OSMOTIC STABILITY OF HUMAN ERYTHROCYTES IN SEPSIS AND/OR SEPTIC SHOCK

ESTABILIDADE OSMÓTICA DE ERITRÓCITOS HUMANOS NA SEPSE E/OU CHOQUE SÉPTICO

Alice Vieira da Costa[1]
Mario da Silva Garrote Filho[1]
Patrícia Terra Alves[2]
Lucas Moreira Cunha[1]
Rodney Coelho da Paixão[1]
Wener Barbosa-Resende[1]
Thúlio Marquez Cunha[3]
Luiz Ricardo Goulart Filho[2]
Nilson Penha-Silva[1*]

RESUMO: A sepse é uma síndrome caracterizada pela exacerbação das respostas metabólicas e imunológicas a uma infecção e o aumento na distribuição de volume das células vermelhas do sangue (*RDW*), pode ser um marcador de mortalidade em pacientes com sepse e choque séptico. **Objetivo:** Avaliar a estabilidade osmótica de eritrócitos em indivíduos com sepse ou choque séptico. **Métodos:** Noventa e nove homens, 49 indivíduos saudáveis, 25 com sepse e 25 com choque séptico, participaram deste estudo. A estabilidade osmótica da membrana eritrocitária foi caracterizada pela concentração de NaCl em que a lise hiposmótica se inicia (H_0) e atinge 50% (H_{50}) e 100% (H_{100}) da população eritrocitária, e pela variação da concentração salina entre H_0 e H_{100} (dX). **Resultados:** Valores mais baixos de H_0, H_{50} e H_{100}, o que significa maior estabilidade osmótica dos eritrócitos; e menores níveis de hemoglobina, contagem de eritrócitos e valores de hematócrito, hemoglobina corpuscular média e/ou concentração de hemoglobina corpuscular média, indicando tendência à anemia ou anemia; e valores mais elevados de *RDW*, foram encontrados na sepse e no choque séptico. Foram encontradas associações positivas entre variáveis de estabilidade eritrocitária, anemia e inflamação, e entre valores de *RDW* e risco de morte. **Conclusão:** A maior estabilidade osmótica dos eritrócitos nesta população foi associada à anemia e inflamação, e valores mais elevados de *RDW* foram associados a maior chance de morte.

Palavras-chave: Glóbulos vermelhos; Índices eritrocitários; Membrana celular; Inflamação.

[1]Biophysicochemistry Laboratory, Institute of Biotechnology, Federal University of Uberlândia, Brazil
[2]Nanobiotechnology Laboratory, Institute of Biotechnology, Federal University of Uberlândia, Brazil
[3]Pulmonary Function Laboratory, Health Sciences, Federal University of Uberlândia, Brazil
[*]Corresponding author: nspenha@gmail.com

ABSTRACT: Sepsis is a syndrome characterized by exacerbation of metabolic and immune responses to an infection and the increase in red-cell distribution width (RDW), could be a marker of mortality in patients with sepsis and septic shock. **Objective:** To evaluate the osmotic stability of erythrocytes in subjects with sepsis or septic shock. **Methods**: Ninety-nine men, 49 healthy subjects, 25 with sepsis and 25 with septic shock, participated in this study. The osmotic stability of the erythrocyte membrane was characterized by the concentration of NaCl in which hyposmotic lysis begins (H_0) and reaches 50% (H_{50}) and 100% (H_{100}) of the erythrocyte population, and by the variation in saline concentration between H_0 and H_{100} (dX). **Results**: Lower values of H_0, H_{50} and H_{100}, which means greater osmotic stability of erythrocytes; and lower levels of hemoglobin, erythrocyte counts and values of hematocrit, mean corpuscular hemoglobin and/or mean corpuscular hemoglobin concentration, indicating a tendency to anemia or anemia; and higher values of RDW, were found in sepsis and septic shock. Positive associations were found between variables of erythrocyte stability, anemia and inflammation, and between RDW values and death risk. **Conclusion**: The higher osmotic stability of erythrocytes in this population was associated with anemia and inflammation, and higher RDW values were associated with a greater chance of death.

Keywords: Red blood cells; Erythrocyte indices; Cell membrane; Inflammation.

1 INTRODUCTION

Sepsis is a syndrome characterized by exacerbation of metabolic and immune responses to an infection that affects the functioning of various organs. The septic profile has high morbidity and mortality in the Intensive Care Units, mainly in developing countries (BONE; BALK; CERRA; DELLINGER *et al.*, 1992; SINGER; DEUTSCHMAN; SEYMOUR; SHANKAR-HARI *et al.*, 2016). Around 5.3 million people in the world die each year from sepsis; in Brazil this number would correspond to 233,409 adult patients (FLEISCHMANN; SCHERAG; ADHIKARI; HARTOG *et al.*, 2016; MACHADO; ASSUNÇÃO; CAVALCANTI; JAPIASSÚ *et al.*, 2016).

