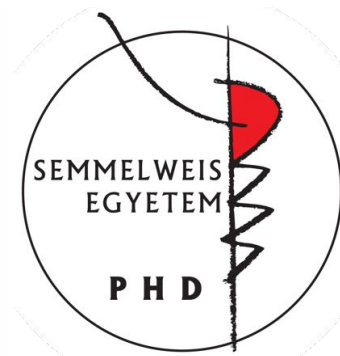# Investigating the hepatoprotective effects of limb remote ischemic perconditioning

Ph.D. Thesis

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LIST OF ABBREVIATIONS

Abbreviations used in text in alphabetic order.
Each figure has a separate list of abbreviations in the figure legends.

ALT	Alanine aminotransferase
ANOVA	Analysis of variances
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
bwkg	Body weight Kilogram
cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin gene related peptide
eNOS	Endothelial nitric-oxide synthase
ERK	Extracellular signal-regulated protein kinase
ET	Endothelin
FN	Femoral nerve
HSP	Heat shock protein
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
IPC	Ischemic preconditioning
IPOST	Ischemic postconditioning
IR	Ischemia-reperfusion
JNK	c-Jun N-terminal kinase
K^+ATP *Channel*	ATP-sensitive potassium channel
LDF	Laser Doppler flowmeter
LLL	Left lateral lobe
MAP	Mean arterial pressure
MDA	Malondialdehyde
miRNAs	Microribonucleic acid
ML	Median lobe
MODS	Multiple organ dysfunction syndrome
MP	Maximal plateau
mPTP	Mitochondrial permeability transition pore

mRNAs	Messenger ribonucleic acids
NO	Nitric-oxide
NR	Nerve resection
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
RA	Reperfusion area
RIPC	Remote ischemic preconditioning
RIPER	Remote ischemic perconditioning
RIPOST	Remote ischemic postconditioning
RISK	Reperfusion injury salvage kinase
ROS	Reactive oxygen species
rpm	Revolutions per minute
SAFE	Survivor activating factor enhancement
SECs	Sinusoidal endothelial cells
SH-group	Thiol groups
SN	Sciatic nerve
SOD	Superoxide dismutase
STAT	Signal transducer and activator of transcription
STDF	Stromal derived factor
tBi	Total bilirubin
TGF	Transforming growth factor
THVO	Total hepatic vascular occlusion
TK	Tyrosine kinase
TNF	Tumor necrosis factor
TXA2	Thromboxane A2

1. INTRODUCTION

Over the past few decades there has been a progressive decrease in both morbidity and mortality following major liver resections, liver transplantations, and other liver surgical procedures involving vessel occlusions. Mortality rate associated with major resection has decreased by half compared with the 1990s and is currently between 3-7%, depending on the study conducted [1,2].

It should be noted, however, that major liver resections performed in patients with chronic liver diseases, cirrhosis or severe co-morbidities are still carrying a significant mortality risk (up to 16%) [3]. Duration of liver exclusion is one of the most significant determining factors, which affects mortality within a homogenous patient population requiring major liver surgeries. Therefore, numerous surgical and pharmacological methods have been developed in order to reduce liver ischemia-reperfusion (IR) injury [4,5].

Classic local surgical conditioning techniques, such as ischemic preconditioning and postconditioning, have proved useful in reducing the degree of IR injury in many organs, including the liver [5-7]. A new theory regarding surgical conditioning techniques emerged in 1993, the concept of remote organ conditioning. The essence of this technique lays in the observation that target organ protection can be achieved by brief IR cycles applied to a distant organ [8].

Remote ischemic perconditioning (RIPER) *refers to the application of brief, remote ischemic and reperfusion cycles instituted* <u>*after*</u> *the induction of sustained target organ ischemia but* <u>*before*</u> *reperfusion.*

This novel technique is capable of reducing myocardial or cerebral IR damage according to various cutting-edge experimental and clinical studies [9]. Although, perconditioning has proved to be effective in various cases, the underlying mechanisms behind the protective effect have not been sufficiently explored yet.

This introduction chapter of the present doctoral thesis aims to give the reader an overview on different aspects and mechanisms of liver IR injury. Various small animal models and clinical scenarios with IR injury of the liver, as well as specific features and underlying mechanisms of different conditioning techniques, are discussed.

1.1. Ischemic-reperfusion injury of the liver

Ischemic injury is a phenomenon induced by interruption of blood flow to the organs, tissues. Tissue damage is paradoxically accentuated following revascularization causing the pathophysiological responses termed as reperfusion injury. Ischemic-reperfusion injury is a frequent and, in several cases, inevitable sequel of major liver resections and transplantations. Studies of Toledo-Pereyra et al. shed light for the first time on the possibility of paradox damage occurring during reperfusion of transplanted canine livers in 1975 [10,11]. Afterwards, countless new studies have revealed several important details and molecular mechanisms of liver IR injury [12-14].

Figure 1. Mechanisms of liver ischemia-reperfusion injury.
Abbreviations used: EC, endothelial cell; ET, endothelin; UPR/ER, unfolded protein response/endoplasmic reticulum; IRE1, inositol-requiring enzyme 1; PERK, PKR-like ER kinase; SLP, secretory leukocyte protease inhibitor; ICAM, intracellular cell adhesion molecule; VCAM, vascular cell adhesion molecule; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; INF, interferon; TNF, tumor necrosis factor; PAF, platelet-activating factor; LTB4, leucotriene B4; KC, Kupffer cell; X/XOD, xanthine/xanthine oxidase; Cyt c: cytochrome c.
(From: Mendes-Braz et al. (2012) J Biomed and Biotech, doi: 10.1155/2012/298657. Epub ahead of print.)

During ischemia oxygen supply to hepatocytes becomes insufficient as a result of the absent blood flow. Consequently, adenosine triphosphate (ATP) depletion induces deficiencies in active ion transport mechanisms and initiates the activation of anabolic glycolysis [15]. In parallel with the unfolding ATP deficit, reduced activity of Na^+/K^+ ATPase results in increased cellular influx of Na^+ [16]. Prominent intracellular acidosis during ischemia, caused by metabolites of anaerobic glycolysis, facilitates Na^+/H^+ Antiporter-1 activity contributing to the Na^+ influx. As a result of intracellular Na^+ accumulation, physiological function of Na^+/Ca^{2+} Exchanger is disturbed presented as Ca^{2+} influx and mitochondrial Ca^{2+} accumulation [17]. Cellular ion homeostasis disturbances will end up in cellular swelling and cell-death (Figure 1.).

Functional unit of the liver is the Rappaport's hepatic acinus [18]. A hepatic acinus implies the vascular bed of one small perilobular arterial and venous branch (originating from the hepatic artery and from the portal vein, respectively) situated along the short diagonal of the rhombus shaped lobule. Opposite vertices of an acinus are central veins and Glisson-triads (branches of the portal vein, hepatic artery and bile duct). The acinus, considered as functional unit, can be divided into 3 zones based on its biochemical and functional inhomogeneity (Figure 2.).
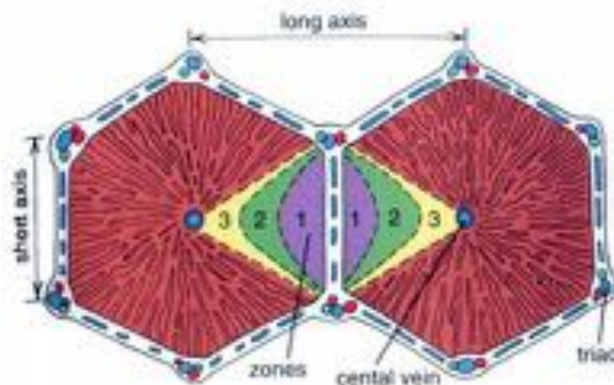
Figure 2. Hepatic acinus according to Rappaport
(From: URL: http://studydroid.com/imageCards/0a/k1/card-11143124-back.jpg)

While nutritive blood supply of hepatocytes in zone 3 is poor, the closest region to the portal vessels (zone 1) is well-oxygenated. Accordingly, activity of oxidative metabolism is much higher in hepatocytes of zone 1; therefore, they are more resistant against the detrimental effects of IR injury and have better regenerative potential when compared to zone 3 [18]. Periportal necrosis is a characteristic sign of liver IR injury.

The mitochondrion is an essential component in injury during ischemia-reperfusion and the main source of extensive reactive oxygen species (ROS) production. During reperfusion, an important initiator of cell-death is the opening of mitochondrial giant-channel, known as mitochondrial permeability transition pore (mPTP) [19]. Fast restoration of normal pH during reperfusion, ROS, high concentrations of Ca^{2+}, and oxidant compounds induce mPTP opening, whereas low pH, cyclosporin A, and Mg^{2+} block it [19].

Besides hepatocytes, sinusoidal endothelial cells (SECs) are also particularly sensitive to IR injury [20]. Membrane potential changes of SECs, cell swelling, disturbances in cytoskeleton organization are characteristic signs of IR injury. These aforementioned changes, combined with the imbalance between decreased nitric-oxide (NO) levels and increased endothelin (ET) and thromboxane A2 (TXA2) production, are the main factors in endothelial cell dysfunction. Further steps, such as leukocyte infiltration, platelet aggregation as well as hepatic stellate cell contraction induced sinusoidal narrowing, are all contributing to the observed reperfusion microcirculatory failure [20].

During the early reperfusion phase, selectin family members of adhesion molecules (P-,E- and L-selectin) are highly expressed by SECs [12,21,22]. Subsequently, integrins, essential for firmer leukocyte-endothel interactions, are upregulated [12] (Figure 3.). Activated neutrophil granulocytes are playing a key role in free-radical production and oxidative liver damage during the late phase of reperfusion (predominantly after 6 hours of reperfusion) [23,24].
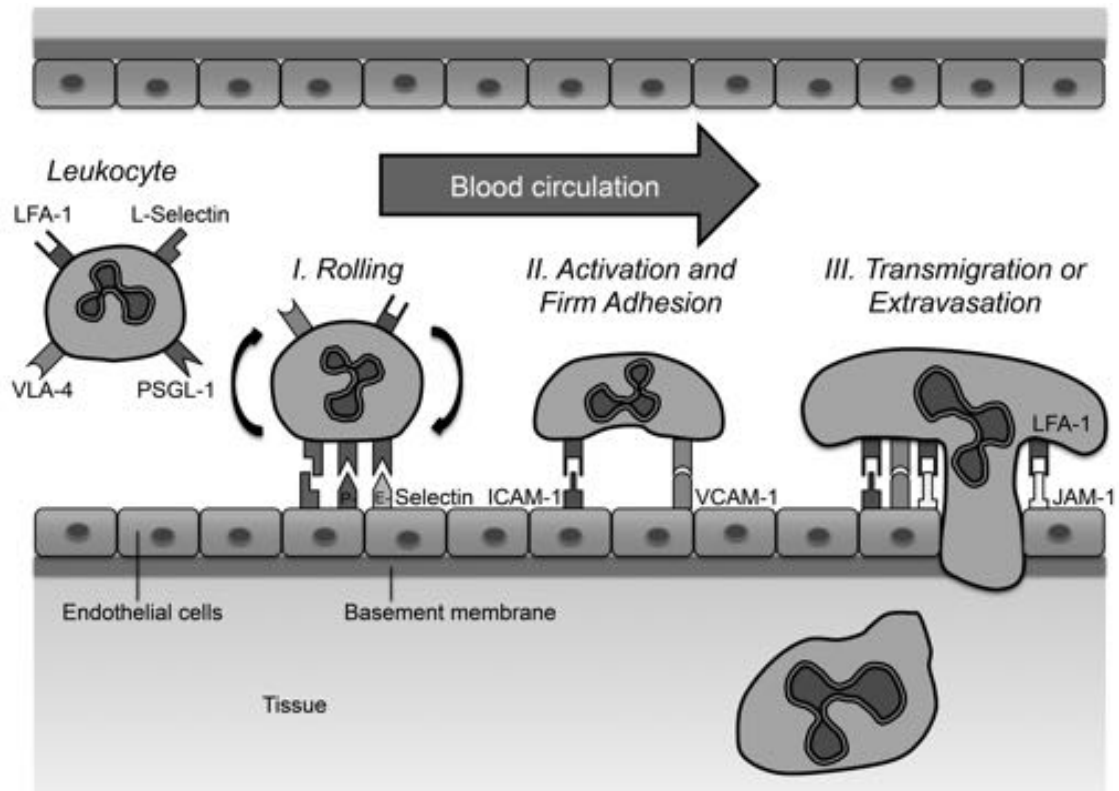
Figure 3. Leukocyte rolling, adhesion and extravasation during IR injury.
Abbreviations used: LFA-1, leukocyte function associated antigen-1; VLA-4, very late antigen-4; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; PSGL-1, P-selectin glycoprotein ligand-1; JAM-1, junctional adhesion molecule-1.
(From: Iwasaki, Czigány et al. Adhesion Molecules: Therapeutic Targets for Allograft Rejection and Ischemia-Reperfusion Injury. In: Chen (editor), Current Immunosupressive Therapy in Organ Transplantation. Nova Science Publishers, New York. 2015: 369.)

As discussed above, cellular ATP depletion during ischemia results in accumulation of adenosine, hypoxanthine and xanthine [12]. During the initial phase of reperfusion, cellular metabolism is still shifted towards anaerobic pathways; thus the immediately increased oxygen delivery generates free-radicals (superoxide, hydrogen-peroxide, peroxynitrite) [12]. In this very early phase of injury, tissue-specific macrophages, the Kupffer-cells, are believed to be the main source of ROS production. Infiltrating neutrophils take a more dominant role later on [25,26].

A complex cross-talk between different cytokines is also essential in the mechanism of liver IR. Extensively studied cytokine and chemokine mediators are TNF-α, IL-6, IFNs, IL-10, CXCL-10, etc. [12].

TNF-α, predominantly originated from activated macrophages during ischemia-reperfusion, has a crucial role in the mechanism of injury [14]. It has pleiotropic effects in post-ischemic injury and inflammation, albeit it thought to be an initiator molecule of liver regeneration as well, together with IL-6 [14]. Very soon after reperfusion IL-1 and TNF-α levels are markedly increased [27,28]. IL-1 is induced by TNF-α [14]. IL-1 can intensify neutrophil ROS production, while TNF-α has a positive feedback loop resulting in more TNF-α release [27,28]. Delayed elevation of IL-6 levels can also be observed.

Figure 4. Remote organs affected by the systemic consequences of liver IR injury.
(From: Nastos et al. (2014) Oxid Med Cell Longev, doi: 10.1155/2014/906965. Epub ahead of print.)

Up to this point, the author has discussed several crucial steps in the mechanisms of *local* injury observed in liver ischemia-reperfusion. Although, liver IR injury following sustained hepatic exclusions is a well-recognized phenomenon, injury of other *remote* organs, affected by the systemic effects of liver IR, has not been widely explored yet (Figure 4.). It has been reported that IR injury of the liver might be responsible for several remote organ injuries in various surgical scenarios, including major resections and transplantations [29]. Acute kidney injury [30], lung damage and acute respiratory distress syndrome [31], gut barrier failure and consequential bacterial translocation [32], pancreatic, [33] and adrenal injury [34] as well as myocardial damage [35] might occur in experimental settings or in clinical liver surgery (Figure 4.). Among several mechanisms, free-radicals (ROS) and inflammatory pathways (cytokines and leukocytes) are thought to play an essential role in the unfolding multi-organ dysfunction syndrome (MODS) following major liver surgeries [29].

1.2. Vascular exclusion techniques in liver surgery

Taking the fact into account that IR injury of the liver occurs mainly when we are forced to apply sustained exclusions of vascular pedicles during surgical interventions, in the following part of the doctoral thesis we attempted to compile the most important vascular control techniques used in clinic.

1.2.1. Pringle-maneuver

The best-known inflow occlusion technique to control blood loss during surgical interventions is linked inseparably with the name of Dr. James Hogarth Pringle [36] (Figure 5.). Pringle published his approach in 1908 to control bleeding from the liver in patients with severe abdominal trauma via clamping the hepatoduodenal ligament (Figure 6.). However, all of his 8 patients described in his seminal study had died postoperatively, the principles of vascular control during liver resections became accepted predominantly after Ichio Honjo and Jean-Louis Lortat-Jacob (Figure 5.) reported the first successful major anatomical liver resections [37,38].
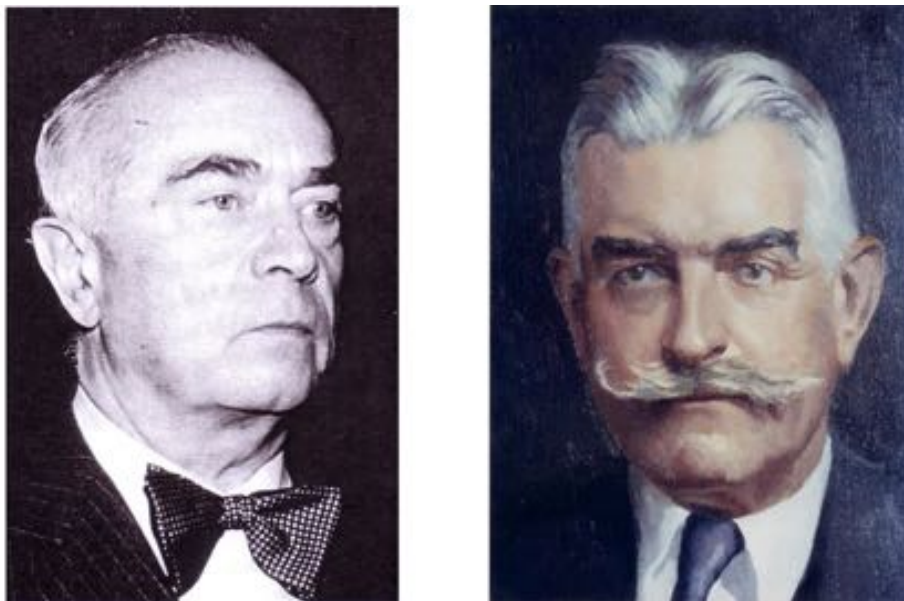
Figure 5. Jean-Louis Lortat-Jacob (1908-1992) and James Hogarth Pringle (1863-1941)

However, the aforementioned vascular control approach is attributed to Pringle and known as Pringle-maneuver in international literature, the Hungarian surgeon, Jónás Báron suggested and reported the use of temporary occlusion of the hepatoduodenal ligament to minimize blood loss, earlier, in 1876 [39].

Although, hepatic pedicle clamping and reperfusion have significant hemodynamic effects, the exclusion is well-tolerated thanks to the uninterrupted caval flow [40,41].

The literature is controversial, whether inflow occlusion should be applied continuously or intermittently [40,41]. Intermittent clamping is usually applied as 15-30 min ischemia interrupted by 5-15 min reperfusion [42]. In case of a good functioning liver, continuous clamping of the hepatoduodenal ligament can safely be implemented for up to 60 minutes [43].

Belghiti et al. performed a randomized study to compare continuous portal clamping with intermittent portal exclusion [44]. They found a significant increase in blood loss, although they detected a markedly reduced parenchymal damage in the intermittent clamping group compared with continuous clamping. They have concluded that intermittent clamping is beneficial, especially in patients with abnormal liver parenchyma. This approach allows us to extend net ischemic time safely to 120 min.

