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REVIEW

Understanding red blood cell rheology in sepsis and its role in clinical practice. From biomolecular aspects to possible therapeutic interventions

Emanuel Moisă[1], Silvius Negoiţă[1,2], Dan Corneci[1,2]
[1] *Anaesthesiology and Critical Care Department, Elias Clinical Emergency Hospital, Bucharest, Romania*
[2] *Carol Davila University of Medicine and Pharmacy, Bucharest, Romania*

Correspondence to:
Emanuel Moisă, MD, Department of Anaesthesia and Critical Care, Elias Clinical Emergency Hospital, 17 Marasti Bd, Bucharest, Romania
E-mail: emanuelmoisa@gmail.com

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Abstract

Erythrocyte rheology is of interest in understanding microcirculation and oxygen delivery and consumption alterations induced by sepsis and septic shock. Several mechanisms are proposed: (i) direct or indirect RBC membrane alterations, (ii) abnormal intraerythrocytic homeostasis, (iii) RBCs interaction with other cells and extracellular molecules, (iiii) increased reactive species production and altered redox homeostasis. In this review, we describe in part these mechanisms and what's the impact of these hemorheological disturbances on the outcome and mortality rate. Also, we outline the possible therapeutic interventions and further perspectives regarding sepsis and septic shock management.

Introduction

In 2016 The Third International Consensus for Sepsis and Septic shock defined sepsis as "a life-threatening organ dysfunction caused by a dysregulated host response to infection" [1]. The concept of severe sepsis is not actual anymore [1], but in this review we also collected data about red blood cell rheology in context of severe sepsis (as it was defined by the previous definitions in 2001) [2]. Sepsis and septic shock still represent the most common cause of death

and critical illness worldwide [1]. Pathophysiology of sepsis is complex and involves alterations particularly observed in microcirculation and blood constituents homeostasis [1]. Of interest in this review is the way sepsis alters erythrocyte rheology and function, the actual possible therapeutic tools and future perspectives regarding the treatment of these alterations.

Basic principles of hemorheology. Sepsis and septic shock induced altered red blood cell (RBC) rheology

Hemorheology is a branch of fluid dynamics studying and describing flow and deformation behaviour of blood and its cells (i.e., erythrocytes, leukocytes, platelets) [3]. Blood rheology still represents an area of interest, after decades of studies and, yet, there are still many aspects that need to be elucidated, like aggregation's (one major determinant of erythrocyte rheology) implications in pathophysiology of several diseases [4, 5]. But, in this review, we will focus on RBC rheology and how sepsis and septic shock influences it.

Blood viscosity

Blood is a non-Newtonian two-phase fluid. Plasma represents the suspending phase for blood cells and is considered a Newtonian fluid, thus alterations of its viscosity will lead to changes in whole blood viscosity independent of blood cells properties (changed or not) [3,6]. Plasma viscosity can be increased in acute phase of inflamatory diseases (increased mostly by plasma proteins, like fibrinogen and other acute phase reactants) and by extension, in sepsis [3,7].

Added to plasma, blood cells make whole blood viscosity higher than plasma viscosity and this has implications in blood flow behaviour (fluid transition from Newtonian to non-Newtonian) [3] . Viscosity can be expressed as the ratio between shear stress and shear rate [8]. In terms of viscosity, shear rate determines RBC behaviour, at high rates causing RBC dispersion and preventing aggregation, and at low rates incresaing their chance to aggregate [8]. Microvascular stasis (low flow state) in sepsis may lead to low shear rates and microthrombosis (capillary-stopped flow) [8,9].

Hematocrit can seriously alter blood viscosity as seen in hyperviscosity syndromes which can associate severely decreased tissue perfusion pressures [10]. On the other hand, a low hematocrit (anemia) leads to kyperdynamic circulation [11]. In sepsis, development of a hyperviscosity syndrome is controversial and less probable [7]. Systemic inflamatory response syndrome (SIRS) is frequently associated with increased acute phase reactants which, in theory, leads to increased plasma viscosity [3], sepsis and septic shock associate seriously altered microcirculation (vasodialation) [1] and by extension, a hyperdinamic status, and also, in time, a progressive anemia [1,7,12] which, in turn, reduces blood viscosity [3,8]. Blood viscosity in sepsis can maybe be characterized by an increased variability [7] caused first and foremost by the high volumes of fluids following aggressive volemic resuscitation [12]. Given this, a hyperviscosity syndrome is unlikely to develop and or it can be at least, prevented [13]. Hematocrit is the only hemorheological parameter included in the Acute Physiology and Chronic Health Evaluation (APACHE II) predictive score [14]. In sepsis, hematocrit value can be falsely increased with anemia [12], and this can modify studies results in blood rheology and also lead to false lower APACHE II scores in ICU patients assesment. Moreover, blood viscosity can be influenced by blood transfusions in sepsis [7]. Finally, knowing that dialysis increases blood viscosity and alters RBC rheology [15,16] patients who require renal replacement therapy may present further altered hemorheology. *Piagnerelli et al* concluded in their RBC rheology in sepsis review that blood viscosity is influenced mainly by plasma visosity, shear rate, deformability and aggregation [8].

Aggregation

Capillary-stopped flow is specific for sepsis [9], and RBC rheologic changes like increased aggregability and adhesion are incriminated as possible mechanisms [8,9,17]. Of course, this phenomenon is much complex and must be integrated in the overall pathophysiological mechanisms of sepsis.

Aggregation can be described as the equillibrum between aggregating and disaggregating forces [3]. The dissaggregating forces (electrostatic repulsion between cells, RBC membrane elastic energy and fluid share forces) are dependent of RBC integrity and describe the concept of RBC aggregability, while aggregation is determined by large macromolecules (i.e., fibrinogen) [3,4,6,18]. The way this imbalance between aggregating and disaggregating forces may appear and how it can be ameliorated will be discussed later in this review.

