Investigating ischemic tolerance after acute lower limb vascular occlusions

Ph.D. Thesis Outline

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1. INTRODUCTION

Acute long-lasting arterial occlusions represent serious clinical problems due to their frequent occurrence (incidence: 15/100000) and severe complications. Even recent studies put post-operative mortality between 10-20%, Prompt and proper diagnosis is therefore important. Due to the deprivation of blood flow tissues distally to the site of the occlusion suffer ischemic damage. Restoration of blood flow however paradoxically can lead to further, reperfusion injuries, therefore this phenomenon is called the ischemic reperfusion injury.

After a long-lasting ischemic period, revascularization can result in serious consequences which manifest not only locally (rhabdomyolysis, muscle cell necrosis, microvascular damage), but has systemic aftermath as well. Toxic metabolic products and pro-inflammatory agents can be released from the injured muscles which could induce a systemic inflammatory response and potentially can lead to the development of multiple organ injury or even failure.

The development of the severe local and systemic complications following a lower limb vascular occlusion depends mostly on the degree of the ischemic injury. Thus the early determination of the degree of ischemic injury is crucial for the selection of proper therapeutic approaches to minimize complication rates. Unfortunately however there are no methods available so far to achieve this task, only gross approximation of the severity of the injury is possible with inadequate sensitivity.

Decreasing the degree of the ischemic-reperfusion injury could be a suitable therapeutic tool to minimize serious consequences of lower limb ischemia. Postconditioning is a potent surgical method which might possess the capability to decrease the extent of ischemic-reperfusion damage in case of long-lasting lower limb vascular occlusions thus improving mortality rates and patient quality of life.

2. OBJECTIVES

The goal of this investigation series was to test a novel method (developed by our team) under experimental and clinical conditions which has the potential to assess the degree of ischemic injury with proper sensitivity in a clinically relevant time frame. Another goal was to test the effects of postconditioning on skeletal muscle ischemic reperfusion injury in a clinically relevant animal model of long-lasting lower limb vascular occlusion. Preliminary experiments were performed to evaluate the suitability of our animal model.

The asked questions were as follows:

1. How does the degree of injury develop in case of infrarenal aortic injury in contrast to the application of tourniquet?
2. What kinds of collateral vessel can be identified behind the existing residual perfusion?
3. How does increasing time of ischemia effect the light and electron microscopic morphology of the muscle tissue?
4. How do the various muscle fiber types react to ischemia?
5. Is our novel method able to detect the degree of ischemic injury with sufficient sensitivity?
6. Does a short period of reperfusion affect the sensitivity of our method?
7. Is our method capable of detecting the fine differences among the various muscle fiber types?
8. Is our technique reliable under clinical circumstances to the detection of histological injury?
9. Is it possible to foretell the clinical outcome of the disease with our method?
10. Is postconditioning able to decrease the extent of histological muscle injury after a long-lasting vascular occlusion?
11. Can postconditioning improve muscle viability?
12. How does postconditioning effect inflammatory parameters?
13. Is postconditioning able to alter the dynamics of injury development?

3. METHODS

3.1. Experimental Studies

Male Wistar rats weighing 220-250 grams were used. The animals were kept under specific, pathogen-free conditions in 12-hour day-night cycles at 22–24 °C with unlimited access to commercial pellets and water. Each experiment was started at the same time of day to avoid the effects of circadian rhythm.

3.1.1. Experimental groups

3.1.1.1. Investigation of the importance of residual perfusion

	3 hours of ischemia	4 hours of reperfusion	n
Infrarenal aortic occlusion	+	+	10
Application of tourniquet	+	+	10
Corrosion casts	-	-	10

3.1.1.2. Investigation of muscle fiber viability

Group		Ischemic period (h)				Reperfusion (2h)	n
		4	6	8	9		
Untreated	Control	-	-	-	-	-	6
Ischemia	4I	+				-	6
	6I		+			-	6
	8I			+		-	6
	9I				+	-	6
Reperfusion	8IR			+		+	6
	9IR				+	+	6

