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Early hemorheological changes in a porcine model of intravenously given *E. coli* induced fulminant sepsis

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Abstract. The pathophysiology of hemorheological and microcirculatory disturbances in septic process -mostly during the early hours- still not clarified in all the details, yet. In anesthetized pigs living *E. coli* (ATCC 25922 strain) was administered intravenously with an increasing concentration and the animals were observed for 8 hours. Before the intervention and in every 2 hours arterial (cannulated femoral artery) and venous (cannulated external jugular vein) blood samples were collected for hemorheological laboratory tests: blood and plasma viscosity, ESR, leukocyte anti-sedimentation rate, erythrocyte deformability (together with osmoscan parameters) and erythrocyte aggregation (using light-transmission and laser back-scattering methods) Control animals were stable over the 8-hour anesthesia, while septic animals died by the 6th hours in a fulminant sepsis. Over the experimental period, the tendency of impairment in erythrocyte deformability (together with osmotic gradient ektacytometry parameters) and the controversial decreasing of erythrocyte aggregation values (declining all aggregation index values, elongating $t_{1/2}$) were well detected in this porcine model during the early hours (4–6) of fulminant sepsis. The *in vitro* effect of these bacteria on erythrocytes' micro-rheological parameter was similar: decreasing red blood cell deformability and lowering aggregation. Further studies are needed to clarify the early micro-rheological changes of bacteremia and the developing sepsis.

Keywords: Red blood cell deformability, red blood cell aggregation, E. coli, sepsis, animal model

1. Introduction

Sepsis still means a serious challenge in the clinical practice. It is estimated that 13 million people become septic each year, the mortality of severe sepsis is about 30–50% and of the septic shock it is over 50% [23, 40, 47]. The pathophysiology of the septic process (colonization – infection – bacteremia and with systemic inflammatory response syndrome turning towards sepsis – severe sepsis – septic shock and multiorgan failure) is very complex and not completely clarified, yet. The sepsis pathophysiology includes numerous links to the rheology of blood since sepsis is considered to be a disorder of the microcirculation [24, 42, 45].

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The disturbed microcirculation in sepsis can be resulted by numerous factors including blood and plasma viscosity alterations, disturbed micro-vascular blood flow and vascular resistance, decreased red blood cell deformability, increased red blood cell aggregation among others [4, 16, 24, 42]. It is known that fibrinogen concentration and plasma viscosity increases, and red blood cell deformability impairs and their aggregation is enhanced significantly both in experimental sepsis and in clinical manifestations [4, 7, 8, 34, 38]. Furthermore, filterability of neutrophils also significantly decreases, while their aggregation markedly increases in severe sepsis and septic shock [11, 48, 47].

However, the magnitude of changes and the dynamics of micro-rheological alterations as well as the 'mystified border' between reversibility and irreversibility are still unclear. Numerous experimental models are known using various animal species and different methods to induce the sepsis [17, 18, 37]. Besides the limitations of the experimental model and sometimes the difficult comparability, these *in vivo* studies are highly important to reveal and understand the pathomechanism. Intravenously administered live bacteria method is one of the ways, by which an increasing magnitude of bacteremia can be produced, being comparable with some clinical situation [3, 12, 18, 37, 44]. However, the complete hemorheological characteristics of the very early hours of induced bacteremia and of the response reaction is not known completely.

In our study we aimed to investigate whether *E. coli* bacteremia of increasing concentration results in sepsis and whether micro-rheological parameters do alter during the early hours of the septic process in a porcine model.

2. Materials and methods

2.1. Experimental animals and protocol

The experiments were carried in accordance with the European Community guidelines and State Regulations with the approval of the University of Debrecen Committee of Animal Welfare (reg. Nr.: 21/2013. DEMAB). Nine juvenile female Hungahib pigs (bodyweight: 17.8 ± 2 kg) were subjected into Control ($n = 4$) or Septic Group ($n = 5$). Under general anesthesia (15 mg/kg ketamin, i.m.+1 mg/kg xylazin i.m.) tracheostomy was performed for assisted ventilation, and the left external jugular vein and femoral artery have been cannulated for sampling and hemodynamic measurements. The animals did not receive anticoagulant.

In *Sepsis group Escherichia coli* suspension (2.5×10^5/ml; strain: ATCC 25922, Department of Medical Microbiology, University of Debrecen) was intravenously administrated in a continuously increasing manner as the followings: 2 ml in the first 30 min, then 4 ml in 30 min and afterwards 16 ml/h for 2 hours (so a total of 9.5×10^6 *E. coli* within 3 hours) (Fig. 1). In the *Control Group* the anesthesia was maintained for 8 hours, infusion was administered as a similar volume of isotonic saline solution and no other intervention was made. At the end of the experimental period the animals have been over-anaesthetized.

At the beginning of the procedure (prior to *E. coli* administration) blood samples were collected in parallel from the cannulated external jugular vein and the femoral artery (base samples) directly into tubes containing K_3-EDTA (1.5 mg/ml) for all the laboratory tests, except for sedimentation measurements of venous blood, where 0.109 M sodium-citrate was used as anticoagulant. Further arterial and venous blood samples were collected at 2, 4, 6 and 8 hours after the time point when the infusion started. At the same times, body temperature (central venous, T [°C], mean arterial pressure (MAP [mmHg]), heart rate (HR [1/min]) and oxygen saturation (Sat [%]) were recorded.

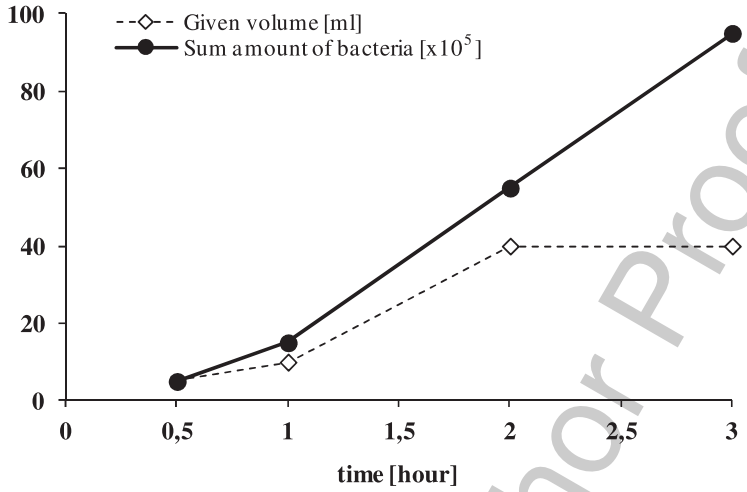

Fig. 1. Protocol of the intravenous administration of living *E. coli* suspension (2.5 x 10^5/ml). Dashed lined plot shows the step-wisely increased volume of the suspension [ml], the continuous black line represents the sum amount of the administered *E. coli* bacteria (x10^5) during the first 3 hours of the experiment.

2.2. In vitro study (effect of the presence of living bacteria on blood rheology)

Venous blood samples taken from two animals at the beginning of the experiment (base or normal blood sample) were divided into two sub-samples. Those were mixed with suspension of *E. coli* bacteria at half and total dose that was presented *in vivo* (according to the totally given amount of bacteria described above and the circulating blood volume, as 60 ml/kg).

Within 15–20 minutes after the mixing and after 2 hours of incubation at room temperature micro-rheological tests (red blood cell deformability, osmotic gradient ektacytometry, red blood cell aggregation) were performed.