Deformability

The normal erythrocyte is a biconcave disc shaped-like anucleated cell, approximately

7 μm in diameter and 2 μm thick [9]. Its primary function (considered the only one for a long time) is to transport oxygen from lungs to the tissues, but in the past years, erythrocyte was described not only as an oxygen carrier, but also as an oxygen sensor [19,20]. Of course, with recent findings there is much more we know about RBCs function and it will be described in this review. Deformability represents erythrocytes ability to deform when exposed to blood flow and this is of utter importance for physiologic cappilary circulation, where RBCs can undergo extreme deformability in order to pass and maintain homeostasis [8,9,21,22]. RBC deformability depends on (i) membrane surface area-to-volume ratio also known as erythrocyte geometry, (ii) elements forming cytoskeletal proteins (spectrin, ankyrin, band 3 protein domain, 4.1 protein and 4.2 protein) affecting RBC membrane mechanical features and RBC shape and (iii) intraerythrocytic water and ion homeostasis. [21,22,23,24]. Red blood cell deformability was recently reviewed by Huisjes et al [21].

RBCs deformability can be an early marker of sepsis [7,25,26,27] and also a marker of severity [28]. Furthermore, altered deformability can be a prognostic factor being associated with outcome [29] and progression to organ dysfunction [30]. Finally, less probable, it can be an element of differential diagnosis between septic and non-septic critically ill patients [7,31]. RBC deformability is altered through several proposed mechanisms we will discuss later in this review along with how therapeutic interventions ameliorated deformability and changed outcome. Erythrocytes increased sphericity, variable volume and deformability alterations are factors which may alter red blood cells distribution width (RDW, a basic paraclinical tool studied routinely with every complete blood count laboratory examination) along with decreased hematocrit and hemoglobin [9,11]. In fact, RDW is a measure of RBCs grade of anisocytosis [9,11], and it was found to be changed in septic patients by several studies [?]. Increased RDW was associated with poor immediate [32,33] and short term prognosis: increased 28-day [34,35], 30-day [36] and 90-day mortality [34]. It it also considered, that RDW value can be helpful in differentiating the severity of sepsis [33,37] and is associated with MDA and TNF-alfa levels during the first week of sepsis [33].

Mechanisms explaining RBC rheology in sepsis Erythrocyte membrane alterations in sepsis RBC membranc sialic acid (SA) content and bacterial compounds action on RBC membrane

The way RBC content of sialic acid in septic patients changes RBC shape was described by Piagnerelli et al. including in their studies non-septic critically ill patients, septic patients and healthy volunteers as control group [38,39]. Piagnerelli et al aim was to observe if there is any relationship between RBC membrane sialic acid content and RBC shape using flow cytometry. They reported significantly lower values of SA content in septic patients compared with non-septic patients and healthy volunteers, and this low values have been correlated with RBC shape; RBCs were more spherical in septic patients and also had a decreased capacity of sphericity in hypo-osmolar solution. [38,39]. Just recently, Qadri et al prooved that artificial desyalation of RBCs with Clostridium perfringens derived neuraminidase induced increased phosphatidylserine exposure and increased intraerythrocyitic calcium levels [40]. Changes in RBC shape due to sialic acid content and sialidase activity was also described in patients with diabetes. Diabetic patients presented lower quantities of sialic acid and an increased sialidase activity [41,42] and also, a higher percent of senescent erythrocytes [42]. RBCs in diabetic patients present a lower deformability [42] and increased aggregability [41]. Moutzouri et al [43] reported that sepsis and diabetes have an additive effect on red blood cell deformability with significantly higher rigidity indices in patients with diabetes and sepsis compared with septic but not diabetic patients, diabetic but not spetic patients and healthy volunteers [43].

Another proposed mechanism was a direct effect of bacterial virulence factors on RBC membrane sialic acid content. [8]. Milligan et al [44] and later, Mattingly et al [45], reported that serotype III isolates of group B Streptococci from infants having the disease produced more extracellular neuraminidase than serotype III isolates of group B Streptococci in those colonized and asymptomatically. Neuraminidase production was not an absolute condition for infants to develop the disease accordingly to Mattingly et al [45]. These findings are of big importance in understanding the pathologic molecular mechanisms given the fact that sialic acid is attached to A glycophorin (the most abundant sialoglycoprotein on erythrocytes) and is also a monosaccharide derivative of

neuraminic acid which makes it susceptible to glycoside hydrolysis action of neuraminidase, which leads to a reduction of membrane surface negative charge [9]. As described above, smaller RBC sialic acid content leads to a decreased deformability [38,39]. A low negative charge in membrane surface will also reduce the repelling forces between RBCs and vascular endothelium, therefore increasing their susceptibility to aggregation [3,4,6]. Liukkonen et al [46] studied Streptococcus suis (a bacterial pathogen causing severe infections in young piglets and meningitis in humans) erythrocyte binding specificity of sialic acid. In presence of sialidase, hemagglutination of erythrocytes exposed to Streptococcus suis was abolished [46]. It was observed that resialytation of desialytaed RBC with beta 1-3(4)GlcNAc alpha 2-3-sialytransferase caused a strong hemagglutination. Also, they found that Streptococcus suis binds to Band 3, Band 4.5 proteins and glycophorin A. [46] Parkkinen et al [47] findings about S-frimbriated Escherichia coli effects on erythrocytes are comparable with Liukkonen et al, reporting Escherichia coli's specificity of binding to a proteic sequence of the O-linked oligosaccharide of glycophorin A; hemagglutination of bacteria was abolished when erythrocytes were treated with neuraminidase and trypsin [47].

