Ph.D. THESIS

EXAMINATION OF ACUTE LIMB ISCHEMIA-REPERFUSION IN EXPERIMENTAL MODELS. PRESERVATION POSSIBILITIES OF THE VIABILITY OF LIMB AMPUTATES FROM TRAUMATIC ORIGIN.

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1. INTRODUCTION

The demand for preserving injured limbs as a consequence of the ever increasing number of industrial and traffic accidents means a real challenge for surgical teams (traumatologists, hand-surgeons, plastic-surgeons). Microsurgical proficiency and the knowledge of required techniques allows the revascularisation of partially or totally amputated limbs, the suture of nerves, and the continuously improving surgical techniques assure the attendance of bones, muscles, tendons and skin defects.

The amputation of a lower limb is usually caused by a severe trauma, replantation and revascularisation are often sources of serious further problems. Nevertheless – especially in Europe – an increasing number of limb preserving operations are performed; however their complexity makes them serious challenge for practitioners of medicine. What used to be a wonder some hundred years ago became a reality in the light of surgical techniques – however it is accompanied by a large number of difficulties and questions yet unclarified.

Ischemic duration is critical in these interventions, as well as the amount of muscle mass involved. The occurrence of muscle necrosis, renal failure, ARDS (acute respiratory distress syndrome) and other systemic influences is highly probable. Local reaction after reperfusion means partly the swelling of the limb, which can increase tissue damage and it may as well cause systemic reactions, MOF (multiple organ failure) and ultimately death. It is often necessary to amputate, so that complications can be prevented.

Cooling might be an important part in saving the injured limb, either during transportation from the accident location, or during replantation surgery, however the optimal cooling temperature and time period are not clarified yet.

In a number of cases it is not agreed upon, whether cooling really brings advantage, and if it is so, in what circumstances can it cause unwanted effects?

Notwithstanding clinical observations and laboratory test follow-up of acute ischemia-reperfusion, the pathomechanism of reperfusion syndrome, a

large amount of experimental and clinical data regarding outcome and treatment, there are still many questions to be answered.

These questions inspire numerous new experiments. Local and systemic effects of ischemia-reperfusion are only partly known from microcirculatory, hemorheological and hemostaseologial points of view. The effect of local cooling on these parameters is still not fully clarified. Contradictionary data and experience are available in reference to the fact, that however cooling improves ischemic tolerance, a number of circulatory parameters might change abnormally. In connection with these facts, the sometimes necessary vascular clamping and declamping during surgical interventions, as well as vascular damages and occlusions of traumatic origin mean a special issue.

Does any form of cooling bring advantage or drawback in case of amputated limbs, in both prehospital care and daily clinical routine? Neither practice, nor literature has an unambiguous answer for this question, although it is vital concerning the patient's further fate.

Is it possible to draw long-term consequences with the help of recent laboratory methods? What kind of laboratory parameters could be appropriate to detect local and systemic changes, and to forecast outcome? Is there one or more parameters, that help in the matter of cooling?

Is it possible to answer these particular questions for everyday practice with the simplest experimental model, so that the safety of attendance increases?

To improve surgical security and to answer the above mentioned questions animal experimental models were set up. First of all the capability to reproduce these simple models were aimed at. Relevant physical, morphological, functional and laboratory parameters were searched for, so that they might be appropriate to accurately describe the gives status and to interpret damages caused by ischemia-reperfusion. Relationship between experimental models and their clinical relation were looked for.

2. OBJECTIVES

1. After a brief review of pathophysiological pathways of ischemia-reperfusion, the first objective was to summarize own clinical experiences on traumatic injury of extremities, reporting some representative cases and raising questions for the experimental research.
2. According to the questions and problems raised in the clinical practice, development of *experimental surgical animal models* for studying of local and systemic effects of ischemia-reperfusion.
3. *In laboratory small animal model*, analyses of hemodynamic and acid-base changes in the early phase of *reperfusion* after *2-hour hind limb ischemia*, in respect of possible arterio-venous differences, as well.
4. *In laboratory large animal model,* investigation of 3-hour ischemia and following reperfusion of hind limb using microcirculatory, histo-morphological examinations and laboratory analyses (hematological, hemorheological, hemostaseological, routine biochemistry) of *systemic blood* samples, in order to reveal the parameters with indicative force.
5. In the laboratory large animal model, investigation of the *efficacy of cooling* with the indicative parameters measured in systemic blood and *local blood* samples from the *excluded limb*.