During the sepsis the body recruits immune mediators and defense cells, such as lymphocytes and monocytes, and stimulates the production of cytokines to fight inflammation. These changes alter vascular permeability, increase the levels of reactive oxygen species and modify the deformability capacity of erythrocytes (BATEMAN; SHARPE; SINGER; ELLIS, 2017; DONADELLO; PIAGNERELLI; REGGIORI; GOTTIN *et al.*, 2015).

In sepsis, erythrocytes are exposed to different microenvironments. Changes in the shape, decrease of 2,3-bisphosphoglycerate and increased affinity of hemoglobin for oxygen affect the function of erythrocytes. Another observed change is that patients with sepsis present an increase in hemolysis, with a consequent increase in free hemoglobin, and this has been associated with organic dysfunction and increased mortality

(BATEMAN; SHARPE; SINGER; ELLIS, 2017; BATEMAN; WALLEY, 2005; JANZ; BASTARACHE; PETERSON; SILLS *et al.*, 2013).

An important hematologic parameter associated with the prognosis of sepsis is the red cell distribution width (RDW). Several studies have shown that the increase in RDW could be a marker of mortality in patients with a variety of conditions, such as sepsis and septic shock (JO; KIM; LEE; KANG *et al.*, 2013; MARTIN; DESAI; NANAVATI; COLAH *et al.*, 2018).

The aim of this study was to evaluate the osmotic stability of erythrocytes in sepsis and septic shock, and its possible associations with hematimetric and biochemical blood variables to better understand the effects of sepsis on red blood cells.

2 MATERIAL AND METHODS

2.1 POPULATION

The study plan was approved by the Local Ethics Committee on Human Research (nº 153.331/2012). Blood samples were obtained after 48 hours after the diagnosis of sepsis or septic shock in tubes containing K_3EDTA (for hematologic analysis and determination of erythrocyte stability) and in tubes without anticoagulant (for biochemical analysis) (Vacutainer; Becton Dickinson, Juiz de Fora, MG, Brazil). These collections were carried out in patients admitted to the Clinical Hospital of the Federal University of Uberlândia, between 2015 and 2016. The study included 49 healthy volunteers (control group), 25 patients with sepsis (sepsis group) and 25 patients with septic shock (septic shock group).

2.2 DETERMINATION OF THE OSMOTIC STABILITY OF HUMAN ERYTHROCYTES

The determination of the osmotic stability of erythrocytes was performed as described by Penha-Silva and colleagues (PENHA-SILVA; FIRMINO; DE FREITAS REIS; DA COSTA HUSS *et al.*, 2007). Initially, duplicate sets of test tubes containing 1 mL of 0.1-1.5 g/dL NaCl solutions (Labsynth, Diadema, SP, Brazil) were preincubated at 37 °C for 10 min. After addition of 10 μL of whole blood and gentle shaking, the tubes were incubated at 37 °C for 30 min. After centrifugation at 1500 x *g* for 10 min (Hitachi Koki™, model CFR15XRII, Hitachinaka, Japan), the supernatants were used to evaluate the optical density at 540 nm (A_{540}) on a UV-VIS spectrophotometer (Shimadzu™, model

UV1650TC, Japan). The graphs of A_{540} versus NaCl (X) concentration were adjusted by non-linear regression, according to the Boltzmann equation:

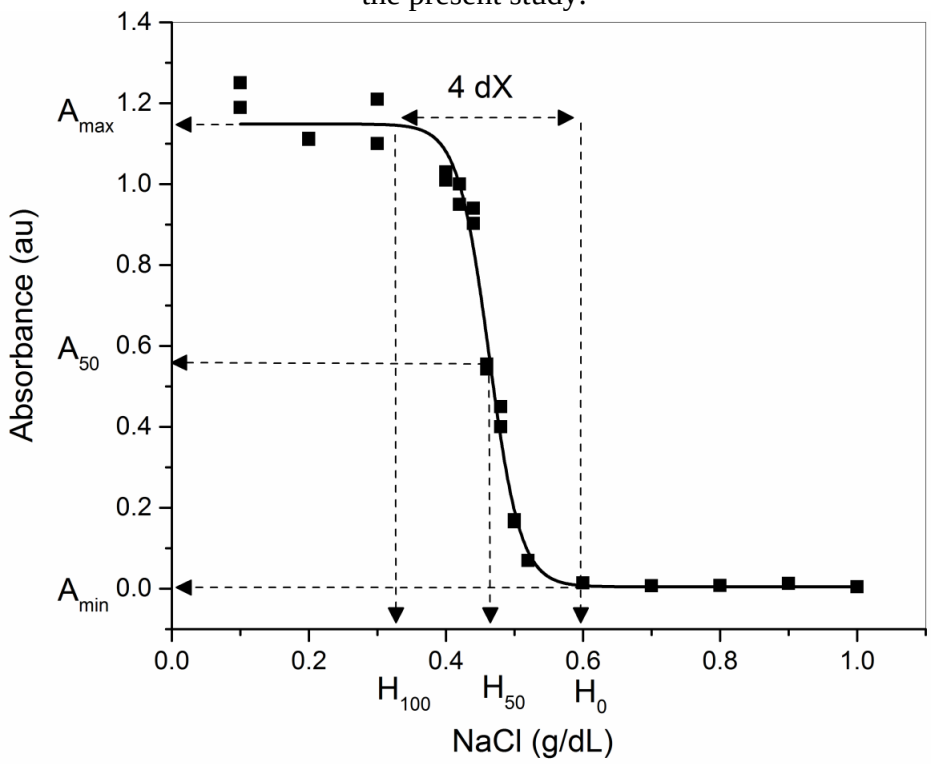
$$A_{540} = \frac{A_{max} - A_{min}}{1 + e^{(X - H_{50})/dX}} + A_{min} \quad (1)$$