Other bacteria have the capacity of producing neuraminidase: Pseudomonas aeruginosa [48], Clostridium perfringens [49], Streptococcus pneumoniae [50], Vibrio cholerae [51], leading to rheological changes in RBCs. Piagnerelli et al studied neuraminidase activity in non-septic critically ill ICU patients and septic patients, reporting increased levels of free sialic acid in plasma and increased blood neuraminidase activity [38,49]. RBCs incubation with Clostridium perfringens neuraminidase using different concentrations led to shape alterations of RBCs that became more spherical and this change was correlated with higher sialic acid hydrolysis rates [49]. Also, authors reported increased RBC levels of 2,3-DPG known to alter RBCs shape [52,53] and increased RBC levels of lactate, but it was not possible to determine if these shape changes were due to increased 2,3-DPG or the spherical shape determined an abnormal RBC glycolysis leading to increased 2,3-DPG levels (2,3-DPG was measured after at least 10 hours of incubation and RBCs shape was already changed) [49].

The importance of RBC sialoglycoproteins in sepsis can be correlated with Lizcano et al recent findings about erythorcytes importance in suppresing neutrophil activation and apoptosis in blood [54]. The authors reported that sialoglycoproteins from RBC surface are important in neutrophil suppresion through Siglec-9 (a receptor known to recognize sialic acid and to decrease innate imune cell activation), both ex vivo and in vitro [54].

Phosphatidylserine redistribution on the outer RBC membrane leaflet is associated with increased eryptosis and erythrocyte clearence in sepsis

Dinkla et al [55] observed that once SIRS is developed in experimental endotoxinemia (LPS administered IV), membrane lipid remodeling appears in both human and animal experiments, leading to increased phosphatidylserine exposure. An interesting observation was thatin experimental endotoxinemia are not observed the exactly same effects as seen in sepsis [55]. In sepsis, bacterial virulence factors, others than neuraminidase were associated with membrane externalization which is intercepted as an erythrocyte senescence signal in the reticuloendothelial system leading to increased eryptosis and RBC clearence, explaining, in part, sepsis induced anemia [56]. Different bacterial compounds like pyocianin [48], α-hemolysin [57], listeriolysin [58], peptydoglicans [59], lipopetides [60] and sphyngomyelinase [61] were associated with membrane externalization through exposure and redistribution of phosphatidylserine on the outer RBC membrane leaflet. Eryptotic RBCs adhesion is increased by bacterial peptidoglycans and it seems to follow a CXCL16-dependent fashion as it was reported by Abed et al [62]. Qadri et al exposed in vitro human RBCs to the virulence factor pyocianin released by Pseudomonas aeruginosa and reported altered intraerythrocytic homeostasis of water (dehydration with cell shrinkage) and calcium (increased RBC cytosolic calcium), increased RBC reactive oxygen species (ROS) concentration and increased phosphatidylserine redistribution on the outer RBC membrane [48]. Pyocianin is redox-active metabolite capable of producing ROS and also, can inhibit catalase, which, in turn can lead to increased hydrogen peroxide levels [9] (it's effects are described later in the sepsis induced oxidative stress chapter).

Phosphatidylserine redistribution is influenced by other factors too, like altered intraerythrocytic calcium homeostasis and sepsis induced oxidative stress [9,48,63,64,65,66,67]. Calcium is important in regulating RBC membrane phosphatidylserine distribution [63] and calcium homeostasis is profoundly disturbed in sepsis [64,65]. Oxidative stress imbalances

have an important contribution on membrane damage too, through lipid peroxidation and protein oxidation which determines increased RBCs inner leaflet membrane exposure to the extracellular space [9,48,66,67]. Eryptosis is also associated with increased ceramide formation in sepsis [68].

Sepsis induces RBC membrane pro teins alterations

Band 3 protein phosphorylation

Phosphorylation of band 3 protein is of importance in sepsis because it plays a significant role in intraerythrocytic metabolic alterations of glycolysis and oxygen delivery, and by extension influences RBC deformability (through several mechanisms) and RBC NO release behaviour [9,69,70,71,72]. Band 3 protein phosphorylation and dephosphorylation was reported in both in vitro [70,71,72] and in vivo [72] studies, using tyrosine kinase inhibitors. Recently, Lin et al in a murine cecal ligation puncture (CLP) sepsis model reported increased cytoplasmic domain of Band 3 protein (cdB3) proteolysis via Western Blot testing. Furthermore, cdB3 proteolysis was higher in mice undergoing severe CLP compared with those undergoing sham CLP [73]. Spolarics et al [74] and Condon et al [72] observed that band 3 phosphorylation was associated not only with reduced RBC deformability but also with altered anion activity.

Band 3 phosphorylation and dephosphorylation depends on several intraerytrocytic alterations: calcium concentration [75], oxidative damage on RBC membrane [76], low oxygen partial pressure leading to increased deoxyhemoglobin levels [77]. These alterations are already described in sepsis and can, in part, explain this phenomenon. Altered calcium homeostasis in erythrocyte leads to phosphotyrosine phosphatase dissociation from band 3 allowing its phophorylation in a reaction catalyzed by the enzyme phophotyrosine kinaze [75]. Band 3 phosphorylation then determines increased cytosol concentration of glyceraldehyde dehydrogenase, phosphofructokinase and aldolase (glycolitic enzymes anteriorly inactive and attached to cdb3) and enhanced glycolysis [75]. Band 3 phosphorylation is also determined by deoxyhemoglobin which through binding to cdb3 enhances glycolisis via the same mechanism described above [77]. Sepsis hypoxic status reduces drastically the hexose monophosphate pathway and further, NADPH concentration, which is vital in producing reductants in order to protect RBCs from oxidative damage [78,79]. Thus, ROS produced from the increased hemoglobin autooxidation processes along with other sources of ROS, in a low oxidative defense state, will lead to increased glycolysis via hydrogen peroxide [76] in the same fashion with calcium and deoxyhemoglobin. Enhanced glycolysis will lead to increased ATP efflux [80]. ATP will bind to the P2Y receptors, inducing NO synthesis, which will further alter RBC deformability [81]. Moreover, glycolytic pathway stimulation, decreased levels of intraerythrocytic reductants (altered NO-glutathione interaction), band 3 phosphorylation and dephosphorylation influences intraerythrocitic NO chemical reactions and modifies its bioavailability and release from RBCs [69]. Finally, band 3 protein phoshphorylation in presence of high fibrinogen levels determines a higher NO release from RBCs [82,83].

