SURGICAL METHODS FOR REDUCING REPERFUSION INJURY AFTER REVASCULARISATION INTERVENTIONS

PhD Thesis

By
László Sínay MD

Supervisor
Gábor Jancsó MD, PhD

Head of Ph.D. Program
Erzsébet Rőth MD, PhD, DSc

University of Pécs, Faculty of Medicine
Department of Surgery

Pécs 2009

CONTENTS

ABBREVIATIONS

ATP:	adenosine triphosphate
BK:	bradykinin
BAX:	Bcl-2 associated X protein
Bcl-2:	B-cell CLL/lymphoma 2
cGMP:	cyclic guanosine monophosphate
COX:	cyclo oxygenase
COX-2:	cyclo oxygenase-2
CR:	controlled reperfusion
Cx43:	connexin 43
DNA:	desoxyribonucleic acid
ECG:	electrocardiogram
EDTA:	ethylenediaminetetraacetic acid
eNOS:	endothelial nitric oxide synthase
GSH:	reduced glutathion
JNK:	jun kinase
H2O2:	hydrogen peroxide
HO-:	hydroxyl anion
I/R:	ischaemia/reperfusion
IAA:	infrarenal abdominal aorta
iNOS:	inducible nitric oxide synthase
LDL:	low density lipoproteins
MPO:	myeloperoxidase
mPTP:	mitochondrial permeability transition pore
NFkB:	nuclear factor kappa B
NO:	nitric oxide
O2-:	superoxide anion
OFR:	oxygen free radicals
ONOO-:	peroxynitrite
pERK:	phospho extracellular signal-regulated kinase
pGSK:	phospho glycogen synthase kinase
PI3K:	phosphatidyl inositol 3 kinase

PKC:	protein kinase C
PKG:	protein kinase G
PMA:	phorbol myristate acetat
PMN:	polymorphonuclear neutrophils
PostC:	postconditioning
pp38MAPK:	phospho p38 mitogen activated protein kinase
PTCA:	percutaneous transluminal coronary angioplasty
PUFAS:	polyunsaturated fatty acids
RBC:	red blood cell
RISK:	reperfusion injury salvage kinase
ROI:	reactiv oxygene intermediers
ROS:	reactiv oxygene species
SH:	plasma thiol groups
SOD:	superoxide dismutase
STAT3:	signal transducer and activator of transcription 3
TNF–alpha:	tumor necrosis factor alpha

1. INTRODUCTION

In vascular surgery during the manipulation on the vessels the periferial tissues always suffer from a more or less severe ischaemia. In acute ischaemia the time of ischaemia could be also serious, thus after reconstruction we always have to face with reperfusion injury. The aim to reduce these extent of these reperfusion injury associated pathways has real clinical importance in vascular surgery.

Reperfusion injury is an inherent response to the restoration of blood flow after ischaemia, and is initiated at the very early moments of reperfusion, lasting potentially for days. The extent of the oxidative stress and the consecutive generalized inflammatory response depends on the ischaemic-time, the ischaemic tissue volume, and the general state of the endothelium-leukocyte-tissue functional complex (diabetes, chronic ischaemia, drugs). The pathogenesis of reperfusion injury is a complex process involving numerous mechanisms exerted in the intracellular and extracellular environment.

1.1 PATHOPHYSIOLOGY OF REPERFUSION INJURY

Reperfusion injury is not a mere worsening of the ischaemia-induced damage, but it is secondary to events that are specifically induced by reperfusion. In fact, reperfusion injury is due to complex mechanisms involving mechanical, extra-cellular and intracellular processes. The modern hypothesis of the pathogenesis of reperfusion injury has been reviewed by Piper and al.[1] In patients with acute periferial ischaemia, it is now widely accepted that periodically reopening the occluded artery is accompanied by a reduction of the extent of necrosis and a major reduction of short- and long-term mortality. However, together with a definite protective effect on ischaemic tissues, post-ischaemic reperfusion may bring with it unwanted consequences that may partly counteract its beneficial effects. This phenomenon has thus been named reperfusion injury.

Causes of reperfusion injury

It seems that in the tissue ischaemia/reperfusion (I/R) can induce various forms of cell death, such as programmed cell death, apoptosis, oncosis and necrosis[2]. Apoptosis can be caused by

both prolonged ischaemia/hypoxia and by reperfusion[3]. In contrast to programmed cell death, apoptosis and oncosis, which are pre-mortal processes, necrosis is a post-mortal event. According to this viewpoint necrosis is not a form of cell death but the end stage of cell death processes.

The mechanisms of reperfusion-induced cell death are not completely understood, but it seems that the occurrence of **oxidative stress** related to the generation of ROS may play an important role[4]. ROS have downstream effects, which results in the initiation of a highly orchestrated acute inflammatory response through the release of cytokines, activation of vascular endothelial cells and leukocytes with expression of cell surface adhesion molecules, and up-regulation of a program of pro-inflammatory genes, which contribute to the onset and maintenance of post-ischaemic inflammation[5]. When the occlusion of the artery branch that perfuse the ischaemic tissue is removed, the superoxide anion (O^{2-}) production increases as a result of the activation of various enzymatic complexes. The superoxide anion and other ROS strongly oxidize the myocardial fibres already damaged by the ischaemia, thus favouring the apoptosis[6]. It reacts with the nitric oxide, forming peroxynitrite ($ONOO^{-}$). Therefore, $ONOO^{-}$, represents a sign of a reduced availability of nitric oxide[7] and it participates with O^{2-} in the lesion of tissues[8]. Superoxide anion dependent damages are reduced if O^{2-} is transformed to hydrogen peroxide (H_2O_2) by the superoxide-dismutase. However, since in the presence of Fe^{2+} or Cu^{2+}, the H_2O_2 can be transformed in hydroxyl anion (HO^{-}), which is more toxic than O^{2-} and H_2O_2, an increase in toxicity can occur. Reperfusion injury is also due to cellular Ca^{2+} overload. The Ca^{2+}-overload, which starts during ischaemia, is further increased during reperfusion.

The overload of Ca^{2+} increases the cellular osmolarity favouring swelling (explosive swelling) of skeletal muscle cells; it can also favour the expression of proapoptotic elements from mitochondria[9]. It is noteworthy that altered cytosolic Ca^{2+}-handling during ischaemia may induce structural fragility and excessive contractile activation upon reperfusion, as also indicated from a progressive increase of ventricular diastolic pressure and contraction band necrosis[10].

Ca^{2+}-overload is also considered to be responsible for the opening of mPTP. Although, mPTP opening is strongly inhibited by acidosis during ischaemia, it is favoured by ATP depletion, oxidative stress and high intramitochondrial Ca^{2+} concentrations, conditions all occurring during myocardial reperfusion[11].

Intriguingly, the nuclear factor kappa B (NFkB) plays a double-edge sword role in tissue-protection. Activation of NFkB is essential for late preconditioning, in which NFkB is involved in the up-regulation of iNOS and COX-2 genes. However, in the longer time the role NFkB is also important in reperfusion injury. It contributes to the exacerbation of the tissues lesions sustaining inflammatory reactions. The activation of NFkB is induced from several agents included hydrogen peroxide[12]. NFkB determine an up-regulation of the genes responsible of the production of molecules of cellular adhesion. These molecules favour the adhesion of leukocytes to the endothelium and possibly the migration within the cells[13]. Moreover, the reduced nitric oxide availability determined by I/R participates to the activation of transcription codifying for molecules of cellular adhesion[14]. Therefore, tissue damages during reperfusion among others can be due to the cellular/mitochondrial overload of Ca^{2+}, to the liberation of ROS, to the activation of mPTP, to the reduced availability of nitric oxide and to the activation of the NFkB. The nitric oxide deficiency can also cause vasoconstriction and formation of micro-thrombi into the lumen of the small vessels[15]. These mechanisms, combined with the adhesion of the leucocytes to the endothelium, can lead to the so-called 'no-reflow phenomenon`[16]. In summary, reperfusion injury is due to several mechanisms that include Ca^{2+} overload, ROS generation, reduced availability of nitric oxide, mPTP opening and to the activation of the NFkB, which lead to the augmented expression of molecules of cellular adhesion, leukocyte infiltration and no-reflow phenomenon.