2.3. Laboratory measurements

2.3.1. Hematological parameters

A Sysmex F-800 semi-automated microcell counter (TOA Medical Electronics Co., Japan) was used to determine hematological parameters. In this paper white blood cell count (WBC [x10^3/μl]), red blood cell count (RBC [x10^6/μl]), hematocrit (Hct [%]), mean corpuscular volume (MCV [fl]), mean corpuscular hemoglobin content (MCH [pg]), and platelet count (Plt [x10^3/μl]) are shown for comparisons.

2.3.2. Erythrocyte sedimentation rate and leukocyte anti-sedimentation rate

The tube containing the sodium-citrate-anticoagulated venous blood samples were taken in vertical positions and left for gravity sedimentation. After 1 hour the regular erythrocyte sedimentation rate (ESR [mm/h]) was registered.

To determine the leukocyte antisedimentation rate (LAR [%]) the upper and the lower part of the sedimentation blood column were gently separated and the white blood cell count was measured in both subsamples. The LAR was calculated using the Bogar's formula [9]:

$$LAR = 100 \times (WBC_{\text{upper}} - WBC_{\text{lower}})/(WBC_{\text{upper}} + WBC_{\text{lower}}).$$

This parameter can provide information about the distribution of light and heavy polymorphonuclear leukocytes [9].

2.3.3. Blood and plasma viscosity

Viscosity measurements were carried out at 37°C using Hevimet-40 capillary viscosimeter (Hemorex Ltd., Hungary) within 1 hour after sampling. Plasma was prepared by centrifuging at 800 g for 10 minutes. Whole blood viscosity values (WBV [mPas]) at 90 s^{-1} shear are used for the comparisons, and according to the Matrai formula, correction for 40% hematocrit has been also performed: $WBV_{40\%}/PV = (WBV_{Hct}/PV)^{40\%/Hct}$, where $WBV_{40\%}$: corrected for 40% Hct; WBV_{Hct}: whole blood viscosity measured at the native Hct; PV: plasma viscosity; Hct: actual hematocrit value [%] of the sample [26].

2.3.4. Red blood cell deformability

Red blood cell deformability was determined by a LoRRca MaxSis Osmoscan device (Mechatronics BV, The Netherlands). Blood sample of 5 μl was taken into 1 ml of isotonic polyvinyl-pyrrolidone solution (360 kDa PVP in normal phosphate buffered saline; viscosity = 27 mPas, osmolality = 290–300 mOsm/kg; pH 7.3) and mixed gently. Based upon the laser diffraction pattern changes the elongation index (EI) values were determined in the function of shear stress (SS) in a range of 0.3–30 Pa. The EI is equal to (L-W) / (*L* + W), where L is the length and W is the width of the diffractogram [19]. EI increases with red blood cell deformability. The measurements were carried out at 37 °C. Comparing EI-SS curves Lineweaver-Burk analysis was performed, calculating the maximal elongation index (EI_{max}) and the shear stress values at half EI_{max} ($SS_{1/2}$ [Pa]): $1/EI = SS_{1/2} / EI_{max}$ x $1/SS + 1/EI_{max}$. Furthermore, $EI_{max} / SS_{1/2}$ ratio was also calculated [6].

For the osmotic gradient ektacytometry (osmoscan) test [15] 250 μl of blood was gently mixed in 5 ml PVP solution. At constant shear stress of 30 Pa the elongation index values were continuously determined while the osmolality was changing (rising gradually from 0 to 500 mOsmol/kg). Among the device-given parameters we analyzed the minimal elongation index values measured at low-osmotic environment (minimal EI), the maximal elongation index values (maximal EI), the belonging osmolality vales (minO and maxO as 'optimal' osmolality), and the area under the individual elongation index-osmolality curves (AUC). Additionally, we calculated further parameters describing the phase between minEI and maxEI in the function of osmolality: ΔEI, as the difference between maximal and minimal EI values; ΔO, as the difference between osmolality values at maximal and minimal EI; and ΔEI / ΔO [31].

2.3.5. Red blood cell aggregation

Based on light-transmittance method, a Myrenne MA-1 erythrocyte aggregometer (Myrenne GmbH, Germany) was used for determining aggregation index values: M (at shear rate of 0 s^{-1}) and M1 (at shear rate of 3 s^{-1}) 5 or 10 seconds after disaggregation. The indices (M 5 s, M1 5 s, M 10 s, M1 10 s) increase with enhanced red blood cell aggregation [19]. The LoRRca device was also used to measure red blood cell aggregation parameters by syllectometry determining the following parameters: amplitude (Amp [au]), aggregation index (AI [%]) and the aggregation half-time ($t_{1/2}$ [s]) [19].

2.4. Statistical analyses

Data are presented as means ± standard deviation (S.D.). According to the normality of data distribution, for inter-group (Control vs. Sepsis) comparison Student *t*-test or Mann-Whitney RS test, for

intra-group comparison (vs. base values) one-way ANOVA tests (Dunn's or Bonferroni's method) were used. A p value less than 0.05 was considered as statistically significant.

3. Results

3.1. Hemodynamical changes and mortality rate

In the Septic group, as expected, all animals developed fulminant sepsis and died within by 3-7 hours (2 animals in 3–4 hours, and 3 in 6–7 hour). In the septic animals the heart rate rose and mean arterial pressure dropped, their ratio increased significantly compared both the base values (at the 6th hour: $p < 0.001$) and the Control group ($p = 0.004$). The control animals showed stable condition over the 8-hour anesthesia (Table 1).

3.2. Hematological parameters

Table 2 summarizes selected hematological parameters. Total leukocyte count showed a continuously decreasing tendency in the Sepsis group, both in arterial and venous samples. By the 4th and 6th hours the values were significantly lower than the base values and that of the Control group ($p < 0.001$). The Control values were constant showing only an elevation by the end of the experimental period. The monocyte-granulocyte% showed slight increasing in both groups, but without significant differences.

Red blood cell count was almost constant in both groups, however, by the 6th hour it was increased together with the hematocrit in the septic animals. Intergroup difference reached a significant level at the 4th and 6th hours, both in arterial and venous blood. MCV values were slightly higher in Septic group compared to the Control.

Platelet count dominantly decreased in septic animals, reaching the significance level by the 6th hours compared both the base and control values ($p < 0.001$).

3.3. Erythrocyte sedimentation rate and leukocyte anti-sedimentation rate

Erythrocyte sedimentation rate (ESR) remained constant in the control animals during the entire experimental period. In septic animals the ESR values increased stepwise till the 4th hours, and then decreased (Table 3).

Table 1
Body temperature (Temp), heart rate (HR) / mean arterial pressure (MAP) ratio and oxygen saturation values of the Control and the Sepsis groups during the experimental period

Variable	Group	Base	2 h	4 h	6 h	8 h
Temp [°C]	*Control*	36.73 ± 0.47	37.43 ± 0.41	37.83 ± 0.7	38.76 ± 1.35	39 ± 1.47
	Sepsis	37 ± 1.21	37.85 ± 0.97	38.2 ± 0.42	38.5 ± 0.28	–
HR / MAP [1/mmHg.min]	*Control*	0.86 ± 0.21	1.09 ± 0.34	0.98 ± 0.17	0.98 ± 0.21	1 ± 0.2
	Sepsis	0.82 ± 0.18	1.48 ± 1.24	1.31 ± 0.24 *	3.09 ± 0.82 *#	–
Saturation [%]	*Control*	97 ± 1.8	96.7 ± 0.9	94 ± 2.7	95.2 ± 1.5	95 ± 1.4
	Sepsis	94.2 ± 3.1	91 ± 4.35	93.6 ± 6.1	90 ± 15.5	–

means $\pm$ S.D., $^{*}p < 0.05$ vs. base, $^{\#}p < 0.05$ vs. Control; at 4 h and at 6 h $n = 3$ in Sepsis Group (used test: Bonferroni's method of one-way ANOVA on rank test, Mann-Whitney rank sum test).