3. MATERIALS AND METHODS

3.1. INVESTIGATION OF LIMB ISCHEMIA-REPERFUSION IN LABORATORY SMALL ANIMAL MODEL

3.1.1. Experimental animals

Twenty-four male Wistar Kyoto rats weighing 400-500 g (455.71±57.47 g) were subjected to the study. The experiments were approved by the institutional Ethical Commission (National Institute of Traumatology).

Animals were housed in groups of 2, and had free access to water 24 hours before the experiment. Anesthesia was induced with sodium-pentobarbital (Nembutal®, 35 mg/kg, i.p.) and maintenance doses were given (15 mg/kg/hour), as required.

3.1.2. Operative technique

The right external jugular vein was prepared and cannulated for the possible fluid supplementation. The cannula was filled with heparinized saline solution (50 IU/ml). The right femoral artery and vein were exposed and separately catheterized for direct blood pressure monitoring and blood sampling.

Venous catheter was advanced approximately 1-1.5 cm above the confluence of the internal iliac veins. Heart rate (HR), systolic, diastolic and mean arterial pressure (MAP) were monitored (CardioStar, Experimetria Ltd., Hungary), and body temperature was permanently measured using a rectal probe.

Animals were divided into two experimental groups:

(I) Ischemia-Reperfusion group (I/R, n=16). After a stabilization period (30 min), the contralateral, left hind limb was undergone to 2-hour ischemia using a tourniquet around the thigh, near to the inguinal ligament. The method was described in details in earlier study. Before the ischemic period (Base), five minutes before releasing the tourniquet (R-5') and during the first hour of the reperfusion (5', 10', 15', 30', 45' and 60') small volume blood samples (0.2-0.3 ml) were taken into heparinized tubes from the right cannulated femoral artery and vein. Physiological saline solution (0.5 ml) was used to replace the removed blood after each sampling.

(II) Control group (n=8). In this series no tourniquet was applied. They were considered as sham operated control animals. Arterial and venous blood samples were collected after the preparation and stabilization period (Base), and at 2, 2.5 and 3 hours of the experimental period. Fluid intake was similar to the Ischemia-Reperfusion group.

3.1.3. Laboratory tests

3.1.3.1. Determination of acid-base parameters

Both in arterial and venous samples blood gases (pO_2, pCO_2 [mmHg]) and pH values were determined by a Radiometer-Koppenhagen ABL330 instrument.

3.1.3.2. Determination of hematocrit and leukocyte count

Regarding the extremely small sample volumes, hematocrit was determined in microcapillary tubes after centrifugation (Janetzky). The rest of the removed blood was used for leukocyte counting in Bürker's chambers.

3.2. INVESTIGATION OF LIMB ISCHEMIA-REPERFUSION IN LABORATORY LARGE ANIMAL MODEL

3.2.1. Experimental animals

The experiments were approved by the Committee of Animals Research at University of Debrecen (UDCAR) (Permission Nr.: 50/2001. UDCAR). Twenty-four mongrel dogs (age: 3-4 years, bodyweight: 23.79±4.05 kg) were subjected to the study. The animals were fed with commercially available mixed food, had free access to water and were housed in individual standard cages (temperature: 18-22 ºC; light cycle was as daylight), under veterinary observation and care.

The anesthesia was induced with ketamin (10 mg/kg) and xylazin-hydrochloride (1 mg/kg) intramuscularly, used in half-doses per hour under control using electrocardiograph. Under anesthesia the left external jugular vein was prepared and cannulated on each animal.

3.2.2. Operative techniques

The animals were randomly divided into four groups:

(I.) Warm Ischemia-Reperfusion group (warm I/R): incision was made parallel to the right inguinal ligament, and the femoral artery and vein were

exposed. The femoral vessels were clamped for 3 hours using vascular clamps, while a sterile metal steel tourniquet was stretched around the thigh under the prepared vessels, closing out the soft tissues. Four hours after releasing the vessels, the tourniquet was removed, and the fascia lata and the skin were sutured.

