

# **Prevention of complications of acute lower limb arterial circulatory disturbances using pharmacological and surgical conditional methods**

Ph.D. Doctoral Thesis

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## Introduction

Ischemia-reperfusion following lower limb acute arterial occlusions and operation of such injuries can induce local, systemic and remote organ complications.

During skeletal muscle ischemia, due to the cessation of the blood supply, intracellular nutrient and oxygen deficits appear, which can lead to the decline of the cellular ATP production. Due to the low intracellular ATP level, dysfunction of the ATP dependent ion transporters occurs, which finally leads to the increase of intracellular  $\text{Ca}^{2+}$  concentration.

During the reperfusion, the blood supply of the skeletal muscle becomes restored, resulting the nutrient and oxygen supply of the cell, however, paradoxically the muscle fibers become damaged. Intracellular  $\text{Ca}^{2+}$  levels continue to rise and production of reactive oxygen species begins. Different microcirculatory changes (endothelial dysfunction, no-reflow phenomenon) and local inflammation develop.

Mitochondria play central role in the pathophysiology of the ischemic-reperfusion damage, since they produce energy and are important factors in the process of cell death, by cause of their having key role in oxidative stress and necroapoptosis. Opening of the mPTP (mitochondrial permeability transition pore) in the mitochondrial membrane at the beginning of the reperfusion means the initial step of the necroapoptosis.

IR of the skeletal muscle results in rhabdomyolysis, the disintegration of the muscle fibers. Local effects may generalize via restored blood circulation, intracellular metabolites, ions ( $\text{K}^+$ ), enzymes (creatin-kinase, lactate-dehydrogenase), myoglobin, different mediators (pro-inflammatory cytokines, complement-factors, acute phase proteins), free radicals may be released into the systemic circulation. A systemic inflammatory response (SIRS) begins, which leads to multi organ dysfunction syndrome (MODS) and later multi organ failure (MOF), involving the renal and respiratory systems.

There are many literary data on models inducing lower limb IR damage. In order to exclude the arterial flow, experimental models use different methods, such as: application of a tourniquet around the limb, femoral artery clamping, iliac artery clamping and infrarenal aortic clamping. Tourniquet application is the only method that has proved to produce complete ischemia with no residual flow, however infrarenal aortic clamping can induce significant amount of ischemic muscle damage. There is no uniform method to date for the modelling of skeletal muscle IR and we do not know exactly the extent of the differences between muscle damages regarding the various IR models.

For decades, there have been many attempts to prevent lower limb IR damage, however there is no uniform therapeutic method to the present day. We can distinguish between two main preventive methods, surgical and non-surgical (pharmacological) conditional techniques.

The use of surgical conditioning techniques before/during/after ischemia, locally or on a remote organ can influence the reperfusion period via flow-modulation. Postconditioning is a surgical method involving repetitive flow interruption (reperfusion-reocclusion) at the very onset of reperfusion. Since the first demonstration of the protective effect of postconditioning in the myocardium, the procedure has been proved to have a positive impact on other organs as well (e.g., intestine, liver, and kidney). Postconditioning can intervene at several points in

the above detailed pathomechanism of IR injury. Various activator molecules, e.g., adenosine, intracellular mediators (nitric oxide, reperfusion injury survival kinases), and effector molecules (mitochondrial  $K^+$ -ATP-channel, mPTP) play role in the protective effects of postconditioning, which have effect on the tissue pH regulation.

Pharmacological conditional techniques mean such pharmacological compounds, which administered before/during/after the ischemia can specifically reduced the complications of IR damage. NIM-811 (N-methyl-4-isoleucin-cyclosporine) is a potential drug, which is a non-immunosuppressive derivate of cyclosporine-A. Based on literary data, it does not have the well-known side effects, but specifically inhibits the opening of the mPTP by means of binding to the cyclophilin-D molecule. The positive effect of NIM-811 has been demonstrated on several organs (heart, liver, brain, kidney), but there are no studies on its effect during the ischemia-reperfusion of the lower limb.