where A_{max} and A_{min} represent respectively the maximum and minimum plateaus of A_{540}, H_{50} is the concentration of NaCl capable of promoting 50% hemolysis, and dX is equivalent to one-fourth of the variation in NaCl concentration responsible for 100% hemolysis.

The saline concentrations where the hyposmotic lysis begins (H_0) and end (H_{100}) were determined using the equations $H_0 = H_{50} + 4dX/2$ and $H_{100} = H_{50} - 4dX/2$, respectively.

Figure 1 shows a typical sigmoid constructed for determination of the osmotic stability parameters used in this study.

Figure 1. Typical osmotic stability curve of one of the volunteers with sepsis of the present study.



A_{min} and A_{max} represent the minimum and maximum values of absorbance promoted by hemoglobin released in lysis in medium with higher and lower saline concentrations, respectively. H_{50} is the concentration of NaCl capable of promoting 50% hemolysis. dX is the change in NaCl concentration which promotes ¼ of the total lysis of erythrocytes. H_0 and H_{100} are the saline concentrations where *in vitro* hemolysis begins and ends, respectively. **Source:** Prepared by the authors.

2.3 DETERMINATION OF HEMATOLOGIC AND BIOCHEMICAL PARAMETERS

Hematologic parameters were obtained by means of an automated system (Sysmex K4500; Sysmex Corporation™, Mundelein, IL, USA). These parameters include red blood cell count (RBC), hematocrit (Ht), hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red-cell distribution width (RDW), and white blood cell (WBC) and platelet counts (Plt).

Biochemical parameters were measured using an automated analyzer (Architect c8000, IL, USA). These parameters include triglycerides (TGC), total cholesterol (t-C), low density lipoprotein cholesterol (VLDL-C), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), creatinine (Cr), sodium (Na^+), potassium (K^+), lactate, C-reactive protein (CRP) and arterial gasometry, including hydrogen potential (pH), partial pressure of oxygen (PO_2), partial pressure of carbon dioxide (pCO_2), bicarbonate (HCO_3^-), base excess (BE) and oxygen saturation (SaO_2).

The reference values were: RBC, 4.3-5.7 x 10^6/mm^3; Hb, 13.5-17.5 g/dL; Ht, 39-50%; MCV, 81-95 fL; MCH, 26-34 pg; MCHC, 31- 36 g/dL; RDW, 12-15 %; Plt, 150-450 x 10^3/mm^3; t-C, <200 (desirable) e ≥240 mg/dL (elevated); TGC, <150 (desirable) and >201 mg/dL (elevated); VLDL-C, <30 mg/dL (desirable); LDL-C, <100 (optimum) e >160 mg/dL (elevated); HDL-C, <45 (low) e >65 mg/dL (ideal); urea, 10-45 mg/dL; creatinine, 0.7-1.2 mg/dL; Na^+, 135-145 mEq/L; K^+, 3.7-5.6 mEq/L; PCR, <0,5mg/dL; lactate, 0.36-0.75 mmol/L; pH, 7.35-7.45; pO_2, 80 a 100 mmHg; pCO_2, 35-45 mmHg; HCO_3^-, 22-26 mEq/L; and SO_2, 94-98%.

2.4 DETERMINATION OF RISK OF DEATH

The risk of death was evaluated using the SAP3 (*Simplified Acute Physiology Score*) instrument (METNITZ; MORENO; ALMEIDA; JORDAN *et al.*, 2005; MORENO; METNITZ; ALMEIDA; JORDAN *et al.*, 2005) in its validated version for the Brazilian population (MORALEZ; RABELLO; LISBOA; LIMA *et al.*, 2017).

2.5 STATISTICAL ANALYSIS

The Shapiro-Wilk test was used to evaluate the normality of the data in relation to the type of diagnosis. Comparisons between the control, sepsis and septic shock groups were performed using One-Way ANOVA when the variables of the three groups showed

normal distribution. For the variables in which one or all groups presented non-normal distribution, comparisons were made using the Kruskall-Wallis test. The comparisons made only between the sepsis and septic shock groups were made using the Student's t-test for two independent populations when the variables data of both groups presented normal distribution. For the variables that presented non-normal distribution in one or both groups, comparisons were made using the Mann-Whitney test.