Table 2
Changes of selected hematological parameters in arterial and venous blood samples of the Control and the Sepsis groups

Variable	Group	Sample	Base	2 h	4 h	6 h	8 h
WBC [x10^3/μl]	*Control*	A	15.3 ± 2.39	16.61 ± 0.79	17.63 ± 2.05	17.97 ± 0.61	20.45 ± 2.64
		V	15.21 ± 1.97	17.36 ± 1.17	16.95 ± 2.69	16.16 ± 2.68	17.45 ± 7.05
	Sepsis	A	14.88 ± 3.21	13.94 ± 4.98	8.88 ± 3.77 *#	7.05 ± 3.58 *#	–
		V	14.53 ± 2.64	14.49 ± 5.08	8.85 ± 4.09 *#	7.88 ± 3.64 *#	–
Mo+Gr% [%]	*Control*	A	43.25 ± 9.54	41.88 ± 8.84	53.2 ± 10.39	60.21 ± 13.39	68.25 ± 12.3
		V	46.47 ± 10.7	41.42 ± 10.02	52.7 ± 11.26	58.35 ± 10.16	67.31 ± 8.22
	Sepsis	A	48.81 ± 6.77	46.14 ± 11.27	45.73 ± 16.42	59.14 ± 20.49	–
		V	45.85 ± 7.03	45.49 ± 11.7	47.15 ± 20.2	53.15 ± 18.42	–
RBC [x10^6/μl]	*Control*	A	6.21 ± 0.51	6.48 ± 0.47	5.97 ± 0.85	5.99 ± 0.84	6.21 ± 0.71
		V	6.27 ± 0.43	6.45 ± 0.31	5.85 ± 0.83	5.53 ± 1.14	5.78 ± 0.53
	Sepsis	A	6.65 ± 0.44	6.17 ± 0.32	6.7 ± 0.7	6.98 ± 0.89	–
		V	6.44 ± 0.64	6.49 ± 0.33	6.85 ± 0.8	6.63 ± 0.93	–
MCV [fl]	*Control*	A	59.83 ± 4.54	62.76 ± 6.3	60.17 ± 2.82	58.71 ± 1.23	60.5 ± 3.41
		V	57.65 ± 2.8	60.3 ± 3.88	58.26 ± 1.47	60.42 ± 5.72	60.11 ± 1.62
	Sepsis	A	61.99 ± 2.62	65.23 ± 7.88	65.93 ± 4.4	64.26 ± 3.05	–
		V	63.88 ± 4.58	64.81 ± 6.44	63.63 ± 2.07	64.86 ± 3.26	–
MCH [pg]	*Control*	A	15.21 ± 0.87	14.76 ± 0.11	15.42 ± 0.89	15.48 ± 1.4	14.58 ± 0.99
		V	14.91 ± 0.24	15.12 ± 0.39	15.07 ± 0.31	15.03 ± 1.21	15.37 ± 0.33
	Sepsis	A	15.66 ± 0.64	16.08 ± 0.83	17.08 ± 0.85	16.43 ± 1.76	–
		V	16.35 ± 1.39	16 ± 0.86	16.73 ± 1.3	16.81 ± 0.92	–
Plt [x10^3/μl]	*Control*	A	608.5 ± 40.2	612.1 ± 127.4	565 ± 152.1	630.6 ± 105.8	555 ± 131.1
		V	562.1 ± 119.3	562.3 ± 145.1	517.6 ± 114.8	578 ± 83.7	477.6 ± 88.7
	Sepsis	A	470.5 ± 55.1	401.75 ± 125.4	382.3 ± 126.2	221.5 ± 32.5 *#	–
		V	421.6 ± 88.9	384.6 ± 111.6	346.1 ± 108	204.5 ± 42.09 *#	–

means ± S.D., $^*p < 0.05$ vs. base, $^\#p < 0.05$ vs. Control. A = arterial blood sample, V = venous blood sample at 4 h and at 6 h $n = 3$ in Sepsis Group (used test: Bonferroni's method of one-way ANOVA on rank test, Mann-Whitney rank sum test).

Leukocyte anti-sedimentation rate showed a declining tendency in the septic animals: almost every 2 hours it decreased by 15–20%. The values differed significantly from the Control group (at 2 h: $p = 0.031$, at 4 h: $p = 0.05$, at 6 h: $p = 0.049$) (Table 3).

3.4. Blood and plasma viscosity

Whole blood viscosity moderately decreased over the experimental period in the control animals, thanking to the blood samplings and infusion. However, plasma viscosity did not changed importantly. The WBV values corrected for 40% hematocrit were almost constant. In the Sepsis group the whole blood viscosity showed an elevation by the 4th hours while plasma viscosity continuously decreased (Table 3).

3.5. Red blood cell deformability

Red blood cell deformability of the Sepsis group showed worsening; in every 2 hours the $SS_{1/2}$ increased and the $EI_{max}/SS_{1/2}$ ratio decreased both in arterial and venous blood. However, the differences did not reach a statistically significant level. There was no obvious arterio-venous difference (Fig. 2, A-F).

Table 3
Changes of whole blood viscosity (WBV), plasma viscosity (PV), corrected WBV at 40% hematocrit ($WBV_{40\%}$), erythrocyte sedimentation rate (ESR) and leukocyte anti-sedimentation rate (LAR) of the Control and the Sepsis groups.

Variable	Group	Base	2 h	4 h	6 h	8 h
WBV [mPas] at shear rate of 90 s^{-1}	*Control*	2.87 ± 0.6	2.74 ± 0.62	2.31 ± 0.89	2.46 ± 0.39	2.28 ± 0.34
	Sepsis	3.03 ± 0.51	2.66 ± 0.65	3.02 ± 0.71	2.77 ± 0.91	–
PV [mPas]	*Control*	0.98 ± 0.09	0.98 ± 0.03	1 ± 0.11	1 ± 0.21	0.9 ± 0.12
	Sepsis	1.03 ± 0.15	0.95 ± 0.02	$0.94. \pm 0.02$	0.91 ± 0.03	–
$WBV_{40\%}$ [mPas]	*Control*	3.41 ± 1.29	2.98 ± 0.85	2.58 ± 0.81	3.19 ± 1.09	2.57 ± 0.31
	Sepsis	2.95 ± 0.46	2.57 ± 0.65	2.72 ± 0.38	2.54 ± 0.59	–
Htc / WBV [%/mPas]	*Control*	12.96 ± 3.4	14.8 ± 4.08	15.66 ± 3.38	13.77 ± 3.25	12.81 ± 5.13
	Sepsis	13.8 ± 2.5	16.47 ± 4.01	14.73 ± 2.43	16.25 ± 4.26	–
ESR [mm/h]	*Control*	3.5 ± 1	3.5 ± 1	3 ± 1.15	3.75 ± 1.25	3.75 ± 1.25
	Sepsis	3.75 ± 0.5	4.25 ± 0.5	4.66 ± 1.15	3.66 ± 0.57	–
LAR [%]	*Control*	21.98 ± 14.33	27.09 ± 7.33	21.11 ± 6.01	22.02 ± 6.74	19.41 ± 7.62
	Sepsis	17.68 ± 4.9	$12.95 \pm 8.12^{\#}$	$10.48 \pm 4.59^{\#}$	$6.91 \pm 6.52^{\#}$	–

means $\pm$ S.D., $^{*}p < 0.05$ vs. base, $^{\#}p < 0.05$ vs. Control; at 4 h and at 6 h $n = 3$ in Sepsis Group (used test: Bonferroni's method of one-way ANOVA on rank test, Mann-Whitney rank sum test).

