Short Thesis for the Degree of Doctor of Philosophy (Phd)

Investigation of the effect of limb ischaemia-reperfusion on tissue perfusion and the possibilities of reducing damage

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1. INTRODUCTION

Circulatory disorders of the lower limb pose challenges in many areas of medicine. The problem can develop acutely but can also be chronic as a complication of certain diseases. During some vascular, traumatological or orthopaedic surgeries, the limb circulation is damaged, hypoperfusion or ischaemia develops, leading to ischaemia-reperfusion injury with significant morbidity and mortality. The estimated incidence is 1.5 cases per 100,000 people. Therefore, it is important to restore the circulation as soon as possible within the ischaemic tolerance time of the organ or tissue concerned. The critical ischaemic time of human muscle tissue in warm ischaemia is about 2.25 hours, irreversible muscle damage starts after 3 hours of ischaemia and at 6 hours almost complete tissue necrosis is observed. Ischaemic damage can occur not only during limb surgery but also during general surgery such as organ transplantation.

As a result of the disruption of the blood supply, the supply of oxygen and nutrients to the cells is significantly reduced or even stopped, which can lead to cell damage and cell death. Many cellular changes occur during ischemia. Mitochondrial function, ion transport and enzyme activity may be impaired. Rapid dephosphorylation of ATP to AMP also occurs, followed by further degradation. Impaired membrane functions upset the ion balance, H+, Na+ and Ca2+ levels increase. Dysfunction of endothelial cells develops, free radicals are formed, which damage carbohydrates, lipids, enzymes, membrane lipids and DNA. Intracytoplasmic components released from dead cells initiate inflammatory processes and release cytokines (TNF-α, IL-1β, IL-6).

It is extremely important to restore circulation as soon as possible, to start reperfusion. Paradoxically, this can cause further damage. Cells with an altered metabolism are at increased risk of oxidative stress, which is more likely to occur with the arrival of fresh, oxygen-rich blood. Together, these two processes are called ischemia-reperfusion injury.

As orthopaedic trauma surgeon me and my colleagues perform many operations in our daily work. These interventions are performed mostly on limbs, but if necessary, we also perform surgery on other parts of the body. These interventions range from bone fusion surgeries, vascular and nerve sutures to the surgical treatment of extensive soft tissue damage. It’s hard to imagine how many factors affect the success of our work and the recovery of the

patients. In case of prolonged wound healing, complications, or unsuccessful treatment, numerous parameters must be taken into account, the deviation, change, and imbalance of which can affect the success of the treatment.

Haemorheology is the discipline that deals with the flow of blood and the components that regulate it. Micro-rheological parameters (red blood cell deformability and aggregation) show significant changes in many pathophysiological conditions, including conditions such as free radical-induced damage, infectious conditions, sepsis, various metabolic disorders, as well as haematological, cardiovascular and cerebrovascular diseases.

Ischemia-reperfusion damage is of great importance, as it affects the micro-rheological parameters at several points due to metabolic changes, free radical reactions, and acute phase reactions.

Although many studies have examined the consequences of ischemia-reperfusion injury to skeletal muscle and the protective effect of pre- and postconditioning, very few studies have been addressed the changes in the micro-rheological parameters.

2. AIMS

Clinical examination

1. Investigation of the effect of ischemia-reperfusion on hematological parameters, whole blood viscosity and micro-rheological parameters (red blood cell deformability and aggregation) in connection with elective knee surgery performed in tourniquet.
2. Investigation of the intraoperative use of sodium-diclophenac as a nonsteroidal anti-inflammatory drug (NSAID) and the effect of ischemic preconditioning on the above parameters.

Experimental study

1. Evaluation of unilateral lower limb ischemia-reperfusion injury caused by tourniquet in a rat model based on hematological, micro-rheological, microcirculatory, metabolic and blood gas parameters, as well as histological examination.
2. Reducing the damage caused by ischemia-reperfusion using pre- and post-conditioning techniques, and comparison of the effectiveness of the two techniques based on the above parameters.

3. MATERIALS AND METHODS

3.1. Clinical examination

3.1.1. *Patient groups*

Seventeen patients (8 men and 9 women, age: 54.41±22.67 years; BMI: 26.81±4.12 kg/m2) were included in the study (Clinical Ethical Committee approval Nr.: DEOEC RKEB/IKEB 3848-2013). In a goup of patients with primary arthrosis who did not respond well to conservative treatment, a total knee replacement (TEP) was performed. Another group of patients underwent arthroscopic anterior crucial ligament (LCA) replacement using their own tendon graft following ligament rupture suffered during a previous accident or sports injury. The operations were performed in bloodlessness, using a cuff placed on the thigh. The total operative time was 96.94±24.74 minutes, including the ischemic (tourniquet) time: 93.11±25.07 minutes. All patients received intraoperative volume therapy (Isolyte solution infusion 1400 ml±620 ml).

