Ph.D. THESIS

INVESTIGATION OF THE HEMORHEOLOGICAL FACTORS AND THE MICROCIRCULATION IN EXPERIMENTAL LIMB ISCHEMIA-REPERFUSION MODELS

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1. INTRODUCTION

Hemorheology is deal with the rheology of blood, and the word itself was introduced by Alfred Levin Copley in 1951, and concerned with the deformation and flow properties of cellular and plasmatic components of blood with interaction of the vascular system.

The dynamically regulated rheological imprint parameter, the whole blood viscosity is determined by many factors, including physical (shear rate, temperature, stasis), morphological (vasculature), and physiological (hemodynamics, fluid distribution, red blood cell deformability and aggregation, plasma viscosity, fibrinogen level, blood cell count) parameters. Among hemorheological factors parameters described the deformation properties of red blood cells play an important role. The adequate deformation property is essential not only for the required perfusion, but it influences the lifespan of erythrocytes.

Hemorheology is multidisciplinary, extended on large clinical and experimental filed, including ischemic- or ischemia-reperfusion injuries, such as cardio-cerebrovascular affection, traumatological or surgical vessel obturation or closure, and furthermore the tissue- and organ transplantation.

It is generally accepted that blood vessel occlusions of different origin, such as occlusion, obturation by pathological processes or surgical interventions, result in damages on different tissues or organs, causing ischemic or ischemia-reperfusion injuries. Reperfusion of ischemic tissue can lead paradoxically to further changes beyond the damage established during ischemia. This phenomenon is known as ischemia-reperfusion (I/R). Ischemia of extremities may cause injuries not only in the tissues of the affected region but also in farther tissues and organs, and may result in lethal complication. Despite significant development in vascular surgery in last decades, in clinical practice acute limb ischemia remains one of the most common and severe events influencing the function of organs and limbs, while the complex pathomechanism of ischemia-reperfusion injury is not clarified completely.

Ischemia and the following reperfusion may generate several processes in tissues and organs, which changes can alter the rheological properties of blood and the functional and morphological state of microcirculation beside of the local tissue damage. Change in pH, free radicals reactions, mediators, local or systemic deviations of hemodynamics, altered fluid composition and distribution, may affect the hemorheological factors through its several parameters.

Ischemia leads to degradation of ATP to hypoxanthine, which provides a substrate for XO. Normally, more than 90% of the XO in tissue exists in the form of xanthine-dehydrogenase (XD), which cannot transfer electrons to molecular oxygen – mainly during reperfusion – to form superoxide. During ischemia, XD is converted to XO, which uses oxygen as an electron acceptor and generates superoxide. Allopurinol is non-competitive inhibitor of XO enzyme, and through its inhibition can block the development of superoxide free radicals in ischemia-reperfusion.

Red blood cells are among the most susceptible cells for damage caused by free radicals: they are exposed to high oxygen tension, their membrane contains multiple poly-unsaturated fatty acids, and these cells are rich in iron, which promotes the formation of oxygen free radicals. Oxygen-derived free can damage red blood cells on these targets: membranes (e.g. lipid peroxydation), modified enzymes and other protein functions, altered hemoglobin molecules (e.g. Heinz-bodies), causing loss deformability or hemolysis.

Consequently, investigation of hemorheological parameters gives valuable information in several processes, but the applicability in experiments on different animal species sets many problems, mainly in the light of the required blood quantity for tests and species specific alterations.

We are working on different animals species in our department. Due to the correct investigation and follow-up studies hemorheological measurements had to be adapted species by species. This demand of applicability mainly for small laboratory animals (mouse, rat) is mainly on follow-up studies of limb ischemia-reperfusion models in rats and on functional investigation of spleen autotransplants in mice.

Our other field of interest is the tissue microcirculation. Ischemia-reperfusion at microcirculatory level in characterized by vasospasm, swelling of endothelium, increased capillar permeability, interstitial oedema, subsequential thrombus formation and „no-reflow" phenomenon.