1.2.2. Hemihepatic and segmental vascular exclusion

These techniques can minimize IR injury of remnant liver as well as limit hemodynamic effects and splanchnic congestion. Furthermore, they are particularly useful in peripherally located tumors [45]. Hemihepatic vascular occlusion might be combined with exclusion of the corresponding hepatic veins (Figure 6.). Segmental exclusion, using balloon catheters, is a feasible tool of parenchyma sparing surgery. Following balloon inflation, segmental borders are delineated through an ischemic line [46]. This might further be enhanced by injection of methylene blue dye into the corresponding portal branch.
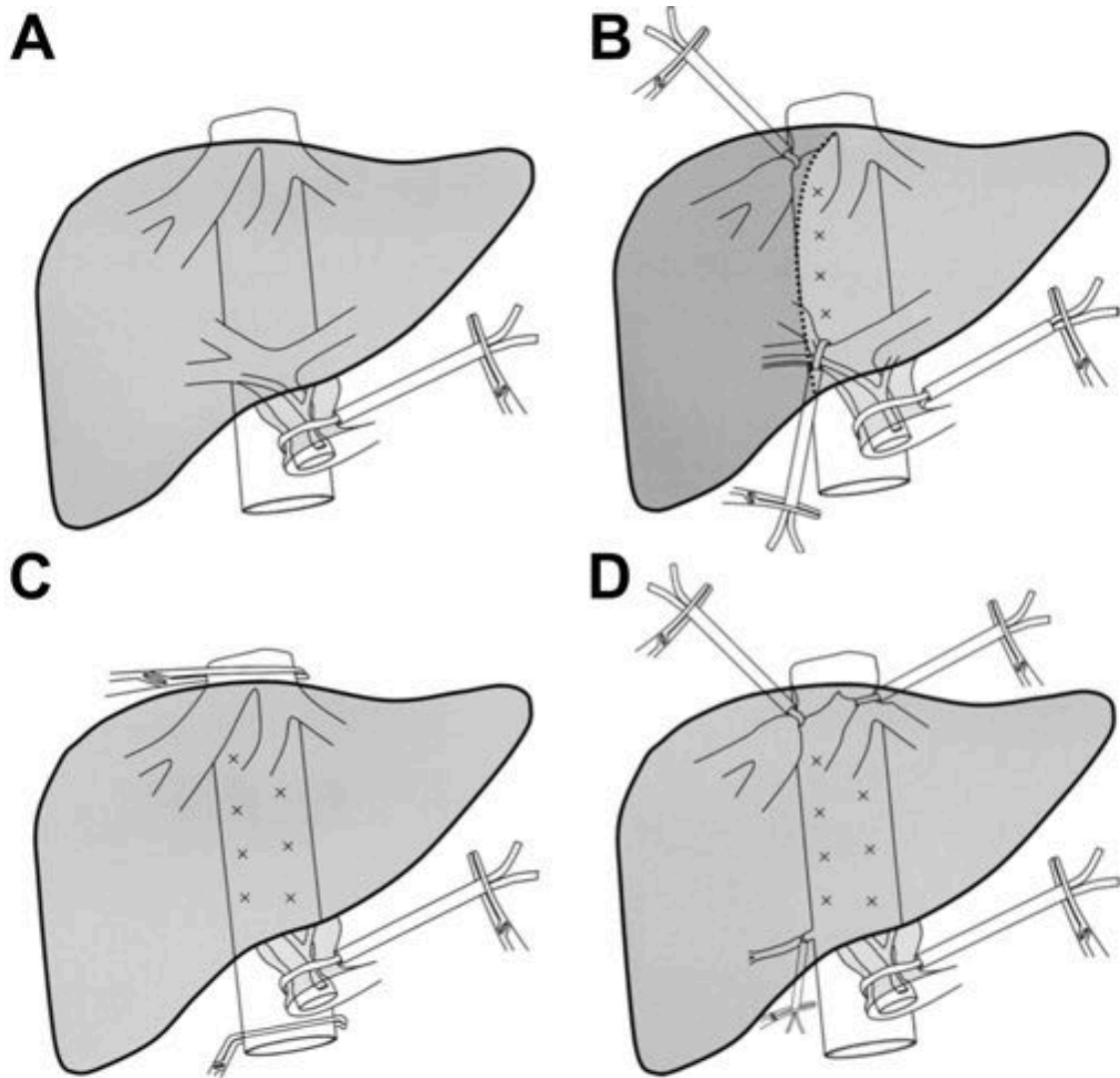
Figure 6. Different vascular occlusion techniques in clinical settings.
A. Exclusion of the hepatoduodenal ligament according to Pringle B. Hemihepatic vascular exclusion C. Total hepatic vascular occlusion D. Total hepatic vascular exclusion with preserved caval flow
(From: Abdalla et al. (2004) Sur Clin N Am, 84: 563-585.)

1.2.3. Total hepatic vascular occlusion (THVO)

This exclusion (Figure 6.) is necessary usually in case of an extensive central tumor mass, which might also involve the inferior vena cava [40,41]. It can also be considered in cases where persistent backflow from the hepatic veins causes an intensive blood loss during resection; however, in such cases, other interventions e.g. lowering the central venous pressure (<5 cm H2O) should be attempted first [41].

THVO has major hemodynamic consequences; therefore, a highly experienced anesthesiologist and careful monitoring are the prerequisites of this procedure [41,47]. THVO is not tolerated by 10-20% of the patients [40,41,47]. Despite the enormous

hemodynamic burden caused by application of THVO, it has several advantages as well. In THVO various complex surgical interventions can be performed. It makes the in situ hypothermic perfusion [48], ex situ resection and auto-transplantation as well as the ante-situm resections possible [49,50].

Combination of inflow occlusion and extraparenchymal control of the hepatic veins (Figure 6.) eliminates severe hemodynamic consequences of THVO [51]. Prevention of air embolism or backflow bleeding without interruption of vena cava inferior flow is the main advantage of this procedure [41]. Nevertheless, it cannot be applied in cases when a tumor mass is involving the vena cava and reconstruction is required, or when the involvement of the cavo-hepatic junction makes the exposure of the hepatic veins complicated [41].

1.3. Small animal experimental models for liver IR injury

Planning and implementing of clinical trials have very strict ethical criteria. After obtaining required ethical permissions the enrollment and follow-up phase as well as data evaluation can take several years. Our access to human tissues and the amount of samples taken are usually limited which can easily lead to underpowered studies. Laboratory research on cell- and tissue-cultures has contributed to scientific advancements regarding subcellular and molecular mechanisms and pathways. In most cases, however, we can only attempt to extrapolate these findings to the in vivo clinical situation with poor certainty.

Pre-clinical/translational research, using animals, has also several drawbacks regarding the translatability of findings to human clinical situations; however, it is still an inevitable approach for the in vivo investigation of pathophisiology responses following complex surgical interventions.

During the last decades several feasible and cost-effective rodent models have been developed to investigate characteristics, mechanisms, and further aspects of liver IR injury. In the present subchapter we have attempted to summarize the advantages and disadvantages of the experimentally and clinically most relevant models. Specific features of global and partial liver IR injury in rodents are described in detail.

1.3.1. Partial liver IR injury models

The reason behind using rodent partial IR injury models is the need for isolated investigation of in vivo effects of liver ischemia-reperfusion. In the literature we can find various models from 10% exclusion models, achieved via exclusion of the caudate lobes, until 70% partial liver ischemia. Figure 7. depicts the most frequently used models based on percentage of the excluded liver volume and shows the anatomical aspects of partial liver ischemia in rats. Essence of the surgical intervention in these models is the selective clamping of the corresponding biliovascular pedicle, using atraumatic clamps.

In this model we can induce ischemia of respective liver lobes, meanwhile leaving uninterrupted blood supply to others. This method can prevent severe congestion of the gastrointestinal tract, which is poorly tolerated by rodents.

However, in the literature there is no general agreement concerning the need for a partial liver resection in this model, most of the authors are removing the non-ischemized lobes right before liver reperfusion. Through this aforementioned partial

Figure 7. Different models for liver IR injury in rats.
Models for partial and total liver ischemia. Grey: ischemized lobes.
Abbreviations used: IRL, inferior right lobe; SRL, surperior right lobe; RML, right mediate lobe; LML, left mediate lobe; LLL, left lateral lobe; ACL, anterior caudate lobe; PCL, posterior caudate lobe.
(Adapted from: Czigány et al. (2015) Eur Surg Res, 55(1-2): 119-138.)

hepatectomy we can namely prevent the blood-stealing effect of the healthy lobes during reperfusion (due to increased vascular resistance of the post-ischemic lobes), ensuring reperfusion of the ischemic damaged liver lobes. In Budapest the reproducible rat model of partial liver ischemia was developed by Kupcsulik et al. in the 70s. This pioneering work provided a basis for the present thesis as well [52].

Advantages of this model are the opportunity to investigate more selectively the in vivo effects of liver IR injury without extreme hemodynamic impairment, severe gastrointestinal tract congestion and consequential bacterial translocation as well as bowel-origin cytokine storm. At the same time, in cases of sustained liver exclusions, the presence of systemic damages and remote organ injuries is probable.

Partial liver IR injury models might have inferior clinical relevance when compared to 100% hepatic exclusion. In clinical practice the most frequently used liver exclusion approach is the so-called Pringle-(Báron)-maneuver, which means the cross-clamping of the hepatoduodenal ligament [36]. Nonetheless, selective balloon catheter occlusion of liver lobes and segments, and hemihepatic vascular occlusion for liver resection can be achieved in clinical settings as well (see Figure 6.) [53,54].

1.3.2. Models of global hepatic ischemia

Global hepatic ischemia in rats and mice can be performed easily via clamping of the hepatoduodenal ligament [13]. This model is optimal to mimic the clinical situation of normothermic liver ischemia with Pringle-maneuver for liver resections. The drawback of this model is the induced very complex multi-organ injury with all of its consequences. Global hepatic ischemia results in major hemodynamic alterations and congestion in the portal vascular bed [19]. Injuries of different remote organs (myocardium, kidney, lungs, gut barrier, adrenals, pancreas etc.) are frequent complications [29].

There are different approaches developed for small animals to decompress the portal circulation in the anhepatic phase [13]. Besides various surgically established porto-systemic shunts [55-57], an interesting technique is the so-called subcutaneous spleen transposition. According to the widely accepted approach, the spleen can be pulled through and fixed in a subcutaneous position via a small incision in the left hypochondrium building a strong collateral network [13,58].

Since the use of a certain portal decompression technique for liver IR injury studies has not been generally accepted within the scientific community, the comparability of findings with numerous different models is questionable (total or partial ischemia, with or without shunt, etc.).

1.3.3. Duration of liver exclusion in rodent models of liver IR injury

Not only the anatomical aspects of liver exclusion but also the duration of ischemia can be varied considerably from one model to another. According to the findings of the comprehensive review article of Karatzas et al. on small animal liver IR injury models, we can conclude that 30, 45, 60, 90 min ischemic periods are most frequently used in the literature [19] (Figure 8.).

Partial liver ischemia models in rats and mice with ischemic intervals no longer than 60 minutes are usually well-tolerated [19].
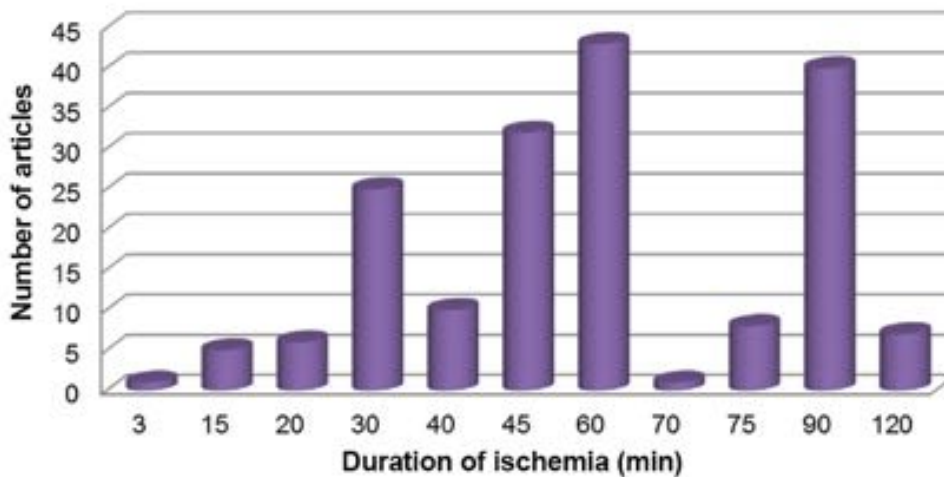
Figure 8. Duration of ischemia in rodent liver IR injury models.
(From: Karatzas et al. (2014) J Surg Res, 91(2): 399-412.)

Kim et al. considered 60 min of 70% partial ischemia of the mouse liver as the optimal model [59]. Shorter ischemic periods could not induce satisfactory tissue injury, presented as minimal elevations in serum transaminase levels. Ischemia over 75 minutes resulted in very poor reperfusion and was not tolerated in their model. Our group made similar observations in rats [60]. In 2006 Szijártó et al. reported the effects of local ischemic preconditioning applied before 30, 45, 60, 90 min of 70% partial liver ischemia in rats. In this study we could detect that a shorter ischemic episode (30 min) is not sufficient to induce a prominent damage in the liver, so preconditioning treatment had no significant effect after this ischemic event. "Restitutio ad integrum" was observed in both control and treated groups. After sustained ischemia (90 min) the severe liver damage was, in general, poorly tolerated; preconditioning could not

considerably improve liver microcirculation and tissue injury. Most remarkable differences we could observe between control and preconditioning groups after 60 minutes of 70% ischemia. This period led to moderate tissue injury, which was significantly reduced by preconditioning treatment. According to the above-described findings, we can say that the optimal model for mimicking sublethal partial liver ischemia in rodents is the 45-60 min liver exclusion, while the "point of no return" can be found between 60-90 minutes.

In the partial ischemia model, animals can be sacrificed after various time points. Based on the biphasic characteristics of liver IR injury (early phase 1-6 h, late phase 9-24 h) 1,3,6 and 24 h intervals after reperfusion are frequently recommended for analysis [19,59].

Regarding global hepatic ischemia (without porto-systemic shunting) the literature is more controversial. For acute experiments, investigating short-term consequences of severe ischemic-reperfusion injury, longer exclusion times are also accepted [19]. However, 60-90 min total hepatic ischemia results in a very poor long-term survival [61]. Higher survival rates can be achieved with 30-(45) min of global ischemia [62-64].

1.3.4. Further concerns regarding liver IR injury models in rodents

During the planning phase of an experimental study using laboratory rodents, several aspects have to be considered, which might have a decisive role to play regarding the reliability of our model and results.

Anesthesia practice for laboratory rodents has markedly changed during the last decades. Modern volatile anesthetics (e.g. isoflurane) and injectable anesthetic combinations (e.g. ketamine/xylazine) are getting more and more popular in rodent surgery [65,66]; nevertheless, the use of obsolete approaches (ether, barbiturates) still can be identified in numerous newer reports.

The assumed conditioning, anti-ischemic effects of different anesthetic drugs, however, should also be considered when our aim is to investigate liver IR injury in a rodent model. Various authors have proved pharmacological conditioning effects of ketamine in different models [67,68]. Guzman et al. reported that ketamine could reduce

mucosal injury in a rat model of intestinal IR injury [67]. Similar effects were found in an in vitro study on human myocardial tissue [68].

According to the findings of Kim et al. isoflurane protects against small intestinal injury, hepatic and renal dysfunction following severe intestinal IR injury via induction of intestinal epithelial TGF-β1 expression [69].

These effects can cause only "internal errors" within our experimental setting, not considerably influencing the between group differences, but we have to be aware of these aforementioned "side-effects" of the used anesthetic agents.

Injection anesthetics must be administered carefully, particularly in studies where sustained liver exclusion is applied (liver transplantation with total hepatic vascular occlusion or IR injury studies with total hepatic ischemia). Significant increment in plasma concentration of several anesthetic drugs, during the anhepatic phase, might result in unexpected mortality [70-72].

A number of distinct age-related differences have been identified in IR injury and inflammatory responses of laboratory rodents [13,73]. In general, older rats show more severe tissue injury following the same surgical intervention [74,75]. Laboratory rats between 200-350 grams are ideal for modeling liver IR injury. Smaller animals are technically more difficult to operate, at the same time, older animals (over 400 g) can also present problems, due to the higher postoperative complication rates and increased intra-abdominal fat [13].

Gender differences might also have an effect on our results, especially on the comparability of the findings [13]. Studies have shown that males and females present differences in susceptibility to reperfusion injury [76-78]. In female animals ischemic tolerance is probably dependent on the estrous cycle, though its role has not been properly clarified yet [76,77].

Small animals are extremely sensitive to changes in body core temperature; therefore, it is very important to monitor and maintain body temperature perioperatively. Heijnen et al. could show significant increment in serum transaminase levels and in histopathological damage in a rat model of 60 minutes partial liver ischemia when the animals´ body temperature increased from 36°C to 38°C [79].

1.4. Ischemic conditioning approaches
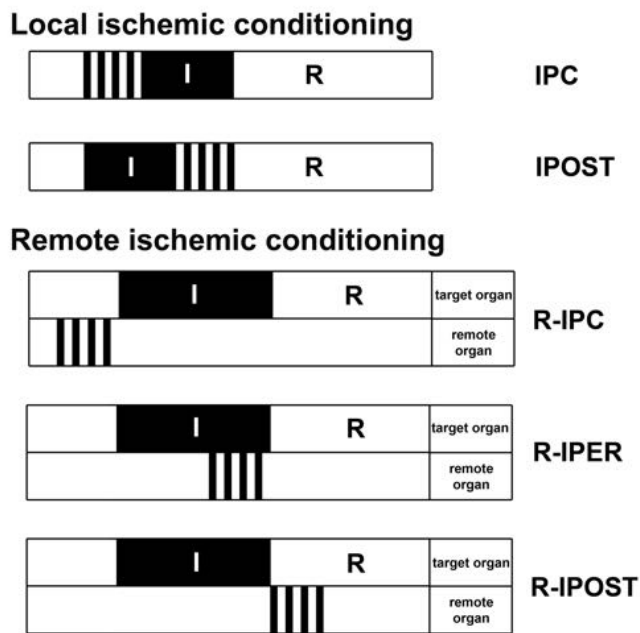
Figure 9. Treatment algorithms of local and remote ischemic conditioning techniques.
Black bands=interruption of circulation; white bands=unimpeded circulation.
Abbreviations used: I, ischemia; R, reperfusion; IPC, ischemic preconditioning; IPOST, ischemic postconditioning; R-, remote; IPER, ischemic perconditioning
(From: Szijártó, Czigány et al. (2012) J Surg Res, 178(2): 797-806.)

