SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Haemodynamic and hemorheological changes in the fulminant sepsis model induced with E. coli suspension

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Introduction

In the last decade the occurrence of sepsis has been showing an increasing trend. In Germany between 2007 and 2013 the occurrence increased by 15% every year, the mortality of the pathological picture is 30.5% with a mere annual decrease of 0.8%, despite the development of the intensive care. Besides the high incidence and mortality numbers the high cost of caring for the septic patients can't be neglected either whose estimated amount in Germany was 9.1 million in 2013 which means 3% of the health budget.

Considering that the data of the developing and low-income countries are available partly only, the global incidence of sepsis and the number of fatalities are not known. Based on a

study published in 2016 there are 31.5 million sepsis cases worldwide, out of these 19.4 million cases are severe which leads to 5.3 million deaths worldwide. In 2019 Jean-Louis Vincent and his work-group published a meta-analysis during which they summarised the data of the European and North-American studies dealing with the mortality frequency of the sepsis and septic shock based on the observations between January 1, 2005 and February 20, 2018 based on the Cochrane database. The average mortality was 37.3% at the ICUs while the hospital mortality was 39%. When the septic shock was diagnosed based on the criteria of the Sepsis-3 consensus, the estimated mortality at the ICU and at hospital level was 51.9% respectively 52.1%.

The septic process is a general reaction given by the body, but the clinical picture is not homogenous at all. Since the exact patho-mechanism of the forming of the sepsis is not known, it can be learned through studies whose objective consists of getting to know more exactly the pathophysiological background and the therapeutic possibilities of the syndrome. The various septic animal models serve for this purpose.

In my thesis - after the summary related to the sepsis and the septic animal models - I am summarising the results obtained through our septic animal model.

Objectives

Our current work is part of a complex research plan whose objective consisted of studying the variation of the haemodynamic, haemostaseological, hemorheological and cerebrovascular components created in the pig model during the sepsis induced by the E. coli. Considering that so far in the septic animal models the high-level haemodynamic monitoring applied during the human sepsis was applied scarcely,

- we aimed primarily to describe the early systemic haemodynamic variations induced with the IV-administered E. coli suspension by using the parameters of the PiCCO monitor.
- The next objective of our examinations consisted of studying the changes in the chosen model during the early sepsis, that concern the microrheological conditions.

Methods

Experimental animals and experiment protocol

The license to experiment with animals was registered and approved by the Animal Welfare Committee of the University of Debrecen (number of experimental license: 21/2013. UD CAW) based on the XVIII/1998 Animal Protection Law of Hungary and the 40/2013 Hungarian Decree which complies with the EU directives (2010/63/EU).

During our experiment we used 31 healthy female Hungahib pigs. The average age of the animals was 10-12 weeks. The pigs were randomized into two groups: in the case of the haemodynamic measurements there was a control group (n=15) and a septic group (n=16); in the hemorheological examinations there was a control group (n=4), and a septic group (n=5). The average weight of the animals was 18.9 kg in the control group (between 16.7 and 20.4), and in the septic group it was 18.9 kg (between 18.9 and 20.6). The average body length was 92 cm in the control group (between 83.5 and 97.2) and 92 cm in the septic group (between 85 and 98.2).

The experiments were performed in general anaesthesia during which the animals received 15 mg/body kg of Ketamin and 1 mg/body kg of Xylazin intramuscularly. The anaesthesia was

sustained based on the vegetative signals occurring for the painful stimuli, and based on the heart pulse and blood pressure increase, if it was necessary, we repeated the Ketamin and Xylasin dose. After inducing the anaesthesia we performed tracheostomy through which we placed an endotracheal tube into the windpipe. After having surgically prepared the left internal jugular vein and the left femoral artery we introduced an arterial and venous cannula through which in the next steps we took the experimental blood samples, and we performed the haemodynamic measuring with these. After inserting the endotracheal tube we performed pressure-assisted mechanical respiration (Airox Legendair Ventilator, PAU CedexFrance) during the experiment. The purpose of the mechanical ventilation consisted of assuring a partial arterial oxygen pressure of 100-130 mmHg and a partial arterial CO2 pressure of 35-45 mmHg. The temperature of the surgery room was about 25 ^{0}C, there were warming cushions under the pigs with which we assured the core temperature of the animals at 37^{0}C. The urination was solved through a cystostomic bladder catheter inserted suprapubically. Besides this the animals did not undergo any therapeutic intervention. The quantity of the administered IV fluid in the septic group corresponded to the volume of the administered E. coli suspension which – in the control group - was replaced with physiological salt solution.

The animals of the septic group intravenously received Escherichia coli culture suspended in physiological salt (2.5X10^5/ml; ATCC 25922, Medical Microbiological Institute of the University of Debrecen), gradually increasing the administered quantity. According to the protocol in the first 30 minutes we administered 2 ml of bacterial culture suspended in physiological salt, then in the next 30 minutes we administered 4 ml of bacterial culture. Then we administered 32 ml of bacterial culture in 120 minutes. Altogether we administered a total dose of 9.5x10^6 E. coli in 180 minutes. Based on the test performed in the local laboratory the number of the live bacteria remained stable after so much time.

We performed the examinations on the animals of the septic group till their death – taking the fatal infection into consideration. We observed the members of both groups for 8 hours, except for the cases when the experimental animal perished earlier. At the end of the experiment the animals were sent to sleep.

During the examination – besides the intravenously administered physiological salt solution and the pressure-assisted respiration – there was no liquid-based resuscitation, medication-based or other therapeutic intervention.