(II.) Cold Ischemia-Reperfusion group (cold I/R): surgical preparation and the conducting of ischemia-reperfusion were similar to the previous group. During the ischemic period, ice bags were placed around the limb for 3 hours, and were removed at the beginning of the reperfusion.

(III.) Warm Sham operated group (warm Sh): preparation of the femoral artery and vein were performed, the wound was covered with a sterile wet textile, and 7 hours after surgical preparation the fascia lata and the skin was sutured.

(IV.) Cold Sham operated group (cold Sh): after preparation of femoral vessels and covering the wound, the method and duration of cooling were similar to the cold Ischemia-Reperfusion group.

Skin temperature on the thigh was regularly measured by a sterile heat probe unit (Haemosys software-hardware configuration, Experimetria Ltd., Budapest, Hungary) in each group. Data of the third hour of the experiment were compared between groups.

All animals were followed-up for 5 postoperative days and were under regular veterinary care. The animals received no anticoagulants, but analgesic drugs were administrated and given by a veterinary doctor.

On postoperative days the behavior and motion activity of the animals were registered on video recordings and observational reports.

3.2.3. Sampling protocol

In each group the basic systemic blood samples were obtained from the cephalic vein by venipuncture. In Ischemia-Reperfusion groups local blood samples were taken from the femoral vein of the excluded limb, at the end of 3-hour ischemic period, just before starting the reperfusion. At the beginning of

the reperfusion and at its 30th and 60th minutes – at parallel time points in Sham operated groups – systemic blood samples were collected from the cannulated external jugular vein. On postoperative period daily blood sampling were performed by puncture of cephalic vein on 1st to 5th days.

3.2.4. Examination of tissue microcirculation

The intraosseal circulation of the distal region – representing the normal circulation, ischemic and reperfusion periods – was monitored with the help of a laser Doppler flowmetry probe (LD-01 Laser Doppler Flowmeter, Standard Pencil Probe, Experimetria Ltd., Hungary) placed into the tibia in a bored hole. The laser Doppler signal was continuously registered (Haemosys software-hardware configuration, Experimetria Kft., Budapest).

3.2.5. Measurement of tissue pressure

In Ischemia-Reperfusion groups following 3-hour ischemic period, and in Shame operated groups 3 hours after the vessel preparation, the tissue pressure (in mmHg) in the anterior muscle compartment of the shin was measured using manometric method.

3.2.6. Hemorheological examinations

3.2.6.1. Measurement of red blood cell deformability

Determining erythrocyte deformability we used classical filtration measurements. For the tests red blood cell suspension in phosphate buffered saline (pH: 7.4, osmolarity: 295±5 mOsm/l) was prepared at 5% hematocrit from each samples anticoagulated with sodium-heparin (143 IU, BD Vacutainer®, Belliver Industrial Estate, U.K.).

A Carat FT-1 filtrometer (Carat Diagnostics Ltd., Hungary) was used to measure the filterability of the red blood cell suspension, based on St. George's filtration technique.

In this device the diluted red blood cell suspension flows through a polycarbonate filter membrane (Nuclepore® filter, average pore diameter = 5 μm, filter diameter = 13 mm, Whatman Inc.) at constant filtration pressure (4 cm of water). Filtration rate is measured at four pairs of light sources and photo-detectors. 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). Note that reduced red blood cell filterability results in decreases of IRFR and increases of RCTT.

According to the standardization guidelines, the filtration measurements were carried out three times for each sample 22±1°C, and were completed within 2 hours after blood sampling.

3.2.6.2. Measurement of whole blood and plasma viscosity

Blood samples were collected into sodium-heparin coated Vacutainer tubes (143 IU, BD Vacutainer®, Belliver Industrial Estate, U.K.) for viscosity measurements. During blood sampling strangulation was not allowed. Plasma was prepared by centrifuging at 1500 g for 10 minutes.