Table 1. Osmotic stability and hematologic and lipid profiles of study populations

Parameters	Control (N=49)	Sepsis (N=25)	Septic shock (N=25)
Age (years)	48.27 ± 14.5	48.88 ± 20.57	47.24 ± 18.37
A_{min} (10^{-2}) (abs)	1.35 (0.45-2.07)	1.03 (0.51-1.65)	0.59 (0.12-1.46)
A_{max} (abs)	1.32 (1.2-1.42) [a,b]	0.7 (0.62-0.87) [a]	0.75 (0.65-0.82) [b]
H_0 (g/dL)	0.48 (0.46-0.49) [a,b]	0.43 (0.41-0.47) [a]	0.43 (0.42-0.45) [b]
H_{50} (g/dL)	0.45 (0.43-0.46) [a,b]	0.39 (0.34-0.42) [a]	0.39 (0.35-0.42) [b]
H_{100} (g/dL)	0.41 (0.4-0.43) [a,b]	0.33 (0.29-0.38) [a]	0.34 (0.31-0.39) [b]
dX (10^{-2}) (g/dL)	1.44 (1.27-1.94) [a,b]	3.09 (0.99-3.49) [a]	2.22 (1.25-2.99) [b]
Hb (g/dL)	15.1 (14.4-15.7) [a,b]	9.25 (8.4-10.7) [a]	8.8 (8-9.8) [b]
Ht (%)	45 (42.2-47) [a,b]	27.9 (25.85-30.85) [a]	27.1 (24.4-30) [b]
RBC (millions/mm^3)	4.86 (4.59-5.2) [a,b]	3.2 (3-3.37) [a]	2.97 (2.89-3.24) [b]
MCV (fL)	92.1 (86.7-95.6)	87.5 (84.2-93.5)	86.5 (84.2-91.4)
MCH (pg)	31 (28.9-32.5) [b]	29.35 (28.4-29.9)	28.9 (27.5-30.2) [b]
MCHC (g/dL)	33.69 ± 0.73 [b]	33.11 ± 1.61	32.58 ± 1.09 [b]
RDW (%)	13.19 ± 0.76 [a,b]	15.32 ± 1.72 [a]	14.47 ± 1.53 [b]
TGC (mg/dL)	121.09 (89.65-151.85)	174.3 (104.48-242.75)	144 (93-230)
t-C (mg/dL)	184.2 ± 36.74 [a,b]	141.45 ± 46.45 [a]	131.5 ± 33.65 [b]
LDL-C (mg/dL)	116.46 ± 33.1 [a]	84.29 ± 50.57 [a]	95.79 ± 28.65
VLDL-C (mg/dL)	24.4 (17.93-30.35)	34.9 (20.6-48.5)	28.8 (18.3-46)
HDL -C (mg/dL)	40.7 (34-48.4) [a,b]	20.7 (13-31.2) [a]	26.4 (15.4-33) [b]

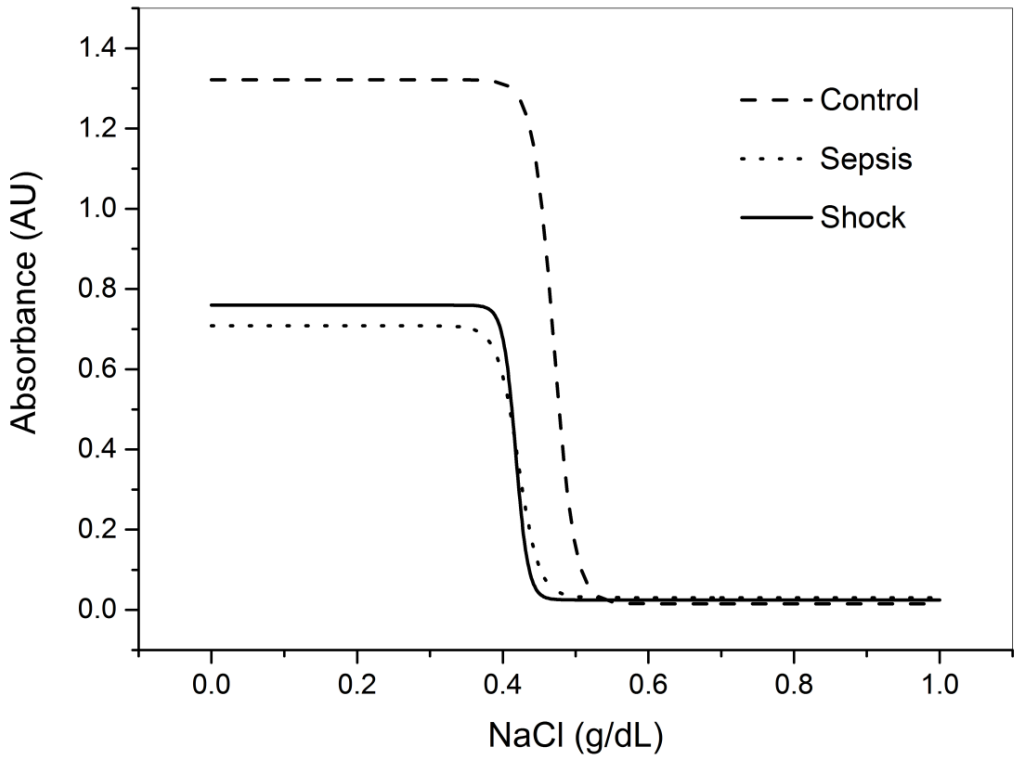
[a,b] Statistically significant differences ($p < 0.05$) are indicated by pairs of the same letter associated with the values of the parameters studied, expressed as mean ± standard deviation or median (25%-75% percentile) for parameters with values normally and not normally distributed, respectively**. Abbreviations:** A_{min}, absorbance at 540 nm associated with initial hemolysis under isosmotic conditions with blood; A_{max}, absorbance at 540 nm associated with total hemolysis; H_0, concentration of NaCl capable of initiating *in vitro* hemolysis; H_{50}, NaCl concentration capable of promoting 50% hemolysis *in vitro*; H_{100} concentration of NaCl capable of promoting total hemolysis *in vitro*; dX, variation of NaCl concentration responsible for ¼ of total hemolysis *in vitro*; Hb, hemoglobin; Ht, hematocrit; RBC, erythrocytes; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red-cell distribution width; TGC, triglycerides; t-C, total cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very low density cholesterol; HDL-C, high-density lipoprotein cholesterol. **Source:** Prepared by the authors.