I: ischemia, IR: ischemia-reperfusion

3.1.1.3. Investigation of postconditioning

Group	Operation type	Postconditioning	Length of reperfusion (h)				n
			2	6	12	24	
$Sham_{2h}$	Sham-operation	-	+				6
2IR	8 hour ischemia	-	+				6
2PC	8 hour ischemia	+	+				6
$Sham_{6h}$	Sham-operation	-		+			6
6IR	8 hour ischemia	-		+			6
6PC	8 hour ischemia	+		+			6
$Sham_{12h}$	Sham-operation	-			+		6
12IR	8 hour ischemia	-			+		6
12PC	8 hour ischemia	+			+		6
$Sham_{24h}$	Sham-operation	-				+	6
24IR	8 hour ischemia	-				+	6
24PC	8 hour ischemia	+				+	6

IR: ischemia-reperfusion, PC: postconditioning

3.1.2. Experimental design

3.1.2.1. Investigation of the importance of residual perfusion

Under general anesthesia the right jugular vein was cannulated for administration of anesthetics (ketamine and xylasine) and saline solution. In one group the infrarenal section of the abdominal aorta was exposed then a microvascular clip was placed on the prepared section for the induction of ischemia (aortic occlusion group). In another group laparotomy was not performed, instead rubber bands were placed on both tighs to induce ischemia (tourniquet application group). Three hours of bilateral lower limb ischemia was established in both groups which was followed by four hours of reperfusion. Blood and muscle (anterior tibial) samples were taken for laboratory tests and histological examination at the end of reperfusion.

3.1.2.2. Investigation of muscle fiber viability

Under general anesthesia the right jugular vein was cannulated for administration of anesthetics (ketamine and xylasine) and saline solution. The experiment was divided into two parts: in the first part the degree of ischemic injury alone was investigated with different exclusion times, while in the second part, the additive effect of reperfusion was studied using long-lasting exclusions followed by reperfusion.

Ischemic experiment: Through a median laparotomy the infrarenal section of the abdominal aorta was exposed. Four, 6, 8 and 9 hours of bilateral lower limb ischemia was established via infrarenal aortic occlusion. After inducing ischemia the abdominal wall was sutured in two layers. No reperfusion was allowed in this experiment. Samples were taken at the end of ischemia from the anterior tibial muscle.

Reperfusion experiment: 8 and 9 hours of infrarenal occlusion were followed by 2 hours of reperfusion. Five minutes prior to reperfusion 60 IU Na-heparin was administered intravenously to mimic the clinical situation. After the specified time of ischemia the aortic occlusion was terminated and the abdominal wall was resutured in two layers. Samples from the anterior tibial muscle were harvested at the end of the reperfusion period. Six additional animals were euthanized without any intervention to serve as untreated controls.

3.1.2.3. Investigation of postconditioning

The animals in this group underwent similar operative procedure till the induction of ischemia. 8 hours of bilateral lower limb ischemia was established by clamping the infrarenal section of the aorta then the abdominal wall was closed in two layers. At the end of ischemia the abdominal cavity was reopened, the microvascular clip was removed and the abdominal wall was re-sutured in two layers. On one group from each reperfusion interval postconditioning (10 sec. reperfusion, 10 sec. re-occlusion in 6 cycles) was applied at the onset of reperfusion. 2, 6, 12 and 24 hours of reperfusion was allowed. At the end of reperfusion muscle (anterior tibial) and blood samples (via left ventricular puncture) were collected. Sham-operated animals underwent the same procedure described above except the aortic occlusion.

3.1.3. Corrosion casting procedure

Animals were sacrificed after painless, lethal anesthesia. Postmortem the abdominal aorta, the superior and inferior mesenteric arteries were isolated and ligated. The supravalvular segment of the thoracic aorta, the distal end of the aortic arch and the right common carotid artery were also ligated. The left common carotid artery and right femoral artery were cannulated then resin mixture was simultaneously injected in order to identify the arterial system of the whole body. The preparations were then submerged into concentrated KOH to remove soft tissues. The finished corrosion casts were microdissected and analyzed under stereomicroscope. Collateral vessels were defined as vessels with visible connection between the upper and lower body half.

3.1.4. Microcirculation

Laser Doppler Flowmeter was placed on the surface of the biceps femoris muscle to assess the alterations in microcirculatory flow during the ischemic period in all groups. Whereas the flow values of the individual animals are different, the obtained values were compared to the baseline levels and expressed in percentage to achieve comparability.

3.1.5. Light microscopy

Samples were collected from the anterior tibial muscle of the left hindlimb and fixed in 4% neutral buffered formalin, embedded in paraffin and stained for hematoxillin and eosin. For semiquantitative evaluation the ratio of injured fibers were calculated and given as percentage of all fibers. Injured fibers were characterized as having broken or "ragged" borders, inconsistent texture and color, presence of intracellular vacuoles and/or detached nuclei. The examining pathologist was not informed about the applied treatment.