Haemodynamic measuring

We measured the haemodynamic variables with the transpulmonary thermodilutional and pulse contour analysis technique. For this we used the PiCCO^{TM} (PULSION Medical Systems AG, Munich, Germany) device. For the haemodynamic measuring we cannulated the left external jugular vein and the left femoral artery. Besides the haemodynamic measurements the cannulas served as sampling points, too. After the surgical interventions the animals underwent a one-hour stabilisation period after which we began the next phase of the experimental protocol. The measuring of the systemic haemodynamic variables took place with thermodilution for which we used 4 F-thick, 8 cm-long PiCCO® catheters (Pulsion Medical System AG, Munich, Germany). During the thermo-dilution we injected 10 ml of cold salt solution (T< 8 ^{0}C) every hour through the central vein cannula, with this we regularly calibrated the pulse contour analysis, too. We monitored the heart rate, the arterial mid-pressure (MAP) invasively through the arterial femoral catheter. In order to create the indices, to determine the pig's body surface we used the Meeh formula:

BSA (body surface area) = 8.58xBW (body weight)

Regarding the PiCCO parameters the cardiac index, the stroke volume variation (SVV), the global end-diastolic volume, the global ejection fraction, the extravascular pulmonary fluid index and the systemic vascular resistance index were recorded.

Data collection

We performed the first measuring before inducing the sepsis, in the so-called rest phase (resting state –RS). Then the measuring took place every 60 minutes after the injection of the E. coli suspension or the physiological salt solution till the death of the animal. The measured data were recorded from T60 to T360. At the T60 moment the injection of 2+4 ml of E. coli suspension or salt solution terminated. At the T120 respectively the 180 time moment we terminated the injection of 2x16 ml of E. coli suspension, meaning that we finished the sepsis-inducing protocol related to the scheduled 38 ml of E. coli solution.

Statistical analysis

During the statistical analysis we used the SPSS 19.0 (SPPS Chicago, IL). In order to verify the normal distribution of the continuous variables we applied the Kolgomorow-Smirnov test. The majority of the parameters did not show a normal distribution, we presented the data in the median and interquartile domain then with the proper non-parametric test (Mann-Whitney Rank Sum Test) we compared the groups. The analysis of the variations appearing in the

group, occurring due to the intervention on the various parameters, took place with repetitive ANOVA measuring. For the calculation of the correlation between the variables we used the Spearman correlation. As a significant deviation we determined the $p<0.05$ value.

Hemorheological measuring

We involved 9 pigs for the hemorheological measuring, where the control group had n=4 cases and the septic group had n=5 cases. The experimental animals did not receive anticoagulant therapy. After placing the central vein cannula and the arterial femoral cannula we took blood samples simultaneously from the two cannulas. This sample (further on: basic sample) served for the basic measuring. The sampling was taken directly into a test-tube that contained K_3-EDTA (1.5 mg/ml), later on we performed every laboratory test from this. The vein-based blood sample used for the sedimentary measuring was an exception where we used 0.109 M sodium-citrate for anticoagulation purpose. The further arterial and vein-based blood sampling took place 2, 4, 6 and 8 hours after inducing the sepsis. At the time of the laboratorial sampling we recorded the core temperature (central vein-based T [^{0}C]), the arterial mid-pressure (MAP [Hgmm]), the heart-rate (HR [1/minute] and the oxygen saturation (Sat [%]), too.

In vitro study: The effect of the presence of the live bacterium on the blood's rheological characteristics

We took samples from two animals for the examination. We divided the samples into two sub-samples to which we added the 50% respectively 100% dose of the E.coli suspension used for the sepsis induction, and calculating with a 60 m/ body kg of circulating blood volume according to the in vivo examination. After adding the E. coli in 15-20 minutes, and after the 120-minute incubation taking place at room temperature we performed micro-rheological examinations – red cell deformability and aggregation, osmotic gradient ektacytometry.

Laboratory examinations (in vivo)

Haematological characteristics

For the determination of the haematological parameters we used the Sismax F-800 semi-automatic microcell counter (TOA Medical Electronics Co., Japan). We compared the white cell count (WBC [x10^3/ μl], red cell count (RBC [x10^6/ μl], the haematocrit (Htc [%]), the

average corpuscular volume (MCV [fl]), the average haemoglobin content (MHC [pg/l] and the thrombocyte count.

Erythrocyte sedimentary rate and the leucocyte anti-sedimentary rate

Placing the test-tubes into vertical position that contain the venous blood samples inhibited to coagulate with sodium-citrate, we performed gravitational sedimentation. After 60 minutes we recorded the regular erythrocyte sedimentation rate. In order to determine the leucocyte anti-sedimentary rate (LAR) we carefully separated the upper and lower part of the blood column and in both samples we determined the white cell count. We calculated the LAR based on the Bogár-formula.

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

Blood and plasma viscosity

The measuring took place with the Hevimet-40 capillary viscometer (Hemorex Ltd. Hungary) within 60 minutes after sampling at 37 ºC. We centrifuged the plasma for 10 minutes at a revolution speed of 800g. We compared the total blood viscosity (WBV [mPas]) values with each other at a shearing stress of 90 s^{-1} and we adjusted it to the haematocrit value of 40% according to the Mátrai formula.

Mátrai formula: $(WBV_{40\%}/PV)^{40\%/Htc}$, where

$WBW_{40\%}$: total blood viscosity adjusted to the haematocrit of 40%

PV: plasma viscosity

Htc: the current haematocrit value of the sample

Red blood cell deformability

The deformability was determined with the LoRRca MaxSis Osmoscan (Mechatronics BV, the Netherlands). We added a blood sample of 5 ul to 1 ml of isotonic polyvinyl-pyrrolidone (PVP) solution (360 KDa PVP normal phosphate puffered salt-solution; viscosity=27 mPas, osmolality=290-300 mOsm/kg, pH=7,3). At a shearing stress between 0,3-30 Pa (SS) we determined the elongation index (EI) according to the variation of the laser diffraction pattern. The elongation index equals the (L-W)/(L+W) ratio, where L corresponds to length, W to width in the diffractogram. The higher the red cell deformability is, the higher the EI value is.