The measurement of the tissue microcirculation can be performed by many indirect and direct, invasive or non-invasive techniques. One of these methods used to examine tissue microcirculation is the laser Doppler flowmetry, which has many positive and negative properties, standardization and measuring problems, even though in other hand it has very sensitive properties to reflect the acute changes in the microcirculation, therefore a

well reproducible and comparable method is required to analyse the effect of ischemia, ischemia-reperfusion on tissue microcirculation.

2. OBJECTIVES

1. Adaptation of hemorheological measuring techniques for different laboratory animal species (mouse, rat, rabbit, mongrel dog, pig), their comparative analyses in the aspect of species specific properties.
2. Harvesting of adaptation method on red blood cell deformability measurement using Carat Ft-1 filtrometer that provide opportunity to perform follow-up studies in small laboratory animals (mice, rats).
3. Investigation the effect of short-term (1-hour) ischemia and the following reperfusion on red blood cell deformability in 1-weekly follow-up study the rat, make a comparison with certain hematological parameters. Testing the possible beneficial effect of xanthine-oxidase inhibitor allopurinol in this model.
4. To analyse the direct effect of 1-hour ischemia on tissue microcirculation using laser Doppler flowmetry with a new test in limb ischemia-reperfusion rat model.
5. Investigation and follow-up of local and systemic hemorheological effect of limb ischemia-reperfusion in a canine model, harvested by one of our work-group.

3. MATERIALS AND METHODS

As hemorheological parameters, red blood cell deformability, whole blood and plasma viscosity, certain hematological parameters and fibrinogen level were investigated. Due to the first, methodical part of the dissertation, the measurement techniques then their adaptation and ischemia-reperfusion models based on the latter are presented.

3.1. Determination of the red blood cell deformability

3.1.1. Sample preparation

For measurement of red blood cell deformability blood samples from large laboratory animals (mongrel dog, pig) were taken into Vacutainer tubes containing sodium heparin (143 IU), while blood from small laboratory animals (mouse, rat) were collected directly into heparinized syringe (10-20 IU/ml). Samples were centrifuged at 2500 g for 10 minutes,

and then plasma and „buffy coat" were removed. The cell suspension was washed in phosphate buffered saline (PBS) (pH: 7.4, osmolarity 300 mOsm/l) and centrifuged. After the centrifugation we washed the suspension in PBS again and determined the hematocrit value (Hct) in Janetzky capillary centrifuge (5 min). According to the measured Hct we added more PBS to adjust the hematocrit of red blood cell suspension (1-5%).

3.1.2. Measurement of red blood cell deformability

To measure the filterability of red blood cell suspension Carat FT-1 filtrometer was used (Carat Ltd., Hungary) based on St. George's filtrometer technique. We performd the test 3 times at each sample. Measurements were carried out within two hours after blood taking and were performd at room temperature (22 ± 1°C). In this instrument diluted red blood cell suspension flows through a polycarbonate filter membrane (Nuclepore® filter, average pore diameter = 5 μm) at constant pressure (4 cm of water). Filtration rate is measured at four pairs of light sources and photodetectors. The unit is interfaced to a computer, which automatically analyses sequential flow rates and determines two calculated parameters: initial relative filtration rate (IRFR) and relative cell transit time (RCTT).

3.2. Measurement of whole blood and plasma viscosity

Blood samples were collected into sodium heparin coated Vacutainer tubes (143 IU) for viscosity measurements. During blood sampling we have avoided the strangulation. Plasma was prepared by centrifuging at 1500 g for 10 minutes. Viscosity measurements were carried out at 37 ºC using Hevimet-40 capillary viscosimeter (Hemorhex Ltd., Hungary) within 2 hours after sampling. The device consists of two capillary tubes plunged into temperate oil chamber (37 ºC). Plasma or whole blood was injected into the capillary tube. The flow of the fluid is detected optoelectronically along the capillary tube and a flow curve is drawn. Shear rate (10-240 s^{-1}) and shear stress is calculated by a computer program, and viscosity values are given in mPas. For presentation of our data whole blood viscosity (WBV) values at 90 s^{-1} shear rate are used. According to the mathematical formula given by Matrai and co-workers, we made correction of whole blood viscosity data for 40% hematocrit ($WBV_{40\%}$) in relation of plasma viscosity (PV) and the actual hematocrit (Hct):

$$WBV_{40\%} / PV = (WBV / PV)^{40\% / Hct}$$

3.3. Determination of the fibrinogen level

Fibrinogen level was determined using Sysmex CA-500 automated coagulometer (TOA Medical Electronics Co., Japan) based on Clauss's method.