• Control group (n=7): no other treatment was performed

• NSAID group (n=5): i.v. sodium-diclophenac (4 mg/kg body weight) was given 5-10 minutes before and 6 hours after the start of reperfusion

• Preconditioned group (n=5): remote organ ischemia preconditioning with a tourniquet (3x15 minutes with 15-minute reperfusion periods on the upper limb) was applied one day before surgery

Each group included both men and women, TEP and LCA replacement surgeries.

3.1.2. *Sampling protocol*

Blood samples were taken from the femoral vein of the operated side (BD Vacutainer® tubes, 1.8 mg/ml K3-EDTA; Becton, Dickinson and Company, USA) before ischemia, at 5 and 10 minutes of reperfusion, and then on the 1st and 2nd postoperative day to determine hematological parameters, whole blood viscosity, red blood cell deformability and aggregation.

3.2. Experimental study

3.2.1. *Experimental animals, anesthesia, surgical protocol*

All procedures were approved and registered by the University of Debrecen Committee of Animal Welfare (permission registration Nr.: 25/2016. UDCAW) in accordance with national and EU regulations [Hungarian Animal Protection Act (Law XVIII/1998) and Directive 2010/63/EU].

Thirty 8-week male Crl:WI rats were included in the experiment, and were kept in standard cages in alternating day and night light conditions in a 12-hour cycle. We provided them with free access to drinking water and conventional rodent chow.

During anesthesia, the rats received ketamine hydrochloride (100 mg/bwkg, CP-Ketamine) and xylazine (10 mg/bwkg, CP-Xylazine) i.p. injection in combination with atropine (0.05 mg/bwkg). In order to maintain anesthesia, one third of the initial dose was administered during the procedure.

A cannula was placed in the right common carotid artery of the animals to monitor blood pressure.

3.2.2. *Experimental groups*

The animals were randomly divided into four groups:

- Control (C) group (n=8, 320.4±9 g): besides the common carotid artery cannulation no other intervention was performed.
- Ischemia-reperfusion (I/R) group (n=7, 376.4±42.4 g): unilateral hind limb ischemia was induced by tourniquet application around the thigh, below the right inguinal region. After 120-minute ischemia the tourniquet was completely released to allow full reperfusion.
- Preconditioned (PreC) group (n=8, 388.6±39.1 g): three cycles of 10-minute ischemia and reperfusion (by tightening then releasing the tourniquet, alternately) was applied before the prolonged ischemia, described in the I/R group.

- Postconditioned (PostC) group (n=7, 386.7±46 g): the same three cycles of ischemia-reperfusion were introduced at the onset of the reperfusion, after 120-minute ischemia, described in the I/R group.

Postoperatively, the animals received Flunixin A.U.V. injection (Norbrook Laboratories Ltd.) i.m. at a dose of 10 mg/bwkg after the 2-hour observation and reperfusion period.

3.2.3. *Measurement and sampling protocol*

During the experiment, blood samples were taken from the lateral tail vein at the beginning of the procedure (before the 120-min ischemia, as Base), then after the removal of the tourniquet at the beginning of reperfusion, in PostC group after the postconditioning, and one week later in all groups. In the Control group the timing of the second blood sampling was set 120 minutes after the preparation and cannulation. Occasionally 0.3 to 0.5 ml of blood was taken (anticoagulant: 1.8 mg/ml K_3-EDTA). Histological samples were taken from biceps femoris muscle one week after the intervention.

3.2.4. *Histological examination*

One week after surgery, tissue samples were taken from the experimental animals under general anesthesia from the biceps femoris muscle. The samples were fixed in 5% formaldehyde, dehydrated in an ascending alcohol series, embedded in paraffin, microtomized into 3-5 μm sections, stained with hematoxylin and eosin (H&E), and then evaluated under an optical microscope. We looked for histological signs of ischemia, such as cell swelling, swelling of cell nuclei, breakdown of normal muscle striation, breakdown of sarcoplasmic myofibrils, inflammatory infiltration.

3.2.5. *Measurement of tissue microcirculation*

A single-channel device (LD-01 Laser Doppler Flowmeter, Experimetria Kft.) operating on the Laser-Doppler principle was used for the non-invasive determination of tissue microcirculation. The device gives the tissue flow in BFU (blood flux unit), which is based on the change in the wavelength of the laser light reflected from moving red blood cells. BFU is a dimensionless number that can be interpreted as the integral of the number (relative concentration) and speed of red blood cells.