Blood and plasma viscosity measurements were carried out at 37 ºC using a Hevimet-40 capillary viscometer (Hemorex Ltd., Hungary) within 2 hours after sampling, with whole blood and plasma viscosity values reported in mPas at a shear rate 90 s^{-1}. Whole blood viscosity is strongly determined by hematocrit, thus, blood viscosity values at native hematocrit were corrected to a hematocrit of 40% using the mathematical formula given by Arpad Matrai and co-workers:

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

where $WBV_{40\%}$ is whole blood viscosity corrected to 40% hematocrit, WBV_{Hct} is whole blood viscosity measured at native hematocrit, PV is plasma viscosity, and Hct is the actual hematocrit value [%] of the native blood.

3.2.7. Hematological measurements

A Sysmex F-800 microcell counter (TOA Medical Electronics Co., Ltd., Kobe, Japan) was used to determine the quantitative and qualitative hematological parameters in whole blood anticoagulated with K_3-EDTA (7.5%, 0.04 ml, BD Vacutainer®, Belliver Industrial Estate, Plymouth, U.K.).

3.2.8. Hemostaseological – blood coagulation tests

For the measurements blood samples anticoagulated with sodium-citrate were used (0.129 M, BD Vacutainer®, Belliver Industrial Estate, Plymouth, U.K.). Prothrombin time (PT, [sec]), activated partial thromboplastin time (APTT, [sec]), thrombin time (TT, [sec]) and fibrinogen concentration (Fbg, [g/l]) in plasma were determined by a Sysmex CA-500 automated coagulometer (TOA Medical Electronics Co., Ltd., Kobe, Japan) using standard reagents (Sigma Diagnostic Inc., St. Luis, USA).

3.2.9. Routine chemistry tests

In respect of the limited volume of blood samples from routine chemical parameters only serum levels of total protein [g/l], and albumin [g/l] were determined by photometry (PraxisLab, at 540 nm, reagents: Total Protein, Albumin, Fabio Ltd., Budapest, Hungary).

3.2.10. Histological examinations

Prior and after the ischemic period, and by the 4th hour of reperfusion, furthermore, under anesthesia on the 5th postoperative day, bilateral muscle biopsies (3x3 mm) were taken from the m. tibialis anterior. For histological examinations, the biopsies were fixed in 4% formaldehyde solution, embedded in paraffin. The sections were stained with haematoxylin-eosin. Histological analyses using light microscopy were performed by a pathologist.

Capillary diameters of the muscle tissue were determined using ocular-micrometer (magnification: 400x) in 10 visual areas of each section.

3.3. Statistical analyses

Results were expressed as means and standard deviations or standard error (means, S.D. or S.E.). Software SigmaStat for Windows 1.0 (1992-1994, Jandel Co., Germany) was used for statistical analyses. Differences were evaluated by ANOVA using Dunnett's tests and Kruskal-Wallis tests for intra and inter-group comparisons. To compare the parameters of the excluded blood Mann-Whitney rank sum test was used; comparing the local blood parameters to base and systemic values Wilcoxon signed rank test was performed. A p value of <0.05 was considered significant.

4. RESULTS AND CONCLUSIONS

4.1. INVESTIGATION OF LIMB ISCHEMIA-REPERFUSION IN LABORATORY SMALL ANIMAL MODEL

In Ischemia-Reperfusion group *mean arterial pressure (MAP)* decreased by the end of the ischemic period, and had further decrease during the reperfusion almost by 20% compared to base values. The lowest values could be found at the 15^{th} minutes of the reperfusion. In the control group MAP showed moderate decline by the end of the experimental period. *Heart rate* expressed slight elevation during the whole experiment, while *body temperature* did not change.

White blood cell count increased by the end of ischemic period in the Ischemia-Reperfusion group, which elevation was significant in arterial samples compared to baseline values. During reperfusion white blood cell count diminished both in arterial and venous samples. In Ischemia-Reperfusion group this decrease was significant both in arterial and venous samples versus base.

Hematocrit values were almost the same in arterial and venous samples, by the end of the ischemic period slightly increased, then showed a moderate decrease.

In the Ischemia-Reperfusion and Control groups the initial *pH values* were almost similar both in arterial and venous samples. Systemic blood pH values slightly increased by the end of ischemic period. In the control animals pH did not significantly change in arterial or venous blood. In Ischemia-Reperfusion group, when the tourniquet was being released, systemic venous pH continuously started to decrease during reperfusion and showed the lowest values in the 60^{th} minute. Arterial pH remained almost unchanged, thus remarkable arterio-venous differences were observed from the 10^{th} minute of the reperfusion.