3.4. Determination of the hematological parameters

The general hematological parameters (quantitative-qualitative) were determined using Sysmex F-800 microcell counter (TOA Medical Electronics Co., Japan).

3.5. Description of the experimental models

Our experiments were approved by the Committee of Animal Research at University of Debrecen. Author's permission number: 6/2000. UD CAR.

3.5.1. Species specific adaptation of hemorheological measurements

3.5.1.1. Experimental animals for comparative analyses

We summarize red blood cell deformability and blood viscosity data of several animal species from normal, control groups of different experiments (before any interventions): A/J inbred mice (23-28 g, n=20), CD outbred [Crl:CD® BR] rats (250-350 g, n=36), rabbits (3.2-3.8 kg, n=32), mongrel dogs (20-25 kg, n=62) and juvenile Durok/Cornwall pigs (30-35 kg, n=13). Blood samples were collected from femoral vein and saphenous vein in rat, with intracardial puncture in mice, from lateral ear vein in rabbits, from cephalic vein in dogs and from femoral vein in pigs, according to the actual experimental protocols.

3.5.1.2. Analysis of the possibility to perform one-weekly follow-up study

Twelve male CD outbred rats (250-350 g) were subjected to a basic one-weekly follow-up pilot study. After anaesthesia the left femoral vein was exposed using microsurgical techniques, and 2-2.5 ml blood was collected by venipuncture, then 3 ml physiological saline solution was injected intraperitoneally. The vein punctured wound was and the skin was sutured. After 1 week the procedure was repeated, and the animals were sacrificed by overdose of pentobarbital. Hematological parameters and red blood cell deformability were determined, for latter using 5% cell-suspension, in base and 1-week samples.

3.5.1.3. Dilution series for comparative deformability measurement

In 10 CD outbred rats (250-320 g) anaesthetized with pentobarbital (35 mg/kg, i.p.) left femoral vein was exposed and punctured with 22 G needle, and 3-4 ml blood were collected, then the animals were sacrificed with an overdose of pentobarbital. Blood samples were divided into 5-5 parts, and were prepared for red blood cell deformability measurements as 5, 4, 3, 2, and 1% cell suspension, then filtration tests were done.

3.5.2. Hemorheological investigation of hind limb ischemia-reperfusion in the rat

3.5.2.1. Operative protocol

According to the above presented technical adaptation of red blood cell deformability measurements we perform a follow-up study on limb ischemia-reperfusion in the rat. Twenty-six male CD outbred rats (250-350 g) were subjected to the study. Anaesthesia was induced with pentobarbital (35 mg/kg, i.p.) combined with atropine (0.01 mg/kg, s.c.). The external jugular vein was cannulated, then the animals were randomised into 4 groups.

(I) In the case of *control* animals no other action was made (Control group, C, n=6). (II) In *sham-operated* group the left femoral blood vessels and their collateral branches were exposed by a parallel incision to the inguinal ligament. We used microsurgical techniques to avoid and decrease tissue injuries. The wound was left open for one hour covering the operating field with sterile wet gauze (Sham-operated group, Sh, n=6), (III) in the *ischemia-reperfusion* group after surgical preparation microvascular clips were used to compress the femoral artery and vein for 1 hour (Ischemia-reperfusion group, I/R, n=6). After the one hour ischemic period the section was closed after the removal of the clips using single interrupted suture line. (IV) Animals of the *allopurinol pre-treated ischemia-reperfusion* group received 50 mg/kg allopurinol intraperitoneally (125 mg allopurinol [Egis Corporation, Hungary] in 20 ml 0.9 % saline solution) 30 minutes before the same intervention, as the ischemic group had (Allopurinol+Ischemia-reperfusion group, AP+I/R, n=8). In every group base blood samples (0.6-0.7 ml) were collected from the cannulated external jugular vein and we injected 2 ml physiological saline solution at body temperature into the abdominal cavity (AP+I/R group received it containing allopurinol). The animals received no anticoagulants. At the end of the experimental period of one week the animals were sacrificed with an overdose of pentobarbital.