In the last 30 years several surgical and non-surgical protective strategies have been developed, with more and less success, to lessen the degree of IR injury (Figure 9.). In 1986 Murry, Jennings, and Reimer described ischemic *pre*conditioning (IPC) [6]. Essence of this adaptive surgical method is to induce short periods of local ischemia and reperfusion before target organ ischemia. There is a vast literature on the strong protective effect of IPC, which has been proved by numerous experimental and clinical studies; however, the technique is limited to elective situations in which the onset of ischemia can be predicted. Local preconditioning cannot be used in acute clinical settings such as acute myocardial infarction, ischemic stroke or acute major vascular occlusions. It therefore became necessary to develop new techniques suitable for providing protection against unpredictable ischemic events. One option is to modify the reperfusion by means of brief coronary artery occlusions and reperfusions applied at the onset of myocardial reperfusion, a phenomenon called ischemic postconditioning (IPOST). Although Na et al. formulated the seminal idea and terminology of IPOST [7,80], the first easily reproducible experimental results were published by Zhao et al. in 2003 [80]. A shortcoming of both pre- and postconditioning is the prolongation in operative time, possibly even for a duration of 15-20 min.
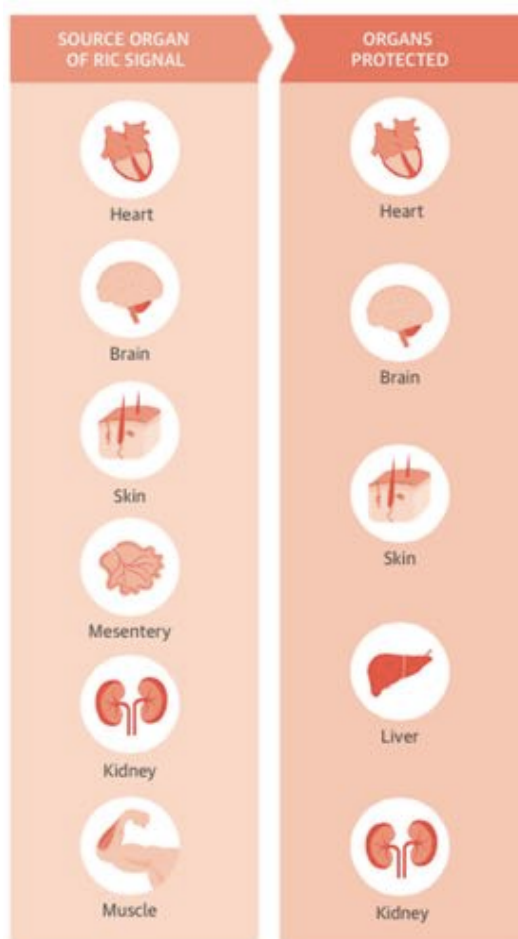
Figure 10. Inter organ protection by remote conditioning.

The figure depicts the numerous different combinations tested to investigate remote conditioning induced inter-organ protection.
(Adapted from: Heusch et al. (2015) J Am Coll Cardiol, 65(2): 177-195.)

Reducing the extent of IR injury via short episodes of ischemia and reperfusion instituted at a remote site is a novel idea. As for local conditioning techniques (IPC and IPOST), remote ischemic conditioning can be applied before target organ ischemia (remote ischemic preconditioning [RIPC]) or at the onset of reperfusion (remote ischemic postconditioning [RIPOST]). When the site of conditioning is remotely located, conditioning cycles can be applied during target organ ischemia; a novel phenomenon known as remote ischemic perconditioning (RIPER) (Figure 9 and 10.).

Przyklenk et al. [8] developed the idea of remote conditioning originally in 1993. The authors described positive effects of regional preconditioning of the myocardium with manipulations in the same vascular bed (territory of the left coronary artery). They demonstrated that transient occlusion of the circumflex artery provided subsequent protection in the myocardial territory of the left anterior descending artery exposed to a sustained, potentially lethal, ischemic insult. This finding assumes the presence of certain transportable mediators and other protective signals delivered via the circulation to a distant myocardium. Over the past 2 decades investigations have demonstrated that short IR episodes applied to kidney, splanchnic area, limbs, etc. may induce organ protection against prolonged ischemic periods of different target organs (Figure 10.) [81,82]. Unfortunately, remote preconditioning shares the major disadvantages of local preconditioning: restriction to elective ischemic conditions and prolongation of operative time. Consequently, it cannot be applied to a wide range of clinical situations. Remote ischemic postconditioning was developed as a protective

strategy bearing similarity to local postconditioning (IPOST); therefore, it extends the range of application of remote conditioning techniques [83-85].

Remote ischemic perconditioning, proposed for the first time by Schmidt et al. in 2007 [86], has plenty beneficial features from a practical point of view. Essence of this strategy is that short remote ischemic attacks are applied after induction of target organ ischemia but before the onset of reperfusion.

1.4.1. Terminology

There is much confusion in literature regarding the concept called "ischemic perconditioning". Three different terms exist for this strategy, meaning "the induction of brief and repetitive interruptions of blood flow at a distance, applied concurrently with sustained target organ ischemia": (1) remote perconditioning [86-88] (2) remote periconditioning [89,90] and (3) remote postconditioning [91-93].

Term "RIPOST" can be excluded first as proper terminus, because it implicates that conditioning cycles take place after ("post") target organ ischemia (Figure 9.). Distinction between periconditioning and perconditioning is more of a semantic question rather than methodical. Greek prefix *peri-* means "around something in time" and is found in the expression "perinatal mortality." In contrast, the meaning of the prefix *"per"* is "over, through, during the course of something" such as "perennial rhinitis." Thus, from a linguistic point of view, the term "perconditioning" is suitable for describing this method. Furthermore, Schmidt et al. [86], pioneers of this procedure, proposed the term "remote perconditioning" as well. Accordingly, "perconditioning" seems to be a suitable term for all cases in which brief ischemic and reperfusion cycles are instituted after the onset of target organ ischemia and completed before reperfusion.

1.4.2. Remote ischemic perconditioning: underlying mechanisms

There is scarce evidence to explain how brief cycles of IR on a distant organ, during prolonged target organ vascular occlusion, are able to provide organ protection. Here, we discuss the literature findings on remote ischemic conditioning in order to understand the underlying mechanisms.

Hausenloy and Lim et al. [81,94] have recently given a detailed review on the underlying mechanisms of RIPC. According to their theory, different humoral, neural

and systemic components hypothetically interact with each other to provide target organ protection from a remote site.

In our hypothesis, there are two main different groups of inter-organ protective pathways [9]: (1) connective mechanisms, which provide linkage between distant and target organs (principally the classical humoral, neural, neuro-humoral, and systemic mechanisms); (2) signal transduction and effector mechanisms, which are mainly molecular, subcellular, and channel-related components (most likely participants of the distal, down-stream pathways of inter-organ protection) (Figure 11.).
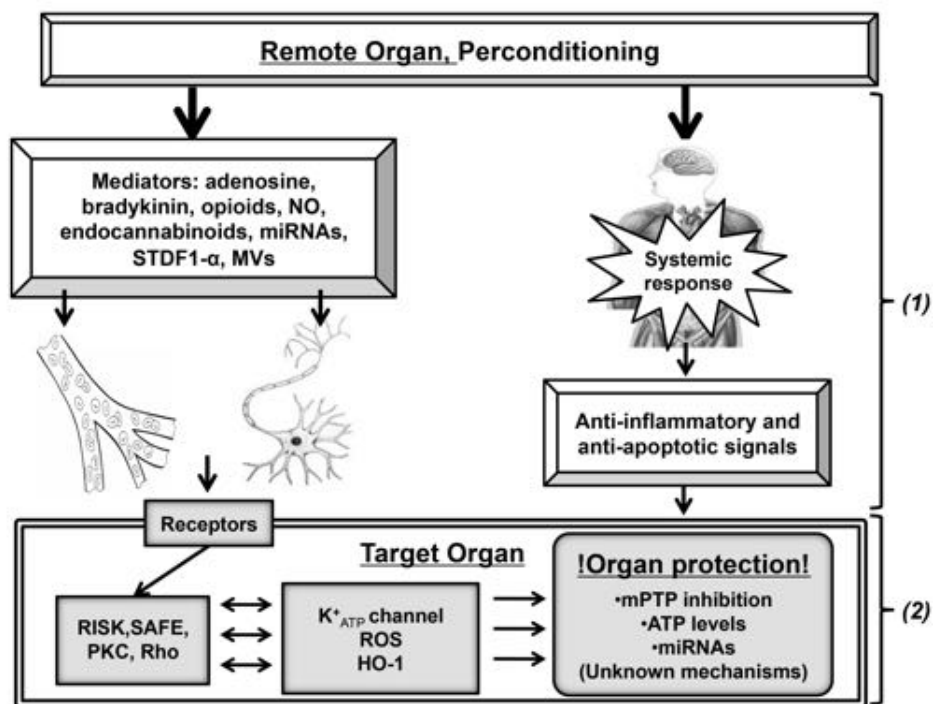
Figure 11. Underlying mechanisms: different components of remote conditioning induced inter-organ protection.
(1) Connective mechanisms between remote and target organ are mostly humoral, neuro-humoral, and systemic. (2) Effector and signal transduction components are predominantly channel-related, subcellular, and molecular mechanisms.
Abbreviations used: NO, nitric-oxide; miRNAs, microribonucleic acids; STDF1, stromal derived factor-1; MVs, microvesicles; RISK, reperfusion injury salvage kinase; SAFE, survivor activating factor enhancement; PKC, protein kinase C; K+ATP channel, ATP sensitive potassium channel; ROS, reactive oxygen species; HO-1, hemoxygenase-1; mPTP, mitochondrial permeability transition pore; ATP, adenosine triphosphate
(From: Szijártó, Czigány et al. (2012) J Surg Res, 178(2): 797-806.)

These mechanisms are related in a complex manner; therefore, neither of these artificial classification models can perfectly reflect the in vivo pathophysiological conditions. Nonetheless, a classification is still essential to understand this complicated phenomenon.

As pointed out in previous studies, concentration or activation status of molecular, humoral, and subcellular participants may be varied significantly among the different remote ischemic conditioning subtypes (RIPC, RIPER, and RIPOST); namely, the timing of these procedures is radically different (before, during or after target organ ischemia) [95]. It is assumed, however, that the main biochemical and signal transduction pathways might be similar among the conditioning techniques, accordingly some comparison between the remote conditioning strategies is inevitable [96].

1.4.2.1. Connective mechanisms

As a result of remote organ ischemia, soluble mediators, suspected to be responsible for the protective effect, are washed into the circulation. McClanahan et al. [97] reported the first evidence of humoral factors. Since presenting their discovery, the role of certain humoral factors has been verified in numerous models [98-103]; investigations on denervated organs, excluding the neural component, have been carried out [104,105]. Cardioprotection was achieved in untreated isolated hearts by means of blood transfusion from remote-conditioned animals to untreated hearts [100]. Among the suspected humoral mediators we can find bradykinin [106], adenosine [107,108], opioids [109], calcitonin gene-related peptide (CGRP) [110], endocannabinoids [111], stromal derived factor 1-α (STDF1-α) [112], microribonucleic acids (miRNAs) [113], extracellular microvesicles [114]. It appears that adenosine has a key role, given the fact that the protective effects of remote lower limb conditioning, during sustained contralateral limb ischemia, could be abolished by the nonselective adenosine receptor antagonist, 8-sulfophenyl theophylline, in mice [91]. In contrast to these results, Hausenloy et al. were not able to demonstrate this adenosine receptor dependent effect on pig myocardium. 8-sulfophenyl theophylline had no influence on perconditioning-induced cardioprotection in their model [95]. Reasons behind these controversial results lay dormant. Differences between various experimental models could be held accountable (different species and target organs); hence, this complex issue needs further investigation. Other studies have implicated the role of opioids and nitric-oxide (NO) [93].

Extracellular vesicles consist of several molecules (mRNAs, miRNAs, proteins, etc.) and participate in inter-organ communication. More recently and for the first time

in literature, a working group of the Semmelweis University (Giricz et al.) has proved the possible role of extracellular vesicles in transfer of remote preconditioning signals [114].

Most of the recent data suggest the involvement of nociceptive fibers, somatosensory system, autonomous nervous system, and the spinal cord in transfer of remote conditioning signals [82]. The potential myocardial infarct size reducing effect could be diminished by the ganglion blocker hexamethonium, suggested the presence of neural factors for the first time by Gho et al. [115]. Further investigations with various receptor-blocking agents indicated that afferent neuron stimulation from a remote organ by humoral mediators, e.g. adenosine, bradykinin, and CGRP, provides the neural path [82,116]. Calcitonin gene-related peptide release from capsaicin-sensitive sensory neurons is presumably caused by NO generation during remote organ IR [117,118]. A study by Ren et al. [83] confirmed the role of CGRP regarding the protective effect of remote conditioning, which could be abolished by neural inhibition with capsaicin. Direct vagal nerve stimulation was also reported to be effective in mimicking of remote conditioning effect [119,120]. Involvement of neural mechanisms, behind remote perconditioning induced responses, remains to be elucidated. In case of RIPER treatment no humoral mediators can reach the target organ via blood flow during the ischemic phase of the target organ; therefore, it is a very interesting question whether other elements can replace humoral signals in this transfer.

It has been confirmed that target as well as remote organ ischemic conditioning is able to change the gene expression profile of the whole body (including target organ and circulating leukocytes) in an anti-inflammatory and anti-apoptotic direction. This is partially due to the down-regulation of specific pro-inflammatory genes and the up-regulation of specific anti-inflammatory genes [121,122]. Secretion of certain cytokines, the tumor necrosis factor receptor signaling pathway, and processes of innate immunity and apoptosis (via repression of caspase-8) are also all suppressed. Anti-inflammatory gene up-regulation also occurs (e.g. in case of heat-shock protein [HSP] 70 or calpastatin) [123]. Inhibition of pro-inflammatory cytokine secretion results in reduced leukocyte-endothelial adhesion and tissue infiltration, which leads to a milder local inflammatory response [124]. Less leukocyte infiltration results in fewer leukocyte-related reactive oxygen species (ROS) production, which is an important factor in the

late phase of reperfusion. This phenomenon suggests a partial explanation for both the observation of Harkin et al. [125] and our findings [126], according to which lower limb ischemic preconditioning and postconditioning can reduce remote lung injury and leukocyte sequestration after local limb IR injury. In these cases, however, the dominating factor is presumably the local IR injury reducing effect of the conditioning strategy, leading to mitigated remote organ damage. Nonetheless, Wei et al. [127] reported a remission in inflammatory responses caused by RIPER. The authors observed significantly diminished macrophage and neutrophil infiltration in rat myocardium after perconditioning.

1.4.2.2. Signal transduction and effector mechanisms

Regarding remote conditioning strategies, the gene regulatory effect is presumably mediated by similar signal transduction cascades. Initially, the ligand binds to its receptor, then the intracellular kinase PKC [128,129] and other signaling mechanisms (NO, reactive oxygen species [ROS], and mitochondrial K^+ATP channels) are activated [87,89,107,130]. Similar signal pathways are present both in IPC [131] and IPOST [132].

It is well-known that application of local preconditioning, postconditioning, and remote organ conditioning results in the activation of certain pro-survival kinase elements of the reperfusion injury salvage kinase (RISK) cascade. However, the mechanisms behind RISK activation during remote conditioning are unclear. The RISK pathway seems to be particularly important in mediating the protective effects of different conditioning procedures. The most clearly defined mechanism is the PI3K-Akt-eNOS-cGMP-PKG pathway, resulting in the opening of mitochondrial ATP-sensitive K^+ (K^+ATP) channels, in case of ischemic preconditioning. In turn it leads to ROS production and more survival kinase activation (p38, JNK, ERK, PKC, Akt, and TK), as a consequence K^+ATP channel opening induces further effector mechanisms [129].

In case of remote ischemic perconditioning, Xin et al. [87] confirmed the activation of certain elements of the RISK pathway. According to their results, RIPER was able to induce significantly higher Akt and ERK1/2 activation compared with the

IR control group. Despite the impressive strides made, the literature is controversial. In their recent study, Hausenloy et al. [95] reported that RIPER could reduce myocardial IR injury in an unknown manner but independent of RISK activation.

Adenosine triphosphate sensitive K^+ channels are actively investigated components of conditioning strategies [86,105,133-135]. Regarding the widely explored local preconditioning, both sarcolemmal and mitochondrial channels have an important role in the mechanisms, but "it is the mitochondrial channels that are sine qua non of the preconditioning effect" [136].

According to Schmidt et al. [86] protective effect of RIPER can be abolished by administration of the K^+ATP channel inhibitor, glibenclamide. Furthermore, Zhao et al. [89] recognized that during remote perconditioning, K^+ATP channel activation is achieved by inhibition of Rho-kinase. This Rho-kinase inhibition is mediated by protein kinase A (PKA), confirmed by Sanada et al. [137]. It is therefore likely for the PKA-induced inhibition of Rho-kinase to result in K^+ATP channel activation.

Although, the exact role of K^+ATP channel activation in the reduction of IR injury is unclear. Its importance in preserving mitochondrial function is suspected by way of moderating ATP depletion [137,138] and inhibiting mitochondrial permeability transition pore (mPTP) channel opening [139]. The mPTPs are nonselective channels in the mitochondrial inner membrane. As the consequence of their opening, the selective permeability of the inner membrane disintegrates; therefore, oxidative phosphorylation is disturbed and mitochondrial integration is completely abolished, leading to cell death [140]. Opioids also have a role to play in the inhibition of mPTP opening via opioid receptor-mediated signal transduction [92,141,142].

A further well-known participant in the mechanism of different conditioning strategies is the so-called survivor activating factor enhancement (SAFE) pathway, in which signal transducer and activator of transcription proteins (STATs) are the suspected key elements. Boengler et al. [143] demonstrated mPTP inhibiting effects, achieved by activation of mitochondrial STAT isoforms (STAT 1 and 3), in mouse myocardium. Based on these data, mPTP channels could represent the final common pathway for both the RISK and SAFE cascades.

Tamareille et al. demonstrated an improvement in cardioprotection with use of RIPER in combination with IPOST, as opposed to IPOST alone [144]. The authors associated RIPER induced protection with the recruitment of the SAFE pathway. Their study indicated the existence of a complex crosstalk between RISK and SAFE pathways. Inhibition of both cascades could abolish the cardioprotective effects of RIPER+IPOST combination. They observed decreased activation of RISK pathway, achieved by administration of SAFE inhibitors, and vice versa, when RISK inhibitors were given. Data mentioned above suggest the presence of many common elements among the various conditioning methods; yet, the specific role of these common mediators in different procedures still needs to be investigated.

According to several studies ROS production plays a key role as a second messenger in the mechanism of conditioning procedures during the early reperfusion phase [145-150]. The protective effects most likely prevail in local preconditioning [151] and postconditioning [152] via activation of the RISK pathway and inhibition of mPTP channels. However, positive effects of ROS production are to be sharply separated from extensive ROS generation observed during reperfusion, which correlates with tissue injury and subsequent cell death [153]. These results are consistent with the findings of Xin et al. [87], who demonstrated reduction of malondialdehyde and superoxide levels related to RIPER. Furthermore, they observed increased survival kinase activation, which suggests an inverse correlation with malondialdehyde and superoxide levels. There is scare evidence on the mediator role of ROS in remote ischemic conditioning of the liver.

2. OBJECTIVES

Ischemic-reperfusion injury of the liver represents a major problem in numerous clinical settings. In extended liver resections performed with Pringle-maneuver the liver undergoes a sustained exclusion and a consequential normothermic liver IR injury. During liver transplantations cold ischemia and warm reperfusion of the liver graft can result in primary graft dysfunction or non-function, especially when we are forced to deal with extended criteria donors (steatotic grafts, elderly donors, etc.).

The main objective of our studies was to investigate the effects of a novel approach on the ischemic-reperfusion injury of the rat liver. Following initial studies on protective effects of remote ischemic perconditioning, we aimed to reveal whether or not neural elements are participating in the RIPER induced protection.

In the present Doctoral Thesis we were looking for answers for the following questions:

1. Is our rat model of hepatic ischemia-reperfusion injury and remote ischemic perconditioning suitable and feasible to test the effects of remote ischemic perconditioning?

