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

Investigation of haematological changes in an experimental sepsis model

by Judit Budai Tóth, MD

Supervisor:
János Kappelmayer, MD, PhD, DSc

UNIVERSITY OF DEBRECEN
KÁLMÁN LAKI DOCTORAL SCHOOL

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Head of the **Examination Committee**:	György Balla, MD, PhD, DSc, member of HAS
Members of the Examination Committee:	László Csernoch, PhD, DSc
	Lajos Bogár, MD, PhD, DSc

The Examination takes place at the library of the Department of Laboratory Medicine, Faculty of Medicine, University of Debrecen, at 12:00, 23rd April, 2018

Head of the **Defense Committee**:	György Balla, MD, PhD, DSc, member of HAS
Reviewers:	Attila Bácsi, PhD, DSc
	Zsolt Molnár, MD, PhD
Members of the Defense Committee:	Lajos Bogár, MD, PhD, DSc
	László Csernoch, PhD, DSc

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 13:30, 23rd April, 2018

1. Introduction and review of the literature

1.1. Definition and etiology of sepsis and septic shock

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection. It is a significant public health problem worldwide, with 15-30 million cases annually and a nearly 20% mortality. The predominant score in current use to assess the severity of organ dysfunction is the Sequential (Sepsis-Related) Organ Failure Assessment Score (SOFA score), which is based on the functions of respiratory, cardiovascular and central nervous systems, the liver and the kidney, as well as the degree of thrombocytopenia. A higher SOFA score is associated with an increased risk of mortality. Sepsis is diagnosed if an acute change in total SOFA score ≥2 points occurs consequent to documented or supposed infection. Septic shock is a subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality. Septic shock can be identified with a clinical construct of sepsis with persisting hypotension requiring vasopressors to maintain the mean arterial pressure ≥65 mm Hg and having a serum lactate level >2 mmol/L despite adequate volume resuscitation.

Sepsis always develops as a result of an infection which is mainly bacteraemia. Before about 1980 most of the septic cases was caused by Gram-negative bacteria, while later Gram-positive bacteria became predominant. Nowadays approximately 25-50% of reported central line-associated bloodstream infections are caused by Gram-negative bacilli. The main causes of nosocomial Gam-negative sepsis are central venous catheters, the respiratory, gastrointestinal and urogenital tract, while the community-onset sepsis is originated mainly from the urinary systems, its most important pathogen is the *Escherichia coli (E. coli)*.

1.2. The pathomechanisms of sepsis

1.2.1. Cytokines and the hypoxia inducible factor-1 (HIF-1)

When microorganisms penetrate into the body, they are eventually be spotted by the innate immune system: monocytes/macrophages, granulocytes and natural killers (NK-cells). The activation of these cells by their pattern recognition receptors (PRRs) occurs after the binding of a wide variety of molecules originating from the infecting microorganism (pathogen-associated molecular patterns, PAMPs) or from necrotic cells (damage-associated molecular patterns, DAMPs). One type of PRRs is the toll-like receptor (TLR) family, the

peptidoglycan of Gram-positive bacteria can bind to TLR2, while TLR4 was identified as the receptor for lipopolysaccharide (LPS, endotoxin) which is a wall-compound of Gram-negative bacteria. The recognition of exodenous PAMPs or endogenous DAMPs lead to a cascade of activation/phosphorylation. In monocytes/macrophages proinflammatory cytokines (tumor necrosis factor-α /TNF-α/, interleukin-1β /IL-1β/), proteases (elastase, collagenase, catepsin) and different mediators – prostaglandins, leukotrienes, platelet activating factor (PAF) – are produced, free oxygen radicals are released, furthermore these cells express tissue factor. Neutrophil granulocytes become activated and they express cell adhesion molecules, roll on the surface of the endothelium, attach to endothelial cells and after extravasation, they migrate to the site of infection and secrete a lot of mediators. Endothelial cells allow activated immune cell to adhere to the vascular wall and enter inflamed tissue. Beyond expression of adhesion molecules and synthesis of chemokines, they also secrete or express procoagulant factors, produce nitric oxide (NO) and the endothelial dysfunction is responsible for increased vascular permeability. These processes are regulated by the balance of pro- and anti-inflammatory cytokines. In sepsis the dysregulated host response to the infection leads to disseminated inflammation and injures own healthy tissues. In the initial hyperinflammatory phase of sepsis the activation of immune system dominates, large amounts of proinflammatory cytokines are released (this is the so-called „cytokine-storm") and the produced NO will result in the hibernation of cells. Over the course of the disease, systemic deactivation of the immune system occures resulting in the anti-inflammatory phase of sepsis. Death is generally caused by septic shock in the hyperinflammatory phase, and immunosuppression and secondary infections in the anti-inflammatory phase.

In sepsis, the HIF-1 is a key player molecule. Its gene is constitutively expressed but the protein is extremely labile: in normoxia it has a rapid degradation but in hypoxia it remains stable. In case of mild hypoxia HIF-1 supports cell adaption via upregulation of gene exression (e. g. VEGF, erythropoietin, NO). As a result, cell metabolism will switch to glycolysis, and enhanced angiogenesis, erythropoiesis and vasodilatation will occur. In addition, NO will inhibit the mitochondrial respiratory chain resulting in cell survival in a hibernated state. In case of severe hypoxia – which causes high mutation rate in the cells – HIF-1 will induce apoptosis. LPS is able to enhance HIF-1 production in macrophages, while IL-1β can increase HIF-1 level even in normoxia.

1.2.2. Bone marrow reaction in sepsis

Bone marrow is known to react to intense bacteraemia by releasing immature cells. The immaturity of granulocytes is indicated by increased immature/total granulocyte ratio (so-called "left shift") which is found either in sepsis, or in SIRS. In sepsis the appearance of immature forms is caused by neutrophil paralysis: as a result of the overproduction of NO, cytokines and chemokines, neutrophil granulocytes fail to adhere to vascular endothelium and fail to migrate to the site of infection. The release of immature granulocytes from the bone marrow is aimed to compensate the functional defect of active neutrophil granulocytes.

Nucleated red blood cell (NRBC) count has been suggested in human studies previously as early indicator of increased risk of mortality. The appearance of NRBCs was found in case of hypoxia, chronic anaemia and severe infections, and they were detected together with the increase of known stimulators of the bone marrow like erythropoietin, IL-3 and IL-6. The concentration of these mediators is elevated in case of hypoxia and inflammation, therefore NRBC may be considered as a parameter that sums up hypoxic and inflammatory changes during sepsis.

The appearance of immature platelets indicates the development of sepsis, the ratio of immature platelet fraction – which reflects the ratio of reticulated platelets – increases 2-3 days before the onset of sepsis, and sunsequently it will decrease according to the bone marrow depression.