1.2. EFFECTS OF REPERFUSION INJURY

Among the outcomes of reperfusion injury are included: (1) endothelial and vascular dysfunction and the sequels of impaired arterial flow, which may concur with the 'no-reflow phenomenon'; (2) metabolic and contractile dysfunction; (3) arrhythmias in case of myocardial I/R; (4) cellular death by cellular swelling, and apoptosis. One may anticipate that effective treatment during reperfusion may reduce tissue injury. However, the complexity of mechanisms suggests that one single intervention aimed to contrast just one or two of these mechanisms may not be sufficient. (*Figure 1*)

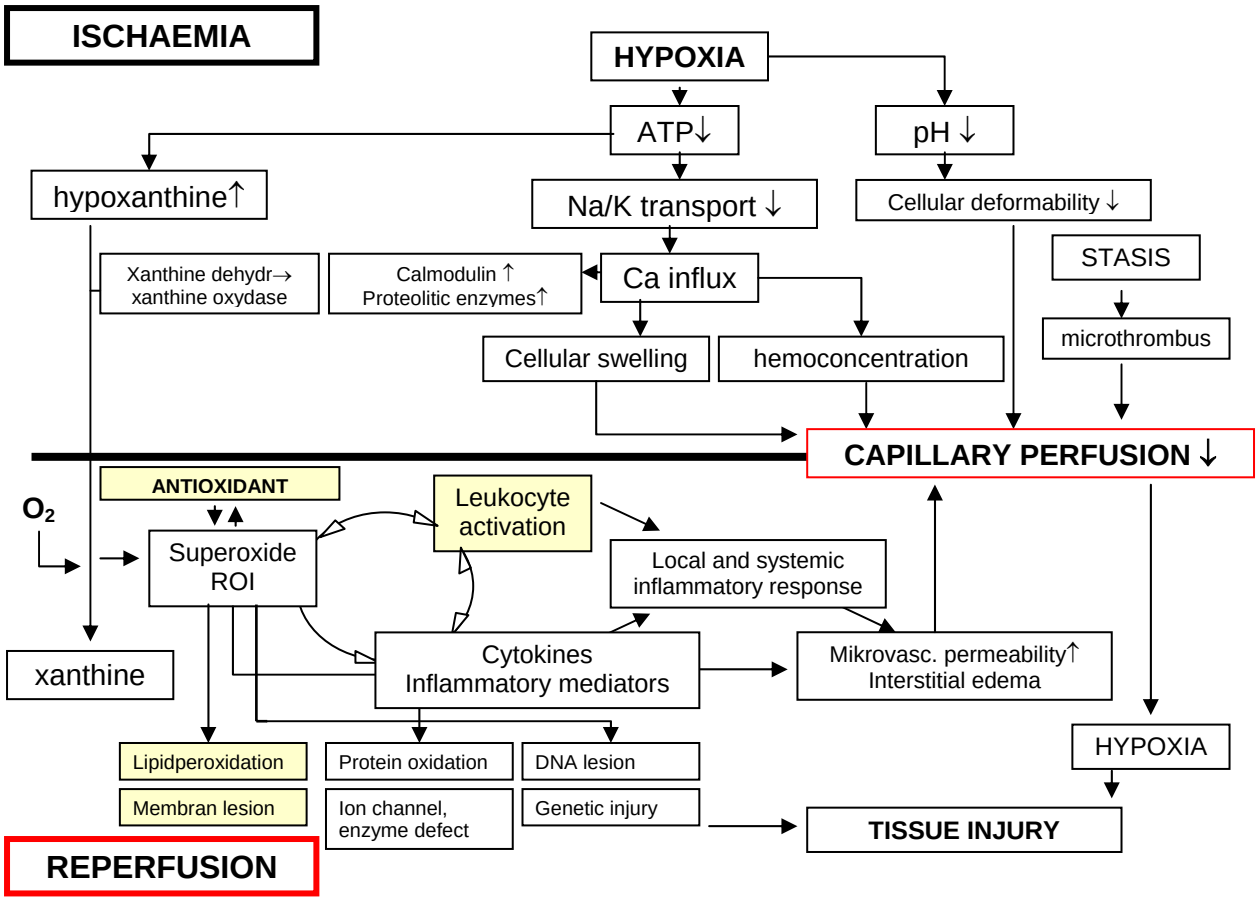


Figure 1: *Simplified presentation of the mechanism of ischaemic-reperfusion injury. Emphasizing, that the engine of reperfusion injury is the ROI – cytokine – leukocyte positive feedback circle.*

Hypoxia leads to intracellular ATP depletion with a consecutive hypoxanthine elevation. In the early seconds of reperfusion, when the molecular oxygen appears in the cell, the - xanthine oxidase catalised – hypoxanthine-xanthine conversion will produce a mass of superoxide radicals. Superoxide radical and the other reactive oxygen intermediates will damage the membrane-lipids (through lipidperoxidation), the proteins (causing enzyme defects and ion channel injury) and the DNA. These are the main pathways of the cellular oxidant injury. The endogenous antioxidant system defends against these radical injuries. [17]

Reactive oxygen species (ROS) will also induce local and systemic inflammatory responses through the inducing of cytokine expression and leukocyte activation. Inflammatory process leads to increased microvascular permeability, interstitial edema, and capillary perfusion depletion. The oxidative and inflammatory pathways will lead to a complex reperfusion injury. [18] *(ROI – reactive oxygen intermediers; ATP – adenosine triphosphate; DNA – deoxyribonucleic acid)*

1.3. DEFINITION OF POSTCONDITIONING

The concept of 'Ischaemic PostC' was first described by Vinten-Johansen's group[19]. This study was performed in a canine model of 1 hr coronary occlusion and 3 hrs reperfusion. In this study the PostC algorithm was 30 sec. of reperfusion followed by 30 sec. of coronary occlusion, which were repeated for three cycles at the onset of reperfusion. Although this seminal study used the term 'Ischaemic PostC', subsequent studies of these and other authors omit the term 'Ischaemic' because it is not clear whether the brief periods of ischaemia, the preceding and/or the subsequent periods of reperfusion, or their combination, provide the key stimulus for cardioprotection. In general, PostC can be defined as intermittent interruption of coronary flow in the very early phase of a reperfusion, which leads to protection against reperfusion injury. The duration and number of these stuttering periods of reperfusion and ischaemia has been one of the aims of early studies on this topic.

1.4. POSTCONDITIONING ALGORITHM

PostC can be obtained by different protocols in terms of duration of the periods of reperfusion and ischaemia and/or in terms of number of cycles of I/R applied after a sustained ischaemia. These different procedures/protocols have been called PostC algorithms by Vinten-Johansen [20]. Virtually in all of the species in which different PostC algorithms have been tested they proved to be protective, including humans.[21] In swine there are two studies: in one study, Schwarz & Lagranha [22] reported that PostC obtained with three cycles of 30 sec. I/R had no beneficial effect in open-chest pigs. In another study it seems that PostC with intermittent cycles of 1 min. I/R is effective also in swine.[23] Although, it has not been specifically studied, it seems that contrasting findings in this species are due to different PostC algorithms.

Nevertheless, these and other studies which used different algorithms to induce PostC stress the importance of the duration of index I/Rs periods as well as PostC I/R algorithm. Another recent study suggested that the cardiac effects of PostC may even be detrimental and that this deleterious effect critically depends on the duration of the preceding period of index ischaemia rather than the employed algorithm[24].

Many studies have sought to identify PostC algorithms that effectively limit infarct size, and, though the 'ideal' PostC regimen has not been established, the emerging consensus is that multiple (3–6) cycles of brief (10–30 sec.) intermittent reperfusion/re-occlusion are associated with protection. The duration of efficient intermittent cycles of I/R seems to be shorter in smaller species (i.e. 10–15 sec in rats and mice) and longer in larger species (30 sec in dogs

and rabbits) and human (60–90 sec)[25]. In the rat hearts a 'classical' PostC algorithm was compared which consisted of five cycles of 10 sec. reperfusion and occlusion with a 'modified' algorithm of PostC which consisted of an initial 15 sec. reperfusion and then in a sequence of progressively shorter (20, 15, 10 and 5 sec.) periods of occlusions separated by progressively longer periods of reperfusions (20, 25 and 30 sec.). *(Figure 2)*

This modified PostC protocol was equally effective as the classical algorithm in reducing the infarct size[26].

Importantly, in all species, the PostC stimulus must be applied immediately upon relief of sustained ischaemia to be protective: if the initial complete reperfusion is allowed to continue for more than 60–90 sec., then the protection against infarct size extension is lost[27].

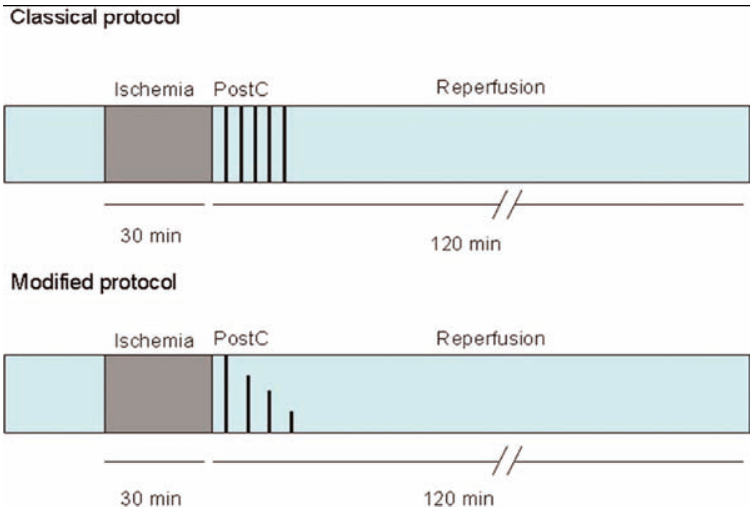


Fig. 2.: Schematic representation of postconditioning protocols. In the modified protocol the decreasing vertical lines represent shorter ischaemia.

1.5. PROTECTIVE EFFECTS OF POSTCONDITIONING

Ischaemic PostC was already examined mostly in myocardium, thus the protective effects are known in the myocardium. Our study was the first to examine the effect of PostC in peripheral tissues.

Depending on species, models and other factors, PostC reduces the infarct size by ~20–70% versus matched controls with matched risk areas. There is an emerging agreement across multiple models and species that PostC may reduce endothelial dysfunction and endothelial activation, thus leading to a reduced endothelial/leukocyte interaction and to a reduced ROS

formation. Reduced incidence of apoptosis and arrhythmias has also been observed. Whether PostC reduces post-ischaemic stunning it has not yet been clarified.

