patients. Nevertheless, in a mice model of sepsis induced by cecal ligation and puncture, Condon et al (22) showed augmented Band-3 tyrosine phosphorylation which led to alterations of Band-3 membrane organization. These biochemistry modifications were contributing to alterations on RBC deformability. NO may prevent these modifications (23). However, observations on RBC rheology from animal models must be interpreted with caution. Indeed, RBC biochemistry, membrane, shape, and thus rheology are completely different from humans (24). By contrast, Kempe et al (25) observed increased fixation of annexin V on the membrane of RBCs from healthy volunteers after incubation in plasma from patients with sepsis, suggesting increased phosphatidylserine exposure from the lipid bilayer's inner leaflet to its outer leaflet. In turn, Baskurt et al (26) observed that RBC exposure to free radicals produced by WBCs led to a decrease in deformability.

Different cell mechanisms may also be responsible for the beneficial effect of preconditioning among the healthy volunteers. Extracellular ATP improves RBC deformability via type P₂Y erythrocyte membrane receptors (8). RBCs exposed to shear stress release ATP molecules via an intracellular signaling pathway involving in particular adenylate cyclase, protein kinase A, Cystic Fibrosis Transmembrane conductance Regulator, and the pannexin-1 channels (27, 28). Ulker et al (11) noted that NO was secreted by RBCs on exposure to shear stress. Although inducing local vasodilation, NO also plays a role in improving RBC deformability, and it is possible that preconditioning may improve RBC deformability via this mechanism. RBC deformability is reduced by oxidative stress. However, in RBCs previously exposed to free radicals, preconditioning still improves cellular deformability. Kuck et al (29) hypothesized that RBC-NOS phosphorylation was restored following preconditioning, so improved RBC deformability following preconditioning could also—at least in part—be explained by these mechanisms, although this requires further studies.

Our study had several limitations. First, we had a limited number of subjects in our three groups. We cannot exclude the possibility of confounding factors such as age and anemia because the small number of subjects does not permit multivariate analysis. However, these factors do not seem relevant in other studies on deformability. These results thus need to be confirmed in a larger population. Furthermore, we included patients during their “ICU stay,” in contrast to the study of Reggiori et al (19) in which patients were included “at admission.” This could in part explain our different results with regard to deformability. A kinetic study of the changes in RBC deformability over the course of sepsis could also be interesting. Second, the statistically significant results obtained after preconditioning among the healthy volunteers only occurred at two shear stress levels, and a wide coefficient of variation exists in measurements at these low levels. However, these errors are valid for all the populations studied and thus should not influence our results. Third, we were mainly interested in studying the membrane in order to explain the loss in the effectiveness of preconditioning, but changes in intraerythrocyte metabolism (such as changes in glycolysis) may also play a role (16). A last limitation of our study is the ex vivo process and not all interactions between the components of the microcirculation are represented such as the important role of the glycocalyx on the deformability of RBCs (30).

CONCLUSIONS

RBC deformability is depressed among septic patients. Preconditioning has deleterious effects on RBC deformability in septic patients, in contrast to what is observed among healthy volunteers where the response to preconditioning was heterogeneous. The effect of preconditioning may be linked to the elasticity of the cell membrane, as seen when it is inhibited by glutaraldehyde. Other mechanisms involving NO and glycolysis may also be involved, although this requires further investigation. A better understanding of RBC rheology alterations in sepsis and their effects on oxygen transport may lead to new therapeutic strategies to reduce organ failure in sepsis and septic shock.

1 Department of Intensive Care, Erasme hospital, Université Libre de Bruxelles (ULB), Brussels, Belgium.

2 Experimental Medicine Laboratory, CHU-Charleroi Vésale, ULB 222 Unit, 6110-Montigny-le-Tilleul, Belgium.

3 *Intensive Care, CHU-Charleroi Marie-Curie, Université Libre de Bruxelles, 6042-Charleroi, Belgium.*

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For information regarding this article, E-mail: Anthony.moreau@erasme.ulb.ac.be

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