1.2.3. Mitochondrial dysfunction and apoptosis

Mitochondrial oxidative phosphorylation is responsible for over 90% of total body oxygen consumption and ATP generation. In sepsis the respiratory chain enzymes (mainly NADH ubiquinone oxidoreductase and cytochrome C oxidase) can be inhibited by LPS, TNF-α and NO. Reactive free oxygen radicals and peroxynitrite impair mitochondrial DNA and membranes. The oxidative mitochondrial damage leads to the release of cytochrom C into the cytosol resulting in apotosis. In case of septic patient the soluble Fas level was also elevated indicating that besides the intrinsic (mitochondrial) pathway of apoptosis, the extrinsic pathway was also activated. In addition, TNF-α can induce apoptosis via the extrinsic pathway. The mitochondrial dysfunction can be reversible in the early phase of sepsis, but severe and persistant oxidative stress may lead to irreversible damage. The sepsis-induced organ failure has been suggested to be at least in part due to mitochondrial dysfunction which results in energy crisis. The mitochondrial dysfunction can affect all cells containing mitochondria. One possible reason for sepsis-related thrombocytopenia can be the enhanced

platelet apoptosis, while lymphopenia can be explained by the apoptosis of CD4+ and CD8+ T-cells, and B-cells.

In the initial phase of apoptosis the phosphatidylserine (PS) exposure of the cell membrane is increased without the impairment of membrane integrity. In platelets the production of mitochondrial reactive free oxygen radicals lead to increased PS exposure, but not integrin activation and granule release. However, platelet activating effect can also result in increased PS exposure and enhanced procoagulant activity. Red blood cells – although they lack nuclei and mitochondria – may undergo apoptosis (eryptosis), which is characterized by cell shrinkage, cell membrane blebbing and PS scrambling. In case of red blood cell damage the eryptosis inhibits haemolysis, which results in haemoglobinuria and consequent renal failure. However, excessive eryptosis may lead to anaemia and impairment of microcirculation, because apoptotic red blood cells adhere to the endothelium. In sepsis, excessive eryptosis was observed and plasma isolated from septic patients was able to trigger cell membrane scrambling and shrinkage of red blood cells from healthy individuals. In sepsis eryptosis can be triggered by bacterial virulence factors (e. g. sphingomyelinase, haemolysin), iron defect related to the infection, enhanced PAF production and oxidative stress.

1.2.4. Oxidative stress markers: lactate and uric acid

The sepsis-associated hyperlactataemia is a strong independent predictor of mortality. Lactate concentration was known as a marker of hypoperfusion and tissue hypoxia, in this case the increased lactate level is the result of anaerobic glycolysis. However, a large body of evidence now supports that the elevation of lactate concentration secondary to activation of stress response is an adaptive mechanism: lactate may well represent an important energy source and may be helpful for survival in sepsis. The adrenergic stimulation accelerates aerobic glycolysis resulted in increased piruvate and lactate production. This lactate can serve as a substrate in highly oxidative cells (heart, brain) or contribute to gluconeogenesis (liver, kidney). In experimental models the lung was found to be the major source of lactate in sepsis.

The uric acid is a powerful antioxidant, it is the final product of purine metabolism. Uric acid occurs predominantly as urate anion under physiologic pH and approximately two-thirds of uric acid is excreted by the kidney. During sepsis free oxygen radicals are released from both activated immune and endothelial cells. As a result, the concentration of the free radical scavenger uric acid will increase. Urate elevation may cause renal failure, endothelial dysfunction, activate some proinfammatory transcription factors, increase cytokine and chemokine production, and decrease NO formation.

1.2.5. Platelets and coagulation in sepsis

In sepsis, inflammation and coagulation are tightly linked and the coagulation is not only triggered by infection, but the coagulation system plays an important role in limiting the spread of infectious agents. The large amount of thrombin, the activated complement system, the proadhesive phenotype of endothelium, the exposure of subendothelial collagen, as well as the pathogen will result in platelet activation. Several bacteria (e. g. *E. coli*) have been shown to mediate platelet activation. Platelet-bacterial interactions can be direct or indirect; mediated via plasma proteins (fibrinogen, von Willebrand factor, complement proteins, immunoglobulins) and/or platelet receptors (Fcγ receptor IIa, glycoprotein Ibα, glycoprotein IIb-IIIa, C1q, C3a and C5a receptors, protease-activated receptor 1, toll-like receptors), furthermore bacteria can bind directly to platelet receptors (TLR2, TLR4, glycoprotein Ibα) or platelets respond to toxins released by bacteria (e. g. Shiga toxin, lipoteicholic acid). Additionally, platelet factor 4 binds to bacteria and reduces the lag time for platelet activation and aggregation. *E. coli* can interact with platelets mainly via platelet TLR4 but a TLR4-independent interaction has been also described. Platelets contain several complement factors and the activated platelets enhance the activation of complement system. In addition to platelet adhesion, aggregation and secretion, platelet-leukocyte interaction also has a meaningful role: platelets support adhesion, extravasation and activation of neutrophils. Platelets can produce several cytokines and they are directly bactericidal through the release of platelet antimicrobial peptides. In sepsis one part of platelets was found to be cytotoxic via their granzyme B secretion resulting apoptosis of the target cell. The activating agents can also lead to procoagulant platelet phenotype. These procoagulant platelets – which are not just highly activated cells, they are undergoing cell necrosis – enhance thrombin and fibrin formation, produce a high amount of PAF and they have an increased reactivity toward neutrophils. Platelet-derived microparticles also have an increased procoagulant activity. While in controlled inflammation platelets support the localisation of infection and the elimination of pathogens, in sepsis the uncontrolled platelet activation leads to hyperinflammation, microthrombus formation and thrombocytopenia, and as a result, disseminated intravascular coagulation (DIC) and multi-organ failure occur. According to the literature, thrombocytopenia – irrespectively of its cause – is an independent indicator of mortality in critically ill and septic patients.

In sepsis the TLR4-activated platelets have been shown to bind to neutrophils and function as the threshold switch for their secretion of nuclear content, forming neutrophil extracellular traps (NETs). In addition, NETs enhance platelet activation. NETs are web-like structures of DNA with proteolytic activity, mainly formed by decondensed nucleosomes and

proteins derived from intracellular granules, such as neutrophil elastase and myeloperoxidase; they can trap and kill microbes in tissue microvasculature. DNA and histones can be released from cells not only during NET-formation, but also in necrosis. Extracellular histones are cytotoxic to the endothelium resulting in endothelial dysfunction, they can activate platelets via TLR2 and TLR4, and enhance tissue factor expression of monocytes which leads to increased thrombin generation. These effects are mainly due to histones H3 and H4. In conclusion, NETs can activate platelets, extrinsic and intrinsic pathways of coagulation, furthermore inhibit the limitation of coagulation via the proteolysis of tissue factor pathway inhibitor (TFPI). As a result of procoagulant and proinflammatory activities of NETs, immunothrombosis will occur in the microcirculation, which can localize infection in controlled form, but it will lead to DIC when it is uncontrolled.

In sepsis mainly the cytokines are responsible for the alterations of coagulation system. The prothrombotic state can be the result of three main mechanisms: (i) activation of procoagulant pathways, (ii) inhibition of natural anticoagulants and (iii) suppression of fibrinolysis. The central step of coagulation activation is the increased expression of tissue factor, mainly by the monocyte/macrophage system. Thrombomodulin, endothelial protein C receptor, protein S and heparan-sulfate expressed on the surface of endothelial cells contribute to the limitation of coagulation. In sepsis, as a result of endothelial dysfunction, the natural anticoagulant pathways (protein C pathway, TFPI and antithrombin) are decreased. The increased synthesis of plasminogen activator inhibitor-1 (PAI-1) and the enhanced activation of thrombin-activatable fibrinolysis inhibitor (TAFI) lead to the inhibition of fibrinolysis.