1.5.1. REDUCTION OF NECROSIS

In their seminal study Vinten-Johansen and coworkers [28] reported that PostC causes massive salvage of the myocardium. The infarct size was reduced by ~45% when the initial minutes of reperfusion were 'stuttered' compared to an abrupt and complete reperfusion. These findings have been confirmed by several laboratories as well[29]. As mentioned, in multiple species and models, PostC reduces infarct size by ~20–70% versus matched controls with matched risk areas[30]. Studies from Przyklenk's laboratory and other laboratories confirmed the infarct size reduction in rat isolated heart model[31]. It has been showed that in hearts perfused with constant flow the infarct size reduction by PostC is greater than that observed in the same model perfused at constant pressure [32].

1.5.2. REDUCTION OF APOPTOSIS

Apoptosis is a genetically programmed cell death that occurs in reperfusion injury[33]. The reduction in apoptosis may involve the inhibition of caspase-3 and caspase-9 and preservation of Bcl- 2/Bax ratio. So far the only study that reported a reduction of apoptosis by PostC is of Zhao et al. [34] in which a reduced apoptosis was detected with TUNEL assay and the presence of DNA ladders in a model of isolated neonatal cardiomyocytes that underwent hypoxic PostC. We also examined anti- and proapoptotic factors in our periferial PostC model.

1.5.3. REDUCTION OF ENDOTHELIAL DYSFUNCTION

The endothelial cell dysfunction is a common characteristic of various heart pathologies[35]. In their seminal study Zhao et al.[36] reported that postischaemic endothelial dysfunction was attenuated by PostC. In this study, incremental doses of acetylcholine were used to evaluate the endothelium dependent vasodilatation of coronary vessels isolated from the post-ischaemic region. The authors demonstrated that vasodilatation of postconditioned vessels was improved with respect to that observed in post-ischaemic control vessels. The vasodilator response was similar to that observed in preconditioned vessels and to that observed in vessels from non-ischaemic region.

1.5.4. REDUCTION OF ENDOTHELIAL ACTIVATION AND NEUTROPHIL ADHERENCE

PostC decreases the expression of P-selectin, an adhesion molecule on the surface of endothelial cells. Moreover, it has been observed both a reduction in neutrophils adhesion on the post-conditioned coronary artery endothelium and accumulation of neutrophils in the area at risk[37]. A reduction in superoxide anion generation in the perivascular area has also been observed in the proximity of risk area of postconditioned hearts[38]. Whether the reduced neutrophil accumulation, the subsequent ROS production and the pro-inflammatory response is a cause or consequence of necrosis, apoptosis and vascular injury is not clear. In fact, PostC exerts marked cardioprotection in leukocyte-free models (isolated buffer perfused hearts and isolated cardiomyocytes)[39].

1.5.5. REDUCTION OF STUNNING

The studies which tested whether PostC may improve post-ischaemic myocardial function after a postinfarction ischaemia report either an improvement [40] or no effect [41] on cardiac performance. In the presence of an infarct, it is hard to distinguish whether the impairment of global function is due to necrosis and/or to myocardial contractile dysfunction or 'stunning' of viable tissue. To date the only study that tested in vivo whether PostC attenuates myocardial stunning in models of short ischaemias (i.e. 10–15 min. ischaemia), which usually induce stunning without cell death, reports that PostC does not prevent myocardial stunning [42]. In an ex vivo preparation of human atrial appendages hypoxic PostC seems to attenuate post-ischaemic dysfunction[43].

1.5.6. ANTI-ARRHYTHMIC EFFECTS

Several investigations have demonstrated that it is mandatory to perform the PostC protocol as soon as possible after ischaemia to protect the heart against ischaemia reperfusion injury. However, Galagudza et al. [44] report that PostC performed 15 min. after the beginning of reperfusion still has a strong effect in limiting persistent reperfusion-induced tachyarrhythmia. The possibility to reduce reperfusion arrhythmias with the reintroduction of ischaemia is not completely novel. In fact, in 1994 Grech and Ramsdale [45] reported that coronary artery recanalization by percutaneous transluminal coronary angioplasty (PTCA) induced an idioventricular rhythm, which was interrupted several times by the reinflation of the balloon and thus restoring sinus rhythm. It has been reported that classical early PostC not only reduces infarct sizes in pigs, but also abolishes reperfusion arrhythmias[46]. Recently,

Kloner and coworkers [47] have reported in a non-infarct regional ischaemia model in in vivo rats that PostC attenuates post-ischaemic ventricular arrhythmia. From these studies it seems that PostC with brief I/R can be used to limit reperfusion arrhythmias. On the other hand, intermittent pacing (intermittent dyssynchrony-induced PostC) during early reperfusion reduces infarct size in two different species and models (isolated rabbit hearts and in vivo pig)[48]. The intermittent pacing was obtained in both models during the first minutes of reperfusion using few seconds of ventricular pacing alternated by few seconds of atrial pacing. It is noteworthy that dyssynchrony- protection is likely induced by modulation graded reperfusion.

1.6. POSSIBILITIES OF POSTCONDITIONING

It has been reported that PostC-induced infarct size reduction persists up to 72 hrs[49]. These are important studies because they demonstrate that the protection by PostC represents a long-term protective effect and not a mere attenuation of event involved in early reperfusion injury. In some studies the protocol of classical preconditioning and PostC were combined in order to see whether or not the protection by these two protocols was additive, relative to the protection of each protocol alone. The results are inconsistent. In a canine model, Halkos et al. [50] showed that the combination of protocols is neither additive for infarct size reduction, ROS production nor for post-ischaemic endothelial dysfunction. Similar results were obtained by Tsang et al. [51] and by us [52] in isolated perfused rat hearts. However, Yang et al. [53] demonstrated in an in vivo rabbit model that the combination of the two protocols reduced infarct size significantly more than either manoeuvre alone. The different results may be due to species difference and/or different I/R and PostC protocols. Recently, Bolli's group reported that cardioprotection induced by late preconditioning is enhanced by PostC via a COX-2-mediated mechanism in conscious rats[54]. It remains to de ascertained whether such additive effect between late preconditioning and PostC can be observed in other species and/or models.

Very few studies tested the differences between male and female hearts with regard to PostC effectiveness. In a specifically designed study it has been reported that while the PostC protective effect against stunning was observed in isolated male rat hearts after both 20 min. and 25 min. ischaemia, the protective effect was present in female rat hearts exposed to 20 min of ischaemia, but absent in those exposed to 25 min. ischaemia[55]. In a preliminary study, it has been observed that after 30 min. ischaemia the PostC protective effect against infarction is less effective in female than in male rat hearts. The importance of PostC warrants further

studies to elucidate the signal pathways and differences in males and female hearts. It has been reported that cardioprotection by PostC is dependent on the PostC algorithm in aged and STAT3 (signal transducer and activator of transcription 3)-deficient hearts. Moreover it seems that the reduced levels of STAT3 with increasing age may contribute to the age-related loss of PostC protection[56].

In clinical practice ischaemic postconditioning seems even as effective as ischaemic preconditioning. Furthermore, PostC could be used after ischaemia, thus it coud be used in acute ischaemia as well. Threre are many more details in the pathogenesis and clinical applicability of PostC, it seems to be an effective tool in cardiology and vascular surgery to reduce reperfusion injury.

2. AIMS

In the first series of our investigations we aimed to examine the protective effects of ischaemic postconditioning on peripheral tissues in a rat model.

After infrarenal abdominal aortic occlusion we applied ischaemic postconditioning and measured the evoked oxidative stress, and inflammatory responses. We aimed to measure the extents of lipid-peroxidation (serum peroxide level) for characterize the reperfusion induced oxidative stress. To feature the reperfusion-induced inflammatory response, we measured cytokine expression (TNF-alpha), the leukocyte activation, serum myeloperoxidase (MPO) levels, and the free radical production of leukocytes.

Furthermore to confirm the protective effect of the applied ischaemic PostC we monitored the activation of intracellular anti- and proapoptotic common signaling pathways (pGSK, pAKT, pERK1/2, pp38MAPK) during the early phase of reperfusion.

In the second series of our investigations we focused on the effect of ischaemic PostC in human revascularization operations. After aorto-bifemoral bypass surgery we applied ischaemic PostC and observed the protective effect.

To describe the oxidative stress we measured the serum malondialdehyde level – to quantify the rate of lipidperoxidation, and the antioxidant enzymes (SOD, GSH, SH). To see the inflammatory changes we measured serum MPO levels, free radical production of leukocytes, and the expression of leukocyte CD11a and 18 adhesion molecules.

In the third series of the investigations we aimed to examine the protective effect against reperfusion injury with controlled reperfusion in animal model. After a long infrarenal aortic occlusion we started the reperfusion for 30 min. with a crystalloid diluted blood perfusion with low pressure to the periphery. We hypothetised, that this low saturated, diluted blood perfusion with a low pressure could reduce the evoked oxidative stress, and thus consecutively the reperfusion injury. We aimed to determine the antioxidant- prooxidant state, the rheological changes in peripheral and diluted blood, and the inflammatory responses.

We aimed to follow the animals up to one week, and to make pathological examinations from tissue samples (skeletal muscle, lung, kidney, heart, liver, small bowels) to see the late effects of controlled reperfusion.