2. Can the applied remote ischemic perconditioning protocol exert any effects
   a, on liver tissue injury?
   b, on systemic hemodynamics and liver as well as lower limb microcirculation?
   c, on redox-homeostasis and systemic inflammation?

3. Is left femoral artery perconditioning also able to exert hepatoprotection in our model, and if yes, does remote organ denervation has any effect on the perconditioning induced hepatoprotection?

4. Is the "tile-based" automated histological image analysis feasible within a real experimental setting?

3. MATERIALS and METHODS

3.1. Experimental settings, surgical procedures

3.1.1. Ethical background and animals

Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Semmelweis University and were in accordance with Government Decree 40/2013. (II. 14.).

Male Wistar rats weighing 200-250 g were used (Semmelweis University Central Animal Facility, Budapest, Hungary) during the studies (Σn=114). The animals were held under standard animal care conditions at 22-24°C, with 12-h day-night cycles. Standard rodent pellets (Toxi-coop Ltd, Dunakeszi, Hungary) and water were granted *ad libitum*.

3.1.2. Circumstances during surgery and anesthesia protocol

All experiments were implemented at the same time of day to avoid disturbing effects of circadian rhythm.

Animals were anesthetized with intraperitoneal injections of ketamine (Calypsol®) (75 mg/bwkg) and xylazine (Xylasin®) (7.5 mg/bwkg), then they were placed on a heating pad connected with a rectal thermometer to maintain body temperature between 36.5 and 37.5°C (Homeothermic Blanket Control Unit; Harvard Apparatus Ltd, Holliston, MA, USA). 22-gauge polyethylene catheter was placed into the right jugular vein for maintenance of anesthesia (25 mg/bwkg/h ketamine and 2.5 mg/bwkg/h xylazine) and administration of saline infusion (3 mL/bwkg/h) as compensation for intraoperative fluid loss.

Postoperative analgesia was achieved by the administration of buprenorphine (0.01 mg/bwkg/24h).

3.1.3. Studies and experimental groups

Protocols for the two different studies are summarized in form of the following flowcharts (Figure 12. and 13.).
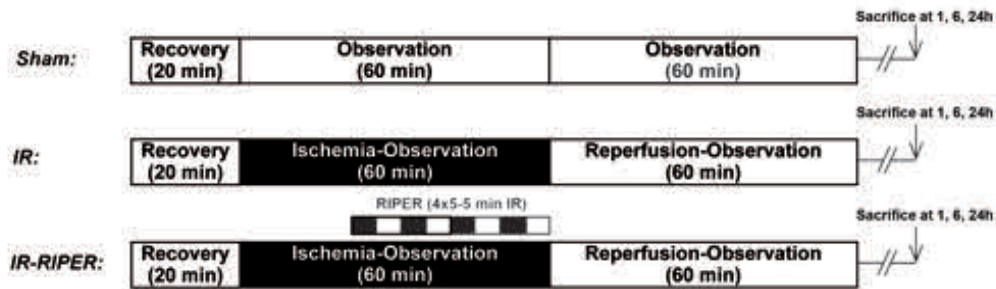
Figure 12. Experimental design for Study I. - Effects of RIPER on IR injury of the liver

Animals (Σn=72) were randomly allocated into three experimental groups (n=24/group). Following 20 min of recovery period all groups were subjected to 60 min of partial liver ischemia or corresponding observation period for the Sham group, followed by 1, 6 or 24 h of reperfusion. RIPER was achieved via exclusion of the infrarenal aorta (four cycles of 5 min of ischemia (I) and 5 min of reperfusion (R) during the last 40 min of liver ischemia). During the first 60 min of reperfusion, animals were continuously observed, blood pressure and microcirculation (liver and lower limb) were monitored. After 1, 6 or 24 h of reperfusion, all animals were sacrificed (n=8/time point) for tissue and blood sampling.
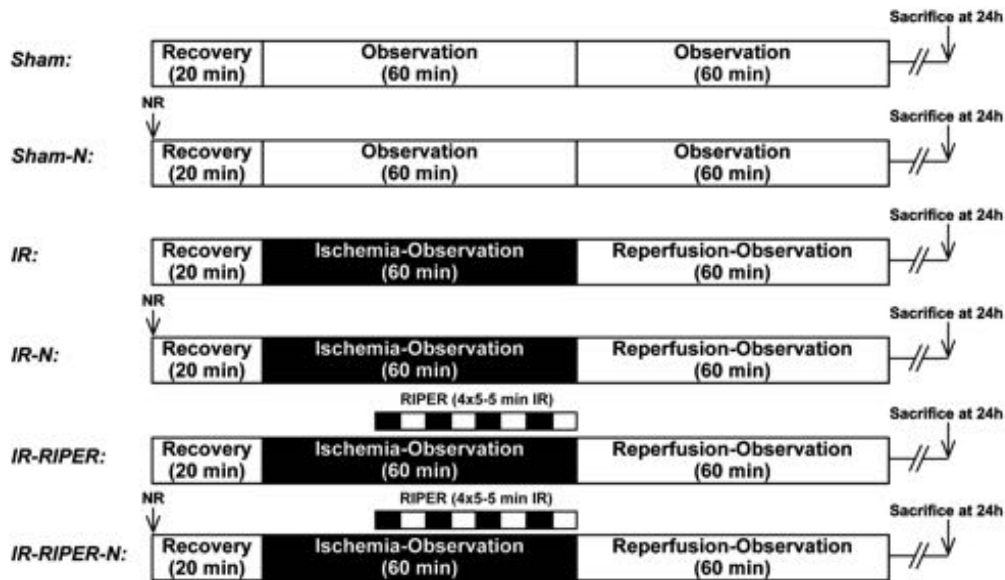
Figure 13. Experimental design for Study II. - Neural elements behind the RIPER hepatoprotection

Animals (Σn=42) were randomly allocated into six experimental groups (n=7/group) and underwent either left femoral and sciatic nerve resection (NR) or only preparation of the left femoral and sciatic anatomical structures. Subsequently, a recovery time of 20 min was allowed before ischemia induction. All groups were subjected to 60 min of partial liver ischemia or corresponding observation period for the Sham and Sham-N groups, followed by 24 h of reperfusion. RIPER was achieved via left femoral artery clamping (four cycles of 5 min of ischemia (I) and 5 min of reperfusion (R) during the last 40 min of liver ischemia). During the first 60 min of reperfusion, the animals were continuously observed, blood pressure and liver microcirculation were monitored. After 24 h of reperfusion all animals were sacrificed for tissue and blood sampling.

3.1.4. Surgical procedure

3.1.4.1. General surgical approaches in Study I. and Study II.
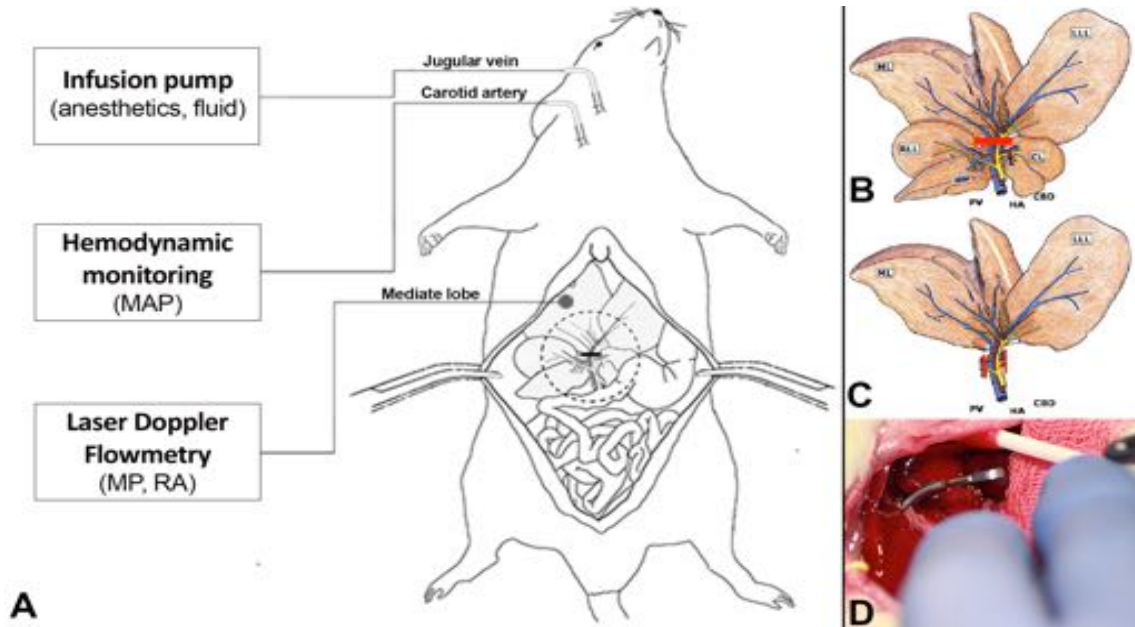
Figure 14. Experimental setup.
A. Experimental setup for partial ischemia of the rat liver with microcirculatory and hemodynamic monitoring. B. 70% liver ischemia. C. Remnant liver after reperfusion and 30% resection. D. Intraoperative photo of the 70% liver clamping.
Abbreviations used: MAP, mean arterial pressure; MP, maximal plateau; RA, reperfusion area; RLL; right lateral lobe; ML, median lobe; LLL, left lateral lobe; CL, caudate lobe; PV, portal vein; HA, hepatic artery; CBD, common bile duct
(Adapted from: Fülöp,…Czigány et al. (2015) J Surg Res, 197(2):307-317.)

After i.p. anesthesia induction, the right jugular vein (for anesthesia, see 3.1.2. Circumstances during surgery and anesthesia protocol) and the right carotid artery (hemodynamic measurements, see 3.2. Assessment of systemic hemodynamics and microcirculation) were cannulated, using 22-gauge polyethylene catheters (Figure 14.).

Laparotomy was performed via a median incision, and the liver was freed from its ligaments. Complete ischemia of median and left lateral lobes (ML, LLL) was achieved by clamping of the hepatobiliary pedicle using an atraumatic microvascular clamp (Aesculap Yasargil FT260T; B. Braun Melsungen AG, Melsungen, Germany) for 60 min (Figures 12. and 13.). Ischemia was induced in approximately two-thirds of the liver, whereas leaving an uninterrupted blood supply to the right lateral and caudate lobes [52,60]. During IR periods the abdomen was covered with a plastic wrap in order to prevent fluid loss through evaporation.

Shunting lobes (right lateral and caudate lobes) were removed (30% liver resection) during the last 10 min of liver ischemia in each group via pedical ligation

method, using a 4-0 silk thread (Resorba Medical Inc, Nuremberg, Germany). At the end of ischemia, the vascular clip was removed and liver reperfusion was allowed.

3.1.4.2. Specificities of surgical procedures in Study I.

Before induction of liver ischemia the retroperitoneal compartment was opened, and the infrarenal aorta was mobilized (Figure 15.). In groups receiving perconditioning treatment, 20 min after onset of liver ischemia, 5 min of bilateral lower limb ischemia and 5 min of reperfusion (four cycles and 40 min in total) were induced by infrarenal aortic cross-clamping, using the same type of microvascular clamp (Aesculap Yasargil FT260T).

After reperfusion and hemodynamic, microcirculatory monitoring the animals were sacrificed (1-h reperfusion groups) or wounds of laparotomy and jugular vein

Figure 15. Perconditioning was applied on the infrarenal aorta.
(From: URL:
http://www.biologycorner.com/resources/rat_circ_artery.gif)

preparation were closed by interrupted 4-0 Vicryl sutures (Ethicon Inc, Somerville, NJ, USA). Thereafter, the animals were returned to their cages for a period of recovery defined by their grouping (6- and 24-h reperfusion groups).

All animals were exsanguinated (6- and 24-h reperfusion groups as well) in anesthesia at the end of the experiment (Figure 12.).

3.1.4.3. Specificities of surgical procedures in Study II.

Preparation of left femoral vessels as well as femoral and sciatic nerves (FN, SN) was implemented as described previously [101]. Briefly, after a small incision at the proximal region near the groin, femoral structures (femoral nerve, vein and artery) were divided with the greatest care. Exposure of the left sciatic nerve was performed by a skin incision at the proximal thigh region followed by blunt preparation of the biceps femoris.

Approximately 0.5 cm segments of the FN and SN were resected in groups subjected to nerve resection (NR) followed by a recovery period of 20 min.

Four cycles, each of 5 min of ischemia and 5 min of reperfusion, were applied as RIPER protocol during the last 40 min of hepatic ischemia achieved by clamping of the left femoral artery with a microvessel clamp (Aesculap BIEMER FD561 R; B. Braun) (Figure 13.).

Following the first post-ischemic hour, wounds (laparotomy as well as vena jugularis and nerve preparations) were closed by interrupted 4-0 Vicryl sutures. Before returning to their cages, animals were treated with subcutaneous injection of normal saline solution (5 mL/bwkg).

After 24 h of liver reperfusion, animals were sacrificed and exsanguinated via right ventricular puncture in anesthesia (Figure 13.).

3.2. Assessment of systemic hemodynamics and microcirculation

In order to carry out hemodynamic and microcirculation analyses, a 125-min period was measured after laparotomy and 20 min of recovery (5 min of pre-ischemic baseline, 60 min of ischemia, and 60 min of reperfusion).

3.2.1. Hemodynamics

Blood pressure was measured by an invasive blood pressure monitoring system (Kent Scientific Corporation, Torrington, CT, USA) and recorded with DasyLab software, version 9.00.02 (National Instruments Corporation, Austin, TX, USA) via cannulated right carotid artery.

Study I: For comparison between groups, the average of the reperfusion mean arterial pressure (MAP) was calculated for the last 20 min reperfusion plateau of the graph of each animal.

Study II: After revealing the characteristics of MAP in Study I., only the most critical time points of reperfusion were evaluated and compared in Study II. (Reperfusion 0 min, 30 min, 60 min).

3.2.2. Microcirculation

Microcirculation was measured using a laser Doppler monitor and a surface probe (DRT4 device with DP1T surface probe; Moor Instruments Ltd, London, UK). The device measures reflection of emitted laser light based on the Doppler effect.

Laser Doppler flowmeter (LDF) probe was placed on a fixed location on the liver's left lateral lobe and held in place. In Study I. a second surface probe was placed on the left femoral biceps muscle to assess alterations in lower limb microcirculation during liver IR and perconditioning.

The LDF monitor registered tissue microcirculation in Arbitrary Units. Due to considerable differences between individuals, certain correction was necessary for the sake of comparability between groups. Details of mathematical transformation, required for correct interpretation of circulation data, were described previously by our team [60].

Integral of the reperfusion graph segments (reperfusion area: RA) and maximal plateau (MP) of the reperfusion section were introduced to characterise microcirculation.

3.3. Light microscopy and automated image analysis

3.3.1. Histological analysis – Study I.

Histological samples were harvested from the left lateral lobe of the liver from identical anatomical sites. The excised liver was fixed in 4% neutral buffered formalin and embedded in paraffin. Slides, 3 μm thick, were stained with hematoxylin and eosin. The examining pathologist was not informed of the applied treatment or grouping. Slides were subjected to semiquantitative histological analysis as described by Suzuki et al. [154]. Sinusoidal congestion, hepatocyte necrosis, and ballooning degeneration were individually graded from 0-4. To simplify this complex scoring, a total score, the sum of the aforementioned individual parameters with a maximum of 12 points/animal, was introduced by other authors [155]. This total score was used when presenting our results. Ten random fields were evaluated per section.

3.3.2. Histological analysis – Study II.

Following the same sample preparation procedure as described above, slides were scanned using Panoramic 250 Flash whole-slide scanner system (3DHISTECH Ltd, Budapest, Hungary) in order to obtain high quality images. Two whole slide images were analyzed from each animal. Necrosis quantification was performed with an experimental automated image analysis software provided by our collaboration partner (Fraunhofer Mevis Institute, Bremen, Germany) [156].
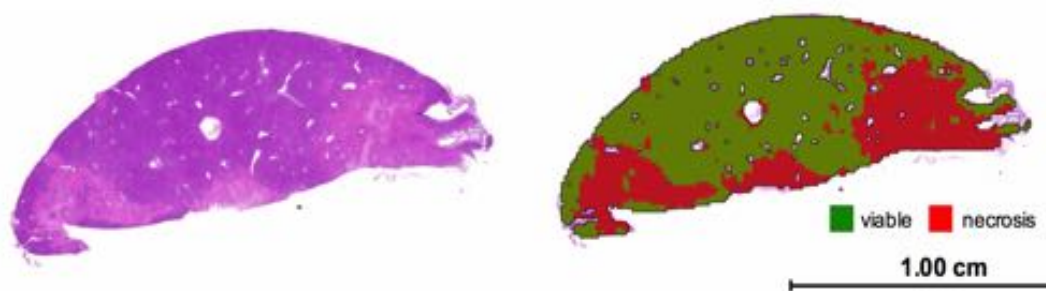
Figure 16. Automated necrosis quantification.
Necrosis was quantified via examining whole slide images, using a novel image analysis software. After a training period the software can automatically differentiate between viable and necrotic liver tissue.

The aforementioned software is an experimental product of the Fraunhofer Mevis Institute. Due to its special algorithm it can be "trained" to recognise special histological alterations and to differentiate necrosis and other pathological signs from the healthy liver tissue in histological whole slide images. Training of the algorithm was performed using healthy and extensively IR damaged negative and positive control slides. Following the software could properly distinguish between healthy and necrotic regions (proportion of false recognized tiles was under 5%), slides of the present study were analyzed using automatic analysis function. With this function huge amount of histological data can be analyzed within a few minutes, depending on the computer performance (Figure 16.).

Results of quantification were revised, and further pathologic alterations were revealed in a blinded fashion by two independent investigators, including a senior pathologist. Area of liver necrosis is given in percentage (necrosis%=necrotic area/[necrotic area+viable area] * 100%).

3.4. Biochemical examination

At the end of experiments, blood samples were collected via right ventricular puncture and then centrifuged (3000 rpm for 2x10 min, at room temperature). Samples were snap frozen in liquid nitrogen and stored at -80°C until analysis. Plasma aspartate aminotransferase (AST) alanine aminotransferase (ALT) and total bilirubin (tBi) levels were analyzed. Measurements were performed using automated clinical chemistry analyzer (Beckman Coulter AU480/2011; Beckman Coulter Inc, Brea, CA, USA).

3.5. Redox-state measurements

Residual liver tissue of left lateral and median lobes were cut into pieces and washed five times with 4°C saline solution to reduce blood content, then homogenized at 4°C using Potter-Elvehjem homogenizer. After the procedure, samples were immediately snap-frozen in liquid nitrogen and then stored at -80°C for assessment. Protein content of each sample was standardized (10 mg/mL) according to the method of Lowry et al. [157]. All reagents used for redox-state measurements were purchased from Sigma-Aldrich Inc (St. Louis, MO, USA) and Reanal Chemical Corporation (Budapest, Hungary).

3.5.1. Measurement of tissue free radicals and antioxidant capacity

A sensitive chemiluminescence assay was adopted to measure tissue reactive oxygen species content. The analysis was performed according to the method of Blázovics et al. [158]. Measurements were carried out using Lumat LB 9051 luminometer (Berthold Technologies GmbH, Bad Wildbad, Germany). Light intensity, which is directly proportional to the concentration of free-radical agents in the sample, was given in relative light unit percentage compared to the background.