## **Aims**

### *1<sup>st</sup> experiment:*

1. Is postconditioning capable of reducing the extent of local skeletal muscle injury in lower limb ischemic-reperfusion model?
2. Can postconditioning reduces the systemic inflammatory response during lower limb ischemia-reperfusion?
3. What is the effect of postconditioning on lung complications following lower limb ischemia-reperfusion?

### *2<sup>nd</sup> experiment:*

1. Are there any differences between skeletal muscle injury caused by aortic cross clamping or tourniquet induced ischemia-reperfusion during the application of similar lower limb ischemic-reperfusion models?

### *3<sup>rd</sup> experiment:*

1. Is NIM-811 capable of reducing the local skeletal muscle injury following 3h tourniquet induced ischemia and 4h reperfusion?
2. Can NIM-811 treatment reduce systemic inflammatory response via influencing TNF- $\alpha$  and IL-6 levels?
3. What is the effect of NIM-811 on renal injury as a remote organ complication following lower limb ischemia-reperfusion?

## Methods

### Surgical procedures and experimental design

#### Groups

*1<sup>st</sup> experiment: Attenuation of skeletal muscle and lung injury to the lower limb following ischemia-reperfusion using postconditioning.*

Atraumatic microclips were applied above the bifurcation of the abdominal aorta and rats underwent a 180-min long bilateral lower limb ischemia. In the PostC group, 3 h after the initiation of ischemia, postconditioning was applied. After removal of the vascular clip, reperfusion was allowed for 240-min. After the reperfusion, local, systemic and lung complications were examined on 10-10 animals per group (Table 1.).

**Table 1.:** Groups of the 1st experiment.

<b>Group</b>	<b>Sham (just median laparotomy)</b>	<b>IR (just ischemia-reperfusion)</b>	<b>PostC (IR + postconditioning)</b>
<b>No. of animals</b>	10	10	10

*2<sup>nd</sup> experiment: Examination of the degree of skeletal muscle damage following the application of infrarenal aortic clamping or lower limb tourniquet.*

Based on the conclusions of the 1<sup>st</sup> experiment, the degree of the local and systemic complications of lower limb IR injury induced by infrarenal aortic cross clamping or lower limb tourniquet were compared. In case of both methods, 180-min ischemia and 240-min reperfusion were induced. After the reperfusion, laboratory and histological sampling were performed on 10-10 animals per group (Table 2.).

**Table 2.:** Groups of the 2<sup>nd</sup> experiment.

<b>Group</b>	<b>A (aortic cross clamping; IR: 3+4h)</b>	<b>B (tourniquet; IR:3+4h)</b>
<b>No. of animals</b>	10	10

*3<sup>rd</sup> experiment: Attenuation of skeletal muscle and renal injury to the lower limb following ischemia-reperfusion using NIM-811.*

Tourniquet was applied around both lower limbs, and rats underwent a 180-min long total bilateral lower limb ischemia. After removal of the tourniquet, reperfusion was allowed for 240-min. Five minutes before reperfusion, NIM-811 was administered in the treated

groups. After the reperfusion, local, systemic and renal complications were examined on 10-10 animals per group (Table 3.).

**Table 3.:** Groups of the 3<sup>rd</sup> experiment.

<b>Group</b>	<b>Sham</b>	<b>NIM-Sham (NIM-811 + solution)</b>	<b>IR (IR + solution)</b>	<b>NIM-IR (IR + solution + NIM-811)</b>
<b>No. of animals</b>	10	10	10	10

### **Preoperative procedures, anesthesia**

Preoperative procedures and anesthesiological features of the 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup> experiments are the same, therefore these are discussed together.

Male Wistar rats (n= 40) weighing 220–250 g were used (Charles Rivers Hungary Ltd., Budapest, Hungary). Animals were kept under specific pathogen-free conditions at 22–24°C, on standard rat chow and water ‘ad libitum’.