Table 4
Changes of selected osmotic gradient ektacytometry (osmoscan) parameters in arterial and venous blood samples of the Control and the Sepsis groups.

Variable	Group	Sample	Base	2 h	4 h	6 h	8 h
minEI	*Control*	A	0.111 ± 0.007	0.115 ± 0.011	0.111 ± 0.009	0.118 ± 0.011	0.126 ± 0.017
		V	0.120 ± 0.011	0.117 ± 0.017	0.121 ± 0.009	0.127 ± 0.008	0.122 ± 0.021
	Sepsis	A	0.105 ± 0.014	0.116 ± 0.023	0.130 ± 0.03	$0.141 \pm 0.016^{*}$	–
		V	0.115 ± 0.024	0.104 ± 0.019	0.131 ± 0.009	0.121 ± 0.02	–
maxEI	*Control*	A	0.502 ± 0.01	0.499 ± 0.01	0.491 ± 0.007	0.493 ± 0.004	0.490 ± 0.004
		V	0.501 ± 0.011	0.500 ± 0.009	0.488 ± 0.01	0.487 ± 0.011	0.484 ± 0.01
	Sepsis	A	0.491 ± 0.012	0.487 ± 0.009	0.491 ± 0.011	0.491 ± 0.016	–
		V	0.490 ± 0.01	0.488 ± 0.011	0.493 ± 0.013	0.490 ± 0.014	–
minO [mOsm/kg]	*Control*	A	187.7 ± 5.8	188.5 ± 8.5	192.5 ± 7.6	191.2 ± 3.6	190.5 ± 3.8
		V	189.7 ± 5.9	191.7 ± 6.8	191.5 ± 4.1	192.7 ± 7	192.2 ± 4.5
	Sepsis	A	194 ± 7.3	200.6 ± 10.4	195.3 ± 3.5	191 ± 3.6	–
		V	199.4 ± 10.7	198.2 ± 10.6	197.3 ± 0.5	181 ± 23.5	–
maxO [mOsm/kg]	*Control*	A	379.2 ± 13.8	380 ± 12.7	380 ± 8.7	369.5 ± 11.2	382.2 ± 22.7
		V	377.7 ± 13.4	388 ± 13.2	383 ± 17	390.7 ± 14.4	391 ± 21.1
	Sepsis	A	379 ± 20.9	390.4 ± 28.1	392.6 ± 8.3	$394.6 \pm 14.3^{\#}$	–
		V	387.8 ± 15.9	391.2 ± 20.4	398.3 ± 12.8	386.3 ± 9.45	–
AUC	*Control*	A	117.7 ± 5.4	112.9 ± 5.1	109.3 ± 6.7	107.5 ± 8.9	109.9 ± 9.2
		V	112 ± 5.3	110.9 ± 5.4	108.3 ± 9.7	105.9 ± 5.9	104.3 ± 7.5
	Sepsis	A	115.3 ± 4.3	106.7 ± 15.3	108.1 ± 7.4	107 ± 11.5	–
		V	105 ± 12.5	104.9 ± 10.6	108 ± 2.6	107.7 ± 11.4	–

means $\pm$ S.D., $^{*}p < 0.05$ vs. base, $^{\#}p < 0.05$ vs. Control. A = arterial blood sample, V = venous blood sample at 4 h and at 6 h $n = 3$ in Sepsis Group (used test: Bonferroni's method of one-way ANOVA on rank test, Mann-Whitney rank sum test).