For analysis of antioxidant capacity three further spectrophotometric measurement protocols were implemented using a Hitachi U-2000 instrument (Hitachi High-Technologies Corporation, Tokyo, Japan). The method of Oyaizu was used to determine global reducing power of samples [159] expressed in ascorbic acid equivalent (mgAA/mL). Hydrogen (H-) donating ability reflects the non-protein-related antioxidant capacity of samples, which was measured on 517 nm in the presence of 1,1-

diphenyl-2-picryl-hydrasyl radical, according to the method of Blois [160] modified by Blázovics et al. [158]. Results are given in inhibition percentage. Thiol (SH-) groups were measured via the approach of Sedlak and Lindsay [161]. Results show the protein-bound reducing power of the samples in micromoles per liter.

3.6. Serum TNF-α levels

Serum tumor necrosis factor alpha (TNF-a) levels were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits according to manufacturer's guidelines (Quantikine Rat TNF-a Immunoassay kit; R&D Systems Inc, Mineapolis, MN, USA). All samples were tested in duplicate. Concentrations were calculated from a standard curve and expressed in picogram per milliliter (pg/mL). Threshold for detection was 5 pg/mL.

3.7. Statistical analysis

Values were expressed as mean ± standard deviation, with "n" representing the numbers of animals or samples studied. Statistical analysis was performed with one-way analysis of variance (ANOVA) and Scheffe's post-hoc correction. Normality and homoscedasticity were tested. For analysis of histological scores Kruskal-Wallis H test was applied. Differences were considered significant when $p < 0.05$. Calculations were performed using IBM SPSS Statistics 20 Software (IBM Corporation, Armonk, NY, USA). Results were plotted using GraphPad Prism 6 Software (GraphPad Software Inc., San Diego, CA, USA).

4. RESULTS

4.1. Study I. – Effects of RIPER on IR injury of the liver

4.1.1. Assessment of systemic hemodynamics and microcirculation

4.1.1.1. Hemodynamics

There was no significant disparity between the pre-ischemic baseline MAP of the animals (Figure 17.). During ischemia the brief aortic cross-clamping periods (perconditioning) are visible as major fluctuation in MAP curve of the IR-RIPER group. Notable MAP reduction was observed in both of the IR-injured groups at the onset of reperfusion. In regard to the late reperfusion, where the mean arterial pressure reached a plateau phase (last 20 minutes of the 60 minute long reperfusion period), a significant difference was detected between the Sham and IR-RIPER groups compared to the IR group (Sham vs. IR: 80.6 ± 1.9 mmHg vs. 67.8 ± 7.6 mmHg, $p=0.026$; IR-RIPER vs. IR: 80.9 ± 6.2 mmHg vs. 67.8 ± 7.6 mmHg, $p=0.012$). There were no significant differences between the Sham and IR-RIPER groups ($p>0.05$).
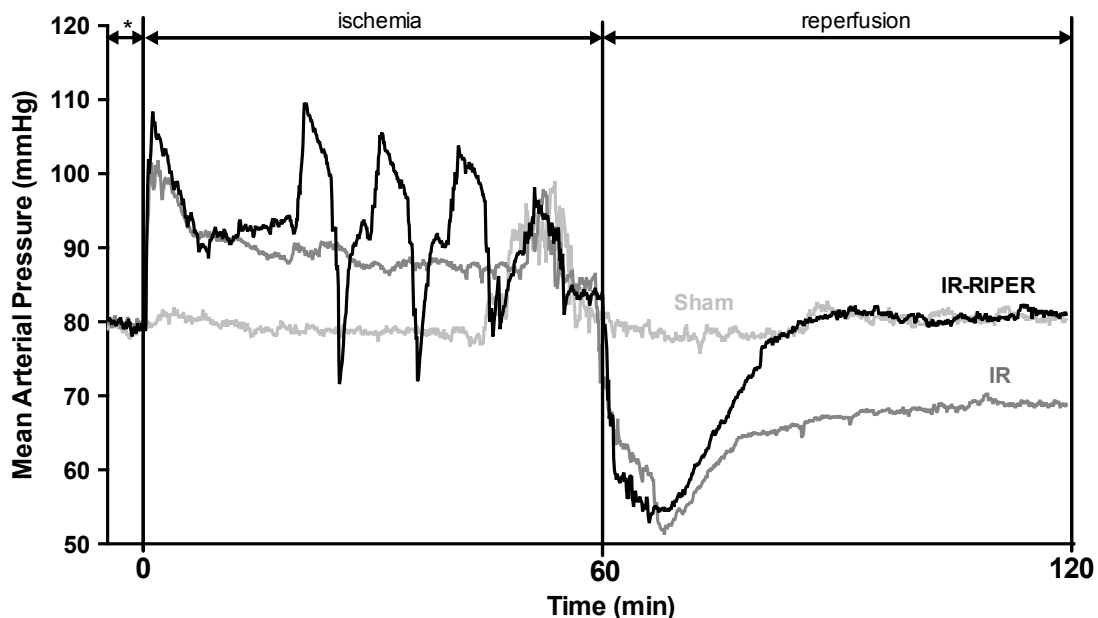
Figure 17. Mean arterial pressure values during the experiment.
Elevated MAP values were detected in the IR injured groups (IR, IR-RIPER) during the ischemic period. Fluctuation in the MAP values of the IR-RIPER group during liver ischemia is associated with the brief aortic exclusions and reperfusions. Prominent hemodynamic impairment was observed in both IR injured groups in the early minutes of reperfusion. During the last two-thirds of the observation period, graph of IR-RIPER group reached the values of the Sham operated group. *:baseline (n=8/group)

4.1.1.2. Microcirculation

Liver microcirculation of the Sham group stayed around the pre-ischemic baseline during the experiment. There were considerable differences between the two IR-injured groups regarding reperfusion liver microcirculation (Figure 18/A.). Significant improvement of reperfusion area and maximal plateau was observed in the IR-RIPER group compared with the IR group (RA: p=0.005; MP: p=0.0002) (Table 1.).

Figure 18/B. shows microcirculation of the femoral biceps muscle. In the IR-RIPER group the applied perconditioning cycles are represented as peaks and valleys during the ischemic phase. Regarding the reperfusion microcirculation of the lower limb, the MP value of the IR-RIPER group was significantly higher compared to the IR group (MP: p=0.038) (Table 1.).

TABLE 1.

Microcirculation data		Sham	IR	IR+RIPER
Liver	**RA (%)**	97.3±2.6	33.4±6.4 a	62.8±13.2 ab
	MP (%)	98.0±2.5	41.6±11.2 a	71.5±4.5 cd
Lower limb	**RA (%)**	98.8±2.9	60.9±21.6 c	79.5±29.1 c
	MP (%)	98.3±3.7	55.6±25.1 c	92.2±26.9 b

[a]p<0.001 vs. Sham; [b]p<0.01 vs. IR; [c]p<0.01 vs. Sham; [d]p<0.001 vs. IR (mean±standard deviation, n=8/group)

A

B
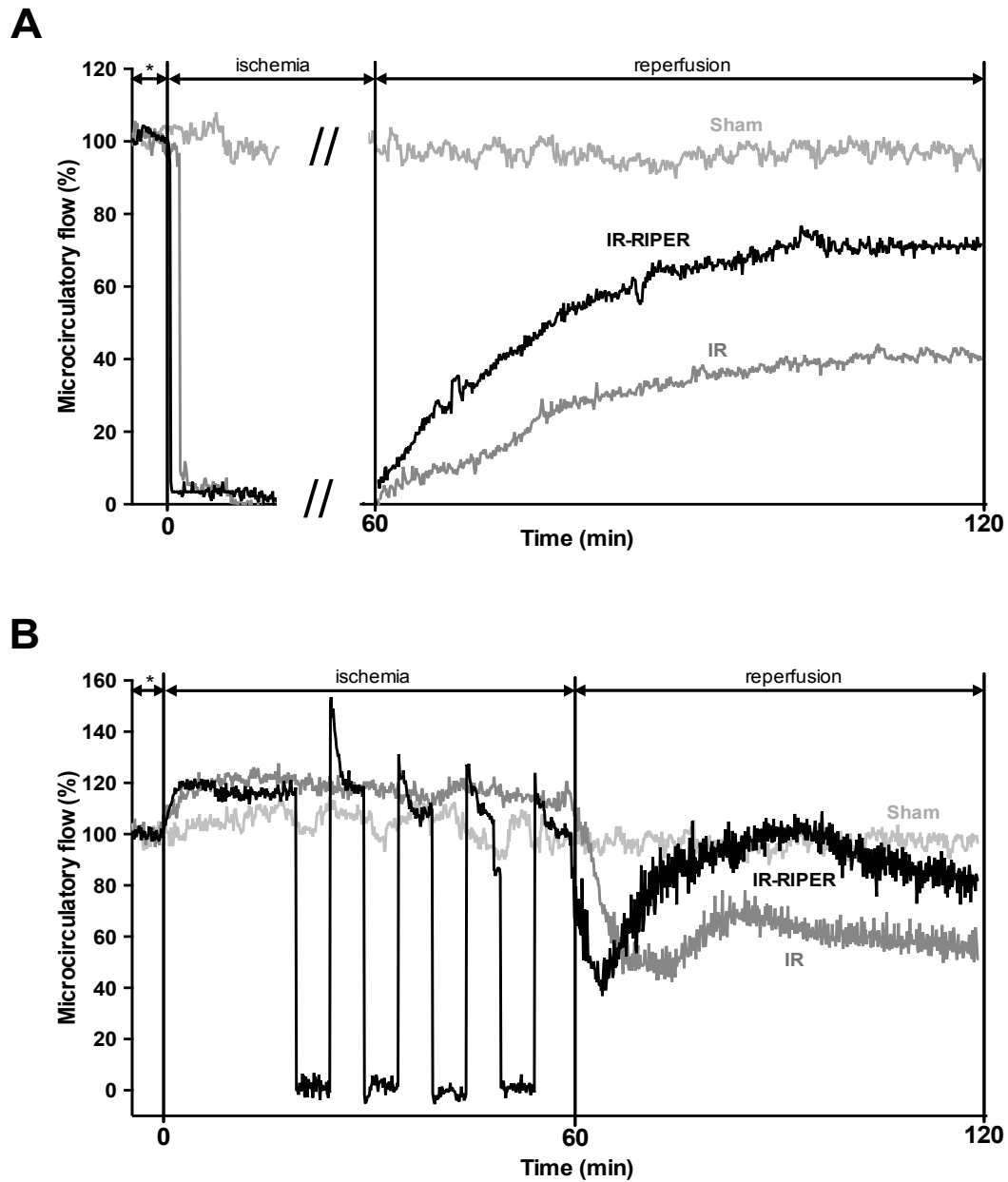
Figure 18. Liver and lower limb microcirculation.
In the IR-RIPER group, the reperfusion microcirculation of the liver (A.) and the lower limb (B.) showed prominent improvement when compared with the IR group. Microcirculation of the Sham group was not changed significantly during the experiment. *:baseline (n=8/group)

4.1.2. Histological analysis

No significant alterations could be observed in the Sham groups. After one hour of reperfusion an intense sinusoidal congestion was visible in the IR_{1hour} group; in contrast, an expressly favorable histological state was detected in the $IR+RIPER_{1hour}$ group (Figure 20.). After 6 hours of reperfusion there were several confluent centrilobular necrotic zones on the sections of the IR_{6hours} group, but in the $IR+RIPER_{6hours}$ group only a few small necrotic lobules were visible (Figure 20.). On the slides of animals, subjected to 24 hours of reperfusion, pronounced necrotic zones were observed in the $IR_{24hours}$ group. Similar pathological alterations were detected in the $IR+RIPER_{24hours}$ group; nevertheless, the overall damage was significantly lower in this treated group than in the $IR_{24hours}$ group (Figure 20.). Accordingly, the total score was considerably lower after each examined reperfusion time point in the IR-RIPER group compared with the IR group (Figure 19.).
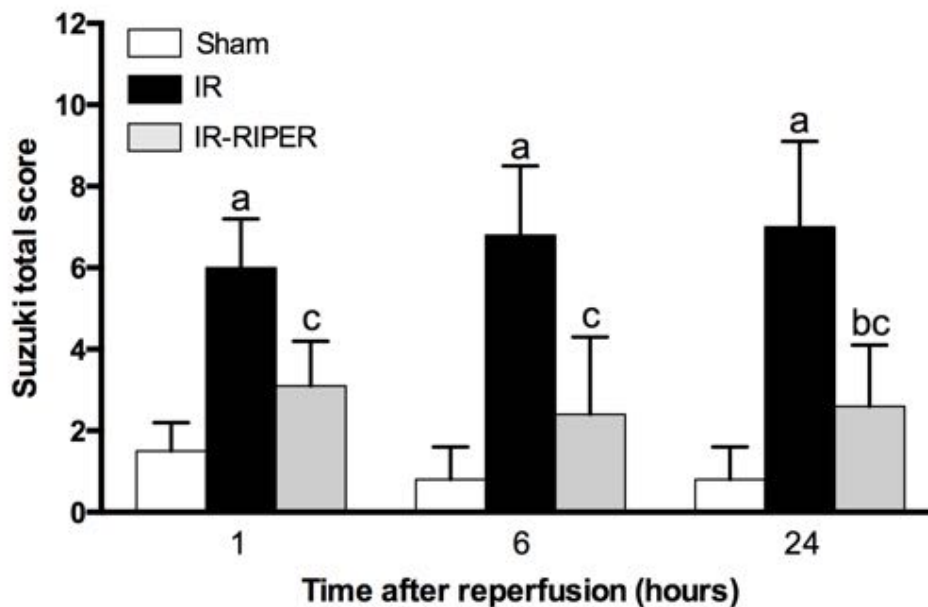
Figure 19. Suzuki total score.
[a]p<0.001 vs. Sham; [b]p<0.01 vs. Sham; [c]p<0.01 vs. IR
(mean±standard deviation, n=8/group)
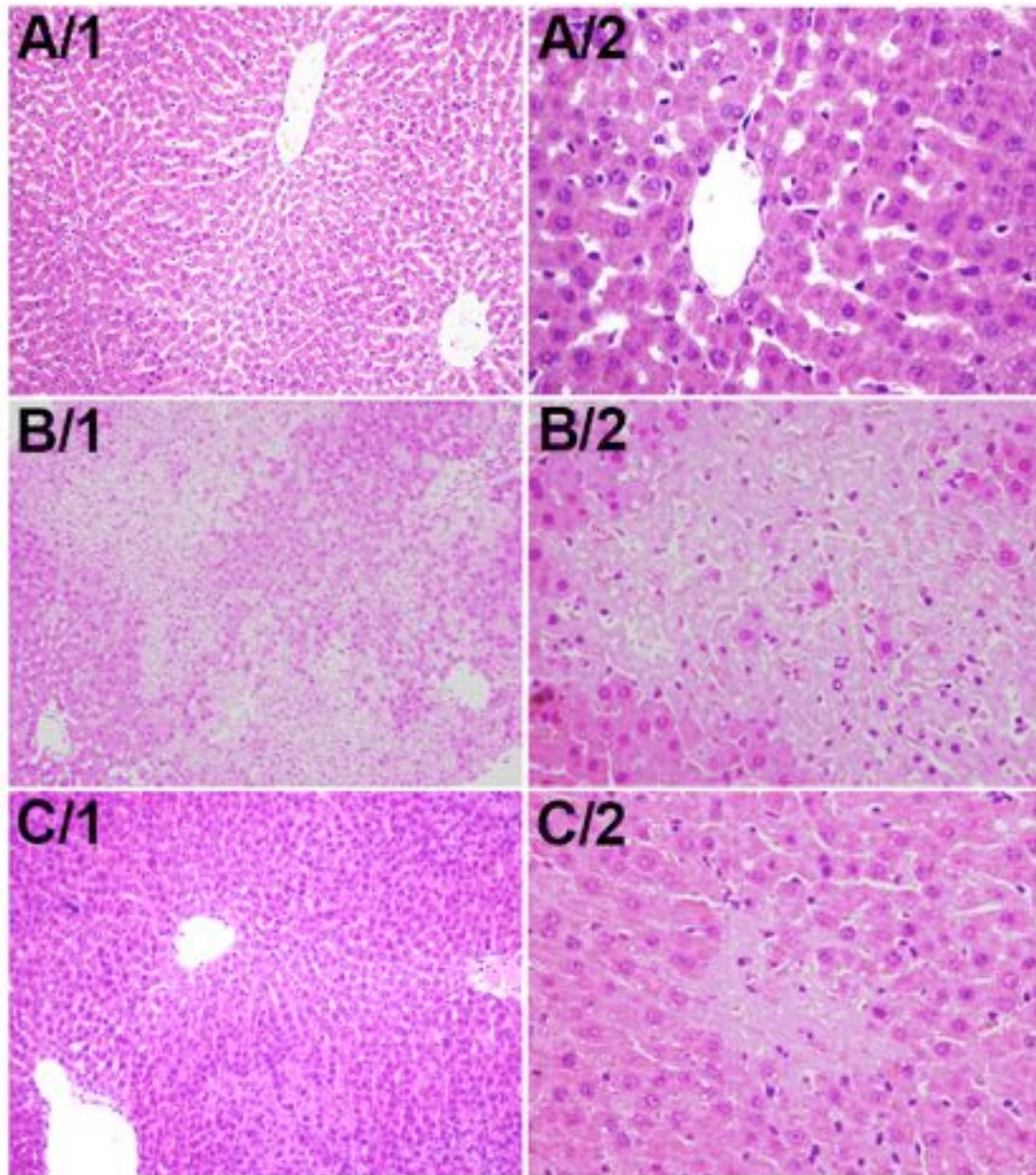
Figure 20. Representative photos, demonstrating the histological alterations in the liver after 60 minutes of ischemia and 6 hours of reperfusion.

A. On the slides of the $Sham_{6hours}$ animals, no remarkable histological alterations could be observed. B. Several confluent centrilobular necrotic zones could be seen on the slides of the IR_{6hours} group. C. In the $IR+RIPER_{6hours}$ group only milder injury, few small necrotic lobules were visible.

Original magnification A/1, B/1, C/1: 200x; A/2, B/2, C/2: 400x.