The measuring head (NP-100 Standard Pencil Probe, Oxford Optronix-Experimetria Kft.) was placed on the lateral foot pad and measured on both feet in the 10th minute before

compression and after release, as well as in the 1st postoperative week. After the signal stabilized, we recorded 10-20 second sections, which were analyzed offline.

3.2.6. *Measurement of sole temperature*

At the same times and points as the measurement of the tissue microcirculation, we also measured the skin temperature on both paws in the central part of the sole, using an ear thermometer (ri-thermo® N professional Clinical Thermometer, Germany).

3.3. Laboratory examinations

Hematological parameters

Hematological parameters were determined using a Sysmex K-4500 microcell counter (TOA Medical Electronics Co., Ltd., Kobe, Japan). Red blood cell count (RBC [106/µl]), hematocrit (Hct [%]), hemoglobin (Hgb [g/dl]), white blood cell count (WBC [103/µl]), platelet count (Plt [103/µl]), mean corpuscular volume (MCV, [fL]), mean corpuscular hemoglobin (MCH, [pg]), mean corpuscular hemoglobin concentration (MCHC, [g/dl]), and mean platelet volume (MPV, [fl]) were also determined.

Whole blood viscosity

For the whole blood viscosity (WBV [mPas]) test, a Hevimet-40 capillary viscometer (Hemorex Kft., Hungary) was used. The values measured at a shear rate of 90 s-1 were analysed. Plasma viscosity was not analysed due to sample volume limitations.

Red blood cell deformability

To test the deformability of red blood cells, we used a LoRRca MaxSis Osmoscan (RR Mechatronics BV, Zwaag, The Netherlands) extracytometer, which determines the elongation index (EI) in the function of shear stress (SS [Pa]). For the tests, polyvinylpyrrolidone (PVP), normal phosphate buffered saline (PBS) was prepared (PVP: 360 kDa, Sigma-Aldrich Co., St. Louis USA; PVP-PBS solution viscosity=29.5 m Pas, osmolality=300 mOsmol/kg, pH=7.2). For comparison, the EI values at 3 Pa of shear stress, and by parameterization of EI-SS curves (Lineweaver-Burk equation), maximal elongation index (EI_{max}), the shear stress belonging to the half EI_{max} ($SS_{1/2}$, [Pa]), and their ratio were calculated. Deformability deterioration is indicated by a decrease in EI_{max} and an increase in $SS_{1/2}$.

Red blood cell aggregation

Red blood cell aggregation was measured by two different methods, the Myrenne MA-1 erythrocyte aggregometer (Myrenne GmbH, Germany) and the LoRRca.

The light transmission method is based on the fact that at a given angular velocity the red blood cells disaggregate, the transmittance of the sample is reduced, and after the rotor stops the cells aggregate again and the transmittance increases. The index parameters M 5s, M 10s, M1 5s and M1 10 s were determined from the results obtained at the 5^{th} and 10^{th} second of the process in the M (stationary rotor) and M1 (slow rotation) modes of the instrument. Their increase indicates an increase in aggregation.

Using a syllectometric approach based on the reflection of laser light from the aggregating blood sample by the LoRRca device, the aggregation index (AI [%), the amplitude of the intensity curves (Amp) and the time to half-amplitude ($t_{1/2}$ [s]) were determined. As aggregation increases, AI and Amp increase, $t_{1/2}$ usually decreases. Assays requiring 1 mL of blood were only tested in the Control and NSAID groups for clinical trials due to technical reasons and sample size limitations.

Blood gas analysis

The Epoc® Blood Analysis System (Siemens Healthineers, Erlangen, Germany) was used to measure blood oxygen saturation (pO_2, pCO_2 [mmHg]), carbon dioxide saturation (pO_2, pCO_2 [mmHg]), pH, various electrolytes (Na^+, K^+, Ca^{2+}, and Cl^-), metabolites (glucose [mmol/L], lactate [mmol/L], and creatinine levels [µmol/L]). The test required 0.1 ml of native blood per sample.

3.4. Statistical analysis

For the clinical study, data were presented as mean±standard deviation (S.D.). Differences within and between groups were analysed by two-way ANOVA test followed by post-hoc Bonferroni test or Dunn's method. A p-value less than 0.05 was considered statistically significant.