The existence of associations between the studied variables was analyzed using the Spearman correlation test, with correlations with p values < 0.05 being considered significant.

3 RESULTS

The baseline characteristics of the control, sepsis and septic shock groups are shown in Table 1. The absence of age difference between the groups indicates that there was good pairing. There was no statistically significant difference between the sepsis and septic shock groups for any of the variables considered, but there were many significant differences between the sepsis and/or septic shock groups in comparison to the control group for several of the studied variables.

Figure 2. Osmotic fragility curves of volunteers from the control, sepsis and septic shock groups, respectively.



Source: Prepared by the authors.

In relation to erythrocyte stability, the sepsis and septic shock groups presented greater osmotic stability than the control group. The lower H_0, H_{50} and H_{100} values found in both the sepsis group and the septic shock group in relation to the control group indicate unequivocally that individuals with these pathological conditions have erythrocytes that are osmotically more stable. This difference in behavior between erythrocytes of the two

diseased populations in relation to the control group can be easily visualized in the graph shown in Figure 2, where the osmotic fragility curve of populations with sepsis and septic shock, constructed from the set of points of all individuals of these populations, are displaced to the left, i.e., towards lower saline concentrations, in relation to the curve obtained for the universe of individuals of the control group.

Some variables were analyzed only in the sepsis and septic shock groups. These variables are shown in Table 2. Also, in relation to these variables, there was no significant difference between the sepsis and septic shock groups. As these two groups did not present differences in their basic characteristics, they were grouped into a single study group to evaluate the associations between the variables studied using correlation analysis.

Table 2. Parameters evaluated in individuals with sepsis and septic shock who participated in the study*

Parameters	Sepsis (N=25)	Septic Shock (N=25)	p
Plt (10^6/ mm^3)	267 (430-155)	212 (357-137)	0.3795
WBC (10^6/ mm^3)	13.51 (22.85-9.54)	14 (20.2-9.9)	0.8315
CRP (U/L)	6.1 (10.7-4.31)	13 (17.15-8.01)	0.1419
Lactate (mmol/L)	1.7 (2-1.2)	1.7 (2.2-1.5)	0.4045
Urea (mg/dL)	53.85 (124.5-37.7)	49 (78-40)	0.7612
Crn (mg/dL)	0.99 (2.62-0.63)	0.83 (1.9-0.6)	0.5090
Na^+ (mEq/L)	142 (144-137)	141 (146-136)	0.9149
K^+ (mEq/L)	4.55 ± 0.96	4.17 ± 0.92	0.1801
pH	7.41 (7.44-7.33)	7.39 (7.43-7.35)	0.6623
pCO_2 (mmHg)	38.5 (48.7-34.5)	36.7 (44-35.5)	0.8445
pO_2 (mmHg)	84.17 ± 19.04	95.94 ± 35.49	0.5090
HCO_3^- (mmol/L)	23.89 ± 4.18	23.43 ± 3.96	0.4389
$SatO_2$ (%)	95.4 (97-94.2)	96.3 (98-94.1)	0.2694
BE (mmol/L)	-0.61 ± 3.77	-1.58 ± 4.26	0.2353
SAPS3	61 (69-56)	61 (75-55)	0.8842

*Values expressed as mean ± standard deviation or median (25%-75% percentile) for variables with normal or non-normal distribution, respectively. **Abbreviations:** Plt, platelets; WBC, leukocytes; CRP, C-reactive protein; Crn, creatinine; Na^+, sodium; K^+, potassium; pH, hydrogen potential; pCO_2, partial pressure of carbon dioxide; pO_2, partial pressure of oxygen; HCO_3^-, bicarbonate; $SatO_2$, oxygen saturation; BE, base excess; SAPS3, Simplified Acute Physiology Score 3. **Source:** Prepared by the authors.

The main correlations involving osmotic stability and hematological variables, especially RBC and RDW, in the three study groups are shown in Table 3.