3.5.2.2. Laboratory investigation protocol

After operations 0.6-0.7 ml blood was collected from contralateral saphenous vein daily for 1 week. Red blood cell deformability in 1% cell suspension and hematological parameters were determined.

3.5.3. Microcirculatory investigation of hind limb ischemia-reperfusion in the rat

3.5.3.1. Operative protocol

Twelve anaesthetised (sodium pentobarbital 35 mg/kg, i.p., atropine 0.01 mg/kg, s.c.) male CD outbred rats weighing 250-350 g were subjected to the study. In the operating room the temperature was controlled and kept on 22-23 °C during the whole procedure. Animals were placed on a heating pad to maintain a constant body temperature of 37 ºC, which was controlled with a rectal probe during the experimental period. In sterile conditions on both sides we have exposed the quadriceps femoris muscle, the femoral artery and vein and their collateral branches using a section parallel to the inguinal ligament. Then we have prepared the right and left external iliac blood vessels from lower transversal laparotomy and ligated the superficial and inferior epigastric arteries and veins. On the left side we compressed the femoral artery and vein for 1 hour using microvascular clips (Ischemic side, I), while on the other side the wound was covered with wet sterile gauze (Control side, C). After the ischemic period of one hour the clips were removed. The animals did not receive anticoagulants. At the end of the experimental period the animals were sacrificed with an overdose of pentobarbital.

3.5.3.2. Laser Doppler flowmetry and the „occlusion”-test

It was Stern in 1975 who described the laser Doppler technique first for objective, non-invasive and well-reproducible analyses of tissue microcirculation, giving relative blood flow units. Many factors may affect the measurement: it depends on the features of the examined tissue, the instrument and external factors. We harvested a special test, the „occlusion”-test using one-channel laser Doppler instrument (LD-01 Laser Doppler Flowmeter, Experimetria Ltd., Hungary). We put the fixed laser Doppler probe (NP-100 Standard Pencil Probe) on medial vastus muscle. After stabilizing the laser Doppler signal we clamped the external iliac vessels for 2-3 seconds with atraugrip clips, while the signal was permanently detected with data acquisition unit. Before and after the interventions

described in chapter 3.5.3.1., 3-3 „occlusion"-tests were performed on both side. The time interval between tests was 10 seconds. Consequently, with fixing the probe and signal stabilization, on Ischemic side tests were made in the first 5-10 minutes of the reperfusion.

3.5.3.3. Analysis of the recorded laser Doppler signal

After „occlusion"-tests in data processing we emphasized on the length as time duration of the downward (descending curve, D) and upward part (ascending curve, A) of the recorded laser Doppler signal-curve, which describe the failure and the restart of perfusion. After inserting of average-line we compared the times in msec at the 50 percent amplitude (T_{50}) of the down- and upward signal, and we calculated the descending/ascending ratio using the T_{50} of downward and upward curves (D/A index = T_{50Desc}/T_{50Asc}).

3.5.4. Hemorheological investigation of prolonged hind limb ischemia-reperfusion in dog

Earlier, our work-group has harvested the present surgical model on hind limb ischemia-reperfusion correlated to certain trauma cases. The local and systemic hemorheological changes are in recent theses, therefore the operative technique is presented briefly.

Eleven mongrel dogs (3-4 years, male and female, 18-26 kg) after anaesthesia (ketamin 10 mg/kg + xylazin 1 mg/kg, i.m.) were subjected to preparation and cannulation of left external jugular vein, and the left femoral artery and vein were exposed, and they were randomised into 2 groups:

I. Sham-operated group (n=6): after the surgical preparation the wound was left open for 7 hours covering the operative field with sterile wet gauze, then the fascia lata and the skin was sutured.