3.1.6. Electron microscopy

After the desired time of ischemia or ischemia and reperfusion the right extremities were perfused through an intra-arterial catheter with 4% paraformaldehyde, followed by cold 2% glutaraldehyde solution for a total of 30 minutes. Approximately 1x1 mm pieces of muscles were cut out and post-fixed in 2% glutaraldehyde (1 hour) followed by 1% osmium-tetroxide for an hour then embedded in araldite. Ultrathin sections were prepared with an ultramicrotome, contrast-stained with uranyl acetate and lead citrate, and analyzed using a Hitachi H7500 transmission electron microscope.

3.1.7. Viability assessment

Muscle samples three μm thick, collected from the left tibialis anterior muscle were cut in a cryostat and stained for NADH-tetrazolium reductase enzyme-histochemical reaction. Morphometric assessment the reaction was performed on histological pictures using Leica QWin Pro software. Viability of all fibers (total fiber viability) was calculated as a proportion of the total area of positive staining and the total area of muscle fibers in each picture. Furthermore, fibers were typed according to their staining characteristics with NADH-TR: lightly stained fibers were categorized as Type IIb (fast-twitch glycolytic), while fibers with intense staining were categorized as Type I (slow-twitch oxidative), then the viability of each type was assessed separately with the software. Viability of a fiber type was calculated as a proportion of the total area of positive staining and total area of corresponding fibers (e.g. Type IIb or Type I) in each picture. Final results were expressed as a percentage of the average of the untreated control muscles.

3.1.8. Laboratory tests

Tests consisted of measuring serum creatine-kinase levels (during the investigation of postconditioning), or serum lactate-dehydrogenase, potassium and TNFα levels (in case of model evaluation). After centrifuging, blood samples (obtained via left ventricular punction), serum was analysed with a clinical chemistry analyzer automate.

3.1.9. Tissue wet content.

Tissue edema was quantified with the utilization of the remaining muscles of the left extremity. Following careful excision, muscles were weighed immediately (wet weight), placed in a drying oven at +80 °C until reaching a constant weight, then the muscles were reweighed (dry weight) [19]. Wet-to-dry ratio was calculated using the following equation: (wet weight – dry weight) / wet weight * 100

3.1.10. Statistical analysis.

All values are expressed as means±SD. The assumption of normality was assessed with Shapiro-Wilk's test. Accordingly, one- or two-way analysis of variance was used for comparison of all groups with Bonferroni's post-hoc correction. Data correlation was evaluated using Pearson's method. A 95% confidence interval was considered as statistically significant ($p<0.05$). Statistical calculations were performed using IBM SPSS Statistics 20.0 software.

3.2. Human study

3.2.1. Study design

The study design was approved by the Scientific and Research Ethics Committee of the Hungarian Medical Research Council and was conducted according to the principles of the Declaration of Helsinki. The enrollment period ranged from June 1, 2011 to June 1, 2012. Patients with unilateral superficial femoral artery occlusion were included in this study regardless of etiology (Figure 1). Patients with proximal or distal occlusion sites, or bilateral occlusion were excluded. Further exclusion criterion was the insufficient distal outflow (i.e. significant popliteal stenosis).

3.2.2. Enrollment

The enrollment period ranged from June 1, 2011 to June 1, 2012. Patients with unilateral superficial femoral artery occlusion were included in this study regardless of etiology (Figure 1). Patients with proximal or distal occlusion sites, or bilateral occlusion were excluded. Further exclusion criterion was the insufficient distal outflow (i.e. significant popliteal stenosis).

3.2.3. Patient population and groups

During the period of this study, 147 patients were admitted to our institute with the initial diagnosis of acute limb ischemia, from which 38 patients (were enrolled in this study. No patient had any known neuromuscular disease. Admitted patients were divided into three main groups: Group A (15 patients, 39.5%) consisted of patients who were suitable for surgical revascularization and were presented without previously known peripheral arterial occlusive disease (PAD), Group B (18 patients, 47.4%) was constituted of patients with a history of existing PAD (of any kind except for significant distal stenosis requiring surgical intervention) and were suitable for revascularization, while Group C (5 patients, 13.1%) consisted of patients with the signs of irreversible injury which required amputation (Figure 2). Groups A and B were subdivided further according to short term outcome: 1st subgroups were constituted from patients (A1: 11 patients, 28.9% and B1: 13 patients, 34.2%) who had successful revascularization, while the patients in the 2nd subgroups required secondary amputation (A2: 4 patients, 10.6% and B2: 5 patients, 13.2%).