After surgical preparation and a stabilization period *pCO_2 values* were almost similar in the groups, respectively in arterial and venous blood. In the Ischemia-Reperfusion group by the end of the 2-hour ischemic period, the values decreased. Values of venous pCO_2 showed a moderate but continuous elevation during reperfusion, while arterial pCO_2 was always lower with a decreasing tendency. In Control group important changes did not appear; the degree of the initial arterio-venous pCO_2 difference did not alter.

In the Ischemia-Reperfusion group arterial *pO_2 values* increased, venous values decreased by the end of 2-hour ischemic period. During reperfusion the arterial pO_2 increased with a plateau between 5^{th} and 45^{th} minutes of the reperfusion, while pO_2 continuously declined in venous samples, resulting in the largest differences between arterial and venous values at 60^{th} minute. In control animals at the 2^{nd} hour of the experimental period pO2 values slightly increased, then did not changed during the rest of experimental period.

We can conclude that: (I) Besides the general effects of the pentobarbital anesthesia, *hind limb ischemia-reperfusion resulted in systemic hemodynamic and acid-base changes in the first hour of the reperfusion, showing arterio-venous differences.* (II) The local metabolic changes and leukocyte accumulation in the

ischemia-reperfusion insulted region may also influence the measured systemic parameters. (III) During the first hour of the reperfusion the *respiratorical compensation* also starts resulting in increase of pO_2 and decrease of pCO_2 values.

4.2. INVESTIGATION OF LIMB ISCHEMIA-REPERFUSION IN LABORATORY LARGE ANIMAL MODEL

4.2.1. Intra-operative examinations

The *intra-operative laser Doppler flowmetry*, monitoring the tissue microcirculation in tibial bone marrow, was a useful method to register the microcirculatory changes caused by clamping or releasing the femoral vessels.

After ischemia the *tissue pressure* in anterior muscle compartment of the shin significantly, almost four-fold increased, compared to Sham groups. This alteration was more enhanced in the case of cooling.

4.2.2. Red blood cell deformability

Generally, *relative cell transit time (RCTT)* showed impressive changes during the operative and the postoperative period.

While RCTT values of the *warm Sham operated* group did not exhibit significant changes during the operative and postoperative period, in the *cold Sham operated* group a significant increase was observed on 3^{rd} - 4^{th} day compared to the base.

During the operative period in the *warm Ischemia-Reperfusion* group essential RCTT alterations could not be observed. Later, increased RCTT values were seen on 2^{nd} and 3^{rd} day, which values were significantly higher compared to the base values. RCTT elevation seems to be normalized for 4^{th} - 5^{th} day.

In the *cold Ischemia-Reperfusion* group remarkable changes were observed during the operative period. RCTT values of the systemic sample just taken after the ischemic period (3h) were significantly higher compared to the

base, and further elevation was found during the forthcoming 1-hour period of the reperfusion. In early postoperative period, by the 1st postoperative day significant increase was observed. The RCTT elevation reached its peak on the 2nd day. The high values on 2nd to 3rd day were significant compared to base and warm Sham group. On the 4th - 5th day RCTT values seemed to be normalized.

4.2.3. Whole blood viscosity

The actual *whole blood viscosity* (WBV) measured at 90 s^{-1} shear rate showed irregular but not significant increase during operative period, and it reached the highest values on 1st postoperative day. On 1st postoperative day the cold Ischemia-Reperfusion group showed the most elevated WBV values. Then WBV values decreased by the 2nd day and showed a further slight decrease during the rest of postoperative period, accompanied by similar changes in hematocrit.

Otherwise, after the correction for 40% hematocrit ($WBV_{40\%}$) values showed a slight increase during postoperative days in each group, except for the warm Sham operated group. The most prominent elevation was observed in the Ischemia-Reperfusion groups, resulting in the highest values on 5th day in the cold Ischemia-Reperfusion group.

4.2.4. Plasma viscosity

The relative homogeneity in *plasma viscosity*, observed in the base values of the four groups, disappeared by the end of the 3-hour operative period.