Inorganic polyphosphates (polyPs) are novel key molecules of the interaction between inflammation and coagulation. PolyPs are linear polymers of 3-1000 orthophosphate residues, larger ones are derived from bacteria, while smaller polyPs are found also in humans, e. g. in dense granules of platelets. PolyPs can activate contact pathway resulting in coagulation activation and in an increased bradykinin generation; they accelerate the activation of factor V; act as a cofactor for thrombin in factor XI activation and also enhance factor XI autoactivation; stabilize fibrin clot; and inhibit the function of TFPI. Contact activation is mediated only by bacterial-sized polyPs, but the other prothrombotic effects can be mediated via smaller polyPs released during platelet activation. Beside haemostasis, they also have an important role in inflammation: polyPs potentiate proadhesive phenotype of endothelial cells; enhance the proinflammatory effects of histones; increase vascular permeability directly and via bradykinin; and activate the classical pathway of complement system. The platelet activating and thrombin generating effects of extracellular histones were found to be polyP-mediated.

1.3. Animal models of sepsis

Clinical studies provide the opportunity to investigate pathophysiological changes in sepsis, but the results of these studies show a high variability according to the different age, underlying disorders, clinical course of disease, administered therapy and the heterogenity of infective agents. Unlike in humans, all these factors can be standardized in animal models, thus results obtained in these studies may be more easily related to the causative agent. Three animal models became widely used: the peritonitis model elicited by coecal ligation and puncture, the induction of sepsis by endotoxin infusion and the introduction of live bacteria to animals. The majority of these latter studies utilize Gram-negative bacteria – mostly *E. coli*. Sepsis induction by administration of live bacteria may better mimic the in vivo pathological process in humans than endotoxin infusion.

2. Aims

The host's response to the infection and resultant hypoxia is intensively investigated but we have less knowledge about the dynamics of these changes. Clinical studies mostly detect hypocoagulability in septic patients, but they fail to demonstrate the initial and short-lived hypercoagulability.

We aimed to study:

- the kinetics of infection and hypoxia induced alterations in relation to sepsis parameters and markers of organ damage,
- the early haemostatic changes during fulminant sepsis, namely the activation of platelets and coagulation, furthermore
- the cell activating effect of soluble substances released during sepsis in in vitro experiments.

3. Materials and methods

3.1. Porcine sepsis model

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). Seventeen juvenile female Hungahib pigs (bodyweight: 19.5±1.6 kg) were subjected into septic (n = 10) and control groups (n = 7). Under general anaesthesia (ketamine and xylazine) tracheostomy was performed for assisted ventilation, and the left external jugular vein and the femoral artery have been cannulated for sampling and hemodynamic measurements. The animals did not receive anticoagulant or any medication.

In the *E. coli*-treated group, sepsis was induced by *E. coli* culture (2.5×10^5/mL; strain: ATCC 25922, Department of Medical Microbiology, University of Debrecen) suspended in physiological saline (API suspension medium, bioMérieux, Lyon, France) was intravenously administered in a continuously increasing manner according to the following protocol: 2 mL of bacterial culture suspended in physiological saline was injected in the first 30 minutes, then 4 mL in 30 minutes and afterwards 16 mL/hour for 2 hours. A total amount of 9.5×10^6 *E. coli* was infused within 3 hours. In the control group the similar volume of physiological saline solution was administered by the same protocol as in the septic group and no other intervention was made. The anaesthesia was maintained for 4 hours by intramuscular administration of 15 mg/kg ketamine and 1 mg/kg xylazine in every 20 minutes within the first hour and in every 25 minutes after it. The depth of anaesthesia was assessed by changes in blood pressure and heart rate values, furthermore by the presence or absence of several reflexes. During the experiment a 37°C heating pad was placed under the animals. At the end of the experiment animals were euthanized.

3.2. Core temperature, haemodynamic parameters and blood gas monitoring

The core temperature was measured via the central venous cannula by PiCCO Monitoring Kit (Pulsion Medical Systems SE, Germany) connected to Philips IntelliVue monitor (Koninklijke Philips N.V., The Netherlands). Heart rate and mean arterial pressure were also monitored invasively by the PiCCO device. The modified shock index – which is a descriptive parameter for the circulation status and a clinically significant predictor of mortality in emergency patients – was calculated as the ratio of heart rate and mean arterial pressure values. The partial pressure of oxygen in arterial blood (p_aO_2) and lactate

concetration were determined by GEM 3500 blood gas analyser (Instrumentation Laboratory, Bedford, USA).

3.3. Blood drawing and sample preparation

Immediately before *E. coli* administration blood samples were collected from the cannulated vein directly into blood collection tubes (Becton Dickinson, San Jose, CA, USA) containing 0.105 M sodium-citrate as anticoagulant and the proportion of blood to the liquid anticoagulant volume was 9:1. The first 3 mL of blood was always discarded. Beside the baseline blood drawing, further blood samples were collected 2 and 4 hours after starting the infusion containing *E. coli*. All samples were transported to the laboratory at 37°C and the measurements were started within 20 minutes after blood drawing. Citrated whole blood was used for automated hematology analysis and blood smear examinations. Coagulation screening tests, thrombin generation and clinical chemical tests were measured in platelet poor plasma (PPP) and PPP was also used in in vitro experiments. Citrated whole blood was centrifuged immediately at 1500g for 15 minutes at room temperature according to the manufacturer's instruction and this single-centrifuged PPP was used for coagulation screening tests, clinical chemical tests and for the dilution of platelet rich plasma (PRP) to obtain a standard platelet count. PPP for thrombin generation assays was prepared by a second centrifugation at 10000g for 10 minutes at room temperature. After centrifugation only the top two third of plasma was used. During flow cytometric measurements and also in a part of the thrombin generation studies, PRP was used which was obtained by centrifugation at 170g for 10 minutes at room temperature, then platelet count was determined by Sysmex XP 300 hematology analyser (Sysmex, Kobe, Japan) and was adjusted to 250 G/L by PPP.

3.4. Automated hematology analysis, light microscopy and coagulation screening tests

Blood cell count was performed according to our routine clinical laboratory procedure on ADVIA 120 hematology analyser (Siemens, Forcheim, Germany) using a special animal software suitable for porcine blood cell counting. Lobularity index value was also calculated by the same analyser. Lobularity index provides an indicator of the „left shift" of white blood cells. During basophil/lobularity method of the analyser, red blood cells and platelets are lysed, then all white blood cells except basophils – which remain intact – are stripped of their cytoplasm using a reagent and increased temperature. Based on shape and complexity of their nuclei, the analyser can categorize these stripped white blood cells as mononuclear or polymorphonuclear cells. Baso X histogram displays the high-angle light scatter information

(nuclear configuration) for this two white blood cell populations, therefore three different values are shown: mononuclear peak, mononuclear/polymorphonuclear valley and polymorphonuclear peak values. The lobularity index is the polymorphonuclear peak value divided by 14, and decreased value indicates the appearence of immature white blood cells in the circulation.