4.1.3. Biochemical examination

As shown in Figure 21., AST and ALT levels were significantly lower in the IR-RIPER group compared with the IR group after each examined reperfusion time point indicating less hepatocellular damage in the perconditioned group.
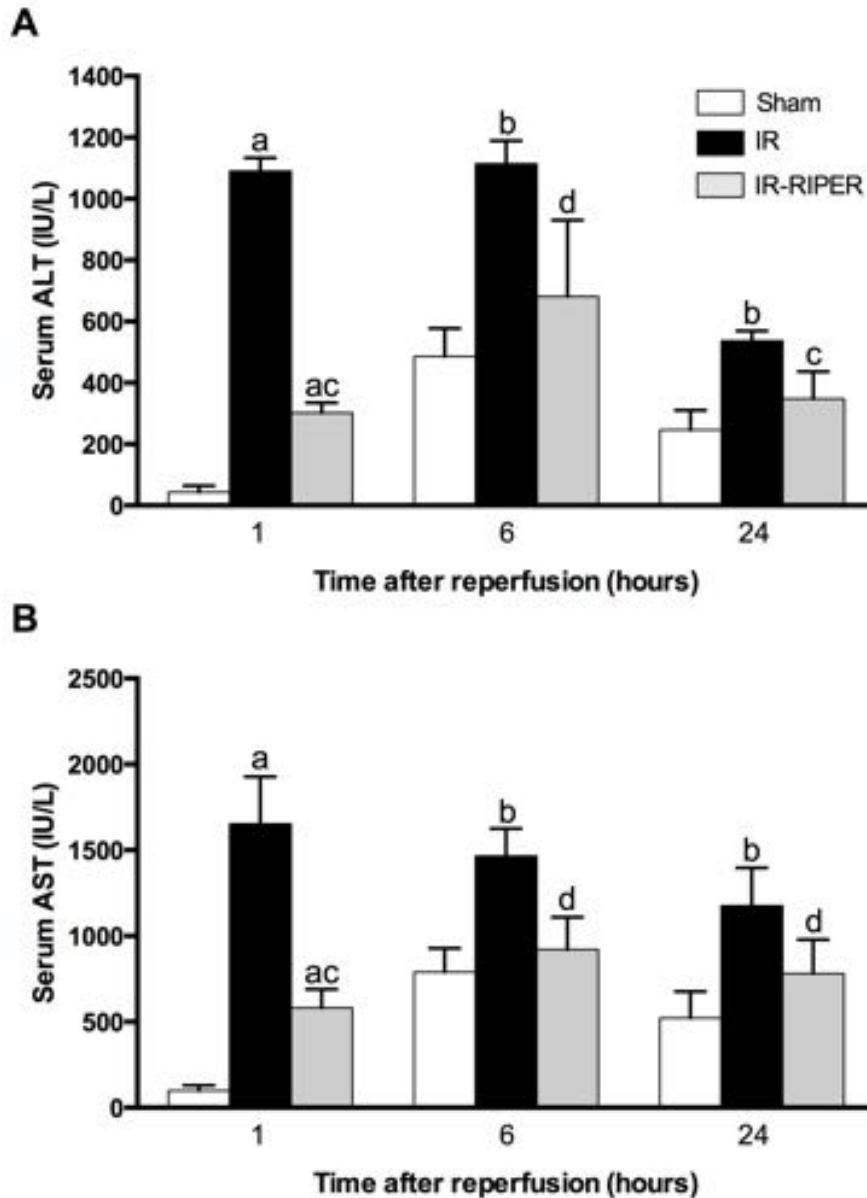
Figure 21. Serum transaminase levels.
A. Serum levels of alanine aminotransferase. B. Serum levels of aspartate aminotransferase.
[a]$p<0.001$ vs. Sham; [b]$p<0.01$ vs. Sham; [c]$p<0.01$ vs. IR; [d]$p<0.05$ vs. IR;
(mean±standard deviation, n=8/group)

4.1.4. Redox-state measurements

The chemiluminescent intensity of the liver homogenate was significantly decreased in the IR-RIPER group compared with the IR group after 6 and 24 hours, but not after 1 hour of reperfusion (IR-RIPER$_{6hours}$ vs. IR$_{6hours}$, p=0.046; IR-RIPER$_{24hours}$ vs. IR$_{24hours,}$ p=0.049) (Table 2.).

Reducing power, referring to liver tissue global antioxidant capacity, was preserved in the IR-RIPER$_{24hours}$ group after 24 hours of reperfusion compared with the IR$_{24hours}$ group (IR-RIPER$_{24hours}$ vs. IR$_{24hours}$, p=0.025). Significantly higher levels of protein-related (SH-groups) and protein-independent (H-donating ability) antioxidants were detected in the IR-RIPER group compared with the IR group with exception of the 1h reperfusion group measurements (Table 2.).

TABLE 2.

Redox homeostasis data				
		Sham	**IR**	**IR+RIPER**
ROS (RLU%)	**1 h**	0.05±0.02	0.14±0.02 a	0.16±0.05 a
	6 h	123.8±16.6	162.3±10.9 b	133.5±11.0 c
	24 h	137.3±23.6	182.0±14.0 a	141.7±18.7 c
Reducing Power (µgAA/mL)	**1 h**	144.9±39.0	106.4±14.2 a	116.6±17.5
	6 h	102.9±5.4	76.4±8.0 a	86.4±4.6 a
	24 h	90.6±6.8	55.2±11.8 b	76.1±11.9 c
SH-groups (µmol/L)	**1 h**	815.9±138.2	493.4±100.4 b	622.7±40.4 a
	6 h	456.1±97.7	249.7±82.6 a	455.1±101.4 c
	24 h	523.1±25.4	349.2±54.9 a	499.9±112.4 c
H-donating ability (inhibition%)	**1 h**	84.6±8.8	68.8±10.3	84.1±11.8
	6 h	43.6±3.0	29.5±4.8 a	41.8±6.4 c
	24 h	46.4±19.7	16.4±7.3 a	37.9±5.1 c

[a]p<0.05 vs. Sham; [b]p<0.01 vs. Sham; [c]p<0.05 vs. IR; (mean±standard deviation, n=8/group)

4.1.5. Serum TNF-alpha levels

Sandwich ELISA, performed for TNF-alpha detection in order to assess the IR induced cytokine response, showed prominent differences. Significant reduction was observed in serum TNF-alpha levels in the IR-RIPER$_{1hour}$ group compared with the IR$_{1hour}$ group after 1 hour of liver reperfusion ($p<0.001$); and there was also significant disparity between the two IR-injured groups (IR-RIPER$_{6hours}$ vs. IR$_{6hours}$) after 6 hours of reperfusion ($p<0.05$) (Figure 22.). At 24 hours of reperfusion we found dramatically reduced TNF-alpha levels in all experimental groups.
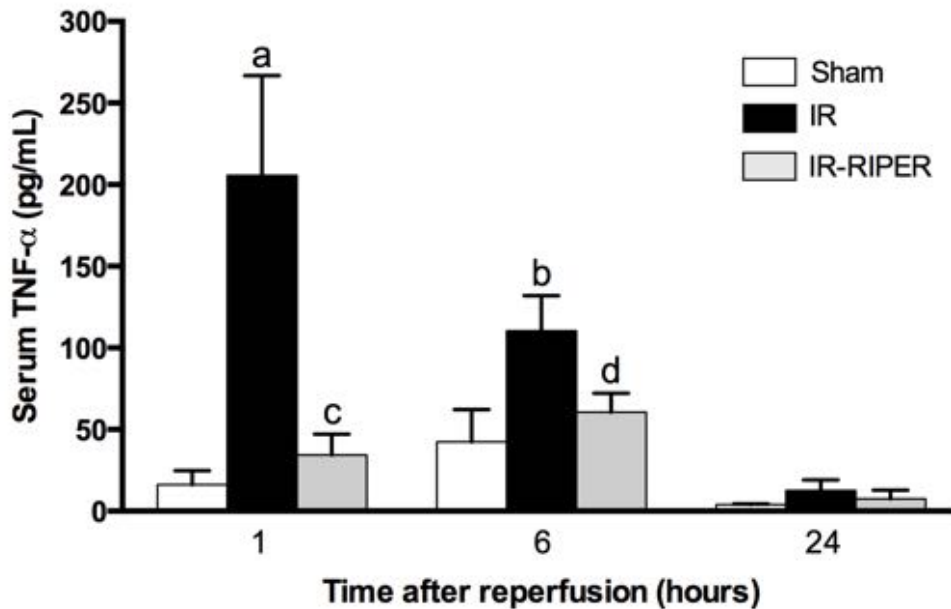
Figure 22. TNF-α levels.
[a]$p<0.001$ vs. Sham; [b]$p<0.01$ vs. Sham; [c]$p<0.01$ vs. IR; [d]$p<0.05$ vs. IR;
(mean±standard deviation, n=8/group)

4.2. Study II. - Neural elements behind RIPER hepatoprotection

4.2.1. Assessment of systemic hemodynamics and microcirculation

4.2.1.1. Hemodynamics

Pre-ischemic baseline MAP did not differ significantly between the experimental groups (Figure 23.). During the ischemic period, no significant differences were detected between groups, values of all animals showed slight fluctuation between 76-86 mmHg. After reperfusion, a severe drop (~35-40 mmHg) in blood pressure was observed in each IR-injured experimental group (IR, IR-N, IR-RIPER, IR-RIPER-N vs. Sham and Sham-N, $p<0.001$) without any conspicuous differences between groups. In the course of reperfusion, MAP was improved in the four IR-injured groups but did not reach the values of the Sham or Sham-N animals during the 60 minutes observation period. Thirty minutes after the start of reperfusion, MAP of the perconditioned animals (IR-RIPER group) was significantly higher ($p<0.05$) compared with the IR and IR-N groups.
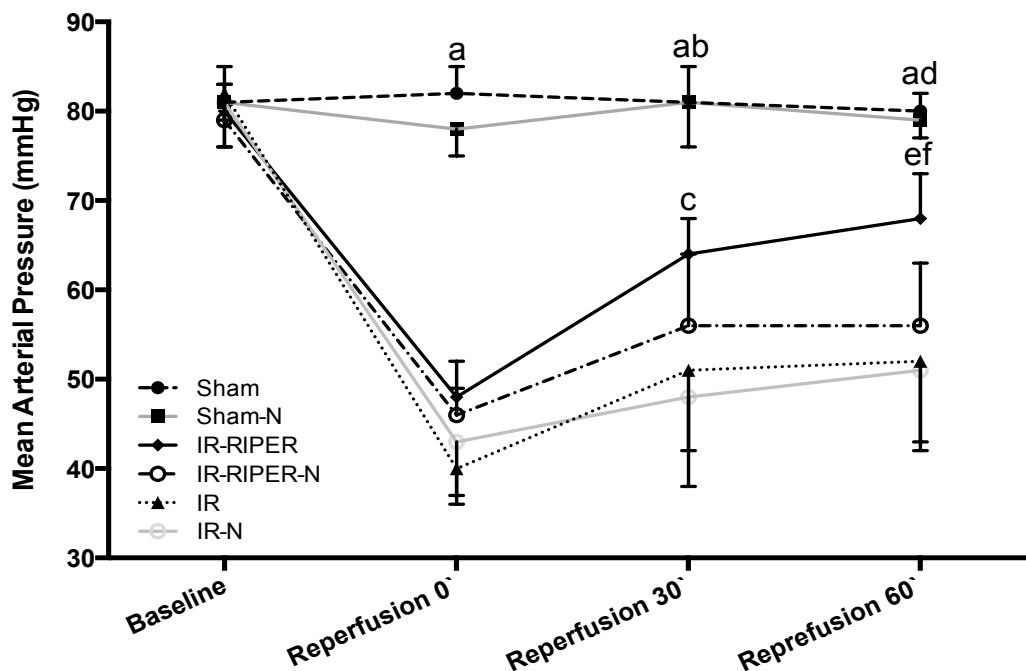
Figure 23. Mean arterial pressure values during Study II.
[a]p<0.001 Sham, Sham-N vs. IR, IR-N, IR-RIPER, IR-RIPER-N; [b]p<0.01 Sham, Sham-N vs. IR-RIPER ; [c]p<0.05 IR-RIPER vs. IR, IR-N; [d]p<0.05 Sham, Sham-N vs. IR-RIPER; [e]p<0.01 IR-RIPER vs. IR, IR-N; [f]p<0.05 IR-RIPER vs. IR-RIPER-N; (mean±standard deviation, n=7/group)

After 60 minutes of reperfusion, MAP values of IR-RIPER animals showed significant elevation in relation to the other three IR-injured groups (IR-RIPER vs. IR-RIPER-N, p<0.05; IR-RIPER vs. IR and IR-N, p<0.01). During the observation period, fluctuation in MAP values of the two sham-operated groups (Sham, Sham-N) showed no notable differences.

4.2.1.2. Microcirculation

During the experiments no significant differences were found between the microcirculation of the sham-operated groups (Sham, Sham-N). Significantly reduced reperfusion microcirculation could be observed in all four IR-injured groups compared with the sham-operated groups (Figure 24.). Nevertheless, perconditioning treatment was able to significantly improve the reperfusion area (RA) and maximal plateau (MP) (IR-RIPER vs. IR, p=0.034, p=0.041, respectively) compared with the IR group (Table 3.). This improvement was completely abolished in the IR-RIPER-N group, namely the reperfusion microcirculation of these animals stayed around the values of the control groups. No significant differences were detected between IR and IR-N and IR-RIPER-N groups (Table 3.).
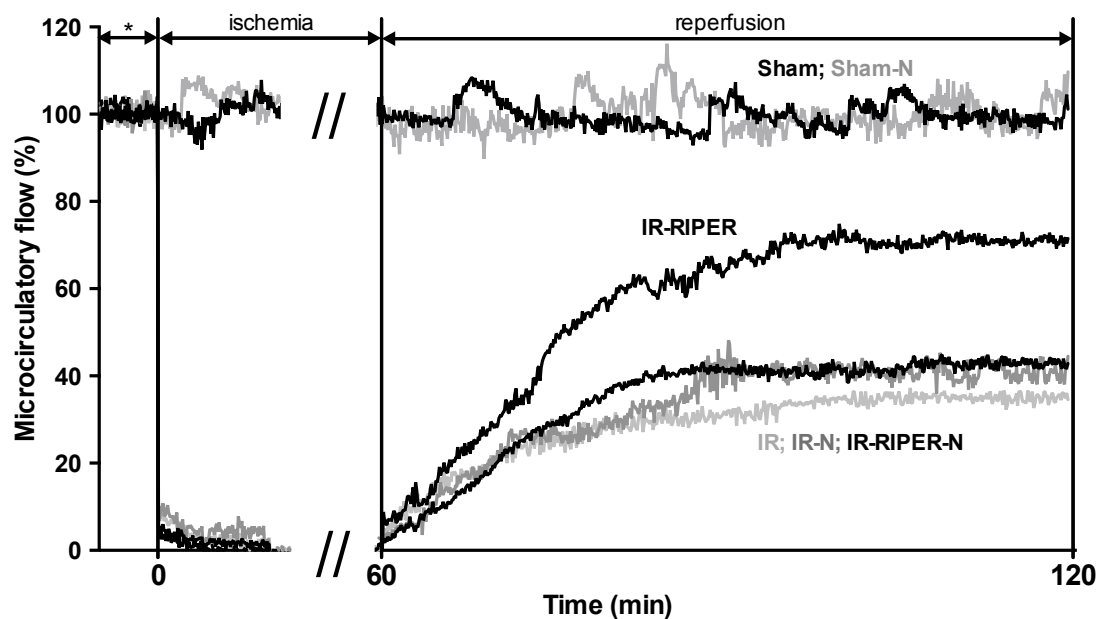
Figure 24. Liver microcirculation.
Liver microcirculation of the Sham and Sham-N groups did not differ significantly during the experiments. Reperfusion microcircualtion of the IR-RIPER group showed conspicuous improvement compared with the IR, IR-N and IR-RIPER-N groups. *:baseline (n=7/group)

TABLE 3.

Microcirculation data

	Sham	Sham-N	IR	IR-N	IR-RIPER	IR-RIPER-N
RA (%)	100.78±1.79	99.45±1.32	28.59±13.61 a	30.58±16.24 a	55.89±14.18 bc	32.62±12.99 a
MP (%)	97.98±4.75	98.83±3.43	34.95±15.44 a	40.01±15.27 a	71.11±16.52 bc	42.89±8.81 a

[a]p<0.001 vs. Sham, Sham-N; [b]p<0.01 vs. Sham, Sham-N; [c]p<0.05 vs. IR, IR-N, IR-RIPER-N; (mean±standard deviation, n=7/group)

4.2.2. Histological analysis

Notable pathological alterations were not observable on the slides of the sham-operated animals (Sham and Sham-N groups). Perconditioning reduced the extent of liver tissue necrosis compared with the IR and IR-N groups (p<0.01) according to the necrosis morphometry (Figure 25/D.).
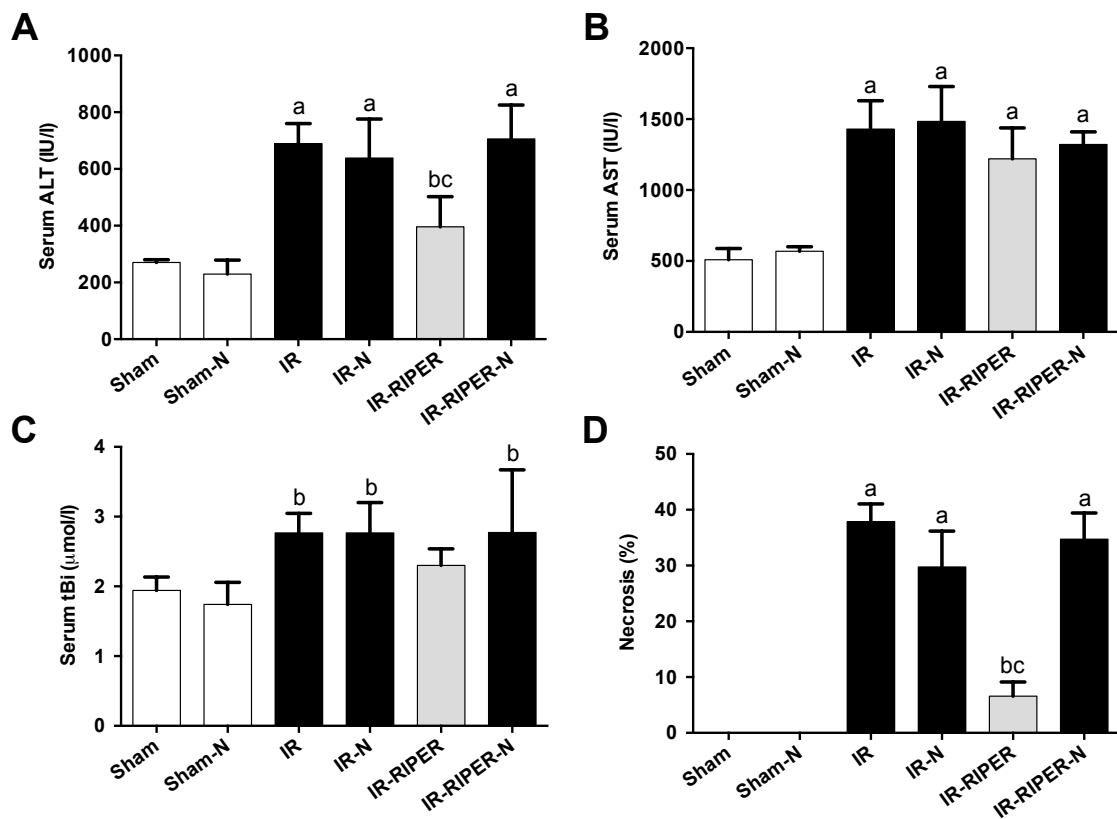
Figure 25. Results of biochemical measurements and necrosis quantification.
A. Serum levels of alanine aminotransferase. B. Serum levels of aspartate aminotransferase. C. Serum levels of total bilirubin.
D. Results of automated necrosis quantification expressed in % (necrosis%=necrotic area/[necrotic area+viable area] * 100%)
[a]p<0.01 vs. Sham, Sham-N; [b]p<0.05 vs. Sham, Sham-N; [c]p<0.05 vs. IR, IR-N, IR-RIPER-N;
(mean±standard deviation, n=6-7/group)

Nerve resection before perconditioning treatment (IR-RIPER-N group) resulted in a significantly higher damage than in the IR-RIPER group (IR-RIPER vs. IR-RIPER-N, $p<0.01$). No significant differences were observed between this group (IR-RIPER-N group) and the IR or IR-N groups. Next to the extended necrotic areas, considerable ballooning degeneration, sinusoidal congestion, and neutrophil cell infiltration were detected in the slides of the IR, IR-N, IR-RIPER-N animals. These alterations were manifested in the IR-RIPER group as well; however, no convincing differences were found concerning the presence of inflammatory cells between groups.