3.2.4. Interventional procedure

Patients after physical confirmation of arterial occlusion, received an intravenous bolus (5000 units) of heparin. Furthermore every patient received at least 1000 ml of physiological saline solution accompanied by 200 mg of pentoxiphylline and 80 mg of papaverine intravenously as well as 1000-U/h infusion of unfractioned Na-heparin were administered preoperatively. For revascularization, Fogarty balloon embolectomy was performed by the femoral approach in patients associated with embolism [7], while in case of in situ thrombosis thromboendarteriectomy procedure was chosen. In every case fasciotomy was performed to ensure proper limb circulation. The exact site of the

occlusion and the appropriate distal outflow was determined intra-operatively. In case of the physical signs of irreversible injury amputation was performed. Samples were taken before the initiation of the revascularization procedure or before the amputation of the limb from the anterior tibial muscle (ischemic sample) and the pectineal muscle (control sample). Primary and secondary amputations were carried out at femoral level in every patient.

3.2.5. Data collection

According to the study protocol, clinical variables concerning patient demographics (age, gender), medical history (diabetes, smoking, hypertension), and associated comorbidities (atrial fibrillation, thrombotic, peripheral occlusive, cardiac, carotid or cerebrovascular disease) were recorded. During operation the collected muscle samples underwent routine light microscopic examination and viability determination as follows:

3.2.6. Light microscopy

Samples were collected before revascularization (in groups A1, B1, A2 and B2) or before amputation (in groups A2, B2 and C) from (1) the pectineus muscle to serve as a non-ischemic internal control and from (2) the anterior tibial muscle to assess ischemic injury. Muscle samples were fixed in 4% formalin solution, embedded in paraffin and stained for hematoxillin and eosin.

3.2.7. Viability assessment

Viability determination was performed in accordance with the procedure described in Chapter 3.1.6. The final result indicating the degree of ischemic injury is expressed as a percentage of the corresponding internal control muscles.

3.2.8. Follow-up

Low molecular weight heparin was prescribed for 10 days (dosage was based on current body weight) to the patients, then aspirin [100 mg/d] therapy was continued for 6 months after the intervention. Follow-up included clinical evaluation of limb circulation during hospital stay and on the 30th post-operative day. Long term follow-up was not performed in this study.

3.2.9. Endpoint

Primary endpoints were to examine histological muscle damage, to assess muscle fiber viability and collate these two parameters to evaluate the potency of muscle fiber viability on determining the degree of ischemic muscle injury.

3.2.10. Statistical analysis

All values are expressed as means±SD. The assumption of normality was assessed with the Shapiro-Wilk test. Accordingly, one-way analysis of variance (ANOVA) was used for comparison of all groups with Bonferroni's post-hoc analysis. Fisher's exact test was used for comparison of patient distribution. A 95% confidence interval was considered as statistically significant ($p<0.05$). Statistical calculations were performed using IBM SPSS Statistics 20.0 software.

4. RESULTS

4.1. Investigation of the importance of residual perfusion

4.1.1. Comparison of aortic occlusion and the application of tourniquet

4.1.1.1. Histologic evaluation

In the aortic occlusion group, moderate pathological lesions were found in the samples. Rhabdomyolytic fibers were observed sporadically. In the tourniquet group a substantial amount of muscle fiber necrosis was seen. Non-necrotic fibers also showed signs of severe ischemic-reperfusion injury.

4.1.1.2. Laboratory tests

The serum creatine-kinase and lactate-dehydrogenase concentrations in the aortic occlusion group were significantly ($p<0.001$) lower compared with the tourniquet group In case of serum potassium levels a similar tendency manifested ($p<0.05$). Serum TNFα levels of the tourniquet group also showed significant ($p<0.001$) elevation compared with the aortic occlusion group.

4.1.1.3. Viability assessment

Aortic occlusion resulted in a marked decrease in total fiber viability. Tourniquet application under similar circumstances caused significantly ($p<0.001$) more pronounced viability reduction compared to the aortic occlusion group.