II. Ischemia-reperfusion group (n=5): the femoral vessels were clamped for 3 hours using vascular clamps, while a sterile steel loop was stretched around the thigh under the prepared vessels to close out the soft tissues. Four hours after releasing the vessels, the steel loop was removed. Finally, this model is consisted of 3 hours of total ischemia and 4 hours of vascular reperfusion (through the femoral vessels).

Blood samples were taken before the operation, from the excluded region after 3 hours, during the first hour of the reperfusion and for 5 postoperative days, and whole blood and plasma viscosity, red blood cell deformability, fibrinogen level and hematocrit were determined.

3.6. Statistical analyses

For statistical analyses software SigmaStat for Windows (SigmaStat 1.0, 1992-1994., Jandel Scientific Co., Germany) and non-parametric tests were used. At *species specific hemorheological measurement adaptation* for double-sample probes paired t-test and Wilcoxon signed rank test was used. To examine the relation between RCTT values and the hematocrit of the elements of the *red cell suspension dilution series* linear regression analysis was preformed. In *rat hind limb ischemia-reperfusion experiments* differences among groups were evaluated by one-way analysis of variance with Student's t-test and Mann-Whitney rank sum test for inter-group comparisons; and Wilcoxon signed rank test for intra-group (before-after) comparisons. In *hind limb ischemia-reperfusion experiments in mongrel dogs*, for intra-group analysis ANOVA and Dunnett's tests, for inter-group comparisons Mann-Whitney rank sum and Kruskal-Wallis tests were used. Significance level was set at $p<0.05$.

4. RESULTS AND CONCLUSIONS

4.1. Species specific adaptation of hemorheological measurements

4.1.1. Comparative analyses of hemorheological parameters

Red blood cell deformability

Almost linear correlation in RCTT values was found in mouse, rat, rabbit, mongrel dog and pig if we make the comparison with the body size. Rabbit was the only exception, where transit time values were lower than in the rat. Comparing these RCTT deviations to the mean corpuscular volume (MCV) of erythrocytes similar tendency was found, except that MCV_{rabbit} is larger than MCV_{rat}, and near the same to the MCV_{dog}, while MVC_{pig} was unexpectedly low. Species specific comparative studies on red blood cell diameter in different animal species similar remarkably distribution can be found.

Whole blood and plasma viscosity

Plasma viscosity in rat, rabbit, dog and pig did not show important difference. Whole blood viscosity was extremely high in rat, which difference remained also after correction for hematocrit (40%).

Data of investigation on hemorheological factors in different animal species corroborate that comparative studies can be done only in the light of species specific properties and using measuring technique adaptation for current species. Conspicuous differences of red blood

cell deformability values can be brought into relation with the cell size and diameter. Similar conclusion is published by Chen's (1994), Baskurt's (1996) and Katyukhin's (1998) work-groups.

4.1.2. Analysis of the possibility to perform one-weekly follow-up study

To clarify the effect of taking the standard quantity of blood (2-2.5 ml) in rat for deformability measurements set at 5% red blood cell suspension, we measured the red blood cell deformability in rat (n=20) in base blood samples (2-2.5 ml) and one week later according to the standard methodical description. RCTT values were significantly lower in one-week samples, on 7^{th} postoperative day compared to the base (p=0.0098), while hematological parameters did not show important differences.

After species like comparative study *methodical adaptation* had to be done *within certain species*. Required blood sample quantity in the original methodical description is too much to use it in small laboratory animals, such as mice and rats, otherwise, planned follow-up studies require even daily blood takings, therefore the quantity have to be reduced. Sampling of 2-2.5 ml blood might means a hemopoietic stimulus that resulted in appearance of high number of young erythrocytes having good mechanical properties. These could be the background of the improved deformability parameters (possible false negative results in follow-up studies) in the samples taken on the 7^{th} day.