NRBCs were counted and platelet morphology was studied on peripherial blood smears using May-Grünwald and Giemsa staining. The microscopic examinations were carried out with Zeiss Axiostar Plus light microscope (Zeiss, Jena, Germany). The NRBC count (G/L) was calculated based on the NRBCs referred to 1000 white blood cells and the white blood cell count of the samples. Giant and vermiform platelets were quantified as the number of these platelets referred to 200 single platelets. The conglomeration of 3 or more platelets was defined as platelet aggregate and it was quantified as the number of platelet aggregates referred to 200 single platelets.

Coagulation screening tests (prothombin time, activated partial thromboplastin time /APTT/, thrombin time and fibrinogen concentration) were determined by BCS coagulometer (Siemens, Forcheim, Germany) according to routine clinical laboratory procedures.

3.5. Clinical chemical tests

Uric acid and creatinine concentrations along with creatine kinase (CK) and glutamate pyruvate transaminase (GPT) activities were determined by COBAS 6000 analyser (Roche, Basel, Switzerland) according to routine clinical laboratory procedure.

3.6. Flow cytometric measurements

Platelet mitochondrium membrane depolarisation was studied by FC500 flow cytometer (Beckman Coulter, Brea, CA, USA) based on the method by Leytin et al adapted to porcine platelets. The principle of this method is that platelets are stained with a fluorescent dye 3,3'-dihexil-oxocarbocianin-jodide ($DiOC_{6(3)}$; Sigma-Aldrich, Budapest, Hungary), and when the mitochondrium membrane is depolarised, $DiOC_{6(3)}$ accumulation in platelet mitochondria becomes impaired and a decreased mean fluorescence intensity (MFI) of the platelet population is measured. PRP aliquots (5µL) diluted 1:20 with phosphate buffered saline (PBS) were stained with 20µL of 1.5µmol/L $DiOC_{6(3)}$. Samples were incubated for 20 minutes at room temperature in the dark and were diluted to 560µL with PBS. Platelets were identified based on light scattering properties, and FL1 histograms were analysed.

Mitochondrium membrane depolarisation was quantified as a decrease of MFI observed on FL1 histograms.

Platelet PS expression was also studied by the modified flow cytometric method of Leytin V et al. PS expression was determined by annexin V binding to platelet surface using FITC-conjugated annexin V. Five μL of PRP was stained with 5 μL of annexin V-FITC in 40 μL of annexin V binding buffer (both from BD Pharmigen, San Jose, CA, USA), and it was incubated for 15 minutes at room temperature in the dark. Samples were diluted to 550 μL with annexin V binding buffer. Platelets were identified based on size and light scattering properties because our previous experiments showed that anti-human antibodies did not cross react with porcine platelets. Investigation of single platelet population was the goal, therefore platelet aggregates were excluded by gating out separate dots of platelets with large forward and sideward scatter properties. The activated platelets appeared as annexin V bright events on FL1 histograms and the ratio of these platelets was calculated as the ratio of annexin V bright events compared to the whole single platelet event count.

3.7. Effect of septic porcine plasma samples on washed human red blood cells

To investigate the effect of *E. coli*-treated and untreated porcine plasma samples on washed human red blood cells in vitro experiments were performed based on previously described methodology. Blood samples from healthy individuals with blood group „0" were collected in EDTA tubes. Red blood cells were separated from white blood cells and PRP by centrifugation at 200g for 10 minutes. Subsequently, red blood cell suspension was washed two times in phosphate buffered saline and once in Ringer solution without $CaCl_2$. The hematocrit of the washed red blood cell suspension was adjusted to 0.1 (10%) by adding calcium-free Ringer solution. Porcine plasma samples obtained before *E. coli* or physiological solution infusion, as well as 2 and 4 hours later, were incubated with washed red blood cells for 24 hours at 37°C in 5% CO_2 in a 0.05 (5%) hematocrit. After 10 minutes and 24 hours of incubation, the PS expression of red blood cells was determined by annexin V binding to red blood cell surface using FITC-conjugated annexin V. The red blood cells were identified based on glycophorin A staining. The ratio of MFI of annexin positive red blood cells was calculated when the MFI of annexin positive red blood cells after the whole incubation time (24 hours) was compared to the MFI of annexin V positive red blood cells after preparing the mixture of porcine plasma and washed red blood cells (10 minutes).

3.8. Effect of TNF-α and LPS on washed human red blood cells

To investigate which component of the septic porcine plasma samples has an effect on washed human red blood cells further in vitro experiments were performed. First, TNF-α and LPS together with autologous plasma were added to washed human red blood cells. The final concentration of recombinant TNF-α (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was always 100 ng/mL, while the concentration of LPS (*E. coli O55:B5*, Sigma-Aldrich, Budapest, Magyarország) was 100 ng/mL, 1 μg/mL or 10 μg/mL. The non-activated samples contained only washed human red blood cells in autologous plasma, but the other samples contained also 100 ng/mL TNF-α; 100 ng/mL, 1 μg/mL or 10 μg/mL LPS; or 100 ng/mL TNF-α together with 100 ng/mL, 1 μg/mL or 10 μg/mL LPS. The samples were incubated for 24 hours at 37°C in 5% CO_2, and after that the PS expression of red blood cells was determined by FC500 flow cytometer, while after centrifugation at 1500g for 15 minutes at room temperature, the LDH activity and haemolysis index of supernatants were measured by COBAS 6000 analyser. In the next set of measurements, TNF-α and/or LPS were added directly to the K_2-EDTA-anticoagulated whole blood, and then samples were incubated for 24 hours (in this case white blood cells and platelets were also present). After 24 hours of incubation, red blood cells were washed and their PS expression was determined, while after centrifugation of the whole blood samples the LDH activity and haemolysis index of PPP samples were measured.

3.9. Thrombin generation assays

Thrombin generation was measured in PRP and PPP using Fluoroscan Ascent FL fluorimeter with Thrombinoscope reagents and software (Thrombinoscope BV, Maastricht, The Netherlands). Assays were carried out according to the manufacturer's instructions.
The PRP-Reagent contained 1 pmol/L recombinant tissue factor and the platelet content of the sample served as phospholipid surface.
The PPP was investigated under three different settings. Regularly, PPP-Reagent 5 pM contained 5 pmol/L recombinant tissue factor and 4 μmol/L phospholipid was used to initiate thrombin formation. All PPP samples were also measured in the absence of any added reagent (instead of PPP-Reagent physiological saline solution was added to PPP). Furthermore, a subset of samples was measured in the presence of phospholipid but without exogenous tissue factor (Microparticle /MP/-Reagent; 4 μmol/L phospholipid). Into each well, 80 μL of PRP/PPP and 20 μL of PRP-Reagent/ PPP-Reagent/ physiological saline solution/ MP-Reagent/ Thrombin Calibrator were pipetted, and after incubation for 10 minutes at 37°C the

thrombin generation was started by adding 20 μL of FluCa (fluorogenic substrate and calcium in buffer). Corresponding to the dynamics of thrombin generation, varying fluorescent signal was detected and thrombin generation curve was drawn. The kinetics of thrombin generation was characterized by lag time, time to peak and start tail parameters, while the generated quantity of thrombin was described by thrombin peak and endogenous thrombin potential (ETP).