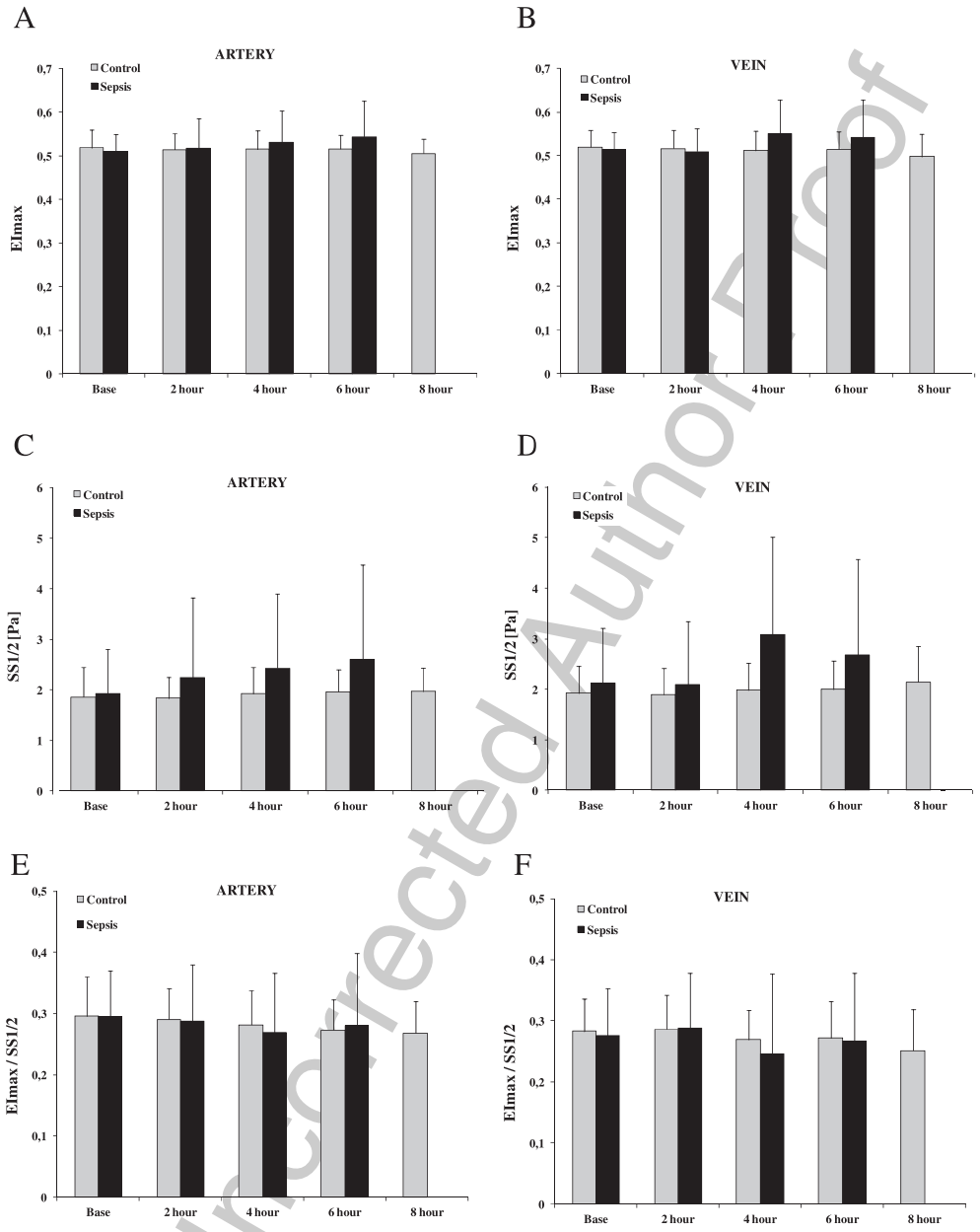

Fig. 2. EI_{max} (A,B), $SS_{1/2}$ [Pa] (C,D), $EI_{max}/SS_{1/2}$ [1/Pa] (E,F) values in arterial and venous blood of Control and Sepsis Groups. means ± S.D., $^{*}p < 0.05$ vs. base, $^{\#}p < 0.05$ vs. Control (at 4 h and at 6 h $n = 3$ in Sepsis Group; used test: Bonferroni's method of one-way ANOVA on rank test, Mann-Whitney rank sum test).

Investigating the osmoscan parameters, the minEI values continuously increased mostly in the arterial blood of the septic animals (at 6 h: $p = 0.017$ vs. base). The belonging osmolality values showed a decreasing tendency both in arterial and venous blood. The maximal EI lowered, the belonging osmolarity slightly increased in the septic animals (at 6 h: $p = 0.047$ vs. Control). The AUC showed larger differences:

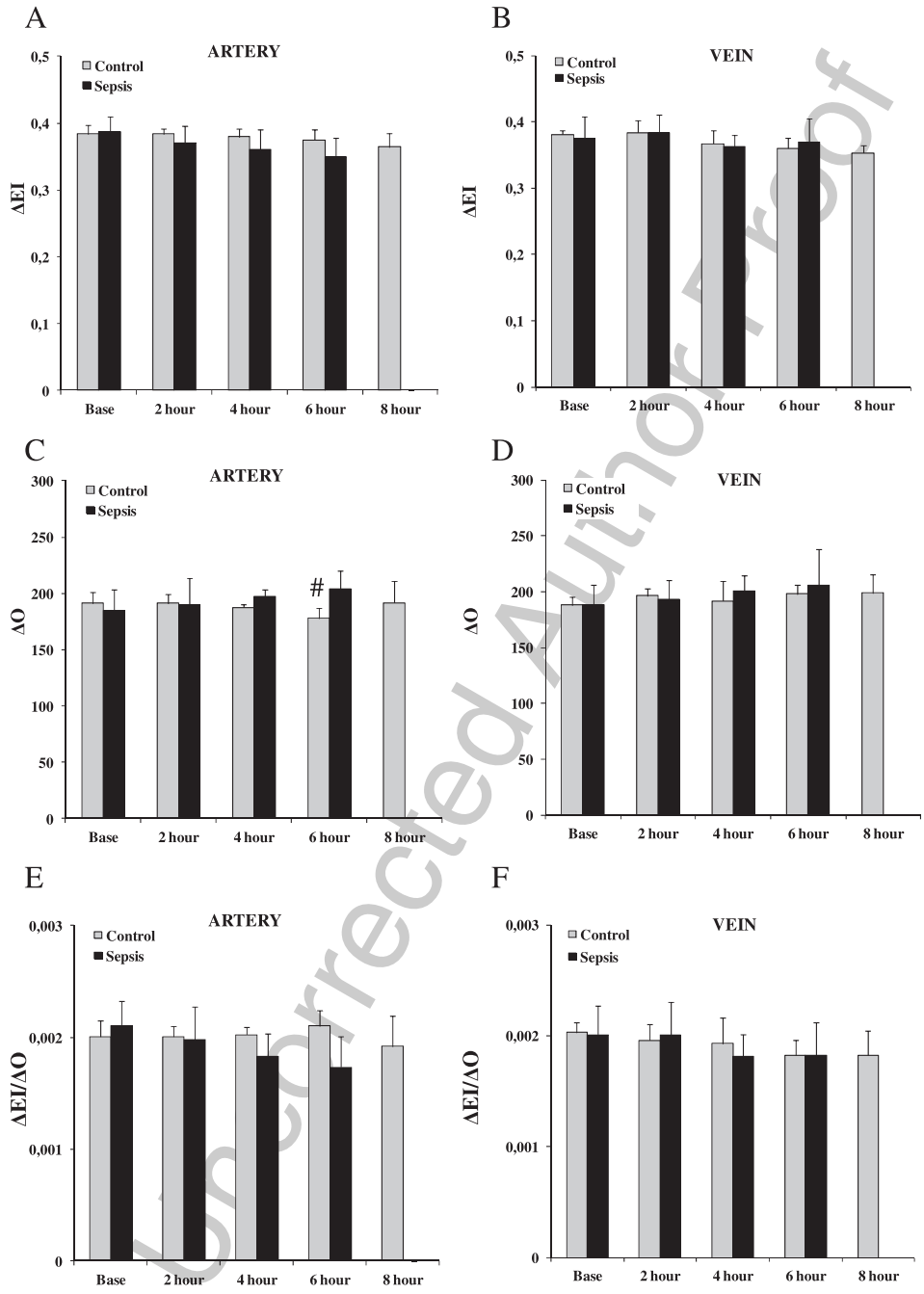

Fig. 3. ΔEI (A,B), ΔO (C,D) and ΔEI/ΔO (E,F) values in arterial and venous blood of Control and Sepsis Groups. means ± S.D., $^{*}p < 0.05$ vs. base, $^{\#}p < 0.05$ vs. Control (at 4 h and at 6 h $n = 3$ in Sepsis Group; used test: Bonferroni's method of one-way ANOVA on rank test, Mann-Whitney rank sum test).

it decreased by the 2nd hour and showed further lowering in the septic animals (Table 4). ΔEI values gradually decreased mostly in the arterial blood of the septic animals, while ΔO increased stepwise till the 6th hour. Their ratio (ΔEI/ΔO) showed more expressed alteration: it gradually decreased mostly in the arterial blood (Fig. 3, A-F).

3.6. Red blood cell aggregation

In Sepsis group the red blood cell aggregation index (AI%) remained almost unchanged till the end of the 4th hour, then declined (at 6 h: $p < 0.001$ vs. base and $p = 0.003$ vs. Control in the arterial blood; $p = 0.011$ vs. base and $p = 0.002$ vs. Control in the venous blood). This alteration was more prominent in the venous blood. The amplitude values showed marked arterio-venous differences in both the Control and Sepsis group. The declination, which was more expressed in venous blood samples, reached the significance level form the 2nd hour (at 2 h: $p = 0.001$ vs. base, at 4 h: $p < 0.001$ vs. base in the venous blood; at 6 h: $p = 0.02$ vs. base and $p = 0.043$ vs. Control in the arterial, and $p < 0.001$ vs. base and $p = 0.029$ vs. Control in venous blood). The $t_{1/2}$ was elongated and rose by the 6th hours in septic animals ($p < 0.001$ vs. base and $p = 0.003$ vs. Control in the venous, $p = 0.02$ vs. base and $p = 0.002$ vs. Control in the venous blood samples) (Table 5).