4.2.3. Biochemical examination

Serum transaminase levels were prominently elevated after 24 hours of reperfusion in the IR-injured groups. Regarding ALT, significantly increased levels ($p<0.01$) were detected in the IR, IR-N, and IR-RIPER-N groups compared with the Sham and Sham-N groups. In the RIPER group serum ALT elevation was significantly milder than in the other three IR-injured groups (IR-RIPER vs. IR, IR-N, IR-RIPER-N, $p<0.05$) (Figure 25/A.).

Characteristics of AST release were different. In case of aspartate aminotransferase, a significant elevation ($p<0.01$) was revealed in all four IR-injured groups compared with the Sham and Sham-N groups; however, the perconditioning treatment had no significant effect on AST levels (Figure 25/B.).

No severe alterations were observed concerning the tBi levels in any of the experimental groups following 60 min ischemia and 24 hours of reperfusion (Figure 25/C.). Significant elevation of tBi can be seen in the IR, IR-N, and IR-RIPER-N groups but not in the IR-RIPER group when compared with the sham-operated groups.

4.2.4. Redox-state measurements

Chemiluminescent intensity – thus tissue free-radical content – was significantly elevated after 24 hours observation period in all IR-injured groups when compared with the sham groups (IR, IR-N, IR-RIPER, IR-RIPER-N vs. Sham and Sham-N, $p<0.001$). Perconditioning treatment potently reduced the level of free-radicals in liver homogenate in relation to the three other IR-injured groups (IR-RIPER vs. IR, IR-N, IR-RIPER-N, $p<0.01$) (Table 4.).

A more complex pattern was observed regarding the different parameters of tissue antioxidant capacity. Global reducing power of the IR, IR-N, and IR-RIPER-N groups was significantly reduced in comparison with the values assessed in the samples of the sham-operated animals (IR, IR-N, IR-RIPER-N vs. Sham and Sham-N, p<0.01) (Table 4.). In the IR-RIPER group, significantly preserved global reducing power was observed when compared with the other three IR-injured groups (IR-RIPER vs. IR and IR-RIPER-N, p<0.01; IR-RIPER vs. IR-N, p<0.05). It should be noted, furthermore, that no significant differences were observed between the IR-RIPER-N as well as IR and IR-N groups in regards to the global redox-state parameters (luminometry, reducing power assessment) (Table 4.).

Considering protein-unbound antioxidants, a significant reduction (p<0.01) was detected in H-donating ability of the IR and IR-N groups in relation to the Sham and Sham-N values (Table 4.). The values of H-donating ability in the IR-RIPER group were significantly higher (p<0.05) than in the IR-N group, but not when compared with the IR group (IR-RIPER vs. IR, p=0.062). H-donating ability of IR-RIPER and IR-RIPER-N groups did not differ significantly, either from each other or from the values of the sham-operated groups. To some extent, a similar pattern was observed in the thiol-group measurements, namely nerve resection was not able to induce significant reduction in the values of thiol-groups (IR-RIPER vs. IR-RIPER-N, p=0.079); albeit, no significantly higher protein-related antioxidant capacity was detected in the IR-RIPER-N group either when compared with the IR and IR-N groups (Table 4.).

Significant differences were not detected in data of the four different redox-state parameters between the Sham and Sham-N, or the IR and IR-N groups.

TABLE 4.

Redox homeostasis data						
	Sham	**Sham-N**	**IR**	**IR-N**	**IR-RIPER**	**IR-RIPER-N**
ROS (RLU%)	6.4±10.5	6.9±11.7	229.2±16.1 a	202.3±20.3 a	131.8±20.8 ab	194.9±16.7 ac
Reducing power (µgAA/mL)	181.3±20.3	171.6±9.2	128.8±11.9 d	134.6±9.0 d	163.4±12.8 ef	131.3±13.6 d
SH-groups (µmol/L)	964.6±108.3	927.9±89.7	480.9±80.9 a	486.8±138.5 a	768.8±92.8 gh	594.3±74.9 a
H-donating ability (inhibiton%)	65.7±5.2	63.9±9.4	47.4±6.5 d	45.7±9.6 d	59.8±7.5 f	56.4±6.7

[a]p<0.001 vs. Sham, Sham-N; [b]p<0.01 vs. IR, IR-N, IR-RIPER-N; [c]p<0.05 vs. IR, [d]p<0.01 vs. Sham, Sham-N; [e]p<0.01 vs. IR, IR-RIPER-N; [f]p<0.05 vs. IR-N; [g]p<0.05 vs. Sham; [h]p<0.01 vs. IR, IR-N;
(mean±standard deviation, n=6-7/group)

5. DISCUSSION

Ischemic-reperfusion injury remains one of the major concerns in extended liver resections and liver transplantations. Sustained liver exclusion and consequential ischemia is characterized by tissue hypoxia and reduced tissue adenosine triphosphate levels, which cannot maintain active cellular functions resulting in cell death [12,19]. The "healing" reperfusion, the restoration of blood flow, is inevitable to keep organ function, nevertheless, it can initiate paradoxically a cascade, which ultimately can cause further cellular damage [12]. IR injury can lead to severe damage and dysfunction of the remnant liver after liver resections [162]. Following liver transplantation IR induced hepatocellular injury is a frequent reason for primary non-function or late dysfunction of the implanted liver graft and might lead to a higher incidence of acute and chronic rejection as well [163]. These issues are even more emphasized when we have to deal with a critically low future remnant liver volume following resection or when using liver grafts from extended criteria donors (e.g. steatotic grafts, elderly donors, etc.) for liver transplantation [164]. Therefore, studying and understanding mechanisms and characteristics of liver ischemia and developing novel strategies are essential in order to increase ischemic tolerance of the liver. Basic and translational research might help us to reduce complication rates in liver surgery and to extend donor pool for liver transplantation.

Since Jennings et al. introduced the concept of reperfusion injury in 1960, several surgical and non-surgical protective strategies have been developed to limit the degree of injury, some more successful than others [165]. After the pioneering work of Murry and Vinten-Johansen, surgical conditioning approaches (local ischemic pre- and postconditioning) became widely used in several settings [6,132]. The concept of remote ischemic conditioning was developed later on by Karin Przyklenk [8]. She postulated that ischemic conditioning might be effective if brief ischemic-reperfusion attacks are applied in a remote vascular bed, distantly from the target organ.

Remote ischemic perconditioning, first proposed by Schmidt et al. in 2007, has several beneficial features from a practical point of view [86]. The essence of this strategy is that brief, remote ischemic attacks are applied after induction of target organ ischemia but before onset of reperfusion.

Considering that RIPER was already tried in different IR injury scenarios of the cardiovascular system, we aimed to use this approach for the first time in a rat model of liver IR injury. Based on our previous experience, the widely used rat model of 60 min normothermic, 70% partial liver ischemia was adopted as basic experimental model [19,60].

Subsequently we had to develop a feasible and effective RIPER protocol for our experimental setting. At the planning phase of our first study 16 articles were available with the use of RIPER in different experimental models and clinical settings [9]. Considering that no studies were published before which would have demonstrated the effects of RIPER in parenchymal organs we had to rely on previous findings of cardiovascular and neurological studies [9].

However, remote conditioning can be applied on various organs to achieve target organ protection [81], among the 16 studies published in the topic of RIPER (at the time point of planning Study I.) 94% of the authors (15/16) used skeletal muscle ischemia-reperfusion attacks (limbs) as conditioning treatment [9]. Conditioning on the lower limbs can be achieved non-invasively (tourniquet, inflatable cuffs, etc.) and invasively (aortic cross clamping, femoral artery clamping, etc.) [9]. In our model we had to perform a median laparotomy to induce ischemic-reperfusion injury of the liver; therefore, we concluded that the easiest and the most feasible way to induce perconditioning of the skeletal muscle in this setting would be the use of an infrarenal aortic cross-clamping.

For determining the optimal conditioning protocol (number of cycles and duration of ischemia-reperfusion) we adopted the previous findings of other authors. An elegant study of Xin et al. investigated the infarct size limiting effects of different RIPER protocols [87]. They reported that fewer (1-2x5 min IR) repetitions or too short cycles (4x1-3 min IR) are not able to exert a significant protection in a rat model of myocardial infarction. The most effective RIPER protocol was the application of 5 minute long IR episodes in 3-4 cycles according to their findings. Potent IR injury limiting effects of this protocol (4x5 min limb IR) were confirmed by others [9,86,89,95,127,166-169]. Further concerns, regarding the used model and the remote conditioning treatment, are described in detail in the Introduction of the present doctoral thesis.

Microcirculatory damage is a pivotal mechanism in liver IR injury [170,171]. Microvascular injury plays an emphasized role in prolongation of the ischemic period and thus in aggravation of cellular injury, due to the no-reflow phenomenon. Main components of microcirculation dysfunction are the deterioration of active ion transport mechanisms, secondary to ischemia-induced ATP deficiency, and the consequential endothel swelling, cellular edema [171]. Results of nitrogen oxide/endothelin imbalance and sinusoidal narrowing, during reperfusion, are worsened by the accumulation of activated neutrophil granulocytes and by reduced red blood cell velocity [170]. In our study we observed a significant improvement in liver reperfusion microcirculation in the IR+RIPER group compared with the IR group. These results are in accordance with previous findings of others who investigated different aspects of microcirculation in order to demonstrate the positive effects of remote perconditioning on target organ circulation [88,89] and on endothelial function [133].

Loukogeorgakis et al. examined flow-mediated dilation in a human study, which is a recognized parameter of endothelial function [133]. These authors proved that remote conditioning treatment, applied during sustained limb ischemia, was able to decrease the extent of endothelial dysfunction. Zhao et al. demonstrated the efficient infarct-size reducing effect of remote perconditioning in a porcine model of myocardial infarction [89]. They also evinced the favorable effect of the mentioned technique on IR induced myocardial no-reflow phenomenon. Noteworthy is the common point between the two aforementioned different studies, namely, both authors have reported the crucial role of K^+_{ATP} channels in the mechanism of remote conditioning induced protection.

Besides target organ (liver) microcirculation, we attempted to follow capillary circulation of the "conditioned organ" in Study I. During the early phase of liver reperfusion, a prominent drop was observed in microcirculation of the left femoral biceps muscle in both IR-injured groups. Nonetheless, skeletal muscle circulation of the RIPER group improved with a greater slope, and the mean microcirculation curve of this group reached higher values compared with the IR group during the observation period.

As previously discussed (see Introduction), liver ischemia has not only local but also global consequences with definitive dysfunction and injury of several organs [29]. The detected drop in lower limb microcirculation in our study, during the early

reperfusion in both IR-injured groups, can probably be attributed to a circulation redistribution, induced by reperfusion of the liver and resulting in reduced peripherial blood volume. There are multiple explanations or hypotheses for the conspicuous characteristic differences between the RIPER and IR groups regarding lower limb microcirculation during the latter phase of the observation period. First, the application of remote conditioning mitigated liver injury in our model, which might result in a reduced liver origin metabolite content in the systemic circulation (cytokines, free-radicals, etc.); therefore, the improved skeletal muscle microcirculation might be, at one hand, interpreted as a result of a reduced systemic response. On the other hand, taking the known effects of different, previously discussed (see Introduction) conditioning techniques into account, we have to consider the possibility of a local conditioning effect behind the aforementioned microcirculatory differences. More exactly, we can assume that remote ischemic <u>perconditioning</u>, applied on the infrarenal aorta, might exert a <u>local</u> <u>pre</u>conditioning effect on the skeletal muscle, protecting against the detrimental systemic effects of liver IR injury.

In Study I. we also attempted to find an answer for the question of whether or not this 40 minute long lower limb ischemia-reperfusion "burden" is able to affect the systemic hemodynamics of the animals. Interestingly, we detected a significantly improved reperfusion mean arterial pressure (MAP) in the perconditioned animals as compared with the IR group. A plausible explanation for this phenomenon could be that in the treated group a milder liver injury resulted in decreased mediator release into the systemic circulation, which might even have cardiodepression or endothelial-dysfunction inducing activity [29]. Such deteriorated systemic hemodynamics [172], myocardial function impairment, and elevation of myocardial enzyme markers following liver IR injury were reported by others [29,173,174].

In 2011, Wei et al. proposed that perconditioning has significant effect on inflammatory reactions, demonstrated by reduction of polymorphonuclear (PMN) leukocyte infiltration, and monocyte chemoattractant protein-1 (MCP-1) expression in their rodent model of myocardial infarction [127]. Nevertheless, the mentioned team could not prove the favorable effects of perconditioning on expression of TNF-α. Sedaghat et al. investigated the effects of RIPER on remote hepatic injury as a

consequence of 45 min renal ischemia in rats [175]. They could not show any significant differences regarding serum TNF-α expression between groups.

In our study we evaluated the changes in serum levels of TNF-α after 1 hour of liver ischemia and 1, 6, 24 hours of reperfusion. In the present model perconditioning was able to significantly reduce serum TNF-α levels, compared with the IR$_{1hour}$ group, after one hour of liver reperfusion. After this observation period there was no significant disparity between the Sham$_{1hour}$ and IR+RIPER$_{1hour}$ groups. Likewise, significantly attenuated TNF-α levels were observed after 6 hours of reperfusion; nevertheless, in the 24 hours reperfusion group serum cytokine levels were remarkably reduced without any noticeable differences between the experimental groups. Our results are correlating closely with the very recent findings of Wang et al. They demonstrated that remote ischemic perconditioning exerts a positive effect on the serum profile of pro- and anti-inflammatory cytokines (TNF-α, IL-6, IL-1, IL-10, ICAM-1, etc.) in a rat model of myocardial ischemia-reperfusion injury [120].

Besides pro-inflammatory cytokine expression, reperfusion associated sinusoidal endothelial injury, imbalance between vasoconstrictive/vasodilatative effects as well as excessive oxidative stress and consequential free-radical production are also represented as key factors in development of microcirculatory impairment and no-reflow phenomenon. Taking the aforementioned facts into account, in our study we aimed to identify the subtleties of the alteration in redox-homeostasis, following hepatic IR injury and perconditioning. Based on chemiluminescency and spectrophotometry, different approaches have been applied for the assessment of tissue free-radical content and antioxidant-capacity [158].

As described in the Results, significantly favorable changes were observed regarding liver tissue free-radical levels as well as antioxidant-capacity parameters in the IR+RIPER group compared with the IR group in our study.

Yet it is noteworthy that the prominent differences in serum TNF-α levels were observed mostly during the early phase of reperfusion, contrary to the more severe disturbances in redox-homeostasis parameters, which found to prevail after 6 and 24 hours of reperfusion. TNF-α-related signaling is activated early after the onset of reperfusion, acting as a trigger mechanism during the early phase of injury [14], thus

elevated serum TNF-α levels can determine the later phase inflammatory responses and redox-state disturbances.

The described redox-state characteristics might be associated with certain liver specific features [170]. Reperfusion injury and free-radical release in the liver can be divided into two distinct phases: the early, acute phase (first 1-6 hours of reperfusion) is dominated by oxidative stress and Kupffer-cell activation, and ensuing free radical production, during the early phase of reperfusion [29,176]. The later phase is characterized by expression of adhesion molecules and consequential polymorphonuclear leukocyte infiltration.

In contrast to the alterations observed in our study after the first hour of reperfusion, representative results were detected after 6 hours concerning all redox-homeostasis parameters with the exception of reducing power. These aforementioned findings would justify further investigations and detailed monitoring of the first six hours of reperfusion to obtain an in-depth understanding of characteristics of redox-state alterations as well as Kupffer-cell and neutrophil activations in this model. In accordance with our findings, Iwamoto et al. and also Kawamoto et al. published similar data obtained from rat models of liver ischemia and reperfusion [177,178]. In their studies, these authors determined free-radical production using different luminometry measurements and reported that post-ischemic free-radical production was drastically increased with liver reperfusion time. They found significantly elevated *blood* ROS content after 90-120 minutes of liver reperfusion. Tang et al. reported increased activity of the protective superoxide dismutase (SOD) enzyme and parallel decreased lipid peroxydation, measured via determining blood malondialdehyde (MDA) levels, with the use of remote conditioning in a rabbit model of myocardial ischemia-reperfusion injury [93]. More recently, Costa et al. have investigated the effects of a similar RIPER protocol in a rat model of 70% partial liver IR injury. Their results have confirmed the findings of our study, demonstrating that RIPER can reduce liver tissue MDA levels and can preserve tissue antioxidant capacity, which they evaluated via trolox equivalent antioxidant capacity measurement [179].

Since the seminal study of Konstantinov et al., we also know that remote conditioning is able to suppress several leukocyte functions (leukocyte chemotaxis, adhesion, and migration), which are essential in IR induced inflammatory responses

[123]. These favorable gene expression profile alterations might also explain the preserved antioxidant defense line and reduced free-radical production by perconditioning confirmed in our present study and also in the latter work of Costa et al. [179]. Summarizing the probable mechanistic steps, perconditioning might be able to hinder intense free-radical production via reducing microvascular injury [89], inflammatory responses [120,127], and leukocyte functions [123]; furthermore, it could initiate activation of free-radical elimination processes [93], resulting in the above detailed favorable redox-state changes.

Various experimental and clinical studies have corroborated the potent necrosis-size reducing effect of remote ischemic perconditioning in myocardium, brain, or skeletal muscle [88,91,180]. In 2012, Basalay et al. published encouraging results regarding bilateral lower limb remote ischemic perconditioning in a rat model of myocardial infarction [181]. These authors reported significant necrosis reduction with application of perconditioning as compared with non-treated animals (a reduction of infarct size by 56%). Based on these data we attempted to evaluate the so far unrevealed hepatoprotective effects of remote ischemic perconditioning. In our study, histopathological alterations were assessed using a semiquantitative scoring system, and serum transaminase levels were also measured. We found that total-score values were significantly lower in the IR-RIPER group, compared with the corresponding IR group, following each examined reperfusion time point. A similar tendency was observed regarding serum transaminase levels measured for estimation of hepatocyte injury following reperfusion. According to our experimental data, significant reduction was detected in serum AST and ALT levels in the perconditioned group compared with the corresponding IR group following each reperfusion period. In addition, a marked disparity was observed between values of the IR-RIPER$_{1hour}$ group in comparison with the Sham$_{1hour}$ group exclusively after this reperfusion period. Considering the results detailed above, our study revealed remarkably mitigated target organ injury upon the application of remote ischemic perconditioning. Our findings were confirmed by subsequent studies of other research groups. Costa et al. and Sedaghat et al. have demonstrated the hepatoprotective effects of remote ischemic perconditioning in their articles published in 2014 [175,179].

In Study I. we obtained complex short- and medium-term experiences with lower limb RIPER in hepatic IR injury. Subsequently, our attention turned to the investigation of underlying mechanisms of RIPER induced hepatoprotection.

As discussed above, several studies have demonstrated the potent protective effects of remote ischemic perconditioning on different target organs such as heart [180], brain [182], skeletal muscle [91], and for the first time by our team on liver; although, our knowledge on the complex mechanisms behind this promising technique is full of obscure details. According to the widely accepted theory, the connection between remote and target organs is provided by certain blood-borne factors and/or neural, systemic elements. These "connective mechanisms" appear to convey the protective signal to the target organ, initiating a complex subcellular protective response [9].