3.10. Statistical analysis

GraphPad Prism 6.0 program was used for the statistical analysis. Data distribution was evaluated by Shapiro-Wilk test. The statistical significance of the differences between septic and control groups was determined by unpaired Student's t-test in case of Gaussian distribution, and by Mann-Whitney test in case of non-Gaussian distribution. The matched septic animals' data and the same of control data were analysed by ordinary one-way ANOVA using Bonferroni correction, or by Friedman test with Dunn's correction, as appropriate. Differences were considered significant when $p<0.05$.

4. Results

In the septic group at 4 hours after *E. coli* administration both the core temperature and the modified shock index were increased significantly compared to baseline data. The absolute lymphocyte count was decreased significantly after *E. coli* administration and a considerable difference was noted between the treated and control group at 2 hours after sepsis induction. The lobularity index of the white blood cells decreased significantly both at 2 and 4 hours referring to the appearance of immature white blood cells in the circulation. In untreated control animals all of these parameters remained constant during the experiments.

The surgical procedure (tracheostomy, assisted ventilation, arterial and venous cannulation) resulted in an acute phase response and muscle damage in both treated and untreated animals as reflected by the elevated neutrophil counts and CK activity values. After *E. coli* administration, the absolute neutrophil count showed a mild, non-significant increase compared to the baseline data, the high maximum and low minimum values correspond to a considerable variance among septic animals. A significant increase was noted in control animals and the CK activities became elevated by the end of the experiments in both treated and untreated animals.

To investigate bone marrow reaction in sepsis, reticulocyte and platelet counts were measured by a hematology analyser, while NRBCs were counted and platelet morphology was studied on peripherial blood smears. The reticulocyte count and the NRBC count of the septic group were increased significantly compared to the control group and to the baseline values of septic animals. These changes were significant as early as 2 hours after sepsis induction indicating a rapid bone marrow reaction in severe sepsis.

The platelet morphology in sepsis showed two types of alterations: heterogeneity in platelets possibly due to release from bone marrow (platelet anisocytosis, giant platelets and vermiform platelets), and platelet activation (platelet aggregates). In the *E. coli*-treated group an early and significant elevation of the number of giant and vermiform platelets was found compared to the control group and to the treated group's baseline data.

To follow the fate of platelets in sepsis, membrane depolarisation of platelet mitochondria was also determined by flow cytometer measuring the emission of a fluorescent dye accumulated in platelet mitochondria. In case of mitochondrium membrane depolarisation the accumulation of the dye and therefore the MFI of the whole platelet population decreased. Depolarisation was quantified as a decrease of MFI observed on FL1 histograms. Significant mitochondrial membrane depolarisation was observed only in *E. coli*-treated animals by 2 hours after sepsis induction.

The *E. coli*-treated animals developed marked hypoxia by 4 hours after sepsis induction. In this group lactate acidosis also occurred and the lactate concentration became significantly elevated by 4 hours into the range that in humans is regarded as severe sepsis. To monitor the response to oxidative stress, uric acid concentration was measured and was found to be continuously elevated. In untreated control animals all of these parameters remained almost constant during the experiments.

To study potential renal and liver damage during the experiments creatinine values and GPT activities were measured. Only creatinine values became significantly elevated by the end of the experiments, but this elevation was much milder than the changes observed in case of uric acid concentrations.

Most of the coagulation screening tests (prothrombin time, APTT and thrombin time) did not show any systematic change during the 4-hour observation period, but showed a considerable variance among individual animals. The fibrinogen level was continuously but non-significantly decreased in case of *E. coli*-treated animals. In controls, after an initial mild reduction a constant fibrinogen level was detected.

Platelet count decreased significantly by 4 hours in both the septic and control group. In the *E. coli*-treated group, this reduction was significant already by 2 hours and the platelet count further decreased up to 4 hours. In untreated animals a moderate platelet count decrease

was seen. In sepsis, intense platelet aggregate formation was detected. Platelet aggregates appeared by 2 hours and a further increase was observed by 4 hours, while only a mild tendency for elevation was observed in untreated animals. To characterize platelet-associated changes in sepsis, we have determined the ratio of activated platelets according to their annexin V positivity. The activated platelets appeared as annexin V bright events, and accordingly the ratio of activated platelets was determined as the ratio of these annexin V bright events compared to the whole single platelet population. In the septic group, a moderate, but non-significant increase was found by 2 hours after *E. coli* administration. Furthermore, we investigated whether *E. coli*-treated and untreated porcine plasma samples have any activatory effect on washed human red blood cells, therefore porcine plasma samples were incubated with washed human red blood cells for 24 hours. The ratio of MFI of annexin V positive red blood cells was calculated when the MFI of annexin V positive red blood cells after the whole incubation time (24 hours) was compared to the MFI of annexin V positive red blood cells after preparing the mixture of porcine plasma and washed red blood cells (10 minutes). This ratio was significantly elevated as a result of incubation with *E. coli*-treated plasma samples obtained by 2 hours after sepsis induction, while no changes were observed in controls.

The result of our in vitro experiment suggested that some component of the septic plasma had an activatory effect on red blood cells, and this component was present in appropriate concentration by 2 hours after sepsis induction. In addition, in the *E. coli*-treated group a significant elevation of LDH activity was also detected by 4 hours. To investigate which component of the septic porcine plasma samples has an effect on washed human red blood cells further in vitro experiments were performed. First, beside autologous plasma 100 ng/mL TNF-α and/or 100 ng/mL, 1 μg/mL or 10 μg/mL LPS were incubated with washed human red blood cells for 24 hours at 37°C, and the PS expression of red blood cells, furthermore the LDH activity and haemolysis index of supernatants were measured. Neither TNF-α, nor LPS activated the red blood cells and resulted in haemolysis. In the next step, TNF-α and/or LPS were added directly to the anticoagulated whole blood, and then samples were incubated for 24 hours. The PS expression of red blood cells did not increase but the LDH activity of plasma samples containing 100 ng/mL TNF-α and 10 μg/mL LPS together increased significantly compared to the LDH activity of non-activated and the other activated samples. In case of haemolysis index, not only the combination of 100 ng/mL TNF-α and 10 μg/mL LPS, but also 10 μg/mL LPS alone resulted in significant elevation.

To confirm platelet and coagulation activation and the consequent consumption after *E. coli* administration, thrombin generation assays were performed. In both PRP and PPP the kinetics of thrombin generation (lag time, time to peak and start tail) as well as the quantity of

generated thrombin (thrombin peak and endogenous thrombin potential) were studied. Thrombin generation was determined in PRP samples where the platelet number has been adjusted to 250 G/L. In PRP samples at 4 hours after *E. coli* administration thrombin generation has started earlier and with higher speed, and has also finished earlier, while the maximum concentration of the generated thrombin and the ETP did not change compared to the baseline data. In the septic group, lag time showed a marked but non-significant shortening, while time to peak and start tail were shortened significantly after 2 hours, corresponding to the platelet and coagulation activation, and after 4 hours they remained constant. In PRP the thrombin peak and ETP showed no change during the experiments.