In the experimental work, data were also presented as mean±standard deviation (S.D.). For those variables where the baseline values showed a large variation, the ratio of changes was also analyzed (in all cases, relative values vs. eigenvalues). For statistical analysis, GraphPad Prism software was used (Windows version 8.0, GraphPad Software Inc., La Jolla, CA, USA). Differences within and between groups were analyzed by two-way ANOVA

followed by post-hoc Bonferroni test or Dunn's method, depending on the result of the normality test. Values $p<0.05$ were considered statistically significant.

4. RESULTS

4.1. Clinical examination

4.1.1. *Changes in hematological parameters*

The white blood cell count (WBC [G/l]) of the control group was significantly elevated at 10 minutes after reperfusion ($p=0.001$ vs. baseline) and further elevated on postoperative days 1 and 2 (both days: $p<0.001$ vs. The NSAID and Preconditioned groups did not show an increase in the early minutes of reperfusion, but increased on postoperative days 1 and 2 (both days $p<0.001$ vs. baseline).

The red blood cell count (RBC [T/l]), hemoglobin concentration (Hgb [g/dl]) and hematocrit (Hct [%)] values decreased in all groups. Significant differences were found in the RBC count of the NSAID group on postoperative day 2 ($p=0.001$ vs. baseline), hemoglobin concentration on day 1 ($p=0.026$ vs. baseline) and day 2 ($p=0.005$ vs. baseline), and hematocrit on day 2 ($p=0.001$ vs. baseline). In the Preconditioned group, initial Hct, Hgb and RBC values were significantly higher than in the Control and Preconditioned groups (both: $p<0.001$). Following intraoperative volume correction, Hct values decreased (R-10: $p=0.003$ vs. baseline) but remained higher throughout (day 1: $p<0.001$ vs. baseline, $p=0.006$ vs. NSAID; day 2: $p<0.001$ vs. baseline, $p=0.005$ vs. NSAID).

Platelet count (Plt [G/l]) showed no significant difference between groups and over the follow-up period, but a decrease was observed in the Preconditioned group.

4.1.2. *Changes in whole blood viscosity*

Total blood viscosity values (WBV [mPas]) at 90 s^{-1} were stable over the observation period in the Control and NSAID groups. Haematocrit in the preconditioned group was initially high, but decreased by postoperative day 1 as a result of volume therapy.

4.1.3. *Changes in red blood cell deformability*

Elongation index values measured at 3 Pa did not change significantly between groups, although the Preconditioned group had higher values throughout. A moderate decrease in EImax values was predominantly observed in the Control group. $SS_{1/2}$ values increased steadily in the Control group, showing a significant difference from baseline on postoperative day 2 ($p=0.025$). Accordingly, EI_{max} and $SS_{1/2}$ decreased in the Control group. This change was observed neither in the NSAID or Preconditioned groups.

4.1.4. *Changes in red blood cell aggregation*

The aggregation index values showed a moderate decrease in the NSAID and Preconditioned groups. The decrease in M 5s values was significant on both postoperative day 1 (NSAID: $p<0.001$ vs. baseline) and day 2 (NSAID: $p<0.001$ vs. baseline, Preconditioned: $p=0.024$ vs. baseline). M 10s values showed similar changes on day 1 (NSAID: $p=0.034$ vs. baseline, Preconditioned: $p<0.001$ vs. baseline) and postoperative day 2 (NSAID: $p=0.011$ vs. baseline, Preconditioned: $p=0.003$ vs. baseline). The values of the Control group remained elevated and even increased further by postoperative day 2 (M 5s: $p=0.004$, M 10s: $p=0.009$ vs. Preconditioned group).

4.2. Experimental study

4.2.1. *Changes in hematological parameters*

White blood cell counts increased immediately after 120 min of ischemia in the I/R ($p = 0.018$ vs. baseline) and PreC groups ($p < 0.001$ vs. baseline), and further increased by the end of the first postoperative (p.o.) week, reaching significant levels in the PreC ($p < 0.001$ vs. baseline) and PostC groups.

Red blood cell count, hemoglobin and hematocrit values showed a modest decrease at the onset of reperfusion compared to baseline values (RBC in I/R group: $p=0.021$, in PreC group: $p<0.001$; Hgb in I/R group: $p=0.009$, in PreC group: $p<0.001$, in PostC group: $p=0.004$). A further decrease was observed in the first postoperative week (RBC, Hgb and Hct in the I/R, PreC and PostC groups: $p<0.001$). The decrease in hemoglobin and hematocrit in the PostC group ($p=0.001$ for both) and the decrease in hematocrit in the I/R group were significant compared to the Control group ($p<0.001$).