Table 3. Spearman correlations involving osmotic stability and hematologic and biochemical variables*

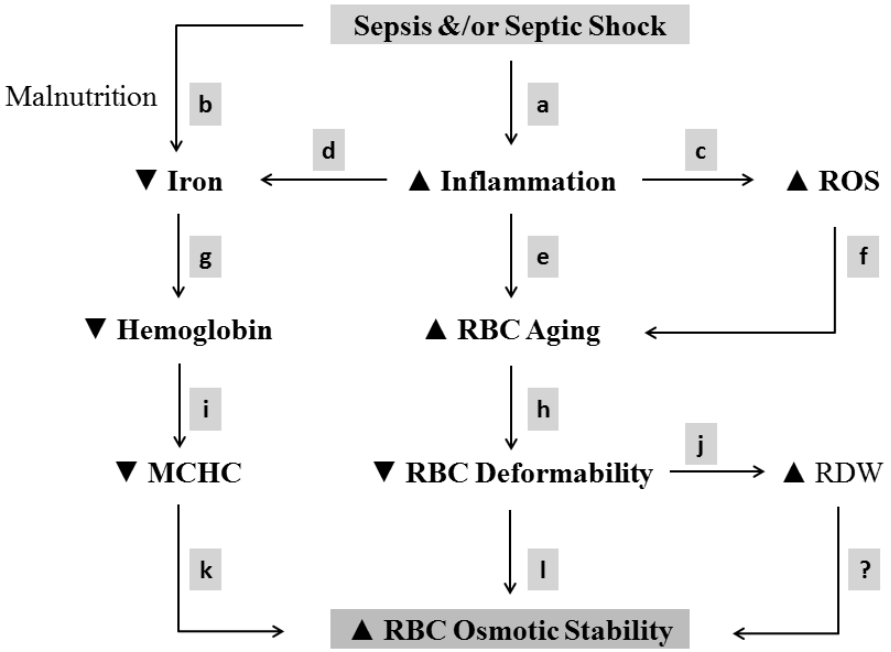
Main	Related	Control Group		Sepsis Group		Septic Shock Group	
		rho	p	rho	p	rho	p
$1/H_{50}$	dX	0.050	0.736	0.408	0.093	0.661	0.002
	A_{max}	-0.022	0.881	0.542	0.020	0.119	0.627
	H_0	-0.868	< 0.001	-0.775	< 0.001	-0.800	< 0.001
	H_{100}	-0.908	< 0.001	-0.905	< 0.001	-0.951	< 0.001
	RBC	-0.136	0.357	-0.580	0.030	0.134	0.595
	t-C	0.289	0.046	-0.297	0.405	0.309	0.385
	LDL-C	0.349	0.015	-0.261	0.467	-0.033	0.932
dX	A_{min}	-0.226	0.118	-0.564	0.018	-0.718	0.001
	H_0	0.335	0.020	0.117	0.645	-0.132	0.591
	H_{100}	-0.374	0.009	-0.719	0.001	-0.828	< 0.001
	RBC	0.340	0.017	-0.207	0.477	0.054	0.832
	RDW	0.472	0.001	-0.288	0.364	0.264	0.290
	MCH	-0.334	0.019	-0.112	0.729	0.136	0.590
Amin	H_{100}	0.087	0.555	0.321	0.209	0.533	0.019
H_{100}	Ht	0.161	0.273	0.534	0.027	0.193	0.443
	t-C	-0.328	0.023	0.103	0.777	-0.248	0.489
	TGC	0.323	0.025	0.220	0.471	0.047	0.879
	LDL-C	-0.385	0.007	0.091	0.803	-0.033	0.932
	VLDL-C	0.292	0.044	0.109	0.749	-0.479	0.162
	HDL-C	-0.311	0.032	0.067	0.855	0.150	0.700
CRP	Amax			-0.429	0.289	-0.673	0.033
	H_0			0.738	0.037	0.067	0.855
	RBC			-0.303	0.364	-0.588	0.074
	Hb			-0.361	0.226	-0.645	0.032
	Ht			-0.313	0.297	-0.588	0.057
	MCHC			0.273	0.391	-0.560	0.073
	t-C			-0.700	0.036	0.551	0.257
	LDL-C			-0.733	0.025	0.600	0.285
	satO2			-0.552	0.063	0.312	0.324
RDW	RBC	0.397	0.005	0.474	0.047	-0.135	0.559
	Ht	-0.093	0.527	0.602	0.008	-0.105	0.649
	MCH	-0.666	< 0.001	-0.149	0.554	0.128	0.582
	MCV	-0.621	< 0.001	0.184	0.465	0.206	0.369
	MCHC	-0.125	0.392	-0.594	0.009	-0.202	0.380
	t-C	0.303	0.034	0.311	0.259	0.107	0.741
	SAPS3			0.756	< 0.001	0.209	0.362
RBC	Hb	0.585	< 0.001	0.816	< 0.001	0.647	0.002
	Ht	0.578	< 0.001	0.908	< 0.001	0.607	0.004
	MCH	-0.613	< 0.001	-0.337	0.171	-0.284	0.212
	MCV	-0.607	< 0.001	0.111	0.632	-0.298	0.190
	MCHC	-0.009	0.952	-0.580	0.006	0.012	0.959

* Gray shading indicates statistically significant correlations ($p < 0.05$). **Source:** Prepared by the authors.