3. THE EFFECT OF ISCHAEMIC POSTCONDITIONING ON THE PEROXIDE FORMATION, CYTOKINE EXPRESSION AND LEUKOCYTE ACTIVATION IN REPERFUSION INJURY AFTER ABDOMINAL AORTIC SURGERY IN RAT MODEL

3.1. INTRODUCTION

After abdominal aortic aneurism surgery or after revascularization of an acute occlusion on the infrarenal aorta, the development of a serious ischaemia-reperfusion injury is a threatening confront and a hard task in peripherial vascular surgery.

Revascularization procedures performed on ischaemic extremities are accompanied by metabolic and functional derangements which may be life-threatening. After the various duration of ischaemia the reflow of oxygen saturated blood induces cellular oxidative stress with a consequent intracellular oxygen free radical (OFR) production. Intracellular OFR-s can damage all macromolecules (proteins, lipids, nucleic acids), impair the membrane ion-channels, disturb the intracellular ion balance, and can lead to cell swelling and cell-death. The early OFR formation induces endothelium activation, cytokine secretion, and leukocyte activation and these activated leukocytes provoke local and systemic reperfusion inflammatory responses. [57]

Despite the wide theoretical knowledge about the reperfusion injury there is not any effective clinical tool in the surgeon's hand to prevent them. The efforts to reduce, or minimize the cellular and systemic reperfusion injury are important and necessary in vascular surgical research. [58 59 60]

In the cardiovascular literature it has recently been published that short interruptions of the early reperfusion with brief ischaemic episodes after the prolonged ischaemic insult may attenuate the total IR injury.[61] This phenomenon has been termed ischaemic postconditioning. In experimental animal models, postconditioning limits infarct size after myocardial ischaemia, reduces tissue edema and polymorphonuclear neutrophil accumulation. In the interventional cardiology the procedure of postconditioning seems to be simple to apply and

potentially effective in reducing reperfusion damages. Cardiovascular researchers anticipate that postconditioning will become part of the standard care of acute myocardial infarction.
The aims of our investigations were to examine the protective effects of ischaemic postconditioning on peripheral tissues.

3.2 MATERIALS AND METHODS

3.2.1. Animal model

24 Wistar rats in both sexes, weighed between 200-250 g were used in the study. The animals were acquired from the university animal house and were housed in individual cages in ambient temperature and light-dark cycle controlled environment with free access to food and water. Food, but not water was withdrawn 12 hours prior to experiment. The present study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996) and was approved by the local institutional Committee on Animal Research of Pécs University (BA02/2000-29/2001).

3.2.2. Aortic ischaemia-reperfusion model

The animals were anaesthetized with an intraperitoneal injection of 50 mg/kg ketamine hydrochloride (Vetalar, Fort Dodge) and were placed on a heating pad. ECG was placed and the carotid artery was catheterized (22 gauge) for blood pressure measurement (Siemens Sirecust 1260, Düsseldorf, Germany). The skin was aseptically prepared and a midline laparotomy was performed. Two ml of warm saline was instilled into the peritoneal cavity to help maintaining fluid balance. The inferior caval vein was gently catheterized for collecting blood sample, fluid equilibration and supplemental anesthetic. The abdominal aorta was exposed by gently deflecting the intestine loops to left. After fine isolation of the infrarenal segment, an atraumatic microvascular clamp was placed on the aorta for 60 minutes. The abdomen was then closed and the wound was covered with warm wet compress to minimize heat and fluid losses. The microvascular clamp on the infrarenal abdominal aorta (IAA) was then removed and IAA was reperfused for 120 minutes. Aortic occlusion and reperfusion was confirmed by the loss and reappearance of satisfactory pulsation in the distal aorta.

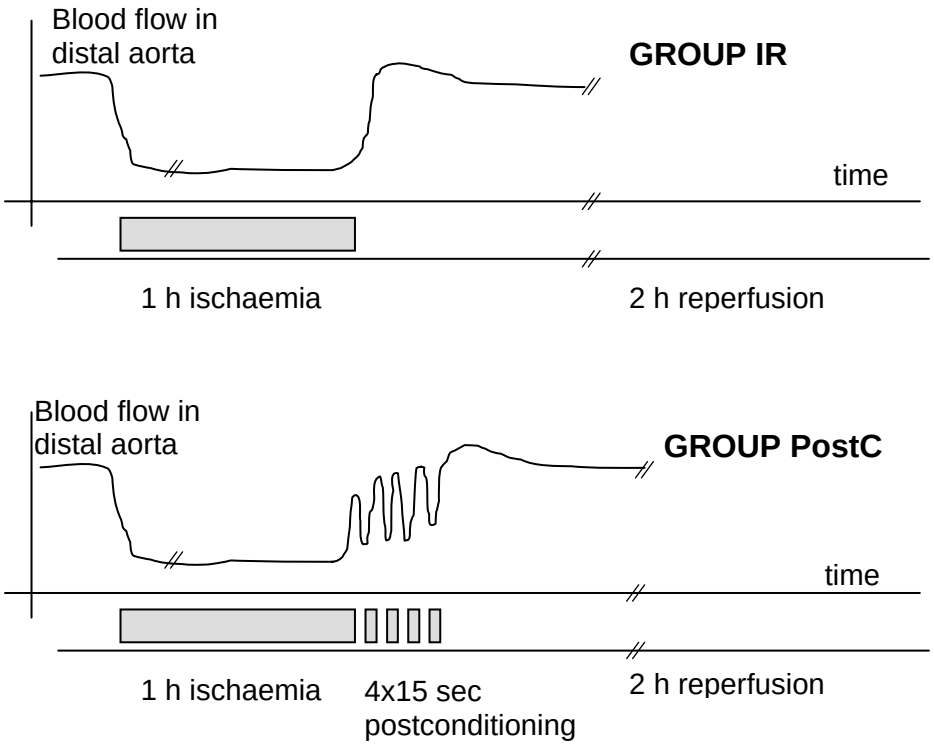


Figure 3.: Experimental protocols with schematic ilustration of distal aortic blood flow. (IR - ischaemia-reperfusion; PostC - ischaemic postconditioning)

3.2.3. Experimental groups

Rats were divided into three groups (8 animals in each group). In the first (ischaemia-reperfusion, IR) group the aorta was closed for 60 min and then a 120 min of reperfusion followed without interruption.

In the second (ischaemic postconditioning, PostC) group the infrarenal aorta was clamped for 60 min, and the early reperfusion was interrupted with 4x15 sec total reclamping of the aorta with an intermittent 15 sec perfusion. The animals then underwent 120 min of reperfusion. (*Figure 3*)

In the third group (control group) animals underwent a sham operation without aortic clamping.

Peripheral blood samples were collected before the operation, and in the early (5; 10; 15; 30; 60 and 120 min) reperfusion periods. The serum samples were harvested and stored at minus 78°C until biochemical assays.

3.2.4. Serum peroxide determination

Low density lipoproteins (LDL) are the most sensitive compounds in the blood for oxidative stress. Oxidation of LDL by oxidative stress in biological systems is principally a free radical process, where polyunsaturated fatty acids (PUFAS) in LDL are converted by lipid peroxidation to lipid hydroperoxides. For quantitative determination of serum peroxides OyxStat (Biomedica Medizinprodukte GmbH, Wien, Austria) colorimetric assay was used, following the manufacturer's instructions.

The results show a direct correlation between free radicals and circulating biological peroxides and thus allow the characterization of the oxidative status in reperfusion.

3.2.5. Serum TNF-alpha quantification

For measurement the TNF-alpha concentration in serum we used Rat TNF-alpha/TNFSF1A ELISA kit (R&D Systems, Inc. Minneapolis, USA), following the manufacturers protocol. This method determines the free i.e. biological active TNF-alpha concentration.

3.2.6. Serum myeloperoxidase assay

Anticoagulated blood was centrifuged with 2000g, and 200 µl plasma was mixed with 1 ml working solution (0,1 M sodium-citrate 10,9 ml, 0,05% Triton-X 100 5 µl, 1mM H_2O_2 1 ml, 0,1% o-dianisidine 100 µl). The mixture was incubated at 37 ºC for 5 minutes, then 1 ml 35% perchloric acid was added. Photometry were done at 560 nm. Plasma myeloperoxidase was expressed as nM/l. Hematologic measurement: Red blood cell count, white blood cell count, platelet numbers, haemoglobin concentration, haematocrit level were measured by Minitron automatic analysator (Diatron Ltd, Budapest, Hungary).

3.2.7. PMA-induced leukocyte ROS production

The induced ROS production of leukocytes was measured in whole blood. The superoxide anion production was induced with 0.2 µg/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich Ltd, Budapest, Hungary), and was detected with luminol 3.33 µg/ml (Boehringer Gmbh Mannheim, Germany;) on a Chrono-log 560-VS lumino-aggregometer (Chrono-log Corp., USA). We have registered the maximum rate of ROS production.

3.2.8. Pro- and antiapoptotic signaling pathways measurements

Western Blot Analysis

Tissue samples were collected from the skeletal muscle, heart, liver, kidney and lung. We examine the effects of ischaemia-reperfusion and PostC on the signaling pathways in these organs as well.