In case of PPP, three different experimental settings were performed. First, thrombin generation was studied by the addition of recombinant tissue factor and phospholipid (PPP-Reagent was added to plasma). In this case, after *E. coli* administration, the thrombin generation has started slightly earlier with constant speed and has finished at the same time as before sepsis induction, while both the thrombin peak and the ETP were significantly lower compared to the baseline values of this group. In septic animals, after 2 hours lag time and time to peak were shortened significantly according to the coagulation activation, and after 4 hours they were slightly prolonged indicating coagulation factor consumption in severe sepsis. Start tail parameters remained constant during the 4-hour period indicating no change in the end-point of thrombin generation. The thrombin peak was continuously decreased during the experiments in treated animals according to the decreased amount of generated thrombin and this difference became significant by 4 hours. ETP showed a significant reduction both in septic and control groups after 2 hours, but in septic animals a more pronounced decrease developed by 4 hours.

In the next set of measurements, thrombin generation assays were performed without addition of any exogenous tissue factor or phospholipid (instead of PPP-Reagent physiological saline solution was added), so thrombin generation was dependent on the TF activity and phospholipid content and further possible thrombin generating substances of the PPP itself. In this case, the baseline values of all parameters referring to the kinetics of thrombin generation were 4-5 times longer than the same values in case of the addition of PPP-Reagent, while the quantity of generated thrombin was 50-80% of the same values measured in the presence of PPP-Reagent. By 4 hours after sepsis induction, the thrombin generation has initiated and finished at significantly shorter time points, while the thrombin peak was higher compared to the baseline values of the treated animals. In the septic group, at 2 hours lag time and time to peak parameters have already been shortened according to the coagulation activation and this change became significant by 4 hours, while start tail showed a significant shortening already by 2 hours. The thrombin peak value increased significantly by 2 hours according to the

increased amount of generated thrombin and also ETP showed a mild elevation at this time-point.

In the last series of experiments, thrombin generation was studied in PPP of selected samples by the addition of phospholipid (MP-Reagent was added to plasma), therefore thrombin generation was determined not only by the clotting factor activities but also by the tissue factor activity of PPP. The characteristic features of thrombin generation curves were comparable to those expected in the absence of recombinant tissue factor and exogenous phospholipid, the only alterations were the slightly shortened kinetic parameters and a mild increase in the quantity of generated thrombin.

5. Discussion

Studying pathophysiological changes in human sepsis is challenging since age, underlying disorders, clinical course of disease, administered therapy and high variability in the characteristics of infective agents may contribute to the variability of data observed in septic patients. Unlike in humans, all these factors can be standardized in animal models. In our porcine model we induced sepsis by intravenous administration of live *E. coli*, and we aimed to study the kinetics of infection and hypoxia induced changes, furthermore we extended to explore early haemostatic alterations.

Bone marrow is known to react to intense bacteremia by releasing immature cells. In our septic cohort we could verify the decrease of lymphocyte count in septic animals versus controls by 2 hours as a part of sepsis criteria and the simultaneous appearance of left shift in neutrophils that is mirrored by the decrease of lobularity index. Both values kept progressing until 4 hours and these changes were not observed in the untreated animals.

In a previous report our research group investigated the changes in microcirculation by laser Doppler tissue flowmetry and found that in septic animals after 1 hour over 40% reduction in blood flow was noticed that further progressed by 4 hours, while controls displayed only an insignificant decrease in flow by 1 hour that normalized in subsequent samples. We hypothesized that this blood flow impairment and resultant hypoxaemia induce bone marrow reactions. Indeed, after 2 hours the absolute reticulocyte count increased and nucleated red blood cells appeared, mostly as polychromatic normoblasts with some orthochromatic cells. NRBC has been suggested in human studies previously as a marker that indicates mortality. These authors concluded that NRBC may be considered as a parameter that sums up hypoxic and inflammatory changes during sepsis. Thus, the appearance of NRBCs in our study confirms previous reports and also identifies NRBC as an early marker

of severe sepsis. Although reticulocyte count also increased as could be anticipated there the values of control and septic animals showed an overlap, while NRBC elevation was more specific for *E. coli*-treated animals.

Similarly to immature red blood cells, immature platelets were also released by the bone marrow. Previously the immature platelet fraction was found to predict sepsis and it was also found that the the immature platelet fraction is useful for discriminating septic patients from non-septic patients, but not for the determination of sepsis severity. We investigated the morphological alterations in platelets that in our case were associated with the enhanced bone marrow response. We rarely observed giant platelets and vermiform platelets in smears of baseline samples and these two dysplastic forms increased several-fold only in the septic group while it did not change at all in the control group. The platelet is a very quickly reacting cell type in its standard function i.e. in hemostatic processes but it has been described that the platelet is also an inflammatory cell. Thrombocytopenia was identified as an independent variable significantly associated with increased mortaliy in a large prospective multicenter observational study. The severity of thrombocytopenia parallels with the severity of inflammation and subsequent mortality. The thrombocytopenia that accompanies the bacteremia also induces the release of young platelets. We thought to look at the mitochondrial function of these platelets that is important from the hypoxemia point of view. Mitochondrial alterations in pathological states can be harmful as well as beneficial for the host. The consensus on the term ’mitochondrial dysfunction’ is still missing. Most studies that investigate this phenomenon require tissue homogenates and isolated mitochondria and the classical method is the measurement of oxygen consumption. Platelets are easily accessible cells and we determined the mitochondrial membrane depolarisation by a flow cytometric method that detects membrane leakage of platelet mitochondria measured by decline in the signal of a fluorescent dye. Mitochondrial membrane potential reflects mitochondrial function and is an indicator of mitochondrial energy status. Besides the production of energy, mitochondria play an important role in cellular survival and apoptotic death. Therefore, mitochondrial membrane depolarisation may reflect not only the abnormality of aerobic metabolism but it can be associated with the mitochondrial pathway of apoptosis. Although during our experiments a slow non-significant decrease was observed also in control animals, only the septic group displayed a significant drop in mitochondrial function by 2 hours indicating mitochondrial dysfunction and potentially platelet apoptosis in sepsis. The same technique was applied previously in humans and was found to correlate with the severity of the systemic inflammatory response. The mitochondrial dysfunction is known to be reversible, but this process is very sensitive even to mild hypoxia, as in our septic group the partial oxygen pressure was decreasing gradually but non-significantly during the experiment

and the increase in the lactate values in our model became significant only after 4 hours. Our results also confirm the hypothesis that not only the sepsis related hypoxia but also the LPS can inhibit the mitochondrial function directly.