In the *warm Sham operated* group plasma viscosity slightly increased during the operative period, and reached its highest values on the 1st postoperative day. The *cold Sham operated* group also exhibited an increase during operative period. After elevation on 1st day plasma viscosity decreased slightly and was significantly elevated again on 5th day compared to the base and the warm Sham group.

Plasma viscosity values of the *warm Ischemia-Reperfusion* group increased by 1st postoperative day and, after the slight decrease on 2nd - 3rd day, a remarkable elevation was found on 4th and 5th days. In the *cold Ischemia-Reperfusion* group conspicuous elevation was observed during the operative period and on almost every postoperative day. The highest plasma viscosity values were measured in the cold Ischemia-Reperfusion group, on 1st and 3rd postoperative 5th day.

4.2.5. Fibrinogen concentration

In *fibrinogen concentration* significant changes were not observed during the operative period. From 1st postoperative day a definite increase was observed in each group, which elevation stabilized between 3rd and 5th days. The most expressed elevation was expressed by the warm Ischemia-Reperfusion group, where fibrinogen level rose almost for the twofold between 1st and 2nd day.

4.2.6. Hematological parameters

Red blood cell count was slightly elevated for 1st postoperative day in each group, and without significant changes it showed moderate decrease on the rest of postoperative days. *Hemoglobin* level and *hematocrit* showed the same decreasing phenomenon. The decrease was significant by the 5th day in the cold Ischemia-Reperfusion group. Mean cell volume, mean cell hemoglobin, and mean cell hemoglobin concentration did not change significantly.

White blood cell count (total) significantly increased by the first postoperative day in each group. In Sham operated groups the normalization was begun from 2nd - 3rd day. The granulocyte-monocyte count was more than 85-90% of total white blood cell count. On the 2nd to 5th day WBC remained elevated, but it was more expressed in the Ischemia-Reperfusion groups: till the 5th day the values were significantly elevated compared to base.

Platelet count showed slight decrease during the operative period, and it decreased by the second postoperative day in each group, and on the 5th day it was expressed in a relatively higher count in the warm Ischemia-Reperfusion group. The *mean platelet volume* did not change markedly.

4.2.7. Changes of blood coagulation factors (PT, APTT, TT)

Prothrombin time (PT) was relatively high in cold Ischemia-Reperfusion group during the first hour of the reperfusion (10-12 sec in average), and on postoperative days, with the highest values on the 3rd and 4th days (16.12±4.98 sec, 18.02±6.25 sec, respectively). Unexpectedly, warm Sham group expressed an increase on 2nd – 3rd postoperative day.

Activated partial thromboplastin time (APTT) showed remarkable alterations in the postoperative period. It has slightly increased by the 1st day in each group, and showed further elevation in Ischemia-Reperfusion groups. The maximal increase of APTT was in cold Ischemia-Reperfusion group on the 2nd day (53.15±13.18 sec), which change was significant compared its base values and versus the warm Sham and warm Ischemia-Reperfusion group also on 2nd day, then APTT decreased. In warm I/R group the elevation in APTT was wider in time, its peak was on the 4th day (42.17±7.82 sec)], but did not reach the highest values of cold Ischemia-Reperfusion group.

Thrombin time (TT) did not change during the operative period, but it showed slight decrease between the 2nd and the 5th days. Warm Ischemia-Reperfusion group expressed an elevation in TT on 1st and 2nd day, while in cold Ischemia-Reperfusion group there was a small elevation only on the 2nd day.

4.2.8. Changes in total protein and albumin level

Both *total protein* and *albumin* changed near similarly in each groups. They have decreased by the 1st postoperative day, and on the following days the levels were partly restored, with the tendentious exception of the lower albumin

levels in both Ischemia-Reperfusion group on 1st to 4th postoperative days. The alterations were not significant.

4.2.9. Properties of the local blood taken from the excluded limb

In blood samples, taken locally from the femoral vein of the excluded limb just before the clamp release after 3 hours of ischemia, *relative cell transit time (RCTT)* was significantly higher in non-cooled, than warm cases. The elevated RCTT values in warm Ischemia-Reperfusion group were also significant compared to the base and versus systemic samples taken just after releasing the clamps.