Up to now, only a handful of data have been published on the mechanisms of remote perconditioning. It should be noted that, however, there are ample similarities and overlap between the mechanisms of different ischemic conditioning procedures, we should also assume the probable existence of subtle differences, namely thanks to the timing of different procedures, in relation to target organ ischemia (pre-, per-, and postconditioning), the concentration and activation status of the participants cannot be similar, which might alter the observed protective responses. Slight differences between protective pathways, triggered by remote pre- and perconditioning, have been proposed by Hausenloy et al. [95]. These investigators reported that the Wortmannin PI3-kinase inhibitor could abolish the protective effect of RIPC but not of RIPER. Therefore, we have to be careful when interpreting our study results and adopting evidence concerning the mechanisms, obtained from other studies using differently timed conditioning procedures (e.g. RIPC).

Gho et al. firstly applied a ganglion blocking agent, hexamethonium, which completely abrogated the myocardial infarct-size reduction achieved by application of brief mesenteric IR attacks as remote preconditioning treatment [115]. Afterwards, further studies confirmed the role of autonomic nervous system behind the RIPC phenomenon. As a subsequent step, other authors have proved that direct vagal nerve stimulation can also reduce myocardial IR injury [183,184]. Furthermore, the potent protective effect of RIPC is absent in the presence of bilateral cervical vagotomy [181].

Recently, Matitskaya et al. have reported their interesting observations. In their study the selective inhibition of the vagal cholinergic pre-ganglionic neurons could likewise effectively extinguish RIPC protection, whilst selective activation of the mentioned neural elements had a strong RIPC mimicking effect. [119]. Presumably, different mediators (adenosine, bradykinin, etc.), released from the remote organ during the short ischemic-reperfusion episodes and stimulating local neural elements, are responsible for triggering these complex neural mechanisms. It has been proposed, as suspected trigger mechanism, that these mediators could activate capsaicin sensitive sensory neurons which can release calcitoning gene related peptide (CGRP) and thus induce a subcellular protection with the participation of complex kinase cascades [117].

Most of the previous findings on the mechanisms of RIPER are obtained from cardiovascular studies. Significantly less data are on hand in regards of the ischemic events of parenchymal organs such as the liver [185]. According to our best knowledge, no data have been reported concerning the liver before, which would investigate the role of neural elements behind the hepatoprotective effects of RIPER. Lim et al. published that myocardial protection, evoked by hind limb remote ischemic preconditioning, is completely abolished after transection of the ipsilateral femoral and sciatic nerves [101]. Based on these data, in Study II., we aimed to investigate the effects of hind limb nerve resection on the efficacy of remote perconditioning against hepatic warm IR injury.

To design a well-elaborated study we had to implement minor modifications in our previous model (see Study I.). We modified the remote conditioning protocol, due to the need to completely denervate the remote (conditioning) organ. In Study II. remote perconditioning was applied on the left femoral artery, after preparation of the left femoral and sciatic nerves. However, in this new model we applied remote conditioning on a significantly reduced vascular bed, but in turn we could easily achieve selective denervation of the conditioned area.

In the present study, we similarly followed liver microcirculatory changes using laser Doppler flowmetry and systemic hemodynamics during the early phase of reperfusion. As a result of IR injury, liver reperfusion microcirculation as well as systemic mean arterial pressure values were strongly reduced in IR and IR-N groups. In contrast, results of the IR-RIPER group were significantly improved during the observation period. It could also be observed that resection of femoral and sciatic nerves

blunted the protection of RIPER, accordingly the circulation of the IR-RIPER-N group stayed around the values of the IR and IR-N groups during observation.

Automated image analysis was used to quantify necrosis caused by IR induced tissue injury. We utilized a novel software developed by our collaboration partner (Fraunhofer Mevis Research Institute). The used experimental-phase software is the first product, which enables the quick and automated quantification of necrosis in entire tissue sections. Details on this "tile-based" automatic analysis software and the exact methods were described by Homeyer et al. [156]. Briefly, necrotic zones can be distinguished from viable areas based on their histological appearance. The mathematical algorithm of the program can recognize the necrosis specific features (dissolution of cellular architecture, "moth-eaten" appearance, shrunken or completely absent nuclei, etc.) after a certain teaching period. This teaching session has to be performed by an experienced investigator using reference slides (with prominent necrotic and viable areas). Subsequently, if the algorithm is able to designate precisely viable and necrotic areas and can distinguish them from background and border areas, we can start to evaluate our study samples. We determined this border as less than 5% of false recognized tiles. Depending on analysis settings and the performance of our computer, enormous amount of histological data can be quantitatively analyzed within a short period of time (minutes). The software is not only able to perform high-throughput quantitative analysis of liver necrosis, but it was also successfully tested using other tissues and staining methods [186].

Extent of histological tissue injury was pronounced in the ischemic-reperfusion injured groups (IR, IR-N), after 1 hour of ischemia and 24 hours of reperfusion in Study II. Femoral artery preconditioning strongly reduced the size of necrotic areas, although such protection could not be observed in the IR-RIPER-N group; nerve resection prior to the conditioning treatment aggravated tissue injury.

In accordance with our results of histological evaluation and microcirculatory measurements, left femoral artery preconditioning was also able to significantly reduce cellular injury indicated by ALT serum activity, which effect was also completely abrogated by nerve resection. In contrast, AST levels were elevated in all four IR-injured groups without characteristic differences among the experimental groups. These confounding results might be ascribed to differing specificity of the two parameters,

whereby ALT is mostly considered as a liver injury specific marker, while AST is also released after injuries of several other tissues such as myocardium and skeletal muscle [187].

Serum total bilirubin levels were measured to roughly estimate liver function (uptake and excretion). Slightly but significantly elevated tBi levels in the IR, IR-N, IR-RIPER-N groups, except in the IR-RIPER group, coincide with the rest of our data.

Concerning liver tissue free-radical levels (investigated via luminometry) and the indirectly proportional reducing power, it can be concluded that RIPER applied on left femoral artery was able to positively influence tissue free-radical burden during reperfusion, thereby preserving antioxidant defense line of the tissue. This protective effect could not be observed following nerve resection. This phenomenon is less obvious in relation to protein-related antioxidants (SH-groups) and H-donating ability. Regarding these parameters, which are only partially representative for tissue antioxidant capacity, nerve resection was not able to completely abolish the effects of perconditioning; consequently, the values of the IR-RIPER and IR-RIPER-N groups did not differ significantly.

Our results of Study II. are in line with the findings of Lim et al. They demonstrated that denervation of the left limb can completely abolish the positive effects of left femoral artery preconditioning on infarct-size, in a murine model of myocardial ischemia-reperfusion [101]. In a more recent study, Wang et al. have tested the protective effects of vagal stimulation perconditioning during experimentally induced coronary occlusion. The authors postulated that electronic vagal stimulation might exert its effects through activation of the cholinergic anti-inflammatory pathway [120]. The observed positive alterations in infarct-size and inflammatory response with the use of this method were comparable with the results obtained with the application of standard ischemic perconditioning in their model. These findings underpin the possible role of certain neural elements behind RIPER induced organ protection. These results do not necessarily mean, however, that neural mechanisms are dominating in the protective response induced by RIPER. We hypothesize that the innate responses behind RIPER might form a redundant, however, also sensitive network with multilateral communication. If there is a certain inhibiting factor (like denervation), disturbing the transfer of protective signals, at one or multiple points, it might be able to

completely suspend these favorable effects. This might be the explanation for the phenomenon that ischemic conditioning is in general less effective in the presence of co-morbidities such as hyperlipidemia, diabetes, which conditions can well-known interfere with the innate protective singaling pathways [188].

An important issue in the field of remote ischemic conditioning research is the minimum amount of tissue necessary to temporarily be excluded from the circulation to achieve a satisfactory protective effect in the target organ [9]. A previous study of our group showed that the infrarenal aortic clamping (used as perconditioning protocol in Study I.) can cause ischemia in the lumbosacral region and in the two lower limbs, representing 42.93 ± 0.58 % of total body muscle weight of the rat, while weight of the musculature of one leg is only 12.16 ± 0.35 % of the total [189].

According to the findings of previous studies, not really the amount of (skeletal muscle) ischemized tissue is determinative, but the optimal repetition and length of the cycles [9,87,190,191]. It was previously reported that even the selective ischemia and reperfusion of canine gracilis muscle can protect against a subsequent IR injury in the contralateral gracilis muscle [191]. In the present doctoral thesis we did not aim to statistically compare the results obtained with application of the two different RIPER protocols (Study I. – infrarenal aortic cross-clamping; Study II. – left femoral artery clamping), because of the fact that relatively long time have passed between the two studies and measurements. An interesting further direction for the present research would be a comparative investigation of the effects of different skeletal muscle exclusions (aorta cross-clamping, femoral clamping, limb tourniquet, inflatable cuffs etc.) on RIPER induced hepatoprotection. Reliability and reproducibility of animal studies represents an actual hot topic in literature [192-194]. Accordingly, it is questionable whether the results of two separate pre-clinical studies with relatively low numbers of individuals enrolled (thus with low power) are comparable. Nevertheless, in general we can say that no conspicuous differences were observed and recorded, regarding the effects of the two different RIPER protocols during our studies. Effects of left femoral artery clamping were similar with those of aortic cross-clamping, regarding liver microcirculation, tissue injury as well as liver tissue redox-homeostasis alterations.

In summary, the present thesis provides the first complex short- and medium-term experiences with lower limb RIPER in a rat model of normothermic liver IR

injury. RIPER is a feasible, available, low-risk method, which could mean a valuable supplementary approach for the clinical practice in several acute and elective situations, when the possibility of severe IR injury should be concerned.

Investigating different connective and effector mechanisms behind RIPER induced organ protection might help us to identify several exact molecular targets of the protective pathway. The end result would be an efficient and practical pharmacologic conditioning technique. In the present thesis we showed that hepatoprotective effect of remote ischemic perconditioning could be almost completely abolished by denervation of the remote ("conditioning") organ; meanwhile, neural transection alone had no significant effect on liver injury. Our results, therefore, imply that protective signals evoked by perconditioning are conveyed to the target organ to a significant extent via participation of certain neural elements, as reported in case of other kinds of remote conditioning treatments as well [9,101,195]. In this study no attempt was made to investigate more fine, cellular, molecular details of the neural pathway behind RIPER, which is a major limitation of our current work.

Accordingly, further studies should be conducted to clarify the still very obscure exact mechanisms and neural components behind the effect of remote conditioning treatment and also the further possible differences or similarities between remote pre-, per- and postconditioning. Furthermore, future multi-centric, randomized clinical trials are necessary to evaluate efficacy and safety of this method in different clinical settings of liver surgery.

6. CONCLUSIONS

As answers to the aims of the Doctoral Thesis formulated under Chapter *2. Objectives*, we can conclude that:

1. Sixty minutes of 70% partial liver ischemia in rats is a feasible experimental model to investigate the consequences and characteristics of liver IR injury and the effects of remote ischemic perconditioning. In this model of moderate liver ischemic-reperfusion injury, application of perconditioning resulted in significant alterations in several parameters.

2. a, After 60 minutes of partial liver ischemia, remote ischemic perconditioning, applied in 4 cycles of 5 minutes of ischemia and 5 minutes of reperfusion, could potently mitigate liver injury, according to the post-reperfusion levels of serum transaminases and based on the semiquantitative histological scoring.

   b, Perconditioning positively influenced systemic hemodynamics (mean arterial pressure) and microcirculation (liver and lower limb) of the animals, manifested in significant differences between reperfusion macro- and microcirculation values of treated and non-treated animals.

   c, Significantly lower tissue free-radical levels and preserved antioxidant capacity were observed with the use of perconditioning after 6 and 24 hours of reperfusion. In parallel, conspicuous reduction was detected in serum values of the pro-inflammatory cytokine, TNF-α, during the early phase of reperfusion.

3. In Study II., left femoral artery perconditioning could also exert potent protection against the deleterious effects of liver exclusion and reperfusion. We could observe significant alteration between treated and non-treated groups in tissue damage (ALT, necrosis%), liver microcirculation and systemic hemodynamics as well as in liver tissue redox-homeostasis. Perconditioning induced hepatoprotection was abolished by denervation of the vascular bed, where conditioning was applied.

4. Automated histological image analysis is an accurate and useful tool for the evaluation of histological samples of a real experiment. Teaching of the algorithm can be achieved easily and analysis of the slides is fast and reliable.

7. SUMMARY

Background: Ischemic-reperfusion (IR) injury of the liver is still a crucial factor in postoperative morbidity and mortality following major liver resections and transplantations. During the last decades, several surgical and pharmacological approaches have been developed to mitigate IR injury of the liver and to improve outcome for patients. The so-called remote ischemic perconditioning (RIPER) imply the application of brief, ischemic-reperfusion attacks on a remote organ after induction of target organ ischemia but before the onset of reperfusion. However, this method might have a potent protective effect, it has not been investigated before in a model of liver IR injury.

Objectives: Our aim was to investigate the effects of remote ischemic perconditioning in a rat model of hepatic IR injury; furthermore, we aimed to demonstrate whether or not certain neural elements are participating in transfer of protective signals between remote and target organs.

Materials and Methods: Two studies were planned to analyze the effects of RIPER in a rat model of liver IR injury. In both studies 60 min of partial liver ischemia (70% of liver volume) was used as basic model. In Study I. animals were randomly divided into three groups: Sham, IR, IR-RIPER (n=24/group). Ischemic perconditioning was applied during the last 40 min of liver ischemia via infrarenal aortic cross-clamping (4x5 min I, 5 min R). Hepatic and lower limb microcirculation as well as systemic hemodynamics, were monitored during the first hour of reperfusion. After 1,6,24 hours of reperfusion (n=8/time point) liver samples were taken for histological and redox-state assessment. Suzuki score was used for semi-quantitative histological analysis. Serum transaminase (AST, ALT) and TNF-α levels were measured. In Study II. rats were randomly allocated into six groups: Sham, IR, IR-RIPER±denervation (n=7/group). Half of the animals underwent left femoral and sciatic nerve resection. The same perconditioning protocol was applied (4x5 min I, 5 min R), but the left femoral artery was clamped. After 24 h of reperfusion all animals were sacrificed. Similar endpoints were used as in Study I. (liver microcirculation, systemic hemodynamics, necrosis quantification, serum transaminases and total bilirubin, redox-state changes).

Results: RIPER could exert a strong protective effect in our model. It improved microcirculation and systemic hemodynamics, reduced free-radical stress, preserved antioxidant defense line, mitigated tissue injury, and reduced systemic TNF-α levels. We could observe a comparable protective effect with the use of remote perconditioning on the left femoral artery in Study II. This effect was abolished when denervation of the remote organ was performed before conditioning.

Conclusion: Perconditioning was able to reduce IR injury of the liver in our model. Presence of a certain inter-organ neural pathway is assumed behind the observed protective response.

8. ÖSSZEFOGLALÁS

A dolgozat magyar nyelvű címe: „A végtagi távoli szervi iszkémiás perkondicionálás hepatoprotektív hatásainak vizsgálata"

Háttér: A máj iszkémiás-reperfúziós (IR) károsodása még napjainkban is meghatározó szerepet játszik a major májreszekciókat és transzplantációkat követő morbiditási és mortalitási mutatók alakításában. Az utóbbi évtizedek során a máj IR károsodásának csökkentésére és a műtéti kimenetel javítására számos sebészeti és farmakológiai módszer került kipróbálásra. Az úgynevezett távoli szervi iszkémiás perconditioniálás (RIPER) egy eltérő szerven, a célszervi iszkémia alatt létrehozott rövid iszkémiás-reperfúziós epizódok alkalmazását jelenti. Habár e technika potens védőhatással bírhat, ezt korábban még nem vizsgálták a máj iszkémiás-reperfúziós állapotai kapcsán.

Célkitűzés: Célunk volt a távoli szervi perkondicionálás máj iszkémia-reperfúzióra gyakorolt hatásainak patkánymodellben történő vizsgálta, továbbá a hatás hátterében álló bizonyos neurális elemek közvetítő szerepének bizonyítása vagy cáfolása.

Anyagok és Módszerek: A RIPER patkánymáj IR károsodásra kifejtett hatásainak vizsgálatára két kísérletet terveztünk. Mindegyik vizsgálatban 60 perc parciális (a máj volumen 70%-a) máj iszkémiát hoztunk létre. Az első vizsgálatban az állatokat 3 csoportba osztottuk: Áloperált, IR, IR-RIPER (n=24/csoport). A máj iszkémia utolsó 40 perce alatt az infrarenális aorta kirekesztésével perkondicionálást végeztünk (4x5 min I, 5 min R). Az első poszt-iszkémiás óra során monitorizáltuk az állatok szisztémás vérnyomását, továbbá a máj és az alsó végtag mikrocirkulációs változásait. Meghatározott idejű repefúziót követően (1,6,24 óra, n=8/időpont) a májakból mintát vettünk szövetteni analízis és redox-státusz vizsgálatok céljából. A szemikvantitatív szövettani analízishez a Suzuki-pontrendszert hívtuk segítségül. Szérum transzamináz és TNF-α szinteket határoztunk meg. A második vizsgálatban az állatokat 6 csoportba randomizáltuk: Áloperált, IR, IR-RIPER±denerváció (n=7/csoport). Az állatok fele bal oldali n. femoralis és ischiadicus reszekción esett át. A korábbiakkal azonos perkondicionálási protokoll ez esetben a bal femorális artérián került alkalmazásra (4x5 min I, 5 min R). 24 óra repefúziót követően az állatokat termináltuk. A második vizsgálatban az első kísérlethez hasonló végpontokat vizsgáltunk (máj mikrocirkuláció, szisztémás hemodinamika, nekrózis kvantifikálás, szérum transzaminázok és totál bilirubin, redox-státusz változások).

Eredmények: A távoli szervi iszkémiás perkondiciánálás modellünkben erős hepatoprotektív hatást volt képes kifejteni. Alkalmazásával javult a mikrocirkuláció és a szisztémás hemodinamika, csökkent szabagyök stressz és megőrzött antioxidáns védelmi vonal volt megfigyelhető. A kondicionálás használata csökkentette továbbá a szöveti károsodást és a szérum TNF-α szinteket. Mindezekkel összevethető védőhatás volt megfigyelhető a második vizsgálatban, a bal femorális artérián alkalmazott kondicionálás esetén is. E kedvező hatások azonban a kondiciánálást megelőző idegátmetszés esetén nem voltak megfigyelhetőek.

Következtetés: Modellünkben a perkondicionálás képes volt csökkenteni a máj IR károsodás mértékét. A protektív hatás hátterében egyes közvetítő idegelemek szerepe feltételezhető.

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10. Bibliography of the candidate´s publications

Articles providing the basis of the doctoral thesis:

1. **Czigány Z,** Turóczi Zs, Bulhardt O, Hegedüs V, Lotz G, Rakonczay Z, Balla Z, Harsányi L, Szijártó A. (2012) Távoli szervi kondicionálás: rövid távú hepatoprotektív hatások patkánymodellben. [Remote ischemic perconditioning: short term effects on rat liver ischemic-reperfusion injury.] Orvosi Hetilap [Hungarian Medical Journal], 153 (40): 1579-1587.

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Further articles:

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