The measuring took place at 37 ^{0}C. We performed the Lineweaver-Burk analysis by comparing the EI-SS curves to calculate the maximum elongation index (EI_{max}) and the shearing stress at half EI_{max} ($SS_{1/2}$ [Pa]: $1/EI=SS_{1/2}/EI_{max}$ x $1/SS+1/EI_{max}$). Then we calculated the $EI_{max}/SS_{1/2}$ ratio, too. (210) For the osmotic gradient ektacytometry or osmoscan test we carefully mixed 250 µl of blood with 5 ml of PVP solution. At the constant shearing stress of 30 Pa we determined the elongation index values continuously in parallel with the gradual increase of the osmolality from 0 to 500 mosmol/kg. Besides the parameters analysed with the device we measured the minimal elongation indices in a low osmotic environment (minimal EI), the maximal elongation index value (maximal EI), the related osmolality values (minO and maxO, as „optimal" osmolality), and determined the area under the individual elongation index- osmolality curve (AUC). Next we determined the parameters with which we characterised the phase between the minEI and maxEI in the osmolality function: deltaEI is the difference between the maxEI and min EI; deltaO, the difference of osmolality between the osmolalities belonging to the maxEI and minEI values; and the deltaEI/deltaO ratio.

Red blood cell aggregation

We used the Myrenne MA-1 erythrocyte aggregometer (Myrenne GmbH, Germany) to determine the aggregation indices whose functioning is based on light-permeability capacity: M (at a shearing ratio of 0 s^{-1}) és M1 (at a shearing ratio of 3 s^{-1}) 5 or 10 seconds after the disaggregation. The indices (M 5 s, M1 5 s, M 10 s, M1 10 s) are increasing with the increased red cell aggregation. We also applied the LoRRca device's syllectometric measuring technique to determine the following red cell aggregation parameters: amplitude (Amp [au]); aggregation index (AI [%]); aggregation half-time ($t_{1/2}$ [s]).

Statistical analysis

We interpreted the obtained data according to the average and standard deviation. According to the normal distribution of the results we performed the comparison between the groups (control versus septic) based on the Student-t or the Mann-Whitney RS test. The comparison of the data obtained within the group with the base values took place with the one-way ANOVA test (Dunn or Bonferroni method). In the case of p we considered the result as significant from statistical perspective at a value below 0.05.

Results

Haemodynamic measuring

Regarding the 16 septic animals two perished after commencing the experimental protocol of the induced sepsis, two animals perished 2 hours later, five pigs after 3 hours, three animals after 4 hours, and one perished after 6 hours. The haemodynamic parameters of the septic and control groups were statistically similar before injecting the E coli suspension. In regards of the control pigs the haemodynamic variables were relatively stable in the examination period. The arterial mid-pressure value decreased in the sixth hour, while the systemic vascular resistance index increased from the fifth hour. In the septic group the septic shock occurred in the 176th minute (IQR:60-263) from the beginning of the injection of the E. coli suspension. When examining the flowing parameters one could observe the decrease of the cardiac index.

The systemic vascular resistance index showed a temporary increase in the third hour, but it returned to the initial value in the later examination period. During the contour analysis of the pulse the stroke volume variance showed a significant increase, along the insignificant decrease of the global end-diastolic volume index (volume parameter). The extravascular pulmonary fluid index showed an increasing trend, while the global ejection fraction decreased during the examination.

Results of the hemorheological examinations

The haemodynamic and mortality characteristics of the pig subgroup participating in the hemorheological measuring

Regarding the hemorheological measuring we analysed the data obtained from altogether nine animals. In the case of each experimental animal of the septic group (n=5) there was fulminant sepsis and the perished in 3-7 hours (2 animals in the 3-4th hour, 3 animals in the 6-7th hour). In regards of the septic group the heart rate increased, the arterial mid-pressure decreased. The heart frequency /MAP ratio showed a significant decrease compared with the initial values in both the control group and the septic group. After 6 hours from commencing the injection of the E. coli solution the p value <0.001 during the statistical analysis within the septic group, and the p=0.004 during the statistical analysis in the control group. Regarding the individuals of the control group we measured stable haemodynamic parameters during the experiment.

Haematological parameters

The total leucocyte count in the septic group showed a continuously decreasing trend both in the arterial and the venal sample. In the 4th and 6th hour it was significantly lower than the initial value and compared to the control group ($p<0,001$). In the case of the control group a steady increase could be observed by the end of the experimental period. The monocyte-granulocyte % showed a slight increase in both groups but this did not reach the significant difference.

The red blood cell count remained almost constant but in the 6th hour of the experiment it increased in parallel with the haematocrit value in the septic group. Between the groups there was a significant difference regarding the red cell count's increase in the 6th hour of the experiment in both the arterial and the venous sample. The MCV values were minimally higher in the septic group compared to the control group.

The thrombocyte count decreased dominantly in the case of the septic animals, there was a significant difference in the 6th hour after beginning the injection of the E. coli suspension between the groups and even within the septic group compared to the initial value.

Erythrocyte sedimentation rate and leucocyte anti-sedimentation rate

The erythrocyte sedimentation rate (ESR) remained constant with the animals of the control group till the end of the experiment. In the case of the septic animals it showed a gradual increase till the 4th hour, then it began to decrease.

During the analysis performed at every second hour the leucocyte anti-sedimentation rate showed a decrease of 15-20%. The extent of the decrease showed a significant difference compared to the control group, too; in the 2nd hour $p=0.031$, in the 4th hour $p=0.05$, in the 6th hour $p=0.049$.

The blood and plasma viscosity

The total blood viscosity (WBV) decreased in medium at the animals of the control group during the experimental period, but in the meantime the plasma viscosity did not change significantly. Regarding the WBV values adjusted for the haematocrit of 40% there was no significant deviation either in the case of the control or the septic animals. In the septic group the total blood viscosity began to rise in the fourth hour, however the plasma viscosity showed continuous decrease.

Red blood cell deformability

The red cell deformability showed a worsening trend with the animals of the septic group. In the arterial and venous blood samples taken every second hour the $SS_{1/2}$ increased gradually, while the $EI_{max}SS_{1/2}$ ratio decreased gradually. At the same time this change was not significant. When comparing the arterial and the venous blood samples we did not perceive significant variation in regards of deformability.