Piagnerelli et al suggested that membrane protein content alterations are not correlated with abnormal RBC rheology seen in sepsis [84]. 25 patients were included in study, 15 with sepsis, 10 non-septic critically ill and 10 healthy volunteers as control group. No signifficant differences were observed in protein content between septic and non-septic patients, but in spectrin content between control group and non-septic critically ill patients, with decreased spectrin levels for the latter. The authors reported decreased levels of band 3, band 4.1 and 4.2, ankyrin, spectrin and increased band 3/ spectrin, Protein 4.2/band 3 ratios, in all critically ill patients, but as long as differences between the two groups were not found, the authors suggested their conclusion presented earlier. [84]

Altered intraerythrocytic homeostasis

2,3-diphosphoglycerate (2,3-DPG)

The erythrocyte's fundamental role is oxygen delivery from lungs to the tissues by binding the oxygen molecule to hemoglobin. 2,3- DPG represents one of the most important organic phosphates in the RBC and is produced through the Rapoport-Lubering shuttle – the side path of glycolysis taking place in RBC. 2,3 DPG is an allosteric regulator of hemoglobin and has the role of reducing hemoglobin's affinity for oxygen by forming a reversible complex with deoxyhemoglobin [9,85]. 2,3-DPG synthesis is increased in the presence of hypoxaemia and acidemia resulting in right shifting of the oxyhemoglobin disociation curve (ODC), decreased oxygen affinity to hemoglobin and increased release of oxygen to the tissues (Bohr effect). Studies results may seem paradoxical as a

first face because some of them suggest that the RBC ODC is shifted to the left [73,86,87], while others suggest both effects [85]. Recently, Lin et al, reported on a CLP murine sepsis model, reduced Bohr effect (increased P50 and increased hemoglobin affinity for O_2) at 3 distinct values for pH (7.2, 7.4, 7.6) and reduced RBC deformability. Chillar et al [85] observed that patients with hypoxaemia but without severe acidemia had increased values of 2,3-DPG and ODC was shifted to the right, but in the presence of severe and persistent acidemia and hypoxia, 2,3-DPG synthesis is low, which, in turn, gives an opposite of Bohr effect – ODC is shifted to the left. Ibrahim et al reported that 2,3-DPG concentration was correlated with acidosis [88], so, we can outline that the most important factors influencing 2,3-DPG concentration, ODC shift and P50 rise or fall are the value of blood pH and hypoxia. Also, 2,3-DPG concentration can be low in the context of hypophosphatemia and 2,3-DPG depleted blood transfusions [9]. Having all these results we can conclude that ODC shift is not paradoxical, but it follows a concentration- and time-dependent fashion (Figure 1), depending mostly on pH value and 2,3 DPG concentration. 2,3-DPG concentration is important in understanding the RBC rheology because a high 2,3-DPG concentration can alter RBC membrane mechanical properties [52,53]. Waugh observed that in Schindler et al [89] and Sheetz and Casaly [90] studies of RBC membrane in the context of high 2,3-DPG concentrations was reported a destabilizing effect on erythrocyte membrane and membrane skeleton. Waugh [52] reported that at high 2,3-DPG concentrations and in non-physiologic conditions, there is an increase in membrane viscosity due to an increase in cellular hemoglobin concentration (MCHC), which, in turn leads to a decrease in membrane shear modulus and deformability. Later Suzuki et al. reported the same increase in MCHC, which in turn, led to an increase in membrane viscosity and a decrease in RBC deformability. RBC deformability greatly improved when MCHC was decreased close to normal values, suggesting that RBC reduced deformability is mainly influenced by internal viscosity, but also by membrane viscoelasticity [53]. Piagnerelli et al reported increased RBC 2,3-DPG and lactate concentrations in presence of desialyation after RBCs from septic patients were incubated with different neuraminidase concentrations [49]. RBC shape transitioned from discocyte to spherocyte after at least 10 hours of incubation. Furthermore, it was not possible to determine if the 2,3-DPG high concentrations modified RBC shape, or if RBCs abnormal shape altered glycolisis because 2,3-DPG was measured only after 10 hours of incubation with neuraminidase and past this moment, RBCs shape was already changed [49].