Platelet counts were lower than baseline values after 120 min of ischemia (Control: $p<0.001$, I/R: $p=0.041$, PreC: $p=0.002$, PostC: $p=0.001$) and increased during the first week of ischemia (Control: $p<0.001$, I/R: $p<0.001$, PreC: $p=0.032$, PostC: $p<0.001$). Values in the I/R

group were higher than those in the Control group at the onset of reperfusion (p=0.002) and at the first p.o. week (p=0.006). The highest values were found in the PostC group one week after surgery (p<0.001 vs. Control).

4.2.2. *Changes in red blood cell deformability*

EI values were lower one week after surgery in the I/R group and more pronounced in the PreC group, but the differences were small. EI values measured one week after surgery showed a significant decrease in the I/R group compared to baseline (p=0.048). When analysing relative changes, larger differences were observed. The magnitude of EI decrease at one week after surgery was significant compared to the post-ischaemia relative values (Control: p=0.012, I/R: p=0.005, PreC: p=0.025). The increase in $SS_{1/2}$ was highest in the I/R group, which was also reflected in the $EI_{max}/SS_{1/2}$ ratio (p=0.002).

4.2.3. *Changes in red blood cell aggregation*

Increased red blood cell aggregation was found in all ischaemic groups (I/R, PreC, PostC) one week after surgery. In the I/R and PreC groups, we observed the highest values for M 5s (p<0.001 vs. baseline for both), M1 5s (only in the PreC group: p=0.038 vs. baseline and p=0.046 vs. control) and M 10s (p<0.001 vs. baseline for both) and M1 10s (p<0.001 vs. baseline for both).

4.2.4. *Changes in blood gas, acid-base parameters, electrolytes and metabolites*

The pO2, pCO2 values did not change significantly. The pH decreased in the PreC and PostC groups at the onset of reperfusion and normalized at postoperative week 1. Sodium, calcium and chloride ion concentrations did not show significant changes. Immediately after ischaemia, potassium ion concentrations increased significantly in all groups compared to baseline, with a greater increase in the ischaemic groups (Control: p=0.01, I/R: p<0.001, PreC: p=0.011, PostC: p<0.001; compared to baseline). Week 1 p.o. potassium concentrations were lower in the I/R and PostC groups compared to Control (p=0.007 and p=0.004, respectively). An increase in glucose concentrations was observed in all ischemia-reperfusion groups. The increase was significant in the PostC group (p=0.002 vs. baseline, p=0.039 vs.

Control). At week 1 p.o. these values were significantly decreased in these groups compared to baseline (I/R: p=0.001, PreC: p=0.001, PostC: p=0.006) and Control groups (I/R: p=0.01, PreC: p=0.004, PostC: p=0.013). Lactate concentrations were significantly increased at week 1 p.o. in the I/R (p=0.019), PreC (p=0.037) and PostC (p=0.041) groups compared to Control. Creatinine concentrations increased significantly only in the PreC (p=0.002 vs. baseline) and PostC (p=0.017 vs. baseline, p=0.008 vs. Control, p=0.038 vs. I/R) groups immediately after reperfusion.

4.2.5. *Changes in tissue microcirculation*

In the ischaemic groups, circulation in the right hind limb was significantly reduced at the end of ischaemia and remained lower at the onset of reperfusion. The decrease was most pronounced in the PreC group. As zero BFU was not recorded in any of the depressed right hind limbs, it can be concluded that complete ischaemia did not develop, presumably due to the presence of collateral circulation.

4.2.6. *Changes in sole temperature*

When examining the changes in the relative values of foot temperature, it was found that the values in the ischaemic groups decreased compared to the control group immediately before and after the lower right limb was released. In the I/R group, a moderate increase was observed in the 1st week after surgery.

4.2.7. *Results of the histopathological examination*

No histological abnormalities due to ischaemia were detected by light microscopy in any of the groups. Post-operative specimens showed preserved striations of muscle fibres, normal contour fibrosis, absence of rounded hypertrophic fibres and normal arrangement of nuclei. In sarcoplasm, disorganization of myofibrils did not occur. No swelling or intense inflammatory infiltration was detected. Necrotic muscle fibre sections were not present and no calibre fluctuation was observed. In the preconditioned animals, signs of subacute inflammation and fresh bleeding in the perimysium were observed, which could be due to the reduction of the lesion.

5. DISCUSSION

Temporary tourniquet is used in many areas of surgery, such as in various orthopedic, reconstructive or vascular surgical procedures including limb revascularisation, replantation, free-limb transfer or elective surgery in the absence of blood. During interventions, skeletal muscles may be subjected to prolonged ischaemia and subsequent reperfusion that can cause serious complications, including muscle necrosis and muscle dysfunction, not to mention the systemic effect when distant organs such as kidneys, liver, lungs may be damaged.