Samples of frozen cardiac powdered tissue (50 mg samples) were sonicated on ice in 200 μl fresh radioimmunoprecipitation assay lysis buffer containing 50 mM Tris HCl (pH 8.0), 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 1 μM NaF, 2 μM Na_3VO_4, 10 μM β-glycerophosphate, and 1 mini protease inhibitor cocktail tablet (Roche). The sonicated samples were then centrifuged at 13,000 rpm for 20 min at 4°C, and the pellet was discarded. Total protein concentrations of the supernatants were determined by using a bicinchoninic acid protein reagent kit (Pierce; Rockford, IL), with bovine serum albumin as a standard. Samples containing equal amounts of protein (30 μg) were prepared and separated by SDSPAGE using the NuPAGE gel system and 4–12% Bis-Tris Gels (Invitrogen; Carlsbad, CA) according to the manufacturer's instructions. The resolved proteins were transferred electrophoretically to nitrocellulose membranes (Invitrolon PVDF, Invitrogen; Carlsbad, CA) by 15 V for 10 h in a cold room using a NuPAGE transfer buffer (Invitrogen) with 10% methanol. The nitrocellulose membranes were incubated for 1 h in TBS-T (0.9% NaCl, 10 mmol/l Tris, and 0.1% Tween-20), supplemented with 5% milk to reduce nonspecific binding and incubated for 2 h at room temperature with a 1:5,000 dilution of anti-Akt antibody (rabbit, Cell Signaling; Beverly, MA), 1:5,000 dilution of anti-phospho-AktSer473 (Cell Signaling), 1:10,000 dilution of anti-ERK1/2 (mouse, BD Bioscience; San Diego, CA), 1:10,000 dilution of anti-phospho-ERK1/ 2Thr202/Tyr204 (BD Biosciences), 1:2,500 dilution of anti-pGSK (Cell Signaling), 1:1,000 dilution of anti-phospho-p38MAPK (Cell Signaling). The membranes were then washed and exposed to secondary antibody (Akt and phospho-Akt, goat anti-rabbit; ERK and phospho-ERK, goat anti-mouse; and pGSK and phospho-p38MAPK, goat anti-rabbit) immunoglobulin G conjugated with horseradish peroxidase (Cell Signaling), diluted 1:5,000 for Akt, phospho-Akt, p70S6K, and phospho-pGSK and 1:10,000 for ERK and phospho- ERK for 1 h at room temperature. Sites of antibody-antigen reaction were visualized, and the detection of signal was determined by using enhanced chemiluminescence (ECL Plus, Amersham, Piscataway, NJ) before exposure to photographic film. The developed films were scanned, and the band densities were quantified by densitometry (NIH Image v1.62 analysis software, NIH). Data for ERK and phospho- ERK represent the sum of the 42- and 44-kDa bands for each sample. For all experiments, control

minigels were run before Western blot analysis and stained with Coomassie brilliant blue (Bio-Rad; Hercules, CA), and several representative bands were quantified by densitometry to ensure equality of loading. Equal protein loading in each lane was confirmed by probing for -actin. Adequate transfer of proteins from the gel to the membrane was confirmed by Coomassie blue staining of the gel and Ponceau red staining (Sigma-Aldrich Ltd, Budapest, Hungary) of the membrane.

3.2.9. Statistical analysis

All values are expressed as means ± SE. Statistical comparisons include two samples Student t-test and one-way analysis of variance (ANOVA). $p<0.05$ was considered significant.

3.3. RESULTS

3.3.1. Hemodynamic Data

Hemodynamic data are summarized in Table 1. Baseline heart rate, systolic and diastolic pressure, and left ventricular rate-pressure product were not significantly different among the various groups, and IR and PostC did not differ from control at any measurement period. The rectal temperature was monitored and did not vary among groups. (*Table 1*)

*Table 1. Hemodynamic variables and body temperature at baseline. Values are means ± SE. No group was significantly different from control at any measurement period. IR, ischaemia-reperfusion; PostC, postconditioning. *p<0.05, statistical significance of difference from baseline.*

	Baseline	Occlusion	Reperfusion
Heart rate, beats/min			
Control	289±7	274±6	305±6
IR	296±5	310±9	302±7
PostC	273±5	311±4	299±4
Systolic blood pressure, mmHg			
Control	104±4	96±3	91±3
IR	104±3	95±4	97±5
PostC	110±4	100±3	89±3
Diastolic blood pressure, mmHg			
Control	83±3	79±2	74±3
IR	80±5	76±4	77±5
PostC	84±3	77±3	68±3
Temperature, °C			
Control	37.9±0.2	38.0±0.2	38.3±0.1
IR	37.5±0.2	38.0±0.2	38.1±0.3
PostC	37.9±0.2	38.2±0.2	38.2±0.3

3.3.2. Serum peroxide results

We measured in our animal model the serum total peroxide concentration during infrarenal aortic cross clamping ischaemia and reperfusion. In both groups we have detected a typical curve of serum peroxide changes with a rapid elevation ($p < 0.05$ vs. before surgery) in the

immediate phase of reperfusion and that was followed with a slow elimination period. Our data showed significant ($p< 0.05$) differences in the extent of early elevation between the two groups. The peroxide concentration was higher in IR group in the 5th (16.91±3.67 µM/l vs 10.04±1.9 µM/l) and in the 15th minutes (20.42±3.17 µM/l vs 13.77±2.84 µM/l) comparing to postconditioned group (PostC). In the depletion phase there was no difference between the groups. (*Figure 4*)

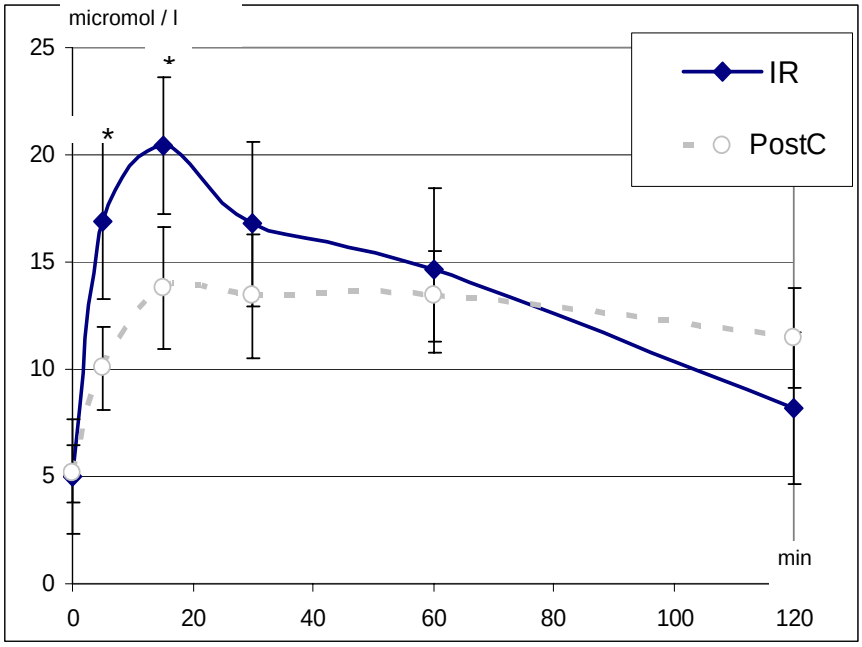


*Figure 4: Serum peroxide (oxLDL-OO) concentration changes ±SE in distal aortic cross clamping ischaemia and reperfusion. * p<0,05 (IR - ischaemia-reperfusion; PostC - ischaemic postconditioning)*

3.3.3. Serum TNF-alpha results

We measured the serum TNF-alpha levels before the ischaemia and after the ischaemia-reperfusion in the experimental groups. We have found that on the end of the reperfusion protocol the serum TNF-alpha concentration was elevated both in the postconditioned (PS) (116.55±12.04 pg/ml vs. 36.31±7.91 pg/ml) and in the non-conditioned (IR) (167.41±21.26 pg/ml vs 38.27±4.31 pg/ml) groups in comparison to the values before the ischaemia.

While both in the postconditioned and in the non-conditioned groups the serum TNF-alpha concentration after the reperfusion was higher than in the control group, in the

postconditioned group the concentration was significantly lower than in the non-conditioned group (116.55±12.04 pg/ml vs. 167.41±31.26 pg/ml). (*Figure 5*)

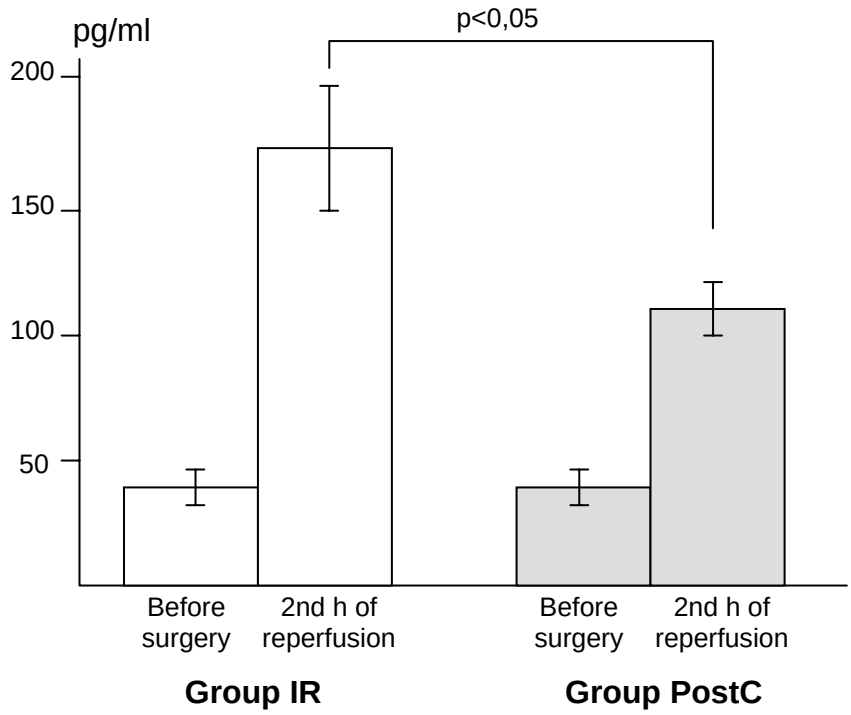


Figure 5: Serum TNF-alpha levels before the ischaemia and after the ischaemia-reperfusion in IR and PostC groups. (IR - ischaemia-reperfusion; PostC - ischaemic postconditioning)