4.1.3. Dilution series for comparative deformability measurement

The results of this pilot study showed that there is no significant difference in the dilution series of 1-5% red blood cell suspension prepared from 10 rats comparing the neighbouring elements, but showed a decreasing tendency toward 1% values (linear regression of RCTT with Htc [5-1%]: 0.2160). Significant difference was observed 1% vs 4% (p=0.0186) or 5% (p=0.0116), and 2% vs 4% (p=0.018) or 5% (p=0.0115). Therefore, according to our opinion, data from the diluted suspension determined consequently at the same suspension hematocrit are comparable safely in rat blood.

The technique of red blood cell deformability measurements theoretically makes it possible to *reduce significantly the quantity of the required blood*, if the red blood cell suspension is set at1% instead of 5%. *According to our work we successfully determined red blood cell deformability in 1% cell suspension*, so, consequently *0.5-0.6 ml blood is enough* for the

tests, and the method became *applicable for follow-up investigations* of red blood cell deformability measured by filtration technique *in the rat*, which possibility can not be found in the literature.

4.2. Hemorheological investigation of hind limb ischemia-reperfusion in the rat

4.2.1. Red blood cell deformability

In the ischemia-reperfusion group (I/R) relative cell transit time (RCTT) showed a remarkably elevated level on the first (vs I/R base: p= 0.006; vs C: p=0.0002) and the second postoperative day (vs I/R postop. 1st day: p=0.0006; vs I/R postop. 3rd day: p=0.0184; vs C: p<0.0001; vs Sham-operated: p=0.008; vs AP+I/R: p=0.0025), while the control and the sham-operated group did not exhibit any significant changes. The group pre-treated with allopurinol expressed a moderate increase compared to the base in a non-significant manner.

We used *1% red blood cell suspension* for filterability measurements in *one-weekly follow-up study* of hind limb ischemia-reperfusion model in the rat. The short-term (*1h) hind limb ischemia and the following reperfusion resulted in well-detectable decrease of erythrocyte filterability*, which was probably caused by free radicals and could be *reduced by allopurinol pre-treatment*.

4.2.2. Hematological parameters

There were no significant differences in *red blood cell count*, *hemoglobin level* and *hematocrit* values, even though they decreased in postoperative period. Values of *mean corpuscular volume (MCV)* and *mean corpuscular hemoglobin (MCH)* expressed slight elevation in postoperative period resulted in significant increase on the 7th postoperative day in I/R and AP+I/R. Although the technical adaptation on filtration technique (using 1% red blood cell suspension in follow-up study) was accomplishable in one-weekly period, hematological parameters showed a moderate tendency toward anaemia.

The *white blood cell count* varied not significantly and not characteristically. The *platelet count* showed a continuous increase in each group resulting in a significantly elevated level both in I/R and AP+I/R groups on the 7th postoperative day – compared to the control group. Increase in platelet count can be a part of the acute phase processes after surgery, and the possible effect of the daily blood takings also are not negligible.

The *mean platelet volume (MPV)* was altered significantly in the ischemic group only on the 2nd postoperative day compared to the control (p=0.0011), and later it seemed to be returned to the base values, showing further decreasing on 4th -7th days similarly in control and sham-operated groups. In 1974, it was first described that platelet count and MPV were inversely related, suggesting that the platelet mass (Plt count x MPV) is near constant. By our data platelet mass increased in each group, but mainly in ischemia-reperfusion group.

4.3. Microcirculatory investigation of limb ischemia-reperfusion in the rat

Characteristic and remarkable changes were observed after the 1-h-long ischemic period on the upward and downward part of the flow-curves representing the restart of the circulation, recorded during „occlusion"-test, compared to the control side or to the state before the intervention. There was no important difference between the time values at 50 percent of the up- and downward line amplitude of the signals ($T_{50Asc,}$ T_{50Desc}) for the two sides before the treatment. After 1-hour ischemia the upward part of the „occlusion test"-curves was significantly longer on the affected side than before the ischemic insult or than the values of the control side (T_{50Asc}, p=0.0007, p=0.0017, respectively). The downward part significantly shortened comparing to the control side (T_{50Desc} before and after the intervention: p=0.0016, p=0.0049, respectively). In the aspect of the D/A index (D/Ai) it means that the index value shifted from a value above 1.00 to a value under 1.00.