When examining the parameters measured by the osmoscan the minEI values showed a continuous increase in the case of the septic animals, more strikingly in the arterial blood samples. In the 6th hour of the experiment and compared with the control group the p value was 0.047. The AUC showed bigger deviations. It began to decrease even in two hours after the induced sepsis, then it kept showing this trend. The deltaEI values decreased gradually, mostly in the arterial blood samples of the septic animals, while the deltaO showed a gradual increase till the 6th hour of the observation period. The deltaEI/deltaO ratio showed a more striking deviation, it decreased gradually, mostly in the arterial samples.

Red cell aggregation

In the septic group the red cell aggregation index (AI%) remained almost constant till the 4th hour after the induced sepsis, then it began to decrease. During the examination of the samples taken in the 6th hour - and comparing with the initial values and the control group's values of the same time – the decrease was significant. The p value < 0.001 in the group, p =0.003 in the case of the arterial samples relative to the control and p=0.11 in the group and p=0.02 in the case of the control venous samples. This change was more pronounced in the case of the venous samples. The amplitude values showed a striking arterio-venous difference both in the septic and the control group. The decrease was more explicit in the venous blood samples, it reached the significance level even in the 2nd hour after the induced sepsis (2nd hour: p=0.001 vs. initial venous sample; 4th hour: p<0.001 vs. initial venous sample; 6th hour: p=0.02 vs. initial arterial sample and p=0.043 vs. control group's arterial sample; 6th hour: p<0.001 vs. initial venous sample and p=0.029 vs. control group's venous sample). The $t_{1/2}$ time got longer, and increased in the 6th hour in the group of the septic pigs (p<0.001 vs. initial sample) and the compared with the control group (P=0.003 vs. control group) both in the case of the venous samples (p=0.02 vs. initial sample; p=0.002 vs. control group) and the arterial samples.

In the case of the parameters measured with the Myrenne aggrometer there was also a decreasing trend in the case of the septic animals in the 4th and 6th hour. The difference proved to be significant in the 6th hour in the case of 5 sec M (arterial sample: p<0.001 vs. initial sample and control group; venous sample: p=0.001 vs. initial sample and p=0.002 control group; arterio-venous difference: p=0.003), in the case of 5 sec M1[1] (arterial sample: p=0,027 vs. initial sample; venous sample: p<0,001 vs. initial sample and p=0.023 vs. control group), in the case of 10 sec M (arterial sample: p=0,022 vs. initial sample, and p=0.028 vs. control group; venous sample: p<0.001 vs. initial sample and control group) index values. In the case of 10 sec M1 we found significant difference only for the venous samples. (p=0.027 vs. initial value; arterio-venous difference: p=0.00053)

Results of the in vitro examination

The half dose of E.coli suspension caused a decrease of 5-6% in the red cell count, while in the case of the full dose this was 13-15%. After the 2-hour incubation the values showed a slight increase. The spreading width of the red cells (RDW-CV%) changed only after the mixing, after the incubation period the increase was of 4-5% after a half bacterial dose, after the full dose there was an increase of 9-10%. The variation of the MCV value did not exceed 2-4%. After a half dose of E.Coli suspension the aggregation parameters decreased slightly, while after the full dose every aggregation parameter began to worsen: the aggregation index % and the Myrenne index values decreased strikingly, which became more explicit after the 2-hour incubation. Simultaneously the red cell deformability worsened at medium level.

Discussion

The sepsis is still a heterogeneous clinical pathological picture that can be differentiated with difficulty, whose mortality is rather high despite the improving therapeutic possibilities. A study of 2016 tried to estimate globally the sepsis incidence and mortality based on the data of the last 10 years. According to the processed data every year and worldwide 31.5 million septic and 19.4 million severely septic patients are expected to be treated which will result in 5.3 million fatalities. In the development of the efficient medication-based therapy a proper animal model might be an important milestone in the case of sepsis which perfectly emulates the human sepsis and its pathomechanism. Unfortunately the majority of the currently used animal models provide misleading findings. One of the main reasons is that during the sepsis the gene-expressions occurring as caused by the trauma or infection differ significantly from

the human gene-expression in the case of the experimental species used most frequently, that is the mouse. Another important factor is that several animal species used for the experiments, such as the mouse or baboon, are resistant to the bacterial lipopolysaccharides, while the human body is rather sensitive to these.

During our current study we created gram-negative sepsis of Hungahib pigs through bacteremia model. The pig model can be used well in the human sepsis research, when comparing the rodents, their anatomic and physiological features are close to the human characteristics. The size of the pigs enables the use of the tools and monitors used in the human clinical area, and because of their proper blood volume they are suitable for the repetitive blood sampling and comparison analyses, without significantly influencing the haemodynamic parameters and the quality of the samples. One should not neglect the fact that the sensitivity of the pigs to the lipopolysaccharides is similar to the human sensitivity, when comparing for example with the resistance of mice. During the study we analysed the early haemodynamic and hemorheological consequences of the sepsis without performing any therapeutic intervention, meaning that actually we wanted to study the spontaneous sepsis. In order to induce the sepsis we applied an E.coli suspension with a $2.5x10^5$ germ count, with a total dose of $9.5x10^6$. There are several septic animal models during which various techniques are applied to induce the sepsis. The bacteremia model used by us is inducing the sepsis through the IV injection of the living E.coli suspension of a continuously increasing concentration. The workgroups of Thorgesen and Castellheim used a similar approach. Castellheim intravenously and intra-pulmonarily injected living E.coli suspension of a low (total dose of $4x10^9$ bacteria) and high (total dose of $16x10^9$ bacteria) dose with an increasing concentration into pigs throughout a relatively short timeframe, with this model the imitated the gram-negative sepsis. Haemodynamically the septic group was characterised by striking pulmonary arterial pressure increase and systemic decrease of blood pressure. The time of the hyperacutely forming pulmonary hypertension was strongly correlated to the beginning of the E. coli infusion and its severity was directly correlated to the dosing rate. The forming of the sepsis was also confirmed – besides the haemodynamic variables – by the increase of the interleukin (IL)-10, IL-12, the vascular endothelial increase factor (VEGF) and the increase of the thrombin–antithrombin complex (TAT) concentration. During the observation performed by us we paid attention to the examination of the haemodynamic and hemorheorological effects of the early fulminant sepsis. In the case of pigs, as far as we know, the early hemorheological deviations were not examined yet on sepsis model induced with intravenous