Animals were anesthetized by intraperitoneal injection of 75 mg/bwkg ketamine and 7.5 mg/bwkg xylazine. Anesthesia was maintained by intravenous (through right jugular vein) administration of 25 mg/bwkg/h ketamine and 2.5 mg/bwkg/h xylazine.

### **Operative procedures**

*1<sup>st</sup> experiment operative description: Attenuation of skeletal muscle and lung injury to the lower limb following ischemia-reperfusion using postconditioning.*

After median laparotomy, the infrarenal part of the aorta was explored. In the IR and PostC groups, atraumatic microclips were applied above the bifurcation of the abdominal aorta and rats underwent a 180-min long bilateral lower limb ischemia. In the PostC group, 3 h after the initiation of ischemia, postconditioning was applied for 2 min: six cycles of 10-s reperfusion and 10-s reocclusions. In both the IR and PostC groups, after removal of the vascular clip, reperfusion was allowed for 4 h. The animals in the Sham-operated group (n= 10) were subjected to the same experimental protocol as described above, but without the application of microclip. Blood gas analysis was performed at the end of the second and fourth hour of the reperfusion from the left carotid artery via a polyethylene catheter. Blood and lung tissue samplings were performed at the fourth hour of reperfusion.

*2<sup>nd</sup> experiment operative description: Examination of the degree of skeletal muscle damage following the application of infrarenal aortic clamping or lower limb tourniquet.*

In group A bilateral lower limb ischemia was performed by atraumatic microclip according to the 1<sup>st</sup> experiment. In group B bilateral hind limb ischemia was induced by the application of an elastic rubber band placed as proximally as possible on the thigh of the

stretched rat legs. Ischemia was maintained for 3 h, followed by 4 h of reperfusion in both groups. At the end of reperfusion, animals were exsanguinated. Blood and muscle samples were taken for laboratory tests and histological examination.

*3<sup>rd</sup> experiment operative description: Attenuation of skeletal muscle and renal injury to the lower limb following ischemia-reperfusion using NIM-811.*

After median laparotomy, a laser-Doppler flowmeter (LDF) probe was placed over the front surface of the left kidney. Following a 1 cm longitudinal skin incision along the lateral side of the thigh, another LDF probe was placed on the femoral biceps muscle to monitor the microcirculation of the left lower limb. Tourniquet was applied around both lower limbs in the femoral region according to the 2<sup>nd</sup> experiment group B, and rats underwent a 180-minute long total bilateral lower limb ischemia. Five minutes before reperfusion, NIM-811 [Novartis International AG, Basel, Switzerland; 10 mg/bwkg; the drug was dissolved in the vehicle, which contained 1.3 mL cremophor oil (Cremophor EL, polyethoxylated castor oil), 0.7 mL ethanol and 8 mL 0.9% saline solution] was administered intravenously to the NIM Sham and NIM-IR groups. After removal of the tourniquet, reperfusion was allowed for 240 minutes. Sham animals received all procedures including anesthesia and laparotomy except for the 180-minute long ischemia. Sampling was performed at the end of the 4<sup>th</sup> hour of the reperfusion.

## **Common endpoints of the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> experiments.**

### **Local skeletal muscle effects**

#### ***Histology***

Samples were collected from the anterior tibial muscle and fixed in 4% neutral buffered formalin solution for one day. Thereafter samples were embedded in paraffin, 3 mm thin cross- and longitudinal sections were cut and stained for hematoxylin and eosin. Histological examinations were carried out with light microscopy. The examining pathologist received no information about the applied pretreatment.