Abbreviations table 3: A_{min}, absorbance at 540 nm associated with hemolysis under isosmotic conditions with blood; A_{max}, absorbance at 540 nm associated with total hemolysis; H_0, concentration of NaCl capable of initiating *in vitro* hemolysis; H_{100} concentration of NaCl capable of promoting total hemolysis; $1/H_{50}$, inverse of NaCl concentration capable of promoting 50% hemolysis; dX, variation in NaCl concentration responsible for ¼ of the total hemolysis; Hb, hemoglobin; Ht, hematocrit; RBC, erythrocytes; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; TGC, triglycerides; t-C, total cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very low density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; CRP, C-Reactive Protein; SAP3, Simplified Acute Physiology Score 3.

Additionally, the associations comprise malnutrition and/or inflammatory aggression, leading to a decrease in the availability of iron in the body and to the need to increase the lifetime of the erythrocyte, with an increase in the population of older erythrocytes and consequent decrease in deformability (Figure 3).

Figure 3. Possible associations, compiled from the literature and/or from results of this study, between sepsis and/or septic shock and osmotic stability of erythrocytes.



a. (BOSMANN; WARD, 2013), b. (KOSALKA; WACHOWSKA; SLOTWINSKI, 2017), c. (MITTAL; SIDDIQUI; TRAN; REDDY *et al.*, 2014), d. (CHERAYIL, 2015), e. (STRAAT; VAN BRUGGEN; DE KORTE; JUFFERMANS, 2012), f. (MOHANTY; NAGABABU; RIFKIND, 2014), g. (ABBASPOUR; HURRELL; KELISHADI, 2014), h. (SUTERA; GARDNER; BOYLAN; CARROLL *et al.*, 1985), i. (PIAGNERELLI; BOUDJELTIA; VANHAEVERBEEK; VINCENT, 2003), j. (ORBACH; ZELIG; YEDGAR; BARSHTEIN, 2017), k. (HLADKY; RINK, 1978), l. (PATEL; MOHANTY; KANAPURU; HESDORFFER *et al.*, 2013). **Source:** Prepared by the authors.

4 DISCUSSIONS

The higher osmotic stability of erythrocytes in patients with sepsis and septic shock, evidenced by the lower values of A_{min}, H_0, H_{50} and H_{100}, as well as by the higher values of dX, in relation to the control group (Table 1), is a remarkable result of this study.

A good justification for this finding should be the existence of tendency to anemia or anemia in the diseased patients, as evidenced by the lower values of A_{max} (Table 1 and Figure 2), hemoglobin levels, RBC counts, and values of hematocrit, MCH and/or MCHC in comparison to the control group (Table 1). Lower concentrations of hemoglobin will exert less osmotic pressure for water intake and higher volume for water occupation (HLADKY; RINK, 1978; SAVITZ; SIDEL; SOLOMON, 1964), making erythrocytes more capable of maintaining their integrity in the hyposmotic environment. The negative correlation observed between dX and A_{min}, not only in the diseased population but also in the control population and in several other clinical conditions (DE FREITAS; MARQUEZ-BERNARDES; DE ARVELOS; PARAISO *et al.*, 2014; MASCARENHAS NETTO RDE; FABBRI; DE FREITAS; BERNARDINO NETO *et al.*, 2014), is possibly a manifestation of this type of mechanism. Erythrocytes with less hemoglobin to release spontaneously in isosmotic conditions with blood (lower A_{min}) should withstand more against the decrease in saline concentration of the medium (greater dX).

It is possible that this anemia is associated with the existing inflammatory state. The observed negative correlations between blood hemoglobin levels and hematocrit values with C-reactive protein (CRP) (Table 3) support this idea.

Another possible cause of this anemia is the existence of malnutrition. Nutritional deficiencies of iron and protein are trivial causes of iron deficiency and/or microcytic anemia. Indeed, the values of mean corpuscular volume (MCV) were lower, although without statistical significance, in the sepsis and septic shock groups when compared to control group. In addition, the strong correlation observed between MCV and MCH shows that this variation in erythrocyte volume occurs due to the influence of hemoglobin levels. In addition, the lowest values of total cholesterol (t-C), LDL-C (LDL-C) and HDL-C (HDL-C) observed in the sepsis and/or septic shock groups are quite suggestive of malnutrition.

In several studies the stability of erythrocytes has been associated with lipidemia (AIRES RODRIGUES DE FREITAS; VIEIRA DA COSTA; ALVES DE MEDEIROS; DA SILVA GARROTE FILHO *et al.*, 2018; BERNARDINO NETO; DE AVELAR; ARANTES; JORDAO *et al.*, 2013; DE FREITAS; MARQUEZ-BERNARDES; DE

ARVELOS; PARAISO *et al.*, 2014) and in fact in this study there was an inverse correlation of H_{50} with blood levels of t-C and LDL-C in the control group, but not in the sick population (Table 3). This means that lipidemia, which helps to modulate the membrane cholesterol content, is not playing a role in the control of erythrocyte stability, which makes sense given the lower levels of t-C and LDL-C observed in the groups with sepsis and shock compared to the control group.