E. coli, respectively the haemodynamic variations were not examined with the PiCCO monitor.

The cardiac output monitoring is a proving method in the clinical area to monitor the haemodynamics of the patients in critical condition and to optimise the fluid, vasopressing and inotropic therapy. For the studying of the haemodynamic deviations of the pigs' sepsis model Schuerholz and his workgroups already applied successfully the continuous pulse contour cardiac output (PiCCO) monitoring but their study aimed to determine the frequency of the calibration required for the accurate functioning of the device. For the induced sepsis they introduced autologous faeces in the abdominal cavity obtained through coecostomia and the forming of sepsis was proved by the positive haemoculture. Barett et al. examined the effect of the polyvalent immunoglobulin with the septic pig model after the intravenous injection of the E. coli during which they observed decrease of arterial mid-pressure and systemic vascular resistance index, as well as the increase of heart frequency. Pranskunas et al. used pulmonary arterial catheter (PAC) to monitor the haemodynamics, also on the sepsis model established with the intravenous E. coli infusion. During their measuring the cardiac index decreased to its half, the systemic vascular resistance doubled after inducing the sepsis. Chvojka and team used the peritonitis-induced pig sepsis model during their experiment. They performed their measuring after the induced peritonitis, after 12, 18 and 22 hours. For monitoring the haemodynamics they also used the pulmonary arterial catheter (PAC). They observed significant deviation in connection with the measuring of the haemodynamic parameters in the 12^{th} hour of the observation which occurred in the form of reduced arterial mid-pressure, cardiac output and systemic vascular resistance and increased pulmonary arterial mid-pressure and pulmonary wedge-pressure. Besides the haemodynamic changes they experienced pH decrease, IL-6 and TNF-alpha increase, too. This peritonitis-induced sepsis model led relatively fast to the forming of the sepsis.

The results obtained by us can be compared well with the previous septic pig studies. During our observation in the process of forming of the septic shock we observed the decrease of the arterial mid-pressure and the cardiac index, with a pulse count of increasing trend. In addition we experienced significant decrease regarding the stroke volume variation value in the case of the septic animals. However it is worth noting that during our experiment we did not administer IV fluid to the animals since we studied the natural process of the haemodynamic changes occurring during the sepsis, without therapeutic interventions. This explains the fact that in the case of the control group's animals we perceived a slow decrease of the arterial

mid-pressure. When we matched the decrease of the arterial mid-pressure of the control animals with the initial value, this proved to be significant from statistical perspective, but the MAP values remained in a relatively stable range of 96-103 mmHg. The animals were anaesthetized with the ketamin-xylazin combo. Since during the analysis of the cardiac index during the experiment we did not perceive statistically significant deviation in the control group, in this way the cardiodepressive effect caused by the medication could be excluded.

Earlier several studies proved that with the intravenous infusion of the living E. coli bacterium early cardiovascular collapsing and the early death of the experimental animal can be caused. Tracey et. al. examined baboons for the role of cachectin (tumour-necrosis factor alpha, TNF-alpha) in the septic shock. For this experiment they used they introduced a lethal dose of (LD_{100}) living E. coli suspension to induce the septic shock. In the animals of the control group hypotension, lethal kidney and pulmonary failure were forming due to this. In the case of the same model the pre-treatment of the animals with TNF-alpha monoclonal antibody improved the animals' haemodynamic parameters and the survival. In the case of small mammals the injection of a low dose of E. coli throughout several hours caused minimal haemodynamic variations but with a bigger dose it caused biphasic reaction. The early increase of the cardiac output was followed by decreased cardiac output in the later phase of the sepsis. Also in the case of baboons, after the injection of a sublethal and lethal dose of E.coli Taylor et. al. observed the forming of the severe disseminated intravascular coagulopathy which was accompanied by complex inflammatory and haemostatic reaction. This included the lesion of the microvascularisatory endothel and the anticoagulation system. In summary the process led to a multi-organic dysfunction. During the study performed by Dehring et al. with pigs they injected E. coli, P. aeruginosa (Gram-negative) and S. aureus (Gram-positive) bacteria and compared the consequences of the induced sepsis. They found that as long as the gram-positive bacteria caused minimal change compared to the initial condition, the E. coli and the P. aueruginosa caused shock and acute respiratory failure. In the case of a septic sheep the E.coli of non-lethal dose provoked hyperdinam cardiovascular reaction with hypotension, increased cardiac output and tachicardia. In the case of dogs both the sublethal and the lethal dose of living E. coli caused severe cardiovascular lesion with hypotension, with very low cardiac output, splanchnicus hypoperfusion and severe metabolic disorder. Based on the mentioned earlier sepsis models it is clear that the haemodynamic reaction caused by the intravenously injected E. coli is influenced significantly also by the animal species used for the experiment.

It is clear that the features of the septic shock caused by the intravenous E. coli infusion differ from those of other septic experimental models. During the fulminant septic shock created by us mono-phase hypodinam circulatory parameters could be observed. Other sepsis models can be characterised with biphasic reaction during which first there is a hyperdinam circulation being formed with increased cardiac index and cardiac output, with low arterial mid-pressure and systemic vascular resistance index. In the later, the so-called hypodinam phase a low cardiac output and systemic vascular resistance can be observed.