#### ***Assessment of muscle fiber viability***

Samples from the left anterior tibial muscle were snap frozen in liquid nitrogen and stored at -80°C until further processing. Cross sections of 3 mm thickness were cut in cryostat. Slides were incubated for 30 minutes at 37°C in a solution consisting of nitroblue tetrazolium (1.8 mg/dL), NADH (15 mg/dL) and 0.05 M TRIS buffer (pH 7.6). Unused tetrazolium reagent was removed using ascending and descending concentrations of acetone. Ten different fields were photographed randomly on each slide with 600x magnification. Viability assessment was performed by detection of NADH-tetrazolium reductase staining of muscles using Leica QWin Pro (Leica Microsystems Ltd, Wetzlar, Germany) morphometric software. Muscle fiber viability was calculated as a proportion of the total stained area and the total muscle fiber area of the slide. Average of the 10 slides was calculated for each animal. The final result is expressed as a percentage of the values of untreated control muscles.

### ***Laboratory tests***

Tests consisted of measuring serum creatine kinase (CK), lactate dehydrogenase (LDH) and K<sup>+</sup> levels. After centrifuging (2x10 min, 1050 g) the blood samples, serum was snap-frozen in liquid nitrogen and stored at -80°C until analysed with a clinical chemistry analyzer automate.

### **Systemic parameters**

#### ***Measurement of TNF- $\alpha$ and IL-6 levels***

Proinflammatory cytokine concentrations were measured by sandwich ELISA kits according to manufacturer's instructions. Absorbance was measured at 450 nm by spectrophotometry.

### **Endpoints of 1<sup>st</sup> experiment: Attenuation of skeletal muscle and lung injury to the lower limb following ischemia-reperfusion using postconditioning.**

### **Systemic parameters**

#### ***Detection of free radical concentration***

Analysis of free radical levels was performed by the luminometry method of Heide-Bögl (modification of Blázovics). The reaction mixture contained hydrogen-peroxide, luminol and microperoxidase. Results were provided in relative light units (RLU%), where the light intensity is proportional with the free radical concentration in the sample.

### **Remote organ effects: lung**

#### ***Histology***

Upper lobes of the right lungs were fixed in 4% neutral buffered formalin for 24 h and then embedded in paraffin. Sections, 3- $\mu$ m thick, were stained with hematoxylin and eosin. All sections were studied using light microscopy by an independent pathologist in a blinded manner. Evaluation of histologic damage was performed in keeping with a score described previously. Sections were assessed according to the following characteristics: (1) alveolar edema, (2) atelectasia, (3) hemorrhage (4) PMN infiltration, and (5) vascular congestion. Each of these features was evaluated by a score as follows: (0: normal, 1: mild, 2: moderate, 3: severe). Lung damage was categorized in accordance with the summary of the scores (0-3: normal, minimal; 4-7: mild; 8-10: moderate; 11-15: severe).

#### ***Lung Wet/Dry (W/D) ratio***

Lung tissues were dried at constant +80°C for 48 h until reaching a constant dry weight. The proportion of wet and dry weights was calculated according to the following formula:  $W/D \text{ ratio} = (\text{wet weight} - \text{dry weight}) / \text{dry weight} \times 100$ . By this means the rate of tissue edema could be determined.

### ***Measurement of lung myeloperoxidase (MPO) activity***

MPO activity, as a marker of tissue leukocyte infiltration, was assessed by modified myeloperoxidase assay from lung tissue homogenizate. The measurement include the oxidation of hydrogen-peroxide depending tetrametil-benzidine, which can be detected by spectrophotometry at 450 nm, 37°C. We can conclude the MPO activity from the protein content of the samples.

### ***Hsp72 expression***

Pulmonary Hsp72 expression was determined by Western blot analysis. Visualization of the bands was performed by enhanced chemiluminescence. Results were quantified by using ImageJ software (NIH, Bethesda, MD).

### ***Arterial blood gas analysis***

Arterial blood samples were taken from the left carotid artery at the initiation of reperfusion, as well as at the second and fourth hours, respectively. Samples were analyzed by Radiometer ABL80 and pO<sub>2</sub> and pCO<sub>2</sub> values were measured in the 0., 2., 4. hours of the reperfusion.