4.1.2. Corrosion casts

On the corrosion cast medial, lateral and epigastrial arterial networks could be identified as collateral arteries capable of supplying the lower extremities in case of an infrarenal aortic occlusion which form the anterior collateral system. Our analysis showed a dorsal, segment collateral tree as another source of potential residual perfusion. During observations a special, so far unknown collateral source could be identified, which revealed anastomoses between the sacral vascular network and the dural arterial system.

4.2. Investigation of muscle fiber viability

4.2.1. Microcirculation

Microcirculatory flow was assessed throughout the ischemic period in all groups to evaluate whether this model is able to produce continuous ischemia without significant variations in flow. After occlusion, the flow dropped to 17.3±7.3% of the baseline flow and remained constant during the course of ischemia even 9 hours after the occlusion (16.5±9.2% of baseline). Flow values did not differ significantly in either of the ischemic groups, and remained below 30% in every animal, indicating that infrarenal aortic occlusion is capable of causing significant ischemia in the extremities with little or no individual variations and remaining constant throughout the whole experiment.

4.2.2. Ischemic experiment

4.2.2.1. Viability assessment

Muscle viability decreased continuously over the time of ischemia with respect to all measurements. Data analysis showed high correlation between length of ischemia and fiber viability in all measurements ($r>-0.98$; $r^2>0.97$; $p<0.001$). Type I fibers suffered greater damage compared to Type IIb fibers, which became significant after 6 hours of ischemia ($p_{6h}<0.05$, $p_{8h}<0.01$, $p_{9h}<0.05$). No significant differences could be found between total fiber viability and any of the fiber types ($p>0.05$).

4.2.2.2. Light microscopy of semithin sections

In order to see if the decrease in muscle fiber viability coincides with the morphological signs of detectable injury, perfusion-fixed, semithin sections stained with toluidine blue were inspected. No detectable damage in muscle fibers of the untreated control animals was observed. After 4 and 6 hours of ischemia no visible pathological changes were present either. Eight hours long ischemia resulted in moderate degree of ischemic damage. Mostly the fibers with higher mitochondria content - supposedly the red fibers - were affected. After 9 hours of ischemia the extent of injury was more pronounced.

4.2.2.3. Electron microscopy

In order to supply a more detailed description of the morphological signs of injury, electron microscopic appearance of the muscle fibers was studied. Electron micrographs of muscle fibers in control animals showed normal morphology and no appreciable

damage. Eight-hours-long ischemia resulted in moderate degree of mitochondrial damage primarily in mitochondria-rich muscle fibers. After nine hours of ischemia, mitochondria showed signs of disruption, with myelin figures observable in some places. The sarcoplasmic reticulum showed moderate degrees of vacuolization and disorganization. Segregation of euchromatic and heterochromatic components of the nucleus became more prominent. The observed changes appeared also in mitochondria-poor muscle fibers in a lower degree.

4.2.3. Reperfusion experiment

4.2.3.1. Viability assessment

Viability showed significant reduction ($p<0.001$) in the 8IR group compared to the 8-hours-ischemia only (8I) group in both assessed fiber types, as well as in total fiber viability. Also, viability after 9 hours of ischemia and 2 hours of reperfusion (9IR) decreased significantly in all measurements compared to the 9-hours-ischemia group ($p<0.001$). In the 8IR group Type I fibers showed significantly decreased viability ($p<0.05$) compared to the Type IIb. No significant differences ($p>0.05$) could be found between the fiber types in the 9IR group.

4.2.3.2. Light microscopic evaluation of semithin sections

Eight hours of ischemia and 2 hours of reperfusion resulted in higher degree of morphological damage in addition to the alterations visible in the eight-hour-ischemia-only group. As in other stages, mitochondria rich fibers showed higher degree of injury. Reperfusion after 9 hours of ischemia showed large numbers of necrotic fibers in semithin sections. Non-necrotic fibers showed the same morphological pattern of injury as in case of the 8IR group.

4.2.3.3. Electron microscopy

After eight hours of ischemia followed by 2 hours of reperfusion myelin figures the disintegration of several mitochondria were frequently observed. Prominent swelling of the sarcoplasmic reticulum was detectable. Similarly to other samples, pathological alterations were more prominent in mitochondria-rich fibers. Two hours of reperfusion

after 9 hours of ischemia resulted in necrosis in the majority of fibers. Non-necrotic fibers showed similar but more pronounced alterations as in the 8IR group.