It seems that the inflammatory reaction caused by the E. coli is influencing directly the contractility of the myocardium. It leads to the decrease of the cardiac index and the global ejection fraction during the process, which can be the leading cause of the hypodinam circulation. Due to the hypodinam circulation reaction - during our experiment – one could observe an immediate microcirculatory lesion, too, even in the early phase of the bacterial infusion. At the same time the cerebral perfusion remained intact. It could also be observed that because of the worsening haemodynamic status the lactate level began to rise which was followed by the increase of the count of the cored red cells which indicated a clear hypoxia at tissue level. In this format the intravenous E. coli infusion caused a fulminant hypodinam sepsis in our pig model which led to hypoperfusion and hypoxia at tissue level. Wester et al. used inactivated N. meningitides for pigs in order to induce the sepsis. In the case of examining the micro-circulation of the tongue and the skin the clear signs of sepsis got formed 200 minutes after the induced sepsis. This incubation period can be matched well with the perceptions in our experiment during which the signs of the fulminant sepsis can be dated by the 2^{nd}-4^{th} hour considered from the beginning of the living bacterial infusion.

Currently the forming of the septic cardiomyopathy is not clarified in full, despite the fact that it has been a known problem for 40 years. Based on several studies the cardiomyopathy occurring during the sepsis can cause a 2- or 3-fold mortality increase. The supposed mechanisms are the impact of the inflammatory cytokines onto the direct myocardium, the myocardium oedema, the increased oxidative stress and the reduced expression of the adrenerg receptors, that lead to systolic and diastolic dysfunction and the dilation of the cavity system. During the experiment conducted by us the decrease of the cardiac index and the global ejection fraction can also signal this, besides the stroke volume index with a decreasing tendency.

Besides the haemodynamic parameters our experiment aims to study the variation of the hemorheological parameters during the early fulminant sepsis. Several studies published

findings in the past decades about the effects of the sepsis onto the microcirculation and the rheological parameters. Among these the majority provides information about the worsening of the microrheorological condition, such as the reduced red cell deformability, increased red cell aggregation. During the sepsis the deformability of the red cells is influenced by several factors. The 2,3-diphospho-glicerate is increasing the inner viscosity and the membrane's viscoelasticity. The Nitrogen-monoxide alters the cell's calcium homeostasis, by increasing the quantity of the intracellular free Ca^{2+} ions. During the sepsis the quantity of the sialic acid is decreasing in the membrane of the red cell by which the cell changes its form, the deformability will worsen and the red cell aggregation will increase. The white cells are releasing free oxygen radicals, peroxidases, proteases that alter the membrane structure, in this way damaging the deformability and increasing the aggregation.

During the experimental sepsis Baskurt et al. intubated septic and aseptic rats' red cells with endotoxin and demonstrated that during the examination of the microhemorheologival features, such as the red cell deformability or the red cell aggregation the temperature – at which the measuring takes place – is influencing the obtained results. During the measuring at 37^0C performed with ektacytometry there was significant decrease in the case of the elongation index. (36) Singh and Stolz examined the red cells of healthy volunteers and proved the significant decrease of the elongation index by reducing the temperature from 37^0C to 5 ^{0}C. Piagnerelli et al. performed flowcytometric observations with septic and healthy red cells. They found that the shape of the septic red cells is less biconcave, it is rather spherical, but the temperature of the sample did not influence the shape of the red cells.

There were several studies to compare the microrheological parameters of the healthy and septic red cells. Reggiori et al. examined the red cells of septic patients and healthy volunteers. The deformability of the red cells was significantly worse than in the case of the septic patients when examining at various shearing stress. The aggregation index increased, the $t_{1/2}$ shortened in the group of septic patients. The size of the elongation index was influenced only by the sepsis and the haematological co-diseases. Piagnerelli et al. published several study results that dealt with the microrheological effects of the sepsis on the red cells. In the case of patients in critical condition, treated at the ICU they described the variation of the proportion of the red cell membrane proteins compared to the red cells of healthy volunteers, but no difference was found regarding the composition of the red cell membrane skeletal protein of the septic and aseptic patients. Also Piagnerelli et al. examined the sialic acid and glicoforin A content of the membrane by involving septic patients and healthy

volunteers. The sialic acid content was determined with high-performance fluid chromatography, the glikoforin A content was determined with antiglikoforin with flow-based cytometry. In addition they evaluated the red cell shapes by applying the spherical index. The shape of the septic red cells is rather spherical instead of biconcave, the spherical index decreased significantly in the sepsis. The sphericity capacity of only the septic red cells decreased in the hypoosmolar environment. In the membrane of the septic red cells the sialic acid content decreased significantly, that can be caused by the increased neuraminidase activity. In addition the membrane's glikoforin A content was higher.

There were several human sepsis studies to explore the variation of the blood's hemorheological parameters and to determine the connection between the pathological status and the outcome. In a prospective study published by Donadello et al. in 2015 they compared the rheological parameters of the red cells measured with laser-assisted optical rotary cell-analyser of the septic and aseptic patients on the 1st and 3rd day of the admission to the ICU. In order to express the red cell deformability they used elongation index based on the shearing stress and besides this the aggregation index was also determined. In the examined group of patients the mortality of the septic patients was 31%. The red cell deformability was worse in the case of the septic patient even when being admitted to the ICU, during the examination this worsened in the case on the non-surviving septic patients. In the non-septic patient group this could not be observed. The values of the aggregation index were higher for the septic patients but the prognostic value of this was not confirmed. The deformability getting reduced in parallel to the therapy time signalled clearly the bad prognoses of the septic patients. Moutzouri and his work-team reported also about the prognostic significance of the red cell deformability in terms of sepsis in a study published in 2007. It was useful in forecasting and even for the monitoring of the severity of the sepsis in the clinical practice. Nozocomial septic patients were selected for the study in 24 hours after the diagnosis. The decrease of the red cell deformability was evident in the patient cohort, whose extent expressed the severity of the clinical picture, too. The extent of the worsening of deformability was provided in the form of the rigidity index. The IR was significantly higher in each patient group, in the case of sepsis higher by 51%, in the case of severe sepsis by 229%, in the case of septic shock by 1285%, in the case of respiratory distress syndrome by 923% compared to the IR values of the healthy control group. The red cell rigidity index showed a strong correlation with the simplified acute physiological point system (SAPS II) and the estimated mortality, too.