## **Endpoints of 2<sup>nd</sup> experiment: Examination of the degree of skeletal muscle damage following the application of infrarenal aortic clamping or lower limb tourniquet.**

The methods of experimental endpoints of the 2<sup>nd</sup> experiment are the same as described in the “Common endpoints of the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> experiments” section.

## **Endpoints of 3<sup>rd</sup> experiment: Attenuation of skeletal muscle and renal injury to the lower limb following ischemia-reperfusion using NIM-811.**

### **Local skeletal muscle effects**

#### **Skeletal muscle Wet/Dry (W/D) ratio**

Tissue edema was quantified with the wet/dry ratio utilizing the remaining tibial anterior muscle. Following careful excision, muscles were weighed immediately after the end of reperfusion (wet weight) and placed in a drying oven set at a temperature of +80°C until reaching constant weight. Muscles were then reweighed (dry weight) and wet/dry ratio calculated using the following equation: (wet weight – dry weight)/wet weight x 100.

#### **Measurement of tissue microcirculation**

Registration of microcirculation of the lower limb skeletal muscle was performed by laser Doppler flowmeter. The probe of the device was placed on the surface of the left femoral biceps muscle. To characterize the microcirculation, reperfusion area (RA) was used, based on the mathematical calculations of our research group. The mathematical transformations required for correct interpretation of the circulation data were described previously.

## **Remote organ effects: kidney**

### ***Histology***

Tissue sampling was performed from the same anatomical location (left kidney) in case of every animal, regardless of group. As in case of skeletal muscle samples, kidney samples were fixed in 4% neutral buffered formalin solution (24 h), followed by embedment in paraffin, then 3 mm thin sections were cut and stained for hematoxylin and eosin. During evaluation of the sections, features of tubular and interstitial injuries were considered. Evaluation of histological damage was performed in keeping with a score described previously. The degree of tubular damage to the kidney tissue was scored by determining the percentage of tubules in the kidney cortex, which showed tubular damage, tubular cell necrosis and cast formation as follows: 0: none; 1: 0%; 2: 10–25%; 3: 25–45%; 4: 45–75%; 5: >75%. Ten randomly chosen, non-overlapping fields per section were examined. Scoring was carried out by an independent pathologist in a blinded manner.

### ***Laboratory tests***

Examination of the blood samples from the animals was the same as described in the “Common endpoints of the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> experiments” section. Levels of Na<sup>+</sup>-, creatinine- and BUN (blood urea nitrogen) were determined, from which renal injury parameters (BUN/creatinine ratio, fractional Na<sup>+</sup>-excretion ( $FENa = \frac{U_{Na+} \times P_{kreat.}}{U_{kreat.} \times P_{Na+}}$ ) were calculated.

### ***Registration of renal microcirculation***

During the experiment, microcirculation of the left kidney cortex was registered with LDF, placing the laser probe on the anterior surface of the left kidney.

### ***Measurement of peroxynitrite concentrations in the kidneys***

Homogenized samples of kidney tissue were analyzed by spectrophotometric method after dilution in 1.0 M NaOH (60:1) solution; absorbance was measured at 302 nm. As control, 100 mM potassium-phosphate (pH 7.4) was added to the sample (60:1). The rate of decline in absorbance was measured on neutral pH.

## **Statistical analysis**

Data are shown as means  $\pm$  SEM. Statistical analysis of data was performed using IBM SPSS Statistics 20.0 software (IBM Corporation, Armonk, NY, USA). Two-way analysis of variance (ANOVA) with LSD posthoc tests, Student’s t-test, Mann-Whitney U-test, Kruskal-Wallis test were used. A 95% confidence interval was considered as statistically significant ( $p < 0.05$ ).