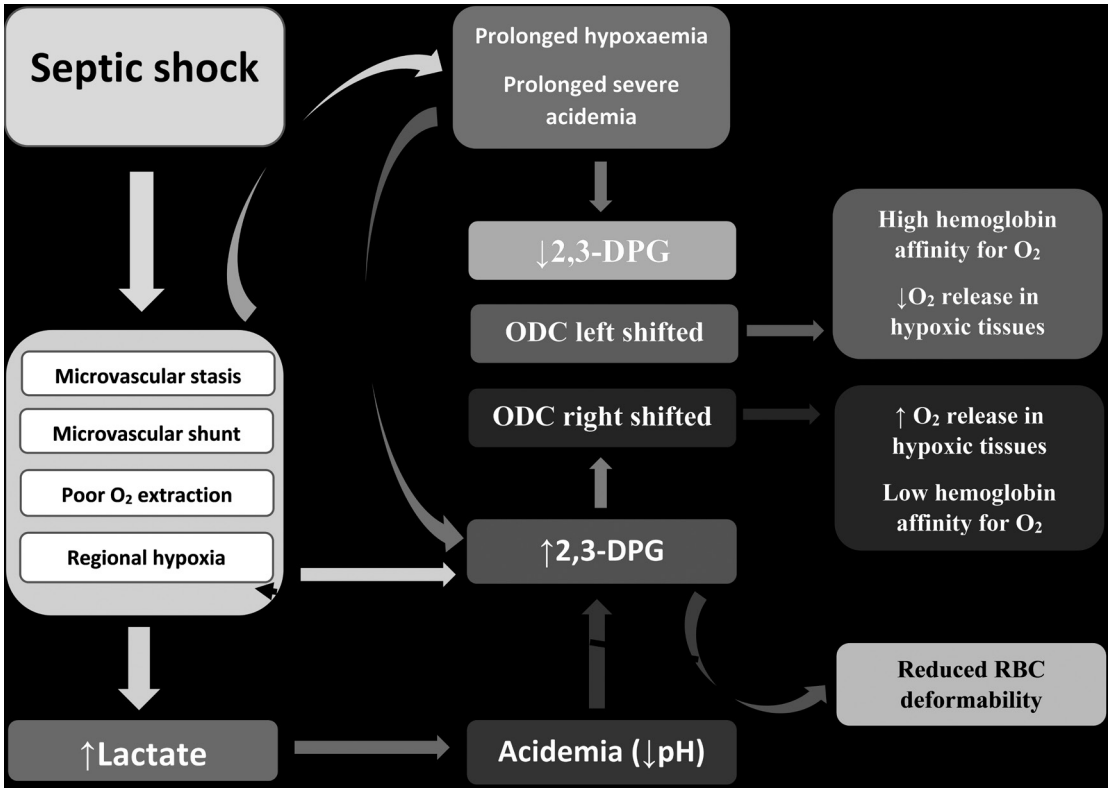

Figure 1. 2,3-DPG and ODC trend in relation with blood pH and oxygen partial pressure

Calcium homeostasis

Calcium homeostasis is profoundly disturbed in septic patients [64]. Totsimon et al reported that hypocalcemia in septic patients can predict mortality [7] and Desai et al found that septic nonsurvivors had lower plasma calcium compared with survivors [65].This alteration is observed both in extracellular [65,91] and intracellular [64,92] calcium physiology. Intracellular calcium has structural (erythrocyte morphology, rheology, adhesion, cellular volume) and biochemical (proteolysis, transglutaminse activity, redox status) effects on RBC [9,63]. Intracellular calcium homeostasis alterations in septic patients were described for liver cells [93], cardiomyocytes [94], lymphocytes [95] and of interest in this review, eryothrocytes [64,92,96,97]. Ruef et al reported increased intraerythrocytic calcium concentration and decreased erythrocyte deformability after RBCs (from healthy donors) were incubated with E. coli lipid A [92]. Erythrocyte deformability was found to be inhibited by administration of verapamil (a calcium channel blocker) and protein kinase inhibitors like staurosporine [92]. Todd and Mollit observed that intracellular calcium homeostasis is altered in vivo (septic patients) and in vitro (whole blood incubated with E. coli endotoxin), respectively [64]. They found that increased intraerythrocytic calcium levels (as free cytosolic calcium measured by fluorescent spectroscopy) is specific for septic patients. These in vivo results were studied also in vitro by incubating whole blood (pretreated with dantrolene, or verapamil, or ATP and posttreated or not with ATP) with E. coli endotoxin in the presence or absence of extracellular calcium [64]. Todd and Mollit reported the same increase in free cytosolic calcium for the in vitro experiment and also found out that intracellular calcium concentration was not influenced by pretreatment with verapamil or dantrolene (a ryanodine receptor blocker known to decrease intracellular calcium), transmembrane calcium channels, intracellular calcium deposits and extracellular calcium concentration [64]. An interesting finding was that pretreatment with ATP led to a smaller increase in intraerythrocytic calcium and this phenomenon may rise the suspicion of an energy deficiency being responsible for the cytosolic calcium increase. Nowadays, RBCs ATP deficit in sepsis is described through many mechanisms we will discuss later and can support Todd and Mollit theory.

WBCs modulation and increased oxidative stress role in RBCs intracelluar calcium homeostasis was reported by Todd and Mollit which incubated anticoagulated whole blood samples from healthy donors with LPS and observed an increase in erythrocyte cytosolic calcium concentration, but this increase was dependent on WBCs presence; in absence of WBCs this change was not reported [98]. WBCs implication in these findings was demonstrated when incubation of whole blood pretreated with xanthine oxidase inhibitors (allopurinol), leukocytes modulators (pentoxifylline) and free radical scavengers (superoxide dismutase) with LPS resulted in smaller increases in cytosolic calcium concentration [98].

Adenosine triphosphate

In conditions of impaired oxygenation as seen in sepsis, erythrocytes are capable of releasing vasoactive molecules like nitric oxide, nitrosothiols and ATP [99] In conditions of hypoxia ATP can be released [100,101] through erythrocyte Pannexin-1 channel [9,102]. The released ATP binds to P2Y receptors and causes vasodialation [9,29,103,104]. Mathie et al reported that nitric oxide hepatic vasodialiton was ATP-induced in rabbits perfused in vitro with Krebs-Bülbring buffer via heaptic artery and portal vein. The study results suggested that ATP binding to P2Y receptors (which are abundant in endothelial cells) induces NO and causes vasodialation in hepatic artery vascular bed. ATP-induced NO release via endothelial cells was reported also by Busse et al [105]. ATP was found to be an important mediator of NO release in isolated perfused rabbit lungs [107,106] and was reported in dogs models too [106]. An interesting and contradictory hypothesis to what was presented until now comes from Serroukh et al [?] suggesting that the decreased functional capillary density can be caused by an ATP-induced vasoconstriction in microvascular bed, secondary to increased ATP release from abnormal erythrocytes, but further investigations are needed to establish this theory.