The leucocytes play an important role not only in the pathophysiology of the sepsis but they have a significant impact on the microcirculation, too. The margination of the white cells is strongly influenced by the axial migration of the erythrocytes. It is a common fact that the red cell aggregation is boosting the margination of the white cells along the endothelium. (During the sepsis Kirschenbaun and his work-group – besides observing the decrease of the red cell deformability and the increase of the red cell aggregation – they also observed the increased neutrophil-thrombocyte interaction and the reduced leucocyte deformability, too. Yodice and his work-group also achieved a similar result, they described an increased white cell aggregation and reduced neutrophil cell deformability in the case of patients with severe sepsis and septic shock. (46)

Naturally the microrheological deviations occur even if being not associated to sepsis. Several studies published hemorheological changes in connection with liver diseases. In the case of patients with alcohol-based liver cirrhosis they described reduced whole blood viscosity and plasma viscosity, as well as lower haematocrit and fibrinogenous concentration. In the case of Hepatitis B patients some publications reported significantly higher whole blood viscosity and red cell aggregation index. Some authors think the bad tissue perfusion perceived during the diabetes mellitus as a complication is due primarily due to the reduced red cell deformability occurred during the disease.

In our own experiment we found a continuously decreasing trend of the red cell deformability in the group of septic animals during the experimental period. This could be perceived in the case of the osmoscan parameters, too. The part of the osmoscan curve belonging to the cell deformability and the membrane stability which corresponds to the area between the min EI and max EI, showed the most striking deviation together with the related osmotic values. The difference between the two points showed a gradually worsening trend. Besides the reduced deformability this may also reflect the cell-damaging effect of the modified osmotic micro-environment.

Differing form the earlier study in our study we observed aggregation parameters with a decreasing trend. Based on the obtained values the speed and intensity of the aggregation process became lower in the 6^{th} hour after the infusing the living E. coli suspension, although the red cell deformability worsened. This contradictory result can be explained by the fact that our study took place in parallel to the forming of the septic shock in the early phase of the fulminant sepsis. In addition one can’t exclude the impact on the aggregation exerted by the

live bacteria that get directly into the blood-flow with an increasing concentration. That is why we tested the impact of the living E. coli suspension with normal blood sample, too. For two normal blood samples we mixed living E. coli suspension with a dosage that corresponds to the total bacterial concentration introduced into the blood system of septic pigs. We determined this based on the pigs' calculated total blood volume. During the in vitro examination we found that the red cell deformability and the aggregation varied according to the in vivo examination, that could still be observed during the 2-hour long incubation period. However it is important to mention that the E.coli strain used by us contained haemolysin which induces beta haemolysis. During the in vivo examination in the examination period we did not perceive change in regards of the red cell count and the MCV. However in the in vitro examination both the red cell count and the MCV began to decrease after the live bacterial suspension got mixed into the blood sample. It is a common fact that the E.coli haemolysin damages the red cell membrane by forming pores in the membrane with ring-shaped heptameter complexes. Due to this the red cells get swollen irreversibly because of the osmosis and this leads to cellular lysis after a certain time. The presence of complements also plays a role in the damaging of the red cells. Keshari and his colleagues investigated the effect of the inhibitor of the C5 lysis, a macro-cyclical peptide with baboon models during the sepsis induced by the E. coli. They observed that - compared with the control group – in the group treated with the C5 lysis inhibitor – the decrease of the red cell consumption and the haematocrit was minimal in the first 24 hours. After suspending the therapy the decrease kept continuing slightly, but without the visible signs of the haemolysis. Even the direct impact of the endotoxins needs to be mentioned as a cause of the damaging of the red cells. Pöschl and ally examined the hydroxymyric acid content and the red cell deformability in the case of sepsis induced by the Gram negative pathogens as well as in the case of incubation with red cell lipid-A or lipopolysaccharides obtained from healthy volunteers. The red cell deformability reduced in the case of the Gram negative septic patients and after the incubation of the washed red cells with lipid A and the total blood with lipopolysaccharide. The extent of the reduction of the red cell deformability was strongly connected to the increase of the membrane's hydroxymyric-acid content which may hint that the connection of the endotoxin to the membrane influences directly the cell's mechanical characteristics. Todd and ally observed that the intracellular Ca-ion level of the septic patients is increasing. The result could be reproduced during the E. coli endotoxin and total blood incubation. In the same study it was proved in vitro that the increased intracellular Ca^{2+} level could not be eliminated with verapamil, but it could be inhibited with ATP pre-treatment. From this they deduced that

an energy-lacking condition can be made responsible for the changed intracellular homeostasis. Ruef et al. incubated red cells of healthy volunteers with E. coli Lipid A endotoxin. They found that due to this the citosol's Ca^{2+} concentration increased, and in parallel to it the red cell deformability worsened. The Lipid A effect could be suspended with verapamil and the protein kinase inhibitor staurosporine.