Red blood cell counts were significantly higher in excluded blood versus base and the systemic values just after the ischemic period. *Hematocrit* showed the same tendency in both Ischemia-Reperfusion groups, with significance between base and excluded blood, even between excluded and systemic blood taken just after ischemia. Hematocrit values of warm Ischemia-Reperfusion group were higher than cold group.

Whole blood viscosity values in excluded limbs were higher than in the base samples, and mostly in the cold Ischemia-Reperfusion group. There was significant difference in WBV values of warm and cold ischemia, which difference remained after the correction of WBV values for 40% hematocrit.

Plasma viscosity did not exhibit significant differences, however, in local blood the values were higher than the base.

Fibrinogen concentration increased in local blood compared to the base or systemic values. In cold ischemia fibrinogen level was higher locally. *Total protein* and *albumin* levels were lower in excluded blood than in base or systemic samples in both of warm and cold Ischemia-Reperfusion groups.

Leukocyte counts were elevated both in warm and cold excluded blood versus base values. In cold Ischemia-Reperfusion group the increase was significant.

Platelet count was extremely low in the excluded local blood of warm Ischemia-Reperfusion group. The difference was significant compared to base and the systemic values at the beginning of the reperfusion, and versus cold local blood. In cold Ischemia-Reperfusion group there was a relative smaller decreasing in excluded blood, however it was also significant compared to its base and systemic values.

Coagulation factors in *warm Ischemia-Reperfusion* group were higher than the base or systemic values. In *cold Ischemia-Reperfusion* group the changes were different: Comparing to the base values prothrombin time was slightly higher in excluded blood, but was elevated in systemic samples taken at the beginning of the reperfusion. Activated partial thromboplastin time was remarkably elevated in excluded blood, while thrombin time was lower in local blood than in base or systemic samples. The changes were not significant.

4.2.10. Results of histological examinations

Besides slight loosening of muscle fibers there were no important changes in *warm Sham operated* group by the end of 7-hour operative period. In contrary, at the same time in *cold Sham operated* group focal myocytolysis and intracytoplasmatic vacuolization were observed. Sections made from biopsies taken on 5^{th} postoperative day showed normal histological picture.

In *warm Ischemia-Reperfusion* group histology of biopsies taken at the 4^{th} hour of reperfusion (7^{th} hour of the operative period) showed focal eosinophilia and incipient leukocyte infiltration. In samples taken on 5^{th} day extensive leukocyte infiltration with eosinophilia of muscle fibers were seen, in some sections thrombotized vessels and aggregated erythrocytes were found. In *cold Ischemia-Reperfusion* group at 4^{th} hour of reperfusion only fine loosening of muscle fibers and appearing of hypertrophic nuclei were observed. In samples taken on 5^{th} day increasing ratio of hypertrophic nuclei without expressed inflammatory signs could be seen.

Capillary diameters in sections hardly differed in warm Sham operated group by the end of operative period and on the 5th postoperative day. In cold Sham operated group the capillary diameters were larger in both sampling time. In warm Ischemia-Reperfusion group significantly larger capillary diameters were observed on 5th postoperative day compared to the warm Sham operated group. The largest capillary diameters were measured in cold Ischemia-Reperfusion group at the 4th hour of reperfusion.

Summary of the changes in examined parameters

According to the information content and indicative force, the examined parameters can be divided into four categories, summarizing their changes:

(1.) *Parameters without important changes:* mean corpuscular volume (MCV), mean corpuscular hemoglobin content and concentration (MCH, MCHC) of erythrocytes.

(2.) *Parameters with moderate and similar changes in each group:* red blood cell count, hemoglobin level, platelet count and the total protein concentration. In both Ischemia-Reperfusion groups prothrombin time, thrombin time and albumin level changed in larger manner.

(3.) *Parameters with significant changes in each group:* white blood cell count (mainly granulocyte+monocyte count) and hematocrit value.

(4.) *Parameters with characteristic changes during or after ischemia-reperfusion:* whole blood and plasma viscosity, and fibrinogen concentration. Within the characteristic alterations there were parameters that expressed significant differences between warm and cold ischemia-reperfusion as well: relative cell transit time (RCTT) of red blood cells, and activated partial thromboplastin time (APTI). As non-laboratory parameter, tissue pressure in anterior muscular compartment of the shin and the capillary diameters measured on muscle biopsy sections also showed significant differences between warm and cold ischemia-reperfusion.