4.3. Investigation of postconditioning

4.3.1. Light microscopy

2 hours of reperfusion resulted in a significant rise ($p<0.05$) in the amount of injured muscle fibers both in the 2IR and 2PC groups compared to the sham-operated animals (Figure 1A, B). After 6 hours the amount of injured fibers rose significantly ($p<0.001$) compared to 2 hours of reperfusion. 12 hours of reperfusion resulted in further significant increase ($p<0.05$) in the number of injured fibers in the IR group compared to the previous time points, as well as morphological signs of extracellular edema appeared on the sections. Presence of polymorphonuclear leukocytes was also detectable. After 24 hours of reperfusion the amount of injured fibers did not advance further significantly ($p>0.05$), as well as similar degree of tissue edema and PMN infiltration could be detected in contrast to the previous measurement point. Postconditioning was able to reduce significantly ($p<0.05$) the rate of injured muscles, the degree of tissue edema and polymorphonuclear infiltration in every measured time points after 6 hours of reperfusion compared to the IR groups.

4.3.2. Viability assessment

8 hours of ischemia and 2 hours of reperfusion resulted in a significant ($p<0.001$) decrease in viability both in the 2IR and the 2PC groups compared to the sham-operated group. It remained at approximately the same level in both groups throughout the experiment. Postconditioning however resulted in a significantly less reduction of viability in every measured time point compared to the corresponding IR group ($p<0.001$).

4.3.3. Electron microscopy

In the 2IR group pronounced mitochondrial swelling could be observed in all animals. In some cases disrupted mitochondria could be found. Nuclei showed signs of marginalization of heterochromatin. Postconditioning resulted in lesser degree of

mitochondrial damage (swelling was reduced, no disruption was detectable) in all animals compared to the 2IR group.

4.3.4. Laboratory measurements

After 2 hours of reperfusion serum CK levels raised significantly both in 2IR and 2PC groups compared to the sham group ($p<0.001$). Following a peak at 6 hours of reperfusion, CK levels fell back to the 2-hour-reperfusion values in both groups to the 12th post-operative hour. Postconditioning was able to reduce creatine-kinase levels of the serum after 2, 6 and 12 hours ($p<0.05$) of reperfusion. At the end of the 24th hour of reperfusion serum CK values decreased in both groups to the level of the sham-operated group; there was no significant difference among any groups at this measurement point ($p>0.05$).

4.3.5. Tissue wet content

8 hours of ischemia followed by 2 hours of reperfusion resulted in a significant increase in edema index in both the 2IR and 2PC groups compared to the corresponding sham group ($p<0.001$). 6 hours of reperfusion resulted in further increase in muscle wet content in the 6IR group compared to the previous measured time point ($p=0.01$), then it did not increase any further. Postconditioning was not able to affect significantly the amount of tissue edema in the first 2 hours of reperfusion ($p>0.05$), however after 6 hours in every measured time point, tissue edema was significantly reduced by this technique in contrast to the corresponding IR group ($p<0.001$). Postconditioning was also able to prevent the further rise in tissue wet content after 6 hours of reperfusion, tissue edema remained on similar levels throughout the experiment.

4.4. Human study

4.4.1. Patient characteristics

The majority of the patients were men (68.4%). Similar gender distribution was found within the groups. The mean age of the patients was 60.8±9.5 years. Group A is significantly younger than Group B ($p<0.001$). All patients were smokers. Half of the patients had hypertension (19 patients). 26.3% of patient population had diabetes (Type II Diabetes Mellitus in every cases). There were no incidence of diabetes in Group A.

Atrial fibrillation (AF) was present in 4 patients (10.5%) from Groups A and C. 10.5% of the patient had the history of cerebrovascular, while 15.8% of thrombotic disease with similar distribution within the groups.

4.4.2. Light microscopy

In Group A1, ischemic muscle samples showed only mild signs of injury. Samples obtained before revascularization in Group A2 indicated moderate ischemic muscle damage compared to the A1 group, while the muscle samples collected from the amputated limbs showed large necrotic areas and severe muscle injury in the non-necrotic fibers in this group. In the ischemic samples of Group B1 only slight vacuolization and mild intracellular edema were visible (Figure 5B) in addition to the atrophic changes seen in the control muscles of this group. Samples harvested from the anterior tibial muscle before revascularization showed signs of moderate ischemic muscle injury in Group B2. The ischemic muscle samples harvested from the amputated extremities of Group B2 however showed expanded necrotic areas with high degree of non necrotic fiber damage. The picture was very similar to Group A2. In Group C the histological damage was similar to the amputated samples of groups A2 and B2.