The role of nitrogen-monoxide has been studied for long regarding the background of the septic hemorheological deviations. The hemorheological variations occurring during the sepsis might be caused by the reduced red cell and leucocyte deformability and the red cell aggregation, the disorder of the coagulation mechanisms and the hypotension caused by the nitrogen-monoxide produced by the inducible nitrogen-monoxide sintetase. The NO might most probably have a multifunctional role regarding the lesion of the microvascular system, it is changing the red cell deformability and in addition it is modulating the signal transduction routes in the red cells. Bateman and his work-group, in an experiment performed with rats, created peritonitis and acute normotensive sepsis in rats with the cecalis ligation and punction model. During this they demonstrated that in the skeletal muscle the „stopped flow capillary" density increased significantly between 3-6 hours after inducing the feculent peritonitis. In the same period the systemic NO concentration increased which got accumulated in the red cells. Besides this the red cell deformability decreased, and a red cell population with reduced deformability got formed. With an in vivo iNOS inhibitor, the aminoguanidin, they inhibited the NO production. It was observed that caused by this the NO concentration maintained its basic value and inhibited the decrease of the red cell deformability and the functional capillary density. Their results hint that the decrease of the functional capillary density occurring during the sepsis is caused by the NO-mediated damaged erythrocyte deformability. The red cell aggregation decrease observed by us is contradicting the data of the worldwide bibliography. The red cell aggregation is a main defining factor of the blood's viscosity, mostly at a low shearing stress. The area influenced most by the red cell aggregation is that of the postcapillary venules where the vein caliper is sufficiently big, and the shearing stress is sufficiently low to form the aggregation. In the area of the capillaries the main defining factor of the flow is the deformability of the red cell. Various animal experiments proved that during the sepsis and septic shock the red cell aggregation inhibits the blood flow in the microcirculation. (64) In the case of the fulminant sepsis model studied by us we observed a decreasing erythrocyte aggregation - compared to the majority of the earlier examinations. The inflammatory diseases, such as the sepsis, too, can be characterised with an acute phase

reaction, during which a big quantity of C reactive protein, fibrinogen, immunoglobulin and alpha-2 macroglobulin get produced. The increasing concentration of the fibrinogen and other high-molecule weight proteins lead to the increasing red cell aggregation. In the case of the fulminant sepsis model studied by us we observed a decreasing erythrocyte aggregation – compared to the majority of the earlier examinations. During the sub-study of the same experiment we saw that the worsening of the skin's micro-circulation occurred in a very early phase, even before the changing of the haemodynamic parameters. This can be explained by the fibrinogenous consumption, the impact of the increased NO concentration, the direct impact of the bacterial endotoxins on the red cells, the changing of the surface glikocalix structure. This result may suggest that in the case of the fulminant sepsis the rheological changes may deviate from the rheological changes that occur during the slowly and gradually forming sepsis process.

Summary

Sepsis and septic shock remains a leading cause of death in patients treated at the general and surgical ICUs. Its entire pathomechanism is not known yet. Despite the fact that in the recent decades novel therapeutic interventions came into light, there is only a moderate improvement in the morbidity and mortality ratios. The pathomechanism is complex, the patient population is heterogeneous, there are no sensitive diagnostic markers available. The combination of these facts makes the planning of experimental sepsis models more complicated. Hence, an ideal sepsis model would be crucial for getting more insight into the pathophysiological background of the syndrome and searching for effective therapeutic interventions. Septic animal models used so far are not standardized, do not cover the whole spectrum of the possible sepsis development and therefore their result may be misleading.

In the present work we aimed to assess the haemodynamic, haemostatic and hemorheologic and cerebrovascular consequences of a sepsis induced by the E. coli. In the first 30 minutes 2 ml of suspended bacterial cultures were given intravenously, followed by a repeated dose of 4 ml in the next 30 minutes. Thereafter 32 ml of bacterial culture was infused within 120 minutes. As a result of this administration a $9.5x10^6$ overall dose of E. coli was administered within a timeframe of 180 minutes. As we intended to assess the natural course of the sepsis development, animals in the sepsis group did not receive any therapy (neither volume therapy) during the study, but anaesthetic drugs. In the first part we aimed to assess haemodynamic changes induced by intravenous administration of E.coli suspension.

Haemodynamic parameters were followed by PiCCo monitoring. We found that mean arterial pressure, cardiac index decreased during the development of septic shock, that was accompanied by a tendency of increased pulse rate. Stroke volume variation decreased stepwise in septic animals during the course of the study. Our fulminant sepsis model appeared to mimic a single-phase hypodynamic septic shock. We suppose that the inflammatory response evoked by E. coli influenced the myocardial contractility directly. This was supported by the reduced cardiac index and global ejection fraction. An immediate microcirculatory damage could be observed as a consequence of the hypodynamic circulation, already in the early phase of the infusion of the bacterial culture, referring to tissue hypoperfusion and hypoxia.

In the second phase of the study we assessed the change of the hemorheological parameters during the septic shock development. A deceased erythrocyte deformability was observed among septic animals, that were mainly indicated by changes of min EI és max EI ranges. The speed and intensity of the aggregation was also lower in septic animals at 6 hours after E.coli infusion, despite the worsening of deformability. It is conceivable that there is a direct effect of E.coli bacteria on the aggregation process. Thus, we tested this possible effect by using normal blood samples and the in vitro tests revealed identical results after a 2-hour incubation period. We conclude that our E.coli-induced septic model is suitable for assessing the haemodynamic and hemorheological consequences.

Own results, individual findings

1. In the international bibliography we were among the first studying the specific haemodynamic and hemorheological parameters that are characteristic during the spontaneous course of the disease, with no therapy, in the fulminant sepsis induced by the E. coli.
2. We found that during the process the cardiac index is decreasing, the systemic vascular resistance and the intravascular pulmonary fluid index is increasing, and the stroke volume variation is decreasing. Based on this the experimental ensemble is suitable to model the hypodinam septic processes.
3. We found that after administering the bacteria the deformability and aggregation of the erythrocytes in the fulminant sepsis model decreased in the first hours of the sepsis. The direct bacterial effect could be confirmed with in vitro tests, too.
4. Considering that in our examinations we studies the spontaneous course of the sepsis, without any therapeutic intervention, our examinations can be the base for further experimental studies that aim to study the hypodinam sepsis, especially for those that aim to test therapeutic interventions.

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Doctoral School: Doctoral School of Neurosciences

List of publications related to the dissertation

1. **Berhés, M.**, Németh, N., Pető, K., Deák, Á., Hajdu, E., Molnár, Á., Árkosy, P., Szabó, J., Fülesdi, B.: Hemodynamic consequences of intravenously given E. coli suspension: observations in a fulminant sepsis model in pigs, a descriptive case-control study.
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