RBC reduced deformability determines ATP release [27,81,106,109] and, in turn, ATP deposits are low in sepsis [100,101]. Decreased intraerythrocytic ATP impairs PMCA activity leading to an increase in intracellular calcium and decreased RBC deformability [8]. Bateman et al [81] and Rozier et al [110] observed ATP release dependent on RBC oxygenation is impaired in presence reduced pH (in a 6 h rat hypotensive sepsis model) [81] and inhibited, at high lactate concentrations, respectively [110].

Another possible mechanism for increased RBC ATP release in sepsis can be related to pore-forming virulence of some bacteria. Recently, Greve et al, in an in vitro study on knock-out mice, reported pore forming in RBC membrane and immediate RBC ATP re-

lease after α-hemolysin is inserted into RBC membrane via binding to P2X1 receptors [111]. This phenomenon was described in vivo in humans and is associated with P2X7 receptors [112]. Hemolysin E. coli producing strains are observed mostly in patients with severe urinary tract infections and Greve et al speculated that P2X receptor antagonists maybe will be useful in urosepsis [111]. Other bacteria can induce the same cellular changes as described above: Staphylococcus aureus via α-toxin [113] and Clostridium perfringens [114] via β-toxin.

Sepsis induced oxidative stress

Sepsis induced oxidative stress represents an important area of interest nowadays for scientific researchers. Molecular studies regarding oxidative stress were eye-opening in understanding sepsis pathophysiology and a more actual concern for scientists is how we can therapeuticaly intervene to correct all these imbalances [115]. Sepsis mechanisms are complex and multifactorial leading to a vicious circle in which disease creates disease. It's effects are of utter importance in terms of microcirculation disturbances and oxygen physiology, from uptake and delivery in (normal/abnormal) RBCs to (abnormal) utilization and consumption in mithocondrion. Several studies emphasize the oxidative imbalance in sepsis and critically ill patients describing a diminished antioxidant activity [9,115-121] and an increased oxidative status [1,8,9,115-117,121]. Oxygen physiology is altered by transformation of oxygen into reactive oxygen species like superoxide ($O_{2}^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), hypoclorus acid (HOCl) and peroxynitrite ($ONOO^{-}$) [115], oxygen free radicals and oxidizing species [9]. In ROS producing and redox homeostasis alteration are implicated many factors and of interest are: neutrophils [115, 122], endothelial cells [9, 115], the erythrocyte itself [123] and plasma oxidants (nitric oxide, xanthine oxidase, malonyldialdehyde) [9].

For this review, ROS and altered redox homeostasis is important because several studies reported altered RBC deformability [124-135] and increased RBC aggregability [124,135] both related to oxidative imbalance in septic patients. Baskurt et al found that intracellular superoxide anion production is responsible for altered RBC deformability and extracellular superoxide anion increased RBC aggregation, when RBCs were exposed to this ROS [124]. RBC exposure to hydrogen peroxide [125,126,132], determines sepctrin-hemoglobin cross-linking [126,132] decreased deformability [126,132], ekynocytes formation [125,126] and increased RBC adhesion [126], while exposure of RBCs to lipid peroxidation products like MDA determines decreased RBC deformability [67]. ROS influence on band 3 phosphorilation was already discussed earlier in this review. (see band 3 phosphorilation).

Of importance is also RBCs capacity to produce ROS under certain conditions [9]. Erythrocyte autooxidation is determined mainly by H_2O_2 and superoxide and is prevented by catalase and superoxide dismutase (SOD) [136]. The most important factor determining RBC autooxidation is superoxide anion production [123] and this it's production is favoured by high temperature, low pH, increased 2,3-DPG [137,138] and a partial oxygenation of hemoglobin [137]. High temperature as a manifestation of systemic inflamation, hypoxia determined by microcirculation alteration and abnormal oxygen physiology, low pH and in turn, increased 2,3-DPG are frequent in sepsis, making RBCs more susceptible to autooxidation, mainly in areas of capillary stopped-flow where oxygen hemoglobin saturations are lower [81,139].

In sepsis, the antioxidant capacity is overwhelmed by the excessive oxidative species production [115]. This hypothesis is sustained by the studies in which administration of antioxidant molecules in septic patients or critically ill patients improved the outcome [116,121, 128,140-146]. Administration of N-acetylcysteine (NAC) alone was found to transiently improve tissue oxygenation [140,141], increase hepatic blood flow and liver function [142], improve static lung compliannce and lower plasma IL-8 levels [141] and decrease lipid peroxidation with a reduction of organ failure at day 10 [143]. Selenium administration alone was found to reduce mortality rate by 25%, decrease inflamatory response [144] and reduce the incidence of acute kidney injury requiring renal replacement therapy [145]. Gadek et al [147] and Pontes-Arruda et al [148] administered a combination of antioxidant molecules (vitamin c, vitamin E, β-carotene, selenium) enteraly in aproximately same concentrations and number of days (4 days [147] versus 4-7 days [148]). They reported mostly the same results: improved oxygenation, reduced length of ventilatory support and ICU stay, less new organ failure [147,148] and reduced mortality [148]. The pitfall of this combination of antioxidant molecules is the imposibility of associating the benefits with a single or specific molecule, as was reported by Marik et al as well [116] who found that co-administration of vitamin C, thiamine and hydrocortisone

prevented organ dysfunction and decreased mortality.