Reperfusion damage occuring during surgery can very well be modeled in experimental circumstances and a large number of clinical and laboratory parameters can be monitored. The results and observations of the above introduced simple and reproducable experimental models proved, that hemorheological parameters and coagulation factors showed significant changes, and they seem to be appropriate to detect and follow the effects of ischemia-reperfusion, and the further studies for the problem of cooling.

Some of the parameter changes and physical conditions of the experimental model can help to answer questions about ischemia-reperfusion and amputation. Based on the results, cooling does not seem to have advantage, especially when the factors regarding composition and rheology of excluded limb blood is concerned during ischaemia. These factors may have influence upon remote tissues and organs of the body during and after reperfusion. Blood coagulation parameters might show signs of serious deterioration, that seems to be more expressed when cooling is applied. Throughout our experiments tissue pressure in muscle compartments increased more in cold ischemia-reperfusion group.

Clinical observations indicate, that the first few– especially the first three– postoperative days are extremely critical from the point of view of complications. Characteristic changes of the experiments' parameters presented themselves during the first three postoperative days.

In our experimental models local cooling of the affected limb did not bring any advantage with respect to most of the examined parameters. These results confirm the urgent need to determine optimal cooling parameters, which requires new experiments and supplemental methods to be developed.

5. SUMMARY OF THE MAIN RESULTS AND CONCLUSIONS

The main results and conclusions presented in the dissertation can be summarized as the followings:

1. *Based on literature data and own clinical experience ischemia-reperfusion events of partially amputated limbs* were summed from primary care, through surgical intervention, till the follow-up the restoration of functionality. This comprehensive assessment served as the basis of experimental work and research planning with the need for *characteristic examination methods that* occupy clinicians most.
2. Based on clinical observations on *traumatic limb injuries,* two experimental animal models were developed to answer questions arisen, to study the pathophysiological aspects of *ischemia-reperfusion.*
3. Besides the general effects of Pentobarbital anesthesia in the *rat experiment, two-hour hind limb ischemia-reperfusion caused systemic hemodynamic and acid-base changes in the first hour of reperfusion, showing considerable arterio-venous pH differences, too.* The first hour of reperfusion was not enough for restitution, however *respiratory compensation* started slowly with the increase of pO_2 values and the gradual decrease of pCO_2.
4. *In the experimental model on mongrel dogs to be able to examine local and systemic effects of 3-hour hind limb ischemia-reperfusion, the exclusion of soft tissues was independent from the exclusion of blood vessels.* That is how it became possible to analyze the effects of „vascular reperfusion" on the main artery of the excluded limb and to monitor the composition of the blood in the excluded limb, in contrast to the tourniquet-models.
5. *The three-hour ischemia and the following reperfusion in the mongrel dog model caused characteristic and significant changes in numerous hemorheological and hemostaseological parameters of systemic circulation*,

while most hematological parameters did not show specific changes.

During the first three postoperative days *red blood cell deformability and activated partial thromboplastin time, as well as from the non-laboratory parameters the intraoperatively measured tissue pressure in the frontal muscle compartments of the leg showed significant changes.*

6. *The so-called local blood of the excluded limb in normothermy proved to be far worse rheologically, than the blood in systemic circulation.* At the beginning of reperfusion, when the blood from the excluded limb entered the systemic circulation, parts of its effect remained observable in systemic circulation.
7. *Local cooling might very well boost specific hemorheological and hemostaseological changes caused by ischemia-reperfusion,* resulting in serious deterioration in red blood cell deformability, whole blood viscosity and activated partial thromboplastin time during the first hour of reperfusion and in the early postoperative period.
8. The paradoxon of cooling with respect to the limb excluded from circulation is still awaiting solution; its efficacy has still to be cleared. *Uncontrolled cooling* requires more attention and caution, since it can boost damage caused by ischemia-reperfusion, and might very well influence outcome with respect to the complex pathophysiological events and current clinical state. Not optimal cooling might decrease the survival chance of tissues at the replantation of partially or fully amputated limbs, during transportation of amputated parts, as well as during surgical interventions.

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