# Results

## Results of the 1<sup>st</sup> experiment

### Local skeletal muscle effects

#### *Histology*

In histological samples taken from skeletal muscle after 3h ischemia and 4h reperfusion, only mild morphological changes could be observed both in the IR and PostC group compared to the healthy histological picture of the Sham group. The morphological picture of the initial characteristics of rhabdomyolysis appeared. Disintegrated muscle fibers and intracellular vacuolisation could be observed, however there was no significant differences between the two groups.

#### *Assessment of muscle fiber viability*

Four hours after starting of the reperfusion a significant amount of muscle fiber damage could be detected in both groups compared to muscle fiber viability of the Sham group. Compared to IR and PostC groups, no significant differences were observable.

#### *Laboratory tests*

There were significant increases in serum necroenzyme (CK, LDH) and K<sup>+</sup> levels both in IR and PostC groups compared to the Sham group. Significant differences between IR and PostC groups were not detectable regarding either of the markers.

### Systemic parameters

#### *Measurement of TNF- $\alpha$ and IL-6 levels*

TNF- $\alpha$  concentrations were significantly increased in the IR group compared with the Sham-operated group. In the PostC group a significant decrease was detectable in comparison to the IR group (p=0,021).

The concentrations of IL-6 were significantly elevated in the IR group and in the PostC group compared with the Sham-operated group. No striking differences were demonstrable in the PostC group compared with the IR group.

#### *Detection of free radical concentration*

Based on luminometry measurements, oxidative stress was found to be significantly higher in the IR group than in the Sham-operated group. Total free-radical level in the PostC group was significantly lower than in the IR group (p=0,032).

### Remote organ effects: lung

#### *Histology*

In the IR group significantly higher scores were found as compared with the Sham-operated group, whereas in the PostC group the histological damage was significantly lower than in the IR group (p=0,017).

### ***Lung Wet/Dry (W/D) ratio***

The wet content of lung tissue was considerably higher in the IR group compared with the Sham-operated group and the W/D ratio was significantly lower in the PostC group than in the IR group ( $p=0,042$ ).

### ***Measurement of lung myeloperoxidase (MPO) activity***

Increased MPO activity was observed in the IR group compared with the Sham-operated group. A decrease was manifest in the PostC group when compared with the IR group, but the difference was not significant.

### ***Hsp72 expression***

Pulmonary Hsp72 expression was not significantly elevated in the IR group compared with the Sham-operated group. In turn, in the PostC group the Hsp72 level was lower in comparison to the IR group, but the difference was not significant.

### ***Arterial blood gas analysis***

Arterial  $pO_2$  was increased in the IR group compared with the Sham-operated group, whereas significantly higher values were found in the PostC group during reperfusion compared with the IR group at the corresponding sampling times (2<sup>nd</sup> h after the reperfusion:  $p=0,028$ ; 4<sup>th</sup> h after the reperfusion:  $p=0,017$ ).

The arterial  $pCO_2$  values manifest in the IR group were increased in comparison to the Sham-operated group at the second and fourth hours of reperfusion, but the differences were not significant. In case of the PostC group the arterial  $pCO_2$  values were found to be decreased compared with the IR group, but without significant differences.

## **Results of the 2<sup>nd</sup> experiment**

### ***Histology***

In group A where aortic cross clamping was applied, at the end of reperfusion, minimal histopathological lesions were found in most samples taken from the anterior tibial muscle. Rhabdomyolytic fibers (showing intracellular vacuolisation and loss of fiber integrity) were observed sporadically, but this observation was not significant.

In the tourniquet group (group B), a substantial amount of muscle fiber necrosis was seen (the integrity of the muscle fibers was lost, there was severe nuclear damage). Non-necrotic fibers which added up to a smaller portion of the fibers were edematous and intracellular vacuolisation was present.

### ***Assessment of muscle fiber viability***

Aortic occlusion resulted in a marked decrease in the viability of the anterior tibial muscle ( $45.13 \pm 7.51$  %) compared with that in healthy control animals (100 %). Tourniquet application under similar circumstances caused a more pronounced reduction in viability. The difference between the aortic occlusion (A) and tourniquet (B) groups was significant ( $p<0.001$ ).