Moreover, several studies reported an inversely proportional relationship between plasmatic levels of antioxidants and oxidants [117,118,120]. Decreased antioxidants like vitamin E [117,118,120], ascorbate [116,117,118], β-carotene [117,118] and retinol [117] were found in septic patients associating important lipid peroxidation activity (increased MDA [118] or other evidence supporting lipid peroxidation [117]. In support of required proper antioxidant levels and in terms of RBC rheology comes evidence suggesting that suplementation in septic patients with vitamin C improved outcome by preventing organ failure [116], determined inhibition of erythrocyte destruction through eryptosis [146] and vitamin E supplementation decreased mortality rate and ameliorated RBC deformability [128].

Nitric oxide

NO release and effects in sepsis and septic shock were described in several studies [27,81,149-156]. Under physiologic conditions, NO is synthetised in endothelial cells from L-arginine catalyzed by e isoform nitric oxide synthase (eNOS), known also as c isoform (constitutive, cNOS) [157]. Produced in small amounts, NO is vital in capillary tone regulation [8]. Of interest in sepsis, is the i isoforme, which is the inducible NOS (iNOS) found to be stimulated excessively in sepsis by different mediators like LPS, cytokines (IL1, IL6, TNF-α, IFNγ) and ATP [8,56]. Recently, Takatani et al reported that iNOS is associated with immune cell migration and hypothermia in the late phase of sepsis [158]. Pentraxin 3 is known to be overexpressed in sepsis and septic shock [159] and is also thought it can blunt endothelial NO production [160]. Another NOS isoform is the neuronal isoform (nNOS) thought to take part in SIRS pathophysiology [161] and more important, it seems to have serious implications in vascular alterations seen in sepsis [162] Another important aspect is gram-positive bacteria possesion of bacterial isoform NOS (bNOS) which was reported to have multiple roles: protection against oxidative stress, antibiotic resistance and host immune response [163,164].

Hemoglobin and NO interaction is important in sepsis. NO affinity for hemoglobin is high [99] (increased affinity for thiol groups or for the iron ion), but NO binding at Hb is dependent on oxygen partial pressure [69, 99]. At arterial oxygen partial pressure (high-pressure, PaO2) NO and oxy-Hb form S-nitrosohemoglobin (SNO-HBO2) through NO binding at the thiol group of cysteine β93 [69], but at low oxygen partial pressures (as it happens in sepsis), NO interacts with the iron ion binding to it and resulting in nitrosylhemoglobin molecules formation [165]. In 1996, Jubelin et al [166] suggested that erythrocytes can be able to produce NO. In 2004 Carvalho et al [167] demonstrated in vitro NO erythrocyte production using the amperometric method. One year later, Kleinbongard et al reported that RBCs posses a functional eNOS [168]. Recently, Lin et al in a murine CLP sepsis model, reported decreased levels of RBC NO content (total RBC NO, FeNO, and SNO content) [73]. RBC NO content was measured using chemiluminescene and was signifficantly decreased, with lower values in severe CLP mice versus sham CLP mice [73]. These results can be correlated with Herdade et al observations on NO efflux behaviour related to acute inflamatory phase [169]. The authors created an in vivo mice model of acute inflamation by injecting platelet-activator factor (PAF) in mice scrotum and reported an inversely proportional relationship between NO efflux and development of acute-phase response, with decreasing rates of NO efflux as the inflamatory response was developed and approximately normal NO values in the recovery phase. An important observation authors made was that RBC deformability was not completely recovered and this can be, in part, attributed to PAF's ability to breakdown RBCs sphingomyelin [169].

In sepsis, RBC NO and nitrosothiols production and release can be increased via band 3 phosphorylation and NO interaction with reactive species resulted from hemoglobin autooxidation [69,99]. These mechanisms were described in more detail earlier (see Band 3 phosphorylation). Simplyfied, it's important to keep in mind that NO is released from RBCs in conditions of hypoxia, which in sepsis has a significant impact on erythrocyte rheology and microcirculation [81].

Figure 2 summarizes many of the mechanisms presented in this review.

Abbreviations: superoxide dismutase (SOD), catalase (CAT), peroxiredoxin (Prx), glutathione peroxidase, vitamin C, E, A (vit. C, E, A), NADPH O (NADPH oxidase), xanthine oxidase (XO), reactive oxygen species (ROS), superoxide ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2), hypoclorus acid (HOCl) and peroxynitrite ($ONOO^-$), interleukin-1, -6 (IL-1, -6), lipopolysaccharides (LPS), nitric oxide (NO), inducible nitric oxide synthetase (iNOS), adenosine triphosphate (ATP), white blood cell (WBC), cytoplasmic domain of band 3 (cdB3),