Because of the different species and natural dietary differences, uric acid may be an even better candidate for following hypoxaemia induced changes in the septic pig model compared to human septic cases. Uric acid values are very high even in healthy humans the values are close to the solubility cut-off of this substance, but urate is exceedingly low in pigs. Thus, in human studies that utilized uric acid as a marker patients could only be dichotomized with ’normal’ or ’elevated’ urate values compared to sex matched reference ranges. Unlike in humans, in our septic porcine model there was a huge difference by 4 hours in the uric acid levels of septic and control animals resulting in non-overlapping urate values. Furthermore, the uric acid level of the septic group showed an early and significant difference from that of the control group – in contrast to lactate levels –, which indicates that uric acid concentration may be a more sensitive marker of oxidative stress than lactate concentration. This increase was largely unrelated to kidney failure as the creatinine values of *E. coli*-treated animals only increased at 2-4 hours after the start of bacterial inoculation. It is also possible that uric acid itself can cause an acute kidney injury due to direct tubular toxicity, or crystal induced injury, or an indirect injury secondary to the release of vasoactive mediators and oxidative stress. During this sepsis model only a borderline creatinine elevation was observed with no change in the GPT activity i.e. the liver specific transaminase marker. Thus, it is important to emphasize, that urate elevation, mitochondrial membrane depolarisation and the bone marrow associated changes in red blood cells and platelets during this fulminant sepsis, all occur prior to the occurence of evident multi-organ failure. We think that further studies are required to confirm the usefulness of these data in severe human sepsis.

In sepsis, inflammation and coagulation are tightly linked. In our sepsis model it could be anticipated that these animals will develop DIC, similarly as was observed in several previous sepsis studies. Coagulation screening assays (prothrombin time, APTT and thrombin time) however did not display systematic prolongation in the septic group but showed a considerable variance possibly corresponding to different kinetics of hemostatic changes among individual animals. These traditional coagulation tests are coarse estimations of the coagulation system. They use clot formation as endpoint which occurs when only about 5% of all physiologically relevant thrombin is formed, and they are insensitive for any prothrombotic state. In a recent study based on thrombin generation tests less than 15% of patients with septic shock presented hypocoagulability which may result in clotting time prolongation. In our study only the fibrinogen levels were decreased non-significantly during the 4-hour observation period. The fibrinogen is known to be an acute phase protein, so its

moderate decrease is probably a net change determined by the consumption during the coagulation activation process and the elevation by the acute phase reaction.

Contrary to coagulation parameters, platelet count decreased significantly in the septic group already by 2 hours – corresponding to the sequestration of activated platelets after *E. coli* administration –, with the simultaneous appearance of platelet aggregates and these changes progressed until the end of the observation period. It is important to note, that unlike humans, the animals in this study received no systemic heparin anticoagulation. Thus, the changes in platelet number and platelet aggregate formation - although in a considerably attenuated form - were also observed in untreated animals by 4 hours, that may have been due to the hemostatic activation associated with the blood vessel preparation and further invasive procedures related to general anesthesia and 'sham treatment' that all occured without systemic anticoagulation. We thought to determine platelet activation by measuring the ratio of PS positive platelets by annexin V binding. We found a non-significant elevation in the PS positive platelet ratio in *E. coli*-challenged animals. Since already at 2 hours the platelet count significantly decreased, it is quite likely that the most activated platelets were sequestrated and thus became undetectable in the circulation. It has been described that – irrespective of the cause – thrombocytopenia is an independent predictor of mortality in critically ill patients and in sepsis. In humans it is difficult to evaluate whether thrombocytopenia represents platelet activation and consumption as a primary pathologic event or merely serves as a marker of disease severity since medications, bone marrow suppression, nutritional deficiencies and infection may all alter the platelet number. The intravenous infusion of live bacteria in our fulminant model ensures that the above variables are not affecting the changes observed in relation to platelets. Thrombin-dependent platelet sequestration is a key event during endotoxemia as was demonstrated earlier in a murine model.

Furthermore, we carried a series of separate experiments where we incubated normal human washed red blood cells with plasma samples drawn at 0, 2 and 4 hours from septic and control animals and observed their PS inducing capabilities. We found that plasma from control pigs have no PS inducing capacity on human red blood cells, while plasma samples drawn at 2 hours from septic animals induced a significant PS expression. This demonstrates that soluble substances that are capable of inducing a procoagulant surface on cells are released during the *E. coli* challenge. Previous publications reported that extracellular histones released in response to inflammatory challenge were able to induce PS exposure on red blood cells in human studies. Beside histone H4, cell-free DNA was described to have an important role in procoagulant features during sepsis. Lang and coworkers were able to induce eryptosis by the supernatant of pathogens. This eryptosis was indicated by the increased PS expression of red blood cells and was correlated with the sphingomyelinase activity of the supernatant.

They hypothesize that during sepsis the bacterial sphingomyelinase and PAF enhance ceramide production in the red blood cells, and this ceramide sensitizes the red blood cells for the increase of the intracellular calcium concentration resulting in eryptosis. Furthermore the release of proinflammatory cytokines (e. g. IL-6, TNF-α) results in endothelial cell, leukocyte and platelet activation, so they may have an effect on red blood cells, too. Oliver et al described that TNF-α had a peak plasma concentration of 2 hours after exposure to endotoxin followed by a rapid decline. Since our septic pigs were treated with *E. coli* for 3 hours and the half-life of LPS is also considerably short, it is possible that TNF-α and/or LPS may have a significant role in the stimulation of red blood cells. In addition, in the *E. coli*-treated group a significant elevation of LDH activity was also detected by 4 hours suggesting the development of haemolysis in sepsis. In our in vitro experiments if only red blood cells and plasma were present, neither TNF-α, nor LPS activated the red blood cells and resulted in haemolysis, therefore none of them seem to have a direct effect on red blood cells. If TNF-α and/or LPS were added directly to the anticoagulated whole blood (in this case white blood cells and platelets were present in the samples), the PS expression of red blood cells did not increase but the high dose LPS resulted in haemolysis. Since in previous studies of our research group the powerful sepsis induction caused death of all animals within a few hours, we studied the effect of high LPS concentrations – corresponding to non-survival patients – in our in vitro experiments. Our result suggested that TNF-α and LPS do not induce the PS expression of red blood cells directly or indirectly. TNF-α does not have any effect on red blood cells, while LPS can induce haemolysis indirectly. As a cause of PS inducing capacity of plasma samples obtained by 2 hours after sepsis induction, some kind of other soluble substances (e. g. bacterial sphingomyelinase, other proinflammatory cytokines, PAF, histones, cell-free DNA) may be hypothesized.

Thrombin generation as a global test of coagulation has emerged in the past decades and has been used in several studies. Thrombin generation studies in sepsis are a lot more sparse. In septic animal models using the introduction of live bacteria to animals such measurements have not been published. Clinical studies mostly detected hypocoagulation in septic patients with a decrease in thrombin peak values and a prolongation of the time to start coagulation and reach the maximum thrombin formation like lag time and time to peak parameters. More recently in a large number of patients Carlier et al investigated thrombin generation pattern in patients with and without DIC and concluded that due to the large overlap between thrombin generation values this test is not useful in clinical practice to study septic patients. A study that evaluated thrombin generation in severely septic patients compared these parameters in survivors and nonsurvivors. They found that nonsurvivors displayed a lower peak thrombin value and shorter lag time and time to peak parameters

compared to survivors at most investigated time points. Our study is a lethal porcine model that mostly mimics the pathology observed in nonsurvivor human septic patients. Similarly to others we have also observed in the PPP samples a significant shortening in lag time and time to peak by 2 hours, furthermore a significant decrease in peak thrombin and ETP by 4 hours was observed when the assay was done in the presence of exogenously added TF and phospholipids. These changes are reflecting an enhanced coagulation activation but almost simultaneously – due to consumption coagulopathy – a decreased ability to generate thrombin in early sepsis. In controls the mild ETP reduction by 2 hours may be interpreted by the initial mild consumption of the extrinsic and common pathway factors.