3.3.4. Serum myeloperoxidase results

To characterize the neutrophyl activation we measured the plasma myeloperoxidase (MPO) level and the induced ROS production of leukocytes. MPO level increased significantly after ischaemia-reperfusion in non-conditioned group (group IR: 1.759 ± 0.239 μM/ml vs 1.108 ± 0.143 μM/ml). In the postconditioned group there was no elevation before and after the ischaemia-reperfusion (1.22 ± 0.126 μM/ml vs 1.179 ± 0.182 μM/ml). To compare the values of postconditioned and non-conditioned groups we found a significant difference (1.759 ± 0.239 μM/ml vs 1.22 ± 0.126 μM/ml $p<0.05$). (*Figure 6*)

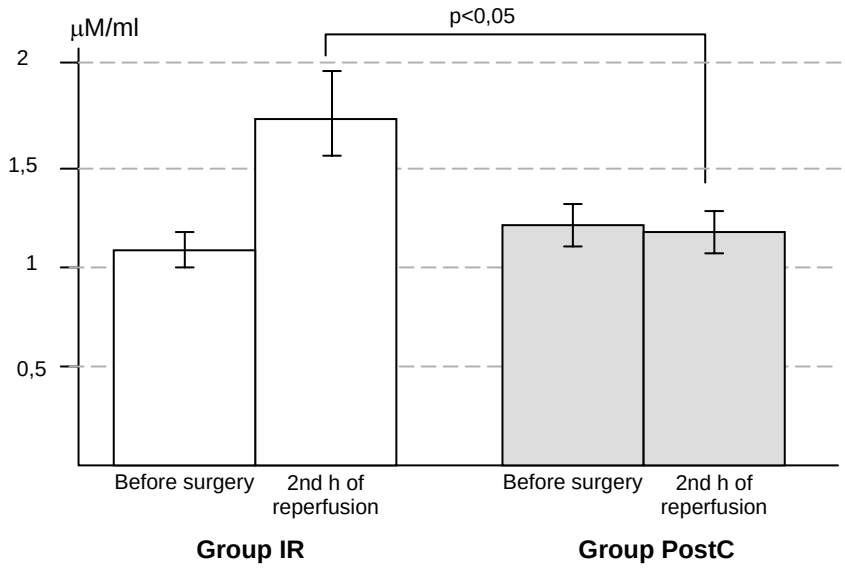


Figure 6: Serum myeloperoxidase (MPO) concentration in postconditioned and in non-conditioned groups. After the ischaemic-reperfusion protocol MPO was significantly lower in postconditioned than in non-conditioned group. (IR - ischaemia-reperfusion; PostC - ischaemic postconditioning)

3.3.5. PMA-induced leukocyte ROS production

The phorbol-myristate-acetate (PMA) induced maximal ROS production of the leukocytes shows their activation level. The results are demonstrated on figure 7. We have found a significant elevation in ROS production in the non-postconditioned group (IR) before and after the ischaemia-reperfusion period (2.59 ± 0.95 AU/10^3 cells vs 5.7 ± 0.96 AU/10^3cells; $p<0.05$). In the postconditioned group there was no significant change detected (3.89 ± 0.94 AU/10^3cells vs 4.63 ± 0.69 AU/10^3cells).

There was no significant change in the white blood cell (WBC) count during the protocol in both groups (IR: 9.25 ± 5.22 x10^3cells/μl vs 13.45 ± 7.23 x10^3cells/μl; PostC: 5.08 ± 0.54 x10^3cells/μl vs 9.38 ± 2.35 x10^3cells/μl).

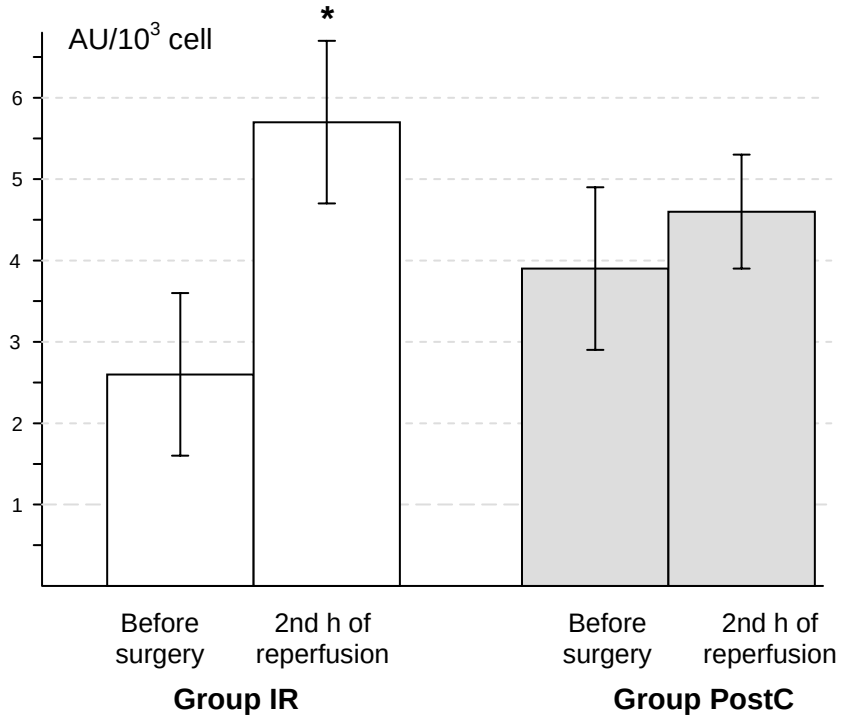


*Figure 7.: The phorbol-myristate-acetate (PMA) induced maximal ROS production of the leukocytes after the experimental protocol. In postconditioned group the activation of the leukocytes was significantly lower than in non-conditioned group. (IR - ischaemia-reperfusion; PostC - ischaemic postconditioning; * $p<0.05$ vs before surgery)*

In the ECG and blood pressure parameters, we did not find any significant differences between the postconditioned and non-conditioned groups. The hematocrit did not show significant changes during the experimental protocol.

3.3.6. Pro- and antiapoptotic signaling pathways

Western-blot analysis shows that in the 2nd hour of reperfusion in the ischaemia-reperfusion groups in skeletal muscle, in myocardium and in the lung pAKT was activated, and ischaemic PostC could further increase this activation in these tissues. In the liver we could not detect real pAKT activation, while in the kidney the activation was detectable, but neither ischaemia-reperfusion, nor PostC increased this.

PGSK in skeletal muscle, and in the heart was increased in I/R group vs controll, and as seen in figure PostC caused further elevation. In the liver, kidney and lung a similar tendency could be seen. (*Figure 8*)

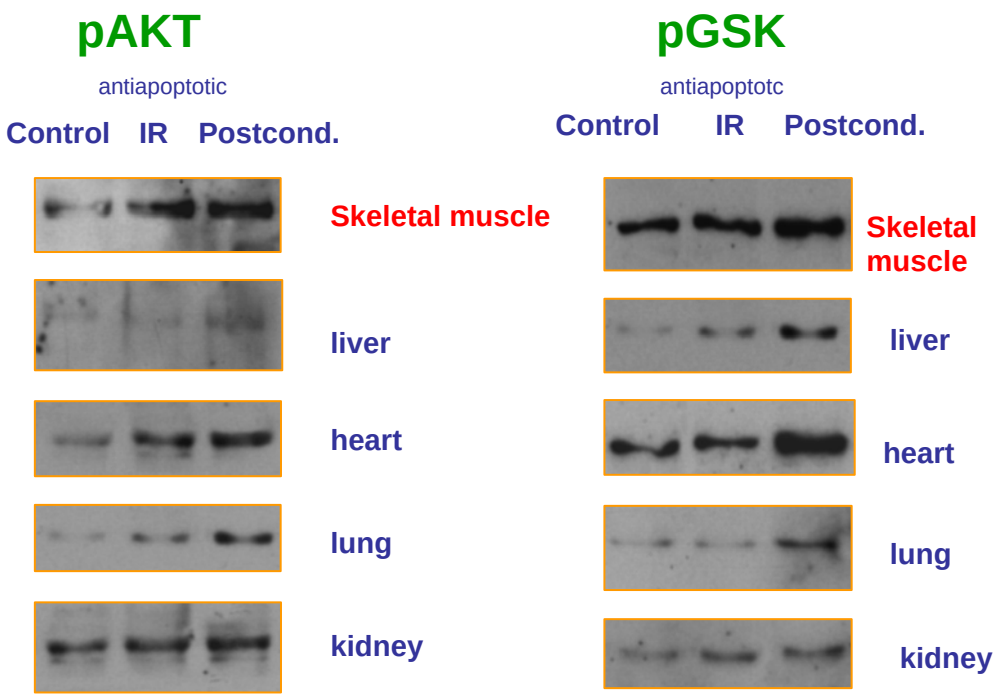


Figure 8: Representative Western blots and relative densities of the phosphorylated (p) form of pAKT (left), and pGSK (right), from tissue samples of the animals removed after 60 min of ischaemia and 2 h of reperfusion in control, ischaemia-reperfusion group and PostC group. Densities show that IR and PostC similarly activate phosphorylation of these proteins.