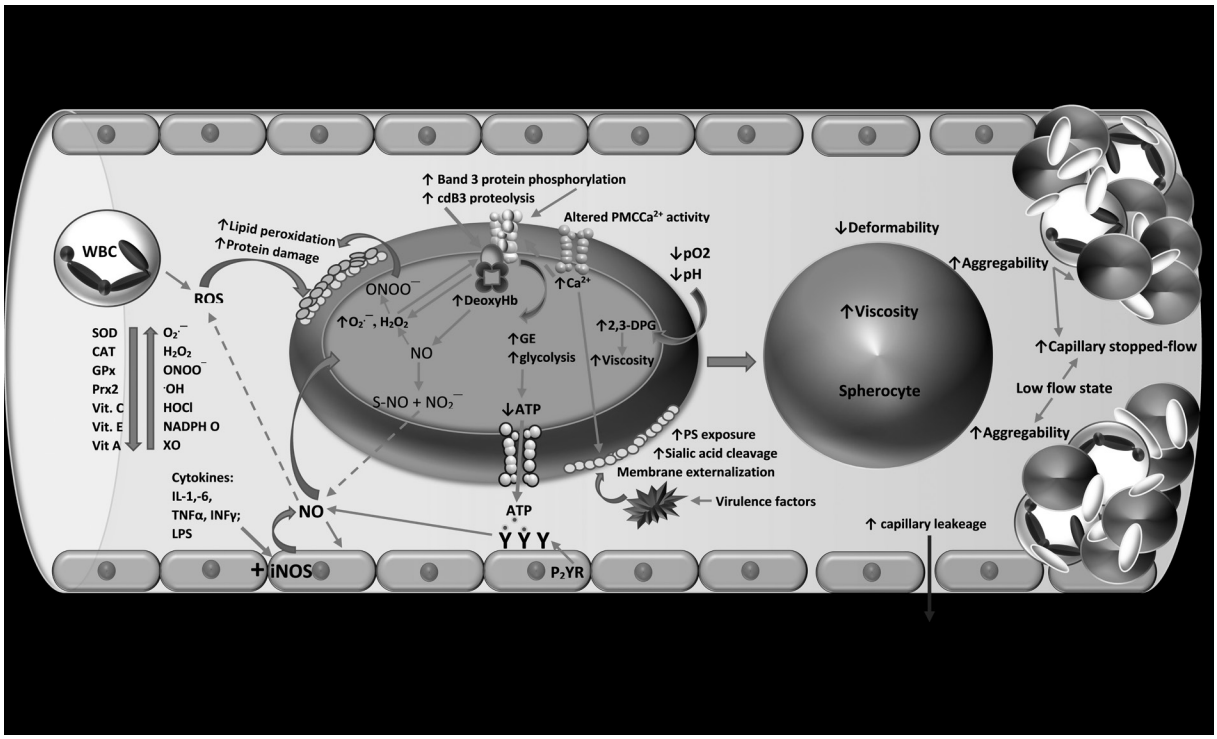

Figure 2. Proposed mechanisms explaining RBC rheology in sepsis and septic shock.

phosphatidylserine (PS), purinergic receptors (P_2YR), glycolytic enzimes (GE), 2,3-diphosphoglycerate (2,3-DPG), oxygen partial pressure (pO_2), tumor necrosis factor-α (TNF-α), interferon-γ (IFN- γ), deoxygenated haemoglobin (DeoxyHb), plasma membrane calcium channel ($PMCCa^{2+}$).

NO role in RBC deformability seen in sepsis was reported in several studies [22,27,69,81,153,169-173]. Petrov et al [174,175] reported presence of soluble guanylate cyclase inside erythrocytes and it is known that NO action is modulated by guanylate cyclase (sGC). Also, it was observed that guanylate cyclase inhibitors like methylene blue and ODQ (1H-[1,2,4]oxadizolo-[4,3-a]quinoxalin-1-one) altered RBC deformability and was reversed by NO donors suggesting, sGC is not the only mechanism involved in NO induced RBC deformability. Moreover, NO and NO metabolites [176-178] alters ion transport across erythrocyte membrane via Na+/H+ exchange [174,175], Na+/K+-ATP-ase [179], K-Cl cotransport [177,178] Ca2+-ATP-ase [180], leading to changes in RBC cytoplasm viscosity [22] and band 3 protein activity [69, 72]. Further studies are needed to completely understand this mechanisms.

Pharmacologic interventions regarding modulation of NO activity in sepsis is of great concern nowadays. In 1993, Korbut and Gryglewski [181], reported decreased RBC (from rabbit) deformability in presence of L-NAME (N-nitro-L-arginine methylester, a NO synthase inhibitor), but increased in the presence of NO donors like sodium nitroprusside and sydonimine (molsidomine metabolite). Bor-Kucukatay et al studied the effects of NOS inhibitors and NO donors on RBC deformability. The authors reported that the nonspecific NOS inhibitors L-NAME and SMT can significantly impair RBC deformability but they observed that this phenomenon can be reversed using NO donors like SNP (sodium nitroprusside) and DETA-NONOate (diethylenetriamine) [22]. Bateman et al in a peritonitis sepsis rat model demonstrated that iNOS inhibitors like aminoguanidine can prevent increased RBC NO levels and reduced deformability [27]. Falkmarken et al studied L-NAME, L-canavanine (selective iNOS inhibitor) and bosentan (endothelin receptor antagonist) effects on hemorheological parameters in endotoxemic rats [173]. The authors found that L-NAME and L-canavanine ameliorated the effects of endotoxin on RBC aggregation, but this didn't happened with bosentan [173]. The effects, others than those on RBC rheology, of NO oxide production inhibition (using methyleneblue, L-NAME and L-NMMA) in sepsis were reported in several studies: increased mean arterial pressure [182-184], increased [Preiser] or decreased [Schneider] output, increased vascular tone [182, 185, 186] , decreased cardiac index and increased resolution of shock [185, 186] but, also, an increase in mortality rate was reported [182,187]. The increased mortality rate was the reason a promising phase III trial was stopped. NO donors, like nitroglycerin, effects

in sepsis are not completly elucidated [188]. Increases in mortality rate were reported, or no effects at all on microcirculation [189], but, in one study authors reported an improvement in microcirculation when nitroglycerin was administered after fluid resuscitation [190]. Actually, nitric oxide pharmacologic interventions are not included in guidelines because there is no strong evidence supporting the use of any of the described agents [12].

Conclusions

The erythrocyte is not seen anymore only as an oxygen carrier, but also as an oxygen sensor and vasoactive molecules producer, playing a key-role in microcirculation alterations seen in sepsis and septic shock. RBCs deformability and aggregation can be influenced through several mechanisms that still need to be completely established, but many of them seem to be interconnected, to augment or inhibit one another, having different trends. In the future, RBCs maybe will be possible targets in sepsis and septic shock management, but further studies are required in order to improve the unfavorable outcome and mortality rate seen in these patients.

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