### ***Laboratory tests***

Among the necroenzyme levels measured after 3 h of ischemia and 4 h of reperfusion, the serum CK concentration in the aortic occlusion group A was significantly ( $p<0.001$ ) lower than in the tourniquet group B, and this difference ( $p = 0.047$ ) was also observable in regard to the LDH levels. A similar tendency was observed for the serum potassium levels, with the

tourniquet group having significantly ( $p = 0.048$ ) higher serum  $K^+$  levels than the aortic occlusion group.

## **Systemic parameters**

### ***Measurement of TNF- $\alpha$ level***

The serum TNF- $\alpha$  levels of the tourniquet group showed significant ( $p < 0.001$ ) elevation compared with those of the aortic occlusion group.

## **Results of the 3<sup>rd</sup> experiment**

### **Local skeletal muscle effects**

#### ***Histology***

As compared with the nearly normal histological picture detectable in case of the Sham and NIM-Sham groups, segmental necroses and disintegrated filaments were observable in the muscle fibers in case of the IR group. The muscle fibers were separated by significant edema. By contrast, in the NIM-IR group, intact conditions were observable.

#### ***Laboratory tests***

Both CK and LDH levels were significantly increased in the IR group compared with the Sham and NIM-Sham groups (values were almost similar in the latter two groups). Both examined parameters were significantly lower in the NIM-IR group than in the IR group (CK:  $p < 0.001$ ; LDH:  $p < 0.001$ ). The serum  $K^+$  level was higher in the IR group compared with the Sham and NIM-Sham groups, and the elevation was significantly lower in the NIM-IR group ( $p = 0.017$ ).

#### ***Assessment of muscle fiber viability***

In the IR group, muscle fiber viability was significantly decreased as compared with the Sham and NIM-Sham groups (where values were nearly 100 percent). Viability in the NIM-IR group was significantly less reduced in comparison with the IR group ( $p = 0.001$ ).

#### ***Skeletal muscle Wet/Dry (W/D) ratio***

Calculations of wet content of muscles revealed a significantly higher value in the IR group as compared with the Sham and NIM-Sham groups. In the NIM-IR group, the amount of tissue edema was significantly lower than in the IR group ( $p = 0.014$ ).

#### ***Measurement of tissue microcirculation***

Calculations of the reperfusion areas displayed significantly compromised microcirculation observable in the IR group when compared with the Sham and NIM-Sham groups. Microcirculation became stabilized at a significantly higher level in the NIM-IR group than in the IR group (RA:  $p < 0.001$ ).

## **Systemic parameters**

### ***Measurement of TNF- $\alpha$ and IL-6 levels***

Both TNF- and IL-6 levels were markedly increased in the IR group in comparison with the Sham and NIM-Sham groups. The TNF- $\alpha$  level was considerably lower in the NIM-IR group than in the IR group ( $p = 0.003$ ). Regarding IL-6 measurements, significantly lower levels were noticeable in the NIM-IR group than in the IR group ( $p = 0.040$ ).

## **Remote organ effects: kidney**

### ***Histology***

In the IR group, swollen tubular cells, intracellular vacuolization, disintegrated necrotic cells and blurred cell borders were observable, as compared with the Sham and NIM-Sham groups. The morphological picture of the kidney was more favourable in the NIM-IR group, cell necrosis could rarely be seen, intact cell borders and nearly normal cell morphology were present.

The applied histopathological score was significantly lower in the NIM-IR group as compared with the IR group ( $p < 0.001$ ).

### ***Urine output and laboratory findings***

Based on the urine volume measured during the reperfusion, diuresis was significantly lower in the IR group than in the Sham and NIM-Sham groups. In the NIM-IR group, urine output was significantly higher when compared with the IR group ( $p = 0.022$ ).