Parameters tested by the Myrenne aggregometer showed again a decreasing tendency in the Septic group at the 4th and 6th hour. The difference was significant by the 6th hours in case of the '5 sec M' (arterial blood: $p < 0.001$ vs. base and Control; venous blood: $p = 0.001$ vs. base and $p = 0.002$ vs. Control; arterio-venous difference: $p = 0.003$), '5 sec M1' (arterial blood: $p = 0.027$ vs. base; venous blood: $p < 0.001$ vs. base and $p = 0.023$ vs. Control), '10 sec M' (arterial blood: $p = 0.022$ vs. base, $p = 0.028$ vs. Control; venous blood: $p < 0.001$ vs. base and Control) as well as in '10 sec M1' (only in venous blood: $p = 0.027$ vs. base; arterio-venous difference: $p = 0.053$) index values (Fig. 4, A-H).

Table 5

Changes of red blood cell aggregation parameters (aggregation index, AI; amplitude, Amp and aggregation half time, $t_{1/2}$) in arterial and venous blood samples of the Control and the Sepsis groups during the experimental period

Variable	Group	Sample	Base	2 h	4 h	6 h	8 h
AI [%]	*Control*	A	67.39 ± 7.75	66.96 ± 3.48	64.65 ± 3.17	64.18 ± 5.4	61.09 ± 7.33
		V	67.28 ± 6.54	68.35 ± 2.93	61.36 ± 4.79	$67.57 \pm 2\ 34$	66.77 ± 4.39
	Sepsis	A	63.64 ± 3.39	61.35 ± 6.24	62.21 ± 6.39	$55.31 \pm 2.85^{*\#}$	–
		V	63.69 ± 2.78	62.75 ± 5.23	63.48 ± 5.12	$51.43 \pm 8.8^{*\#}$	–
Amp	*Control*	A	19.16 ± 1.67	20.61 ± 2.05	18.83 ± 3.43	20.52 ± 3.22	18.73 ± 2.63
		V	$15.37 \pm 0.67^{+}$	$15.4 \pm 1.78^{+}$	$12.81 \pm 2.84^{+}$	$12.96 \pm 3.71^{+}$	$12.16 \pm 2.45^{+}$
	Sepsis	A	20.85 ± 1.08	19.63 ± 2.84	19.96 ± 3.78	$16.39 \pm 5.31^{*\#}$	–
		V	$17.76 \pm 2.39^{+}$	$13.08 \pm 3.03^{+*}$	$10.93 \pm 2.74^{+*}$	$8.82 \pm 1.44^{+*\#}$	–
$t_{1/2}$ [s]	*Control*	A	1.88 ± 0.81	1.8 ± 0.33	2 ± 0.35	2.11 ± 0.59	2.51 ± 0.93
		V	1.85 ± 0.67	1.68 ± 0.27	2.37 ± 0.57	1.75 ± 0.28	1.83 ± 0.43
	Sepsis	A	2.17 ± 0.35	2.49 ± 0.8	2.28 ± 0.64	$3.19 \pm 0.44^{*\#}$	–
		V	2.16 ± 0.3	2.29 ± 0.61	2.23 ± 0.51	$3.85 \pm 1.48^{*\#}$	–

means ± S.D., $^{*}p < 0.05$ vs. base, $^{\#}p < 0.05$ vs. Control;+p<0.05 vs. A. A = arterial blood sample, V = venous blood sample at 4 h and at 6 h $n = 3$ in Sepsis Group (used test: Bonferroni's method of one-way ANOVA on rank test, Mann-Whitney rank sum test).

Table 6
Changes of selected micro-rheological parameters in normal venous blood samples ($n = 2$), and subsamples mixed with half or total dose of living *E. coli* bacteria just after the mixing and over 2 hours of incubation. Results of the supplementary *in vitro* study part

Variable	Base values	With half dose of bacteria after mixing	With total dose of bacteria after mixing	With half dose of bacteria after 2-hour incubation	With total dose of bacteria after 2-hour incubation
EI at 3 Pa	0.362 ± 0.003	0.350 ± 0.012	0.352 ± 0.001	0.342 ± 0.011	0.349 ± 0.001
EI_{max}	0.509 ± 0.004	0.517 ± 0.004	0.510 ± 0.009	0.490 ± 0.008	0.485 ± 0.015
$SS_{1/2}$ [Pa]	1.38 ± 0.06	1.64 ± 0.17	1.55 ± 0.17	1.41 ± 0.16	1.37 ± 0.07
EI_{max} / $SS_{1/2}$ [Pa^{-1}]	0.36 ± 0.01	0.31 ± 0.03	0.33 ± 0.04	0.35 ± 0.04	0.35 ± 0.02
minEI	0.112 ± 0.005	0.106 ± 0.014	0.096 ± 0.001	0.128 ± 0.001	0.103 ± 0.015
maxEI	0.498 ± 0.001	0.496 ± 0.003	0.486 ± 0.014	0.494 ± 0.005	0.482 ± 0.005
minO	192.5 ± 2.1	185.5 ± 2.1	185.5 ± 2	192.5 ± 0.7	189 ± 2.8
maxO	379.5 ± 14.8	361 ± 2.8	356.5 ± 7.7	371.5 ± 10.6	352.5 ± 4.9
AUC	115.9 ± 5.3	121.6 ± 3.8	113.2 ± 10.9	111.7 ± 9.5	109.7 ± 10.1
ΔEI	0.386 ± 0.015	0.390 ± 0.017	0.389 ± 0.023	0.365 ± 0.005	0.379 ± 0.02
ΔO	187 ± 16.9	175.5 ± 0.7	171 ± 5.65	179 ± 11.3	163.5 ± 7.7
ΔEI / ΔO *100	0.207 ± 0.027	0.222 ± 0.011	0.227 ± 0.006	0.204 ± 0.015	0.232 ± 0.023
AI%	59.31 ± 3.46	55.8 ± 2.9	46.12 ± 7.74	59.46 ± 8.45	47.77 ± 4.56
Amp	21.01 ± 0.29	15.87 ± 3.66	10.04 ± 6.87	15.8 ± 10.99	9.5 ± 7.28
$t_{1/2}$ [sec]	2.7 ± 0.44	3.17 ± 0.4	4.76 ± 1.54	2.69 ± 1.07	4.5 ± 0.83
5 sec M	3.06 ± 0.69	2.48 ± 0.65	1.4 ± 1.02	3.7 ± 0.97	1.68 ± 1.28
5 sec M1	6.17 ± 1.38	3.65 ± 1.14	2.7 ± 0.3	2.65 ± 0.78	1.82 ± 1.47
10 sec M	6.78 ± 1.72	7.68 ± 0.75	3.76 ± 1.88	10.5 ± 2.21	5.36 ± 4.68
10 sec M1	14.46 ± 7.63	11.38 ± 2.07	5.78 ± 1.87	10.63 ± 0.79	5.28 ± 4.59

means ± S.D.9.

3.7. Results of the in vitro study

Half dose of bacteria resulted in 5–6% decrease of red blood cell count, while it was about 13–15% when using the total dose. The values were slightly increased after the 2-hour incubation. The red blood cell distribution width (RDW-CV%) did not change just after mixing, while after the incubation period it increased by 4–5% and 9–10% when half or total dose of bacteria was used. The change of MCV did not exceed 2–4%.