Many strategies have been developed in experimental studies to reduce ischaemia-reperfusion injury, but very very few of them have been introduced into the clinical practice. Among surgical approaches, pre- and post-conditioning are promising methods. Their essence is to induce short ischemic insults before or after the prolonged ischemia, which increases the ischaemic tolerance of the target organ.

However, there are still many unanswered questions, not only about the optimal timing of conditioning, but also about the number and duration of cycles. A crucial point is that the applicability of preconditioning in clinical practice is limited, as it can only be used in scheduled surgery. The advantage of postconditioning is that it is easy to use even in emergency situations. Both methods have the disadvantage of prolongation the operative time, which increases the operative risk for elderly patients, patients in poorer physical condition or with multiple risk factors for anaesthesia. It should also be taken into account that, being invasive techniques, they are not free from complications. Their use is not recommended in cases of damaged vessel walls.

Knowledge of the functional and structural changes associated with ischemia-reperfusion injury is essential to clarify the issues involved. Although the literature on limb ischemia-reperfusion injury is quite extensive, very few data are available both on changes in micro-rheological parameters, both in limb ischemia-reperfusion injury and the surgical conditioning procedures to reduce the damage.

5.1. Clinical examination

Given the pathomechanism, the use of anti-inflammatory agents, antioxidant therapy and various conditioning procedures (pharmacological, surgical) may prevent or reduce the ischaemia-reperfusion injury.

Among the many pharmacological agents with different targets (e.g. vasodilators, free radical scavengers, anticoagulants, leukocyte inhibitors), we chose nonsteroidal anti-inflammatory drugs (NSAID) for our study. NSAIDs include salicylates, arylalkanoic acids, 2-arylpropionic acids, phenamic acids, pyrazolidine derivatives, oxycams. We used diclophenac, which is one of the arylalkanoic acids, and has a strong anti-inflammatory, analgesic and antipyretic effect. It was used intra- and post-operatively by intravenous administration.

Among the surgical methods, ischaemic preconditioning (local, remote, early or delayed) has been shown to be beneficial for a number of interventions and target organs. Although there are conflicting results regarding the procedure, several experimental and clinical studies have demonstrated its efficacy in limb ischaemia. However, many questions remain unanswered. The optimal protocol for each organ/tissue or limb is not yet known, either in terms of timing, number of cycles or duration. Leurcharusmee et al. have investigated the combined use of anaesthetics and tourniquet, but no effective dose or protocol has been established. In knee replacement implantation, the protective effect of IPC has been demonstrated, even at the level of gene expression, by triggering early response protective mechanisms.

In our clinical study, we investigated the effect of ischaemia-reperfusion in elective knee surgery for severe arthrosis, due to the bloodlessness required for the procedure, on various laboratory parameters, focusing mainly on haemorheological parameters. In addition, we investigated the effect of a pharmacological method using sodium-diclophenac belonging to the group of nonsteroidal anti-inflammatory drugs, and a surgical method, the remote organ preconditioning, by monitoring the same parameters.

In our study, for precondition we applied tourniquet (3x15 minutes with 15-minute reperfusion periods) on the upper limb one day prior to lower limb ischemia using a blood pressure cuff. Among hematological parameters, white blood cell count was significantly increased in the control group as early as 10 min after reperfusion. This was observed in the NSAID and Preconditioned group only on postoperative days 1 and 2. With respect to micro-rheological parameters, we observed that while the Control group showed a deterioration in red blood cell deformability parameters, this was not observed in the NSAID and Preconditioned groups. For red blood cell aggregation, the aggregation parameters M 5s and M 10s were significantly lower in the NSAID and Preconditioned groups on postoperative days 1 and 2 compared to the Control group, i.e. the degree of aggregation was significantly lower in these patients on postoperative days.

Limitations of the study were the small number of cases, age and sex differences, blood loss and differences in volume therapy.

In conclusion, our clinical study obtained similar results to previous animal studies on limb ischemia-reperfusion, i.e., red blood cell deformability parameters worsened and red blood cell aggregation increased on the first and second postoperative days. Both sodium-diclophenac and remote organ ischemia preconditioning attenuated the deterioration of micro-rheological parameters in the early postoperative period.

5.2. Experimental study

Various models are known for unilateral hind limb ischemia, operating with vascular microvascular clips for clamping the femoral artery, tourniquet or inflated cuff around the thigh. Each method has limitations. Since the collateral circulation from the gluteal region is considerable in rats, clamping the femoral arteries alone does not necessarily lead to complete ischaemia. Tourniquet may compress collaterals as well, but the force applied may cause extended tissue damage.