The *advantages* of our above presented „occlusion"-test are its simplicity, quick and reproducible properties in order to analyze laser Doppler flow curves. According to this method data are independent of the starting and end-point flow unit values, which can be easily altered by many factors mentioned above. Our recent measurements have some *disadvantages*. The tests were performed near blood vessels that condition may disturb the correct measurement of the tissue microcirculation. Further, the anaesthesia with pentobarbital was shown to affect the microcirculation, in addition the surgery-induced soft trauma cannot be excluded. Although, according to the stable measuring point, the response reaction can give valuable information for the quantitative analyses.

A relatively short 1-hour ischemia had already caused detectable changes in the microcirculation of muscle tissue. During the „occlusion"-test, the descending parts of the recorded curves were significantly shorter, and the ascending curve parts were significantly longer than the control states, and the D/A index value-flip was observable. It might mean

that the circulation or perfusion can easily failure and can hardly restore after 1-hour ischemic insult, causing probably by the changed microhematocrit distribution at capillary level, or the locally impaired red blood cell deformability.

The presented test can be applicable in the future for quick, intraoperative examination of the effects of different surgical presses on tissue microcirculation. Our data are preliminary, therefore summarizing and exact mathematical analysis of the recorded curves and safety of the test require more thorough measurements.

4.4. Hemorheological investigation of prolonged hind limb ischemia-reperfusion in dog

4.4.1. Red blood cell deformability

Relative cell transit time (RCTT) of the sham-operated group did not exhibit significant changes during the operative and postoperative period.

During the operative period in ischemia-reperfusion group (I/R) essential RCTT alterations can not be found. Later, remarkable elevated RCTT values are seen on 2^{nd} and 3^{rd} day, which values are significantly higher versus base ($p<0.0001$) and versus sham-operated on 3^{rd} day ($p=0.0023$). RCTT elevation seems to be normalized for 4^{th} – 5^{th} day, values measured on 4^{th} day were significantly lower versus 3^{rd} day ($p=0.0086$).

4.4.2. Whole blood viscosity

The *actual whole blood viscosity* (WBV) measured at 90 s^{-1} shear rate reached the highest values on the 1^{st} postoperative day, and decreased for the 2^{nd} day and showed further slight decrease during the remained postoperative period accompanied by similar changes in the hematocrit values. Otherwise, after *correction for hematocrit (40%)* WBV values expressed slight increase during postoperative days in I/R group, showing the highest values on the 5^{th} postoperative day.

4.4.3. Plasma viscosity

The relative homogeneity, observed in base values of the four groups, disappeared for the end of the 3-hourly operative period. In sham-operated group plasma viscosity slightly increased during operative period, and reached its highest values on 1^{st} postoperative day (1.597±0.068 mPas, vs. base: $p=0.0459$), then decreased under the base values on 2^{nd} to 5^{th} days. Plasma viscosity values of the I/R group increased for 1^{st} postoperative day, and after

the slight decrease on 2nd – 3rd day remarkable elevation was found on 4th and 5th days, latter was significant compared to its base and to sham-operated group on 5th day (p=0.0014, p=0.0007, respectively).

4.4.4. Fibrinogen level

Significant changes in fibrinogen level were not found during the operative period. From 1st postoperative day decided increase was observed in both groups, which elevation stabilized between 3rd and 5th day. The most expressed elevation was expressed by the I/R group, where fibrinogen level has almost risen twofold between 1st and 2nd day (2.806±0.271 → 5.034±0.12 g/l; p<0.0001). The elevated fibrinogen levels were significant versus base values on 3rd – 5th day in sham-operated group (p=0.0154, p=0.0373, p=0.0315, respectively).

4.4.5. Hematocrit

Hematocrit values expressed moderate increase during the operative period, were significantly elevated on 1st postoperative day compared to the base, then decreased on 2nd – 5th days. The most impressive hematocrit decrease was found in sham-operated group between the 1st and 2nd day (p=0.0335). Between 2nd and 3rd day significant changes were not observed, while in I/R group hematocrit significantly decreased between 3rd and 4th day (p=0.,0277).