The activation of pERK1 and 2 could be detected in all examined tissues, and this antiapoptotic marker was increased with ischaemic PostC.

As seen in picture the proapoptotic pp38MAPK was increased in ischaemic-reperfusion group in all tissues, and this activation was less in the PostC group. (*Figure 9*)

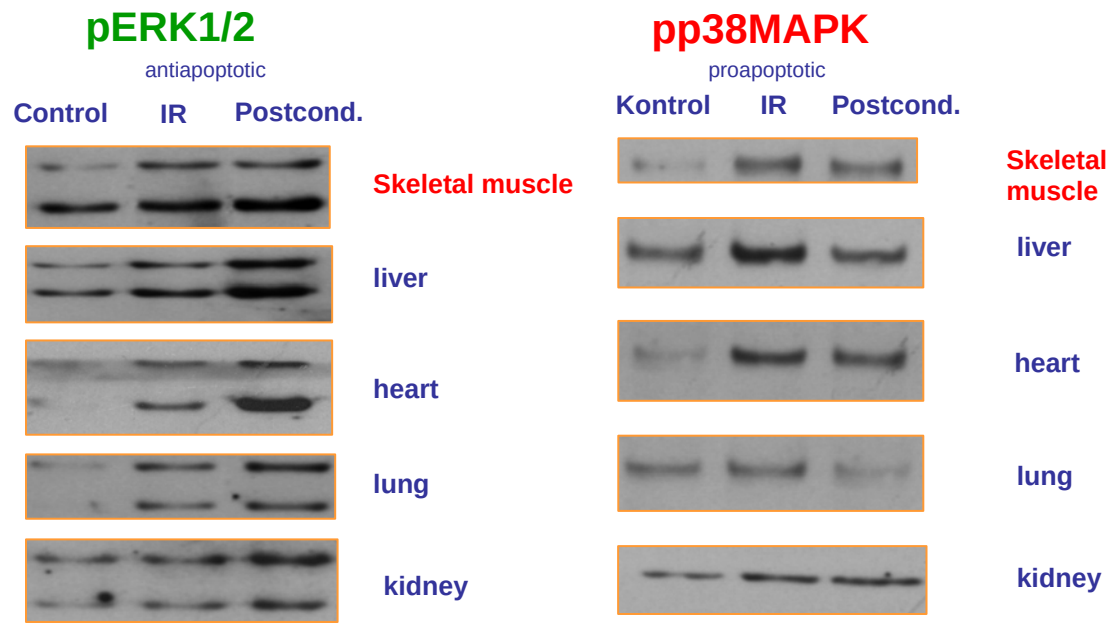


Figure 9.: Representative Western blots and relative densities of the phosphorylated (p) form of pERK1/2(left), and pp38MAPK (right), from tissue samples of the animals removed after 60 min of ischaemia and 2 h of reperfusion in control, ischaemia-reperfusion group and postcond group. Densities show that IR and Postcon similarly activate phosphorylation of these proteins.

4. EFFECTS OF ISCHAEMIC POSTCONDITIONING IN HUMAN VASCULAR SURGERY

4.1. INTRODUCTION

Ischaemic postconditioning was found effective to reduce reperfusion injury not only in experimental animal models, but in humans as well in cardiac interventions. Our first series of examinations has confirmed that ischaemic PostC could also be effective in peripheral tissues, thus has real clinical potential in vascular surgery. To examine the effectiveness of PostC in humans we applied ischaemic PostC during aorto-bifemoral bypass surgery and measured the extent of reperfusion injury.

4.2. PATIENTS AND METHODS

Patient selection for this prospective randomized study performed according to the Helsinki Declaration (1996), considering the statute of Hungarian Ministry of Health (35/2005.(VIII:26.)) with the permission of local ethical board of the Pécs University Medical School (No of permission: 2498). Blood samples were collected in three Vacutainer tube containing trisodium citrate (3.8%) and one containing K3-EDTA (7.5%; Becton Dickinson, UK; blue or purple, respectively), before, and two and 24 hours, then one week after the surgery. All human subjects provided formal informed consent.

4.2.1. Aorto-bifemoral bypass surgery

In general anaesthesia median laparatomy was performed. After physical examination of the abdominal organs we prepared the distal abdominal aorta. Intravenous 7500 IU unfractionated heparine was given. After occlusion of the aorta a 3 cm longitudinal aortotomy was made. High pressure inflow could be detected from the central aorta. Dacron Y-graft (size depending on the diameter of the vessels) proximal end-to-side anastomosis was sutured with 4/0 Premilene (polypropilene monophylament, B-Braun Aesculap, Tuttlingen, Germany).

We isolated the common femoral artery and its sidebranches (deep and superficial femoral artery). 3 cm longitudinal arteriotomy was made on the common femoral artery. Exploration of distal flow was checked Fogarty catheter. The distal branches of Y-graft are tunneled under the inguinal ligament, and on both sides an end-to-side anastomosis was performed to the

common femoral artery with 5/0 Premilene running suture. Followed by haemostasis, drain was placed, and the wound was closed.

All patients completing the study suffered from general atherosclerosis with distal aortic or aorto-biiliac occlusion. All patients received antiplatelet therapy (at least 75 mg Aspirin) before the recruitment. Low molecular weight heparin was administered in the perioperative period. Ten healthy blood donors served as controls for the measurements (Control group). The patients with other chronic inflammatory disease, or gangrene were excluded from the study. After intragroup analysis the patients with significantly deviating results (caused by polytransfusion, extreme intraoperative blood loss, or any postoperative complication) we excluded from the study.

4.2.2. Human ischaemic postconditioning protocol

In the postconditioned group (10 patients) after the completion of the distal anastomosis, before starting the reperfusion we made two cycles of 30 sec reperfusion-reocclusion on the graft. After this two cycles of reperfusion-reocclusion we let the continuous reperfusion to the distal artery.

In the ischaemia-reperfusion group (10 patients) after the distal anastomosis we started the continuous perfusion.

4.2.3. The measurement of oxidative stress parameters:

Measurement of malondialdehyde (MDA):

Malondialdehyde was determined in anticoagulated whole blood, by photometric method[62].

Measurement of reduced glutathione (GSH) and plasma thiol (SH) groups:

GSH and plasma SH levels were determined from anticoagulated whole blood (ethylene diamine tetraacetic acid (EDTA)) by Ellman's reagent according to the method of Sedlak and Lindsay[63].

Measurement of Superoxide dismutase (SOD) activity in washed red blood cell (RBC):

The main principle of this measurement was that adrenaline is able to spontaneously transform to adrenochrome (a detectable colorful complex). This transformation can be blocked by SOD, and SOD containing cells or tissues. The difference in the rate of rise of control and sample curves obtained at 415 nm, are proportional to SOD activity[64].

4.2.4. Measurement of inflammatory response, leukocyte activation

Determination of free radical production from whole blood:

Free radical production was induced by 30 µl phorbol-12 myristate 13-acetate (PMA; 0,2µg/ml) (Sigma Aldrich Budapest); in the mixture of whole blood (20 µl), phosphate buffered saline (1400 µ) and 50 µl luminol (3.33 µg/ml; Boehringer Mannheim Gmbh Germany), and was detected by Chrono-Log Lumino-aggregometer.

Measurement of plasma myeloperoxidase:

The method was described in 3.2.6.

Leukocyte adhesion molecule measurement:

The leukocytes were marked with fluorescein isotiocianide (FITC) labeled antibodies for adhesion molecules (CD11, CD11b, CD18, CD49d, és CD97) (Becton Dickinson Biosciences, Pharmingen, USA), and measurements were performed on BD FacsCalibur (Becton Dickinson Biosciences, Pharmingen, USA) flowcytometer. [65] [66]

4.2.5. Hematology test

Red blood cell count, white blood cell count, platelet numbers, haemoglobin concentration and haematocrit level were measured by Minitron automatic analysator (Diatron Ltd, Budapest, Hungary).

4.2.6 Statistical analysis

Data are expressed as mean ± SE, or percentage. For analysis of data, paired and unpaired Student's t-test, and one-way analysis of variance (ANOVA) were used. Statistical significance was established at $p<0.05$.