Another remarkable result of the present study is the existence of significantly higher RDW values in the sepsis and septic shock groups than in the control group (Table 1). This may be due, at least in part, to the fact that erythrocytes of people with sepsis or shock are less deformable than those of healthy volunteers (MOUTZOURI; SKOUTELIS; GOGOS; MISSIRLIS *et al.*, 2007).

The deformability of erythrocytes may be influenced by the membrane cholesterol content. An excess of membrane cholesterol was associated with decreased erythrocyte deformability (COOPER; ARNER; WILEY; SHATTIL, 1975; MULLER; ZIEGLER; DONNER; DROUIN *et al.*, 1990). But this should not be the case of sepsis and septic shock, given the lower cholesterol levels observed here. Thus, the lower deformability of erythrocytes reported in sepsis and septic shock (MOUTZOURI; SKOUTELIS; GOGOS; MISSIRLIS *et al.*, 2007) should have other associations.

Deformability is a property related to osmotic membrane stability (ORBACH; ZELIG; YEDGAR; BARSHTEIN, 2017). In fact, significant correlations were observed between RDW and osmotic stability in other studies (AIRES RODRIGUES DE FREITAS; VIEIRA DA COSTA; ALVES DE MEDEIROS; DA SILVA GARROTE FILHO *et al.*, 2018; BERNARDINO NETO; DE AVELAR; ARANTES; JORDAO *et al.*, 2013; DE FREITAS; MARQUEZ-BERNARDES; DE ARVELOS; PARAISO *et al.*, 2014) and in the control group but not in the sick population of the present study (Table 3).

The existence of association between increase in RDW and elevation in the population of older erythrocytes had already been suggested (PATEL; PATEL; HIGGINS, 2015). It is important to note that the existence of this predominance of older erythrocytes would occur not because of the acceleration of aging, but because of the organism's need to preserve its erythrocytes longer, especially in view of the existing inflammatory and malnutrition states. Indeed, lower iron availability in the body was associated with exacerbation of inflammation (OSTERHOLM; GEORGIEFF, 2015). In addition, the negative correlation presented by blood levels of hemoglobin with

C-reactive protein (CRP) (Table 3) does in fact allow the association of anemia with the inflammatory state in the present study.

The negative correlation observed between dX and Amin in the sepsis and septic shock groups indicates that lower hemoglobin content would increase erythrocyte resistance against lysis as the saline concentration of the medium decreases. The significant negative correlation observed between blood hemoglobin and C-reactive protein (CRP) levels in the septic shock group indicates that the inflammatory condition of the disease is associated with a decrease in blood hemoglobin levels. The strong correlation observed between mean corpuscular volume (MCV) and mean corpuscular hemoglobin (HCM) illustrates a known association. The inverse correlation of H_{50} with blood levels of t-C and LDL-C in the control group but not in the diseased population shows that the influence of lipidemia on the erythrocyte membrane composition and its behavior in the hyposmotic environment does not appear to occur in the diseased population of this study.

Special attention should be given to the increase in the RDW values of the sick population, since an increase in the RDW has been associated with the prediction of worse clinical outcome in several diseases (BUJAK; WASILEWSKI; OSADNIK; JONCZYK *et al.*, 2015; PATEL; SEMBA; FERRUCCI; NEWMAN *et al.*, 2010; PERLSTEIN; WEUVE; PFEFFER; BECKMAN, 2009; VAYA; SARNAGO; FUSTER; ALIS *et al.*, 2015; ZURAUSKAITE; MEIER; VOEGELI; KOCH *et al.*, 2018). The positive correlation observed between RDW and SAPS3 in the group of volunteers with sepsis of the present study also reveals the existence of an association with a higher chance of death with the highest values of RDW.

The present study presents some limitations that deserve to be highlighted. The first one is the lack of evaluation of glycated hemoglobin and bilirubin in all groups of this study, which would have been useful to estimate the erythrocyte life time, since high levels of glycated hemoglobin and low levels of bilirubin are suggestive of the existence of erythrocytes with longer life (COHEN; FRANCO; KHERA; SMITH *et al.*, 2008; LEWIS; GERSHOW, 1961). The second is the lack of evaluation of oxidative variables, since the exacerbation of oxidative aggression and reduction of antioxidant defenses are factors capable of affecting the erythrocyte membrane structure and RDW values (MOHANTY; NAGABABU; RIFKIND, 2014; SEMBA; PATEL; FERRUCCI; SUN *et al.*, 2010). A third limitation is the lack of information about the body's iron status, which

could sustain the observed results, allowing analysis of the suggested association of iron availability with inflammation.

5 CONCLUSIONS

Despite these limitations, the present study shows that: 1) erythrocytes from the study groups with sepsis and septic shock are more stable in hyposmotic medium than normal volunteers, 2) this increased osmotic stability of erythrocytes is associated with anemia and inflammation, and 3) higher RDW values were associated with a higher chance of death.

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