In addition, we also studied thrombin generation in PPP per se and we have observed a continuous shortening in lag time and time to peak which became significant by 4 hours, and a significant increase in peak thrombin by 2 hours. These results also support the presence of an initial and short-lived hypercoagulable phase in sepsis. Wang and coworkers described increased thrombin generation within 3 hours after cecal ligation and puncture, and marked reduction in thrombin generation by 6 and 24 hours after sepsis induction in their murine abdominal sepsis model.

We suppose that human studies usually fail to demonstrate the initial hypercoagulability in sepsis which might be caused by the delay between early changes in the coagulation system and clinical presentation of septic symptoms; furthermore in thrombin generation assays a coactivation with tissue factor and phospholipid is used. In our study obvious sepsis developed at 4 hours after *E. coli* administration while changes in thrombin generation in PRP and in PPP, furthermore platelet count decrease and increased aggregate formation occurred as early as 2 hours after sepsis induction indicating early coagulation and platelet activation in sepsis. During our studies we used three different settings in the thrombin generation test in PPP and via all series of measurements we could verify coagulation activation as evidenced by shortenings in time related parameters that describe the speed of thrombin formation. However, the ability to detect hypercoagulability with enhanced peak thrombin formation requires that the thrombin generation test is carried out in the absence of any added agonist. If both tissue factor and phospholipid agonists were added, due to the consumption of coagulation factors a hypocoagulability was detectable by the peak thrombin and ETP assays. Another disadvantage of the human studies is that PRP samples frequently can not be studied as platelet count severely drops during sepsis. In our porcine model we also evaluated PRP where the platelet count was adjusted to the same value and have observed similar but somewhat modulated results compared to PPP. The time to peak parameters shortened similarly to PPP at 2 hours and this was maintained until the end of the experiment, however no decrease in peak thrombin and ETP was seen. In PRP samples, the

thrombin generation is elicited only by a tiny amount of synthetic tissue factor and the platelets provide the required phospholipid surface to promote the formation of multiprotein complexes that will eventually result in thrombin generation. Thus, the compensating effect of activated platelets present in the sample may have prevented the decrease of thrombin formation that was observed in the PPP sample.

We have provided evidence that the thrombin generation assay is a useful tool in evaluating true hyper- and hypocoagulability in severe septic conditions but the results strongly depend on the assay setting. This study also confirmed previous reports that global coagulation assays are superior to routine coagulation tests in sepsis. These traditional coagulation tests cannot show the balance of the procoagulant and anticoagulant systems. One reason is that they use clot formation as endpoint which occurs when only about 5% of all physiologically relevant thrombin is formed, so they do not give information about 95% of the generated thrombin. Another reason can be that the natural anticoagulants do not have their normal activity in these systems (e. g. thrombomodulin found on the surface of endothelial cells is needed for protein C activity). In contrast, thrombin generation assays may inform us about both procoagulant and anticoagulant systems, they use less tissue factor and phospholipid concentrations than coagulation screening tests resulting in increased sensitivity, and they are suitable for measuring thrombin generation also in the presence of platelets.

6. Summary

Sepsis is a leading cause of death worldwide, however early diagnosis and adequate therapy can improve prognosis. Use of animal models is a relatively well standardized way to study sepsis. We investigated a lethal porcine sepsis model in which sepsis was induced by the intravenous administration of live *E. coli*. We aimed to study the kinetics of infection and hypoxia induced changes, furthermore we extended to explore early haemostatic alterations during fulminant sepsis. In our sepsis model the appearance of immature red blood cells and platelets, the platelet mitochondrial membrane depolarisation, the elevation of uric acid concentration, as well as platelet and coagulation activation occured prior to the development of manifest sepsis. Results of our flow cytometric measurements suggest that during sepsis the mitochondrial dysfunction may occur even in the absence of significant hypoxia. Urate level was found to be a more sensitive indicator of the oxidative stress than lactate concentration. The early and significant decrease in platelet count together with the increase of platelet aggregates indicate early platelet activation in sepsis. We demonstrated that soluble substances that are capable of inducing PS expression on normal red blood cells were released during the *E. coli* induced sepsis, however the role of TNF-α and endotoxin could be excluded. We have provided evidence that the thrombin generation assay was useful in evaluating true hyper- and hypocoagulability in early stage of sepsis but the results depended on the assay setting. We found that thrombin generation assay was superior to routine coagulation screening for the detection of early haemostatic alterations in sepsis.

7. Main new scientific findings and their clinical significance

1. In our lethal porcine sepsis model the appearance of immature red blood cells and platelets, the platelet mitochondrium membrane depolarization, the elevation of the uric acid concentration, as well as the activation of platelets and coagulation occurred prior to the developement of manifest sepsis.
2. The number of nucleated red blood cells and dysplastic platelets increased only in the septic animals, therefore these parameters are suitable for early detectation of sepsis. The NRBC elevation is more specific for sepsis than the reticulocyte elevation.
3. The membrane of platelet mitochondria became depolarised in early phase of sepsis without significant tissue hypoxaemia, which indicates that during sepsis the mitochondrial dysfunction may occur even in the absence of hypoxia.
4. As a result of oxidative stress, the uric acid concentration increased earlier and more significantly than the lactate level in pigs. This result suggests that the urate level may be a more sensitive marker of oxidative stress than lactate concentration.
5. The early and significant decrease in platelet count together with the increase of platelet aggregates indicated early platelet activation in sepsis. Because sepsis was induced in case of healthy animals not receiving any medication, the decrease of platelet count suggests that thrombocytopenia is the marker of response to the infection.
6. During the *E. coli* induced sepsis, soluble substances that are capable of inducing PS expression on normal red blood cells were released however the role of TNF-α and endotoxin could be excluded. High dose endotoxin was able to induce haemolysis but the presence of white blood cells and platelets was needed for this effect.
7. The thrombin generation assay was useful in evaluating true hyper- and hypocoagulability in early stage of sepsis but the results depended on the assay setting. In platelet poor plasma both hyper- and hypocoagulability could be detected in the presence of agonists, while pure hypercoagulability was found in the absence of them. We found that thrombin generation assay was superior to routine coagulation screening for the detection of early haemostatic alterations in sepsis.

8. List of publications

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Registry number: DEENK/410/2017.PL
Subject: PhD Publikációs Lista

Candidate: Judit Tóth
Neptun ID: TJ0226
Doctoral School: Kálmán Laki Doctoral School
MTMT ID: 10038606

List of publications related to the dissertation

1. **Tóth, J.**, Bekéné Debreceni, I., Deák, Á., Pető, K., Berhes, M., Hajdu, E., Szabó, J., Németh, N., Fülesdi, B., Kappelmayer, J.: Characteristics of thrombin generation in a fulminant in a fulminant porcine sepsis model.
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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