4.4.3. Viability assessment

High values of muscle fiber viability were measured in Groups A1 and B1, where revascularization was successful and amputation was not needed. In groups A2 and B2 viability showed a significant reduction in the ischemic muscles before revascularization ($p<0.05$). In both groups viability values fell under 50%. Viability decreased further in the amputated limbs in groups A2, B2 compared to both Group A1 and B1 ($p<0.01$), as well as the before-revascularization values of Group B2 and A2 ($p<0.05$). In Group C viability indicated similar degree of muscle injury than in the amputated limbs of Group A2 and B2.

4.4.4. Follow-up

The overall mortality was 21.1% and it did not differ significantly within the groups. Overall limb loss was 36.8%. In Group A, amputation rate was 26.7%, while in Group B it was 27.8%. The average hospital stay was 8.1±3.9 days. There was no significant difference among the main groups regarding hospital stay ($p>0.05$). Analysis of the subgroups however revealed significant differences in hospital stay. Our results showed that in groups where revascularization was not successful, therefore secondary amputation was necessary (Groups A2 and B2) had a significantly extended ($p<0.05$) hospital stay than patients with successful revascularization (Groups A1 an B1). In the secondary amputation subgroups (Groups A2 and B2) an average of 2.7±1.4 day elapsed between the revascularization procedure and amputation, without significant difference between the subgroups.

5. CONCLUSIONS

1. Though infrarenal aortic occlusion caused significantly less muscle injury compared to a complete ischemic model (i.e. the application of a tourniquet), it was able however to produce a sufficient degree of ischemic injury even with the utilization of short periods of ischemia.
2. The perfusion of the lower limb reduces pronouncedly to the 20% of the baseline as a result of infrarenal aortic occlusion. According to literature data this kind of flow reduction is sufficient for inducing appropriate degree of ischemic injury which fact is strengthened by our results as well. Anterior (epigastric, medial and lateral) and dorsal collateral networks were identified as sources of this residual perfusion, however the dural anastomoses might have significant contributions as well.
3. With increasing lengths of ischemia ever progressing degree of ultrastructural alteration can be observed in the muscle mitochondria with electron microscopy. After 9 hours of ischemia injury of other subcellular organella is present aside from severe mitochondrial damage. With light microscopy, the signs of injury are apparent only after 9 hours of ischemia.
4. In contrast to literature data the Type I (slow-twitch, oxidative) fibers suffered greater degrees of injury in our model, which might be explained by the differences in the used experimental models.
5. According to the results our new method is able to assess the degree of ischemic injury with high sensitivity, since the obtained values correlate well with the length of ischemia, as well as with the degree of ultrastructural muscle damage.
6. The results obtained by the examination of the brief reperfusion periods further strengthen the suitability of our novel method. The technique was able to determine properly the increased degree of injury after reperfusion, furthermore it was also able to appropriately indicate the degree of ultrastructural muscle damage.
7. Muscle fiber viability was able to appropriately ascertain the damage of the separate fiber types in accordance with the electron microscopic findings. According to the result of both muscle fiber viability and electron microscopy, the Type I fibers suffered higher degree of injury.

8. Fiber viability determination was able to properly assess the degree of histological injury under clinical circumstances, since mild histological injury resulted in mild decrease in viability while, severe muscle damage were represented by a serious drop in viability.

9. Our results indicate that the technique is able to properly approximate the outcome of acute limb ischemia, since high viability values correlated with positive outcome, while low values represented the need for amputation.

10. Postconditioning was able to reduce the histological damage of muscles after the post-operative 6^{th} hour after 8 hours of ischemia.

11. The surgical technique was able to express its positive effects regarding the viability of the muscle fibers after a long-lasting ischemic event.

12. In our study, postconditioning was able to limit the extent of local inflammatory reaction, as well as tissue edema formation.

13. Although postconditioning was not able to affect the injury development dynamics regarding histological muscle injury, muscle fiber viability, or szerin creatine-kinase levels, the method clearly reduced the degree of tissue edema through the alteration of chronological dynamics.

LIST OF PUBLICATIONS

Publications in the subject of the thesis:

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IF: 2.336 * these authors contributed equally to this study

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