Table 6 summarizes the red blood cell deformability (normal and osmotic gradient ektacytometry) and aggregation results. At half dose of the bacteria the aggregation parameters slightly decreased. At total dose of the *E. coli*, all the aggregation parameters were deteriorated: the index values (AI% and all the Myrenne index values) markedly decreased, which declination became more expressed after 2 hours of incubation. At the same time, red blood cell deformability impaired moderately.

4. Discussion

There are numerous animal models of sepsis using various methods to induce bacteremia, or using endotoxins among others [17, 18, 37, 43]. Our current model was based upon recent papers using living *E. coli* colony and administered intravenously in an increasing concentration within a relatively short period

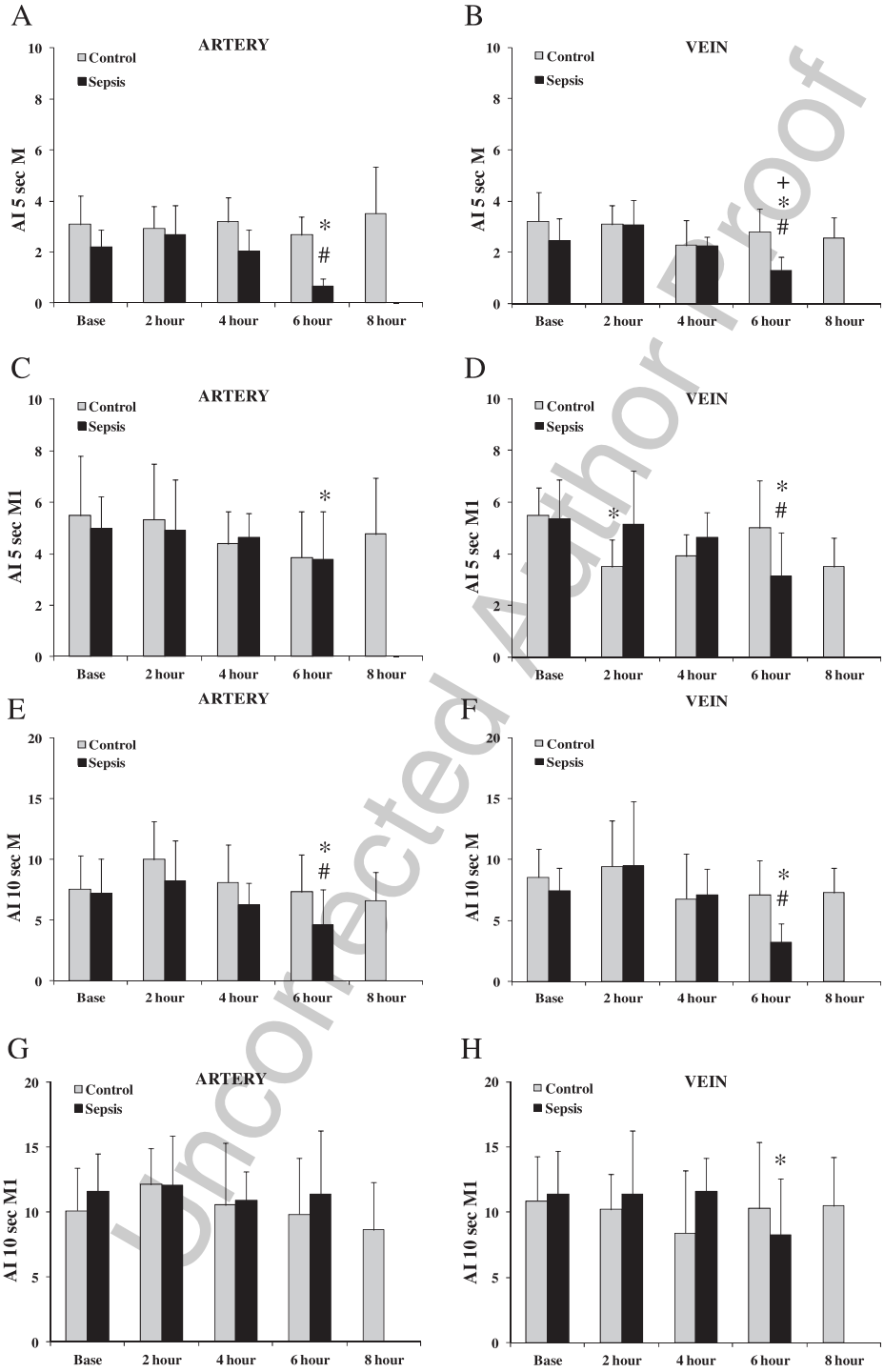

Fig. 4. Aggregation index 5 s M (A,B), 5 s M1 (C,D), 10 s M (E,F) and 10 s M1 (G,H) values in arterial and venous blood of Control and Sepsis Groups. means ± S.D., $^{*}p < 0.05$ vs. base, $^{\#}p < 0.05$ vs. Control (at 4 h and at 6 h $n = 3$ in Sepsis Group; used test: Bonferroni's method of one-way ANOVA on rank test, Mann-Whitney rank sum test).

[3, 12, 44]. We concentrated on the early effects of the developing fulminant sepsis. By our knowledge there is no other paper dealing with the early hemorheological changes during i.v. administered *E. coli* induced sepsis in the pig.

Chvojka et al. used a peritonitis-induced septic porcine model [14]. The measurements were completed 12, 18 and 22 hours after induction of the peritonitis. The hemodynamic parameters reached significant difference (decrease of MAP, increased of MPAP and CO, decrease in SVR, increase of CVP and PAOP) by 12 hours together with decrease in pH and rise in IL-6, TNF-α level and so reflecting the hyperdynamic circulation, the inflammatory response with oxidative and metabolic stress [14]. This was a peritonitis-induced model with a relatively quick development of the sepsis. In our model we used intravenous administration of living bacteria resulting in a rapid, fulminant sepsis. Wester et al. [46] used inactivated *N. meningitidis* for inducing sepsis in pigs. When skin and tongue microcirculation was investigated 200 minutes after sepsis induction, the clinical sings of the sepsis have been obviously developed. This period matches well with our experimental phase: the time period of 2–4 hours after starting the i.v. administration of the living bacteria.

Numerous papers reported important results on microcirculatory and rheological disturbances in sepsis [1, 2, 8, 13, 14, 16, 21, 25, 28, 30, 34, 40, 42, 45]. Most of them concluded significant worsening of the micro-rheological parameters: impaired red blood cell deformability and enhanced red blood cell aggregation is a common finding [e.g., 8, 28, 34, 42, 45].

Red blood cell deformability can be altered by numerous factors in septic process, including 2,3 diphosphoglycerate (by increased internal viscosity), nitric oxide (bound by hemoglobin, modulating membrane properties), increased intracellular concentration (by decreased activity of Ca^{2+} - ATPase pump), sialic acid (signal recognition for capturing by the reticulo-endothelial system, modifying cell shape and increasing aggregation), white blood cells (producing reactive oxygen species, increase aggregation with red blood cells), reactive oxygen species themselves (lipid peroxidation, sulfhydril-crosslinking in proteins, methemoglobin formation) [4, 34].

Baskurt and Mat [5] in a rat model of experimental sepsis clearly demonstrated that the temperature of the measurement (in the laboratory device) has a great importance in detecting the changes of micro-rheological parameters, such as red blood cell aggregation and deformability. While decreased elongation index of septic group has not been detected at 20 °C, 25 °C or even at 30 °C, the significant decrease of red blood cell deformability were found at 37 °C. Also red blood cell aggregation index values were obviously higher at body temperature [5]. Jagger et al. found significant alteration at 25 °C [20]. Piagnerelli et al. found a loss of biconcavity (the red blood cell shape was determined by flow cytometry) in septic patients compared to healthy volunteer. The difference was not depending on the temperature of sample [36].