The serum creatinine level observable in the IR group was significantly higher than the levels found in case of the Sham and NIM-Sham groups. The level manifest in the NIM-IR group was significantly less elevated than in case of the IR group ( $p = 0.001$ ).

### ***Calculated renal functional parameters***

The fractional Na<sup>+</sup>-excretion was significantly higher in the IR group compared with the Sham and NIM-Sham groups. A significantly lower value could be calculated in the NIM-IR group than in the IR group ( $p < 0.001$ ). The serum BUN/creatinine ratio was lower in the IR and NIM-IR groups compared with the Sham and NIM-Sham groups. No statistically significant differences could be seen between the IR and NIM-IR groups, though less decreased values were measured in the latter group.

### ***Renal microcirculation***

After onset of reperfusion, the microcirculatory flow rates of the IR and NIM-IR groups showed a declining tendency in comparison with the Sham and NIM-Sham groups, however, calculations of the reperfusion area (RA) values did not point to any significant differences measurable between the groups.

### ***Measurement of peroxynitrite concentrations in the kidney***

Significantly higher concentration of peroxynitrite could be observed in the IR group when compared with the Sham and NIM-Sham groups. In case of the NIM-IR group, such elevation was preventable by administering NIM-811 ( $p < 0.001$ ).

## Conclusions

### *1<sup>st</sup> experiment:*

1. Based on the results of muscle fiber viability and laboratory tests, skeletal muscle damage could be observed in our experimental model. However according to the light microscopic pictures, postconditioning could not decrease the amount of local skeletal muscle damage significantly.
2. According to the serum inflammatory parameters, postconditioning is a suitable method to attenuate the systemic inflammation. The examined 4h of reperfusion period was long enough to detect serum free radical and TNF- $\alpha$  levels, nevertheless the IL-6 level which appeared in the subacute phase did not show changes after postconditioning.
3. Lung damage following rat lower limb ischemia-reperfusion is effectively reducible using postconditioning. Postconditioning was able to reduce lung tissue leukocyte sequestration and activation as well as lung tissue edema due to decreased capillary permeability. Significantly improved pulmonary oxygen uptake of the animals was observed due to postconditioning during the reperfusion period.

### *2<sup>nd</sup> experiment:*

1. Based on skeletal muscle histology, muscle fiber viability and laboratory examinations, significantly greater damage could be observed induced by lower limb tourniquet compared to occlusion caused by aortic cross clamping. Ischemia induced by lower limb tourniquet resulted in significantly increased TNF- $\alpha$  level, from which we can conclude that complete ischemia causes a much more serious inflammatory reaction. In contrast, infrarenal aortic occlusion does not cause such a serious inflammatory response.

### *3<sup>rd</sup> experiment:*

1. After 3 h ischemia induced by tourniquet and 4 h reperfusion, NIM-811 treatment significantly decreased local skeletal muscle damage. The amount of skeletal muscle edema showed significant decrease and a markedly higher flow was registered during monitoring of skeletal muscle tissue microcirculation.
2. In our experiment NIM-811 was able to reduce serum TNF- $\alpha$  and IL-6 levels, thus the systemic inflammatory response.
3. NIM-811 efficiently reduced kidney damage in the rat lower limb ischemic-reperfusion model. As a result of the applied drug, less developed histopathological changes as well as significantly improved renal function were observed. Our examinations on oxidative stress

support that nitrosative stress of the kidney tissue plays a pivotal role in our experimental model, which was decreased by NIM-811.

## List of publications

### Publications related to the dissertation:

**Garbaisz D**, Turoczi Zs, Fulop A, Rosero O, Aranyi P, Onody P, Lotz G, Rakonczay Z, Balla Zs, Harsanyi L, Szijarto A. (2013) Therapeutic option for managing lung injury induced by infrarenal aortic cross-clamping. *J Surg Res*, 185(1): 469-76.

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