In experimental models the ischaemic time also varies and can differ between species. Durations of 60, 120, 180 and even 240 minutes occur in rat research studies.

Pre- and post-conditioning protocols usually involve three or four cycles, ranging from a few seconds (10, 15 or 30 s) up to 10 minutes.

In our own study, we chose 120 min of tourniquet-induced ischaemia preceded by three cycles of 10-10 min of alternating ischaemia and reperfusion before and after the prolonged in the pre- and post-conditioned groups.

Red blood cell deformability and aggregation are significantly altered in many pathophysiological conditions, including ischaemia-reperfusion injury. These are mainly due to free radical reactions, metabolic changes and acute phase reactions. Free radicals can damage the red blood cell membrane by lipid peroxidation, methemoglobin formation and protein formation by sulfhydryl cross-linking. Metabolic changes may alter the morphological and mechanical properties of red blood cells, which leads to deterioration of their deformability, i.e. their passage through capillaries as well as to increased aggregation. Acute phase reactions may be manifested by an increase in leukocyte count, an increase or decrease in platelet count, hemoconcentration and consequent micro-rheological changes. Impaired deformability and enhanced aggregation of red blood cells elevate blood viscosity, increase vascular resistance and cause perfusion problem in the microcirculatory bed.

In this study we found that hemoglobin and hematocrit decreased significantly after reperfusion and one week postoperatively in all ischaemic groups. In parallel platelet count significantly increased. These changes may be associated with inflammatory processes and acute phase reactions induced by ischemia-reperfusion. The decrease in hemoglobin and hematocrit may also have been due to blood loss caused by surgery and serial blood sampling. We supposed that the alterations observed post-ischemically are mostly due to redistribution changes, and the later alterations can be originated dominantly from the inflammatory processes. White blood cell count also increased in the I/R and PreC groups after ischemia and showed a significant increase 1 week after surgery in the PreC and PostC groups. These differences can also be attributed to the initiation of inflammatory processes.

Similar to our previous studies in other ischemia-reperfusion models micro-rheological parameters has deteriorated during and after ischemia in all ischemic groups. Red blood cell aggregation has significantly increased, mainly due to the increased free radical release, acute phase reactions and inflammatory processes. Interestingly, the most significant increase was observed in the PreC group.

Metabolic alterations affect the morphological and mechanical properties of blood cells, which may result in deterioration of red blood cell deformability and impaired aggregation. Deoxygenated red blood cells show a decrease in deformability and an increase in aggregation, whereas hypoxia leads to cell swelling, which alters the cell surface area/volume ratio and thus deformability as well. When examining deformability, we found that EI values were significantly reduced in the I/R group compared to baseline by postoperative day 7. The extent of the decrease was significant compared to the post-ischaemia relative values in the I/R and PreC groups.

Analysis of blood gas values found that pH decreased in both conditioned groups at the onset of reperfusion and normalized one week after surgery. K+ ion concentrations were lower in the I/R and PostC groups at postoperative week 1 compared to Control. Glucose concentrations were elevated in all ischaemic groups. Lactate concentration also increased significantly in all ischemic groups measured at 1 week postoperatively. Creatinine concentrations increased in the conditioned groups after reperfusion.

However, in our experiment, simultaneous changes in metabolic and micro-rheological parameters were not clearly observed (post-ischaemia vs. 1st p.o. week values). Therefore, given the small sample size, multivariate regression analysis could not be performed. Although mathematically significant changes could be detected, the in vivo significance of the magnitude of change in micro-rheological parameters is still controversial. It is still not

known where the boundary between reversible and irreversible changes lies, nor is it known to what extent a deterioration in red blood cell deformability and/or aggregation leads to perfusion problems.

None of the histological changes typical of ischaemia-reperfusion injury of muscle cells, such as cell swelling, karyopicnosis, karyorrhexis, loss of the ciliary gland, cell fragmentation and the appearance of inflammatory cells, were observed in any of the groups in our study. Although we observed significant changes in laboratory parameters after 120-minute tourniquet and subsequent reperfusion, no detectable differences in muscle morphology were observed. This may be due to the excellent collateral circulation of the rat. Szijártó et al. observed histological signs of irreversible damage only after 8 h of ischemia in a lower limb ischemia model induced by 4, 6 and 8 h of infrarenal aortic occlusion. However, no nuclear abnormalities were observed. If 8 h ischemia was followed by 2 h reperfusion, only mild signs of I/R injury were observed.