Reggiori et al. investigated red blood cell deformability and aggregation values in intensive care unit patients, reporting early alterations of both micro-rheological parameters in septic patients. The elongation index decreased almost at all shear stresses. Aggregation index values increased and $t_{1/2}$ shortened than that of the non-septic patients [39].

Piagnerelli et al. [35] analyzed red blood cell membrane proteins in healthy volunteers, non-septic intensive care unit patients and septic patients (severe sepsis or septic shock). They found that red blood cell membrane skeletal protein content was modified in critical ill patients without additional changes in sepsis [35]. Other cell components have also great importance. Also Piagnerelli et al. [33] investigated erythrocyte shape and sialic acid content of the cell membrane in septic patients (blood samples were collected during the first 24 hours of the sepsis). They found by flow cytometry analyses membrane sialic acid content significantly lowered in septic patients, the glycophorine A content was higher than in the healthy volunteers. They found that spherical index significantly decreased in sepsis. Erythrocyte

of septic patients had a decreased capacity of sphericity in hypoosmolar conditions. It has been shown that red blood cells' membrane sialic acid content significantly decreases in sepsis related to increased neuraminidase activity [33].

Moutzouri et al. reported the evidence about decreased red blood cell deformability as a prognostic marker in sepsis, and also being useful for monitoring the severity of sepsis in the clinical practice [28]. The patients who developed nosocomial sepsis were entered into the study within 24 hours of the diagnosis; consequently the early hours of the septic procedures have not been investigated from the beginning. The impairment of erythrocyte deformability was obvious and expressed a gradual deterioration in sepsis, severe sepsis, ARDS, and septic shock, in this sequence. Red blood cell rigidity showed positive correlation with the projected mortality and the simplified acute physiology score (SAPS) II [28].

Leukocytes play a determining role in the pathophysiology of sepsis, having impact on the microcirculation, too [11, 32, 40, 47, 48]. Their margination is strongly influenced by the rheology: by the magnitude of the axial migration of the red blood cells. The erythrocyte aggregation is known to enhance it [29]. Kirschenbaun et al. demonstrated not only impaired red blood cell deformability and enhanced aggregation but also an increased platelet-neutrophil interaction with decreased leukocyte deformability [22]. Yodice et al. reported increased leuko-aggregation and decreased neutrophil deformability in patients with severe sepsis and septic shock [48].

Micro-rheological abnormabilities are characteristic not solely for sepsis and septic shock. Sordia et al. in a rat model of traumatic shock also found significant increased of erythrocyte aggregability and decreased deformability [41].

In our study we found a continuously decreasing red blood cell deformability of Sepsis Group during the experimental period. The changing in osmoscan parameters also occurred. The phase of the osmoscan curve that is related to the cell deformability and membrane stability (the curve part between min EI and maxEI together with the belonging osmolality values) showed the mostly expressed alterations. The difference between these points clearly showed a gradually worsening tendency. It might reflect not only the decreasing red cell deformability but the susceptibility of the cells to damage by altered micro-environmental osmotic conditions.

Interestingly, in our study we observed decreasing values of the aggregation-related parameters and it seemed that the aggregation process becomes slower and lower in its magnitude within 6th hour after starting the intravenous administration of the living bacteria. However, red blood cell deformability impaired. The systemic inflammatory response reaction was obvious, therefore the presence of sepsis occurred and mortal septic shock has been developed quickly. It is interesting to deal with the data of decreased aggregation of the erythrocytes in this experiment. It is obscure why the aggregation parameters showed an opposite behavior than in other studies. Speculating on this controversial finding it is important to note that our investigations were performed in parallel with the developing septic shock in a very early period of a fulminant sepsis. Furthermore the direct effect of the existing bacteremia (with an increasing concentration) might also have an effect on the aggregation. Therefore, we tested the effect *in vitro* in two normal blood sample that has been mixed with suspension of *E. coli* bacteria approximately in the same concentration as it was presented *in vivo* (according to the totally given amount of bacteria and the circulating blood volume). It was found that the impairment of red blood cell deformability and the decrease of aggregation also occurred *in vitro* and changed further after the 2-hour incubation period. It is important to note that this *E. coli* strain has hemolysin causing β-hemolysis. In the *in vivo* part of the study we did not observe any decrease in red blood cell count or changes in MCV (Table 2). In the *in vitro* study both the red blood cell count and the MCV decreased after mixing the blood with the bacteria suspension.

It is well-known that *E. coli* hemolysin jeopardizes the red blood cell membrane by creating pores with ring-shaped heptamer complexes, thus causing irreversible osmotic swelling. The presence of complement is also important. Furthermore, another way of red cell damage is linked to the endotoxin effect. Pöschl et al. concluded that endotoxin binding may directly affect the mechanical properties of the erythrocytes. The elongation index - shear stress curves were very similar to each other -and being significantly worsened compared to healthy control- in septic blood and blood samples *in vitro* incubated with endotoxin (LPS, *E. coli* O111:B4) [38]. The deterioration of red blood cell deformation (decrease of the elongation index) was associated with an increasing β-hydroxymyristic acid (HMA) concentration in cell membrane. The LPS in washed red blood cells resulted in much lower changes compared to the protocol when whole blood was mixed with LPS. They also suggested that endotoxin might have a maximum effect on red blood cell deformability: as of 25 μg LPS (corresponding to 0.66 μg/ml HMA) bound to red blood cell membrane [38].

Since the late 1990s, the role of nitric oxide (NO) has been widely investigated. The increase of NO in septic shock -but with an initial deficit- is known to contribute to the early microvascular deterioration. Morel et al. [27] used a rat CLP (caecal ligation and puncture) model of sepsis, and found an increased NO concentration in the first 3 hours of the septic shock (peaked at 2 hours). In parallel, microcirculatory flow (tested by laser Doppler flowmetry) gradually decreased over the experiment till 3 hours after the septic shock onset. According to their results, NO deficit does not occur to cause adverse changes in the microcirculation during the very early phase of the septic process [27].

It is also known that NO has an effect on red blood cells improving their deformability and aggregation [4, 7, 10] The effect of NO in the very early hours of the septic process might have an influence on the micro-rheological parameters during septic process. Speculating with this issue, we cannot exclude the NO effect on the red blood cell aggregation when interpret the results: why we found sustained erythrocyte aggregation during the first 2–6 hours of the sepsis. However, this issue should be further investigated with other strains of bacteria, too.

5. Conclusion

Due to the complexity of the findings and also in respect of the case number of this study, we cannot state that these micro-rheological changes are characteristic. However, the tendency of impairment in red blood cell deformability (together with osmotic gradient ektacytometry parameters) and the controversial decreasing of red blood cell aggregation values (declining all aggregation index values and amplitude, elongating $t_{1/2}$) were well detected in this porcine model during the early hours (4–6) of fulminant sepsis that was induced by i.v. administration of living *E. coli*. The *in vitro* effect of these bacteria on erythrocytes' micro-rheological parameter was similar: decreasing red blood cell deformability and lowering aggregation. Further studies are needed for clarifying the early micro-rheological changes of bacteremia and the developing sepsis.

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