4.4.6. Properties of the venous blood obtained from the excluded limb region in I/R group

In I/R group during testing of venous base samples and samples obtained from the excluded limb after 3-hours ischemia just before reperfusion RCTT, whole blood viscosity an hematocrit values were significantly higher in excluded samples compared to base (p=0.0011, p=0.036, p=0.0001, respectively). Whole blood viscosity corrected for hematocrit (40%), plasma viscosity and fibrinogen level did not exhibit significant changes. RCTT values in excluded samples were also significantly higher compared to the systemic values just after releasing the clamps, just after ischemia (p=0.034).

According to our results, 3 hours of ischemia followed by reperfusion resulted in significant changes of hemorheological parameters.

Red blood cell deformability was worse on 2nd – 3rd day. In the previously presented study we found similar changes after 1 hour of limb ischemia in the rat, where RCTT values were significantly high on 1st and 2nd postoperative days. Red blood cells became rigid probably by the harmful effect of reactive oxygen radicals

Increase of *whole blood viscosity* for 1st postoperative day can be in connection with the elevated *hematocrit*, while after the 1st day hematocrit decreased in each group accompanied by lower whole blood viscosity values. Therefore, correction of whole blood viscosity for constant hematocrit is important. Hemoconcentration, increased whole blood and plasma viscosity, elevation of fibrinogen level, accompanied by the changes in leukocyte and platelet count, together, might be a consequence of different surgical interventions (Koppensteiner, 1996). These changes were larger in scale and were significant after ischemia-reperfusion, which means expressive stress, than the sham-operation.

Composition of blood can be altered under ischemia, when anaerobic processes in excluded limb affect the composition of the blood, decrease its pH, which strongly influence red blood cell deformability. During stasis hematocrit increases, the altered fluid distribution results in increased protein concentration (or plasma loss) and plasma viscosity, the volume changes and erythrocyte aggregation may increase the whole blood viscosity.

It is an important aspect that the shear stress resulting from blood at endothelial surface influences the endothelial function, which process might be enhanced by the rheologically altered blood. The appeared endothelial dysfunction after ischemia-reperfusion induces further pathways in the vasculature, so influencing the circulation, microcirculation of the region.

5. SUMMARY OF THE MAIN RESULTS AND CONCLUSIONS

1. We extended our hemorheological investigations for several animal species – mouse, rat, rabbit, dog, and pig – in order to make comparative analysis, too.
2. Certain hemorheological factors showed remarkable differences in the investigated species. The alteration in red blood cell deformability measured by filtration technique is conspicuous, which can be correlated in the size of erythrocytes.
3. According to our adaptation work we successfully determined red blood cell deformability by Carat FT-1 filtrometer in 1% cell suspension, with reduced blood quantity, during follow-up study in the rat.
4. The short-term, 1-hour hind limb ischemia and the following reperfusion resulted in significant decrease of erythrocyte filterability in early postoperative period (1^{st} and 2^{nd} day), which was probably caused by free radicals and could be reduced by administration of allopurinol in 1-weekly follow-up study.
5. After 1-hour ischemia mean corpuscular volume of erythrocytes, platelet count and mean platelet volume significantly increased in the postoperative period.
6. After 3 hours of limb ischemia and the following reperfusion in dogs, red blood cell deformability was worse on 2^{nd} – 3^{rd} day, hematocrit and whole blood viscosity, plasma viscosity and fibrinogen level increased during postoperative period. One part of the changes can be brought into connection with the surgical interventions, and other part with free radical interactions.
7. At the restart of the circulation just after ischemia, rheologically impaired blood - caused by local physical and metabolic conditions – flow into the body, that fact means further injury in farther tissues in the case of pathological vasculature.
8. We harvested a well reproducible, simple test, the „occlusion”-test using laser Doppler tissue flowmetry in order to the acute, prompt changes of microcirculation after ischemic insult. We showed significant impair in the microcirculation of skeletal muscle in the first minutes of the reperfusion.
9. Investigation of hemorheological factors and the microcirculation is informative on pathophysiological processes of limb ischemia-reperfusion.

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