Laser-Doppler studies confirmed reduced microcirculation in the ischemic limbs, but not complete absence, i.e. the tourniquet did not cause complete ischemia, only hypoperfusion ((I/R: 77.3±19.2% vs. C; PreC: 71.1±9.4% vs. C; PostC: 71±11.3% vs. C). There were no significant differences in the conditioned groups either. Adequate strength of compression is essential to induce complete ischaemia, but too much force may cause direct tissue damage, which we wished to avoid. This could be considered a limitation of this research. Considering the collaterals, histological examination of muscles in the lower region (e.g. plantar flexor complex) may be a better choice for future studies.

The changes in sole temperature correlated with the results of the laser-Doppler tests. The sole temperature of both lower limbs decreased significantly not only in the untreated ischemic, but also in the conditioned groups. However, no difference was detected between the left and right sides. This is probably explained by the extensive lower limb collateral vascular network mentioned earlier.

To summarise our results, we found that significant metabolic and micro-rheological changes occurred during the early reperfusion period, but their extent was not sufficient to result in histological, morphological changes.

Although the results were better in both conditioned groups compared to the untreated ischemic group, better micro-rheological values were observed with postconditioning compared to preconditioning. Kocman et al. compared the effect of 3x10-minute early, late, local and remote preconditioning in tourniquet-induced 3-hour hindlimb ischemia in rats. Different enzyme activities, apoptotic and histological assays were performed and it was

found that all methods were effective in reducing the damage, but remote organ application achieved the best effect. There is no clear protective effect in the case of postconditioning either. Some studies showed its protective effect, others did not experience any noticeable change, and even described an increase in damage.

For further studies, longer ischemia (3 or 4 hours), a method that also excludes collaterals, and the use of several different preconditioning protocols would be necessary.

A limitation of this study is that we investigated healthy animals without co-morbidities, with intact vascular system and normovolaemia. Although we observed minimal changes that were not associated with significant consequences, they could potentially have caused more severe reactions. The effects of minor changes may be cumulative and more significant when associated with pathological conditions (atherosclerosis, haemorrhage, shock, malnutrition, etc.). There is no perfect model to test all these aspects. All animal studies have their limitations and many factors need to be taken into account when designing and conducting studies and when evaluating and extrapolating results.

6. SUMMARY OF MAIN FINDINGS

Clincal examination:

1. We have shown that tourniquet-induced ischaemia and reperfusion after releasing caused an increase in white blood cell count, a decrease in red blood cell count, hemoglobin and hematocrit, whereas platelet count was practically unchanged. Total blood viscosity showed no significant change. In the control group, red blood cell deformability was moderately reduced and red blood cell aggregation was significantly increased on days 1 and 2 after surgery.
2. No significant changes were observed in hematological parameters and blood viscosity with NSAID and preconditioning. Red blood cell deformability was not significantly affected by either method, but red blood cell aggregation values were significantly better in both groups compared to the control group. Although both methods had a beneficial effect in reducing reperfusion injury, neither was clearly superior to the other.

Experimental study:

1. In our rat model the 2-hour tourniquet-induced hindlimb ischemia and subsequent reperfusion resulted in significantly reduced hemoglobin and hematocrit values in all ischaemic groups. Platelet count was significantly increased. All these were associated with metabolic changes immediately after ischaemia. Red blood cell aggregation has significantly increased, while red blood cell deformability has decreased.
2. The most significant changes in all examined parameters were observed in the I/R and PreC groups, while only minor changes occurred in the PostC group. Ischaemic pre- and postconditioning resulted in cumulative changes in different ways. Although the effect of postconditioning on micro-rheological parameters was more favourable, it cannot be clearly decided which conditioning protocol is better.

6.1. Authenticated list of *in extenso* publications

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Subject: PhD Publication List

Candidate: Csaba Körei
Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. Turchányi, B., **Körei, C.**, Somogyi, V., Kiss, F., Pető, K., Németh, N.: Beneficial postoperative micro-rheological effects of intraoperative administration of diclophenac or ischemic preconditioning in patients with lower extremity operations: Preliminary data. *Clin. Hemorheol. Microcirc. 79* (4), 557-565, 2021.
   DOI: http://dx.doi.org/10.3233/CH-211200
   IF: 2.411
2. **Körei, C.**, Szabó, B., Varga, Á., Baráth, B., Deák, Á., Ványolos, E., Hargitai, Z., Kovács, I., Németh, N., Pető, K.: Hematological, Micro-Rheological, and Metabolic Changes Modulated by Local Ischemic Pre- and Post-Conditioning in Rat Limb Ischemia-Reperfusion. *Metabolites. 11* (11), 776, 2021.
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