4.3. RESULTS

Plasma malondialdehyde concentration before surgery was similar to the control group. A significant increase was detected in both group right after the reconstruction, but this elevation was significantly higher in the non-conditioned group. Same results were measured 24 hours later and the MDA plasma concentration decreased to the initial values after 7 days. (*Figure 10*)

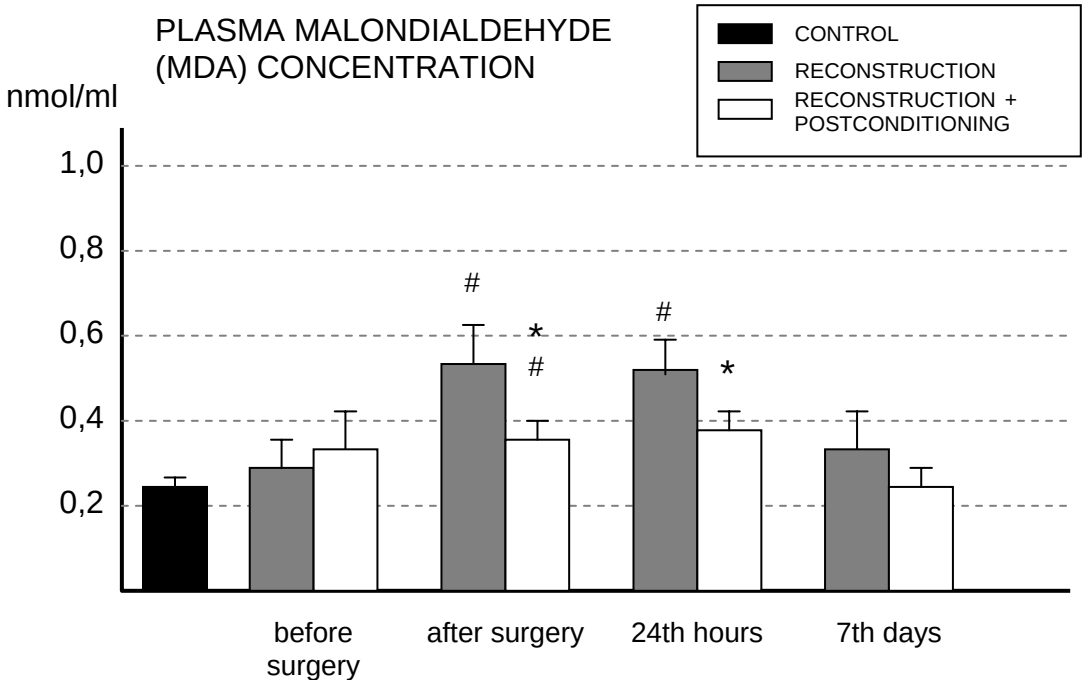


*Figure 10: Changes in plasma malondialdehyde concentration in the patients following the operations. (# p<0.05 vs before surgery; * p<0.05 vs non-conditioned group)*

Measuring the antioxidant enzyme plasma levels we observed that the thiol group concentration in non-conditioned group significantly decreased in the early reperfusion period. The 24 hours values did not show significant changes compared to control and initial values, but after a week in the non-conditioned group a slight decrease was detectable (the second waves of reperfusion injury: mediated by not the ischaemia-reperfusion, but the inflammatory response activated leukocytes[67]).

In the plasma level of reduced glutathion, a significant decrease was detectable in the early reperfusion in both groups. From the first day a continuous elevation was observed until the 7th day and the plasma level in both groups returned to the values before surgery. (*Figure 11*)

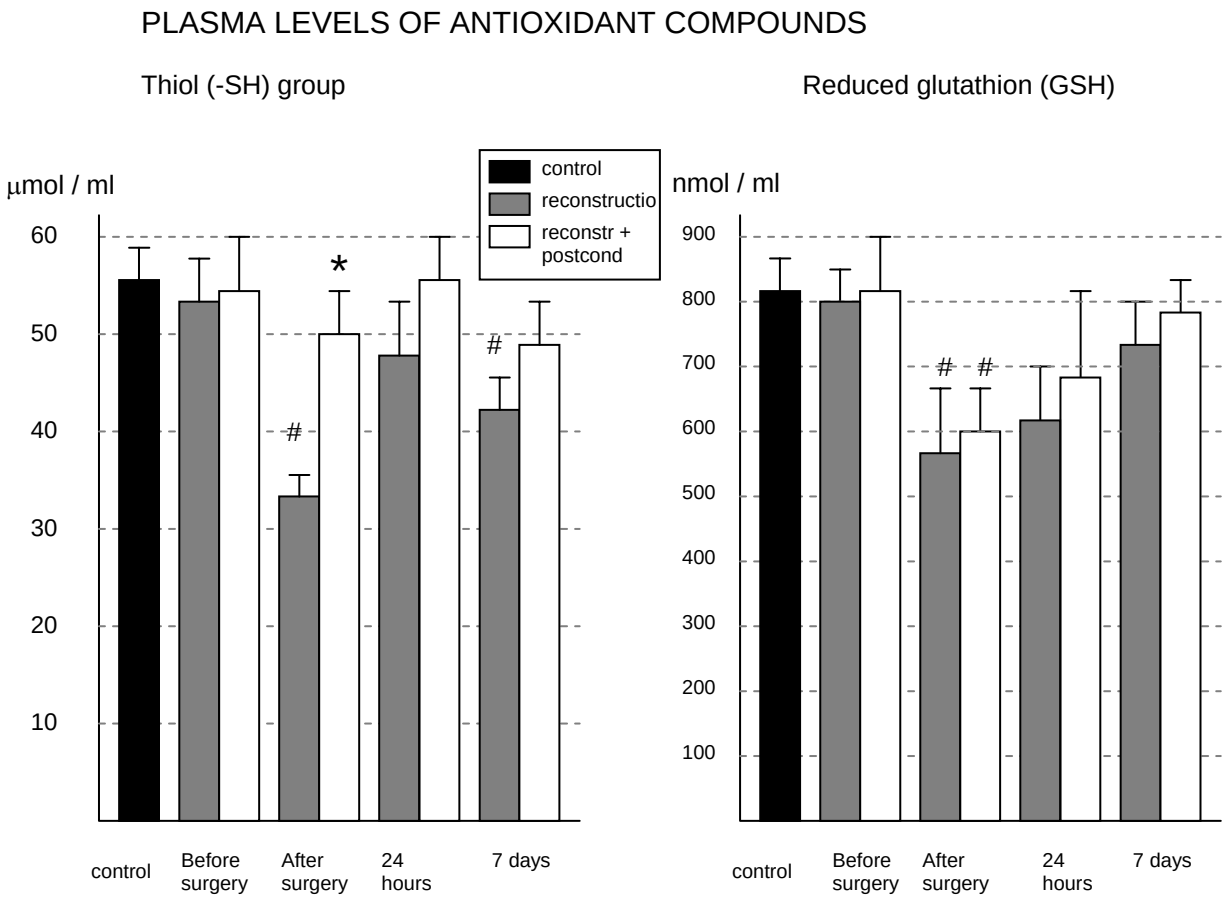


*Figure 11: Changes in antioxidant compounds (thiol group, reduced glutahtion) plasma levels during the examined perioperative period. (# p<0.05 vs before surgery; * p<0.05 vs non-conditioned group)*

The activity of superoxide dismutase before surgery was lower in both groups compared to the control group, and did not show any changes right after the operation. 24 hours later in the non-conditioned group we detected a significant decrease, which disappeared at the end of the week. (*Figure 12*)

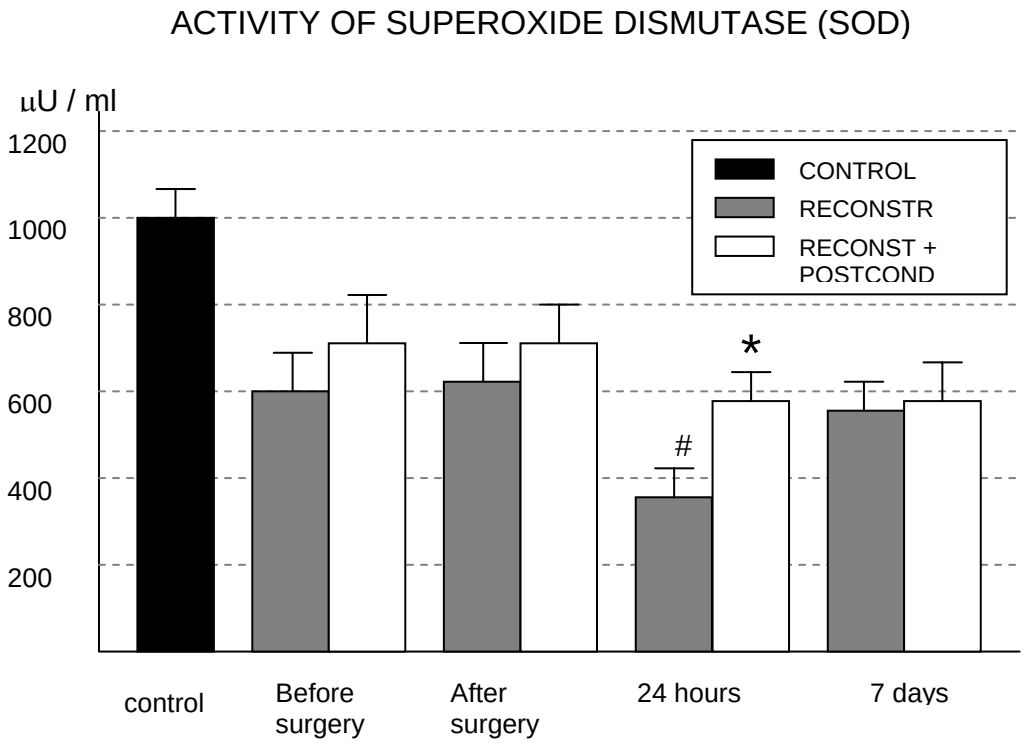


*Figure 12: Changes in blood superoxide dismutase activity during the examined perioperative period. (# p<0.05 vs before surgery; * p<0.05 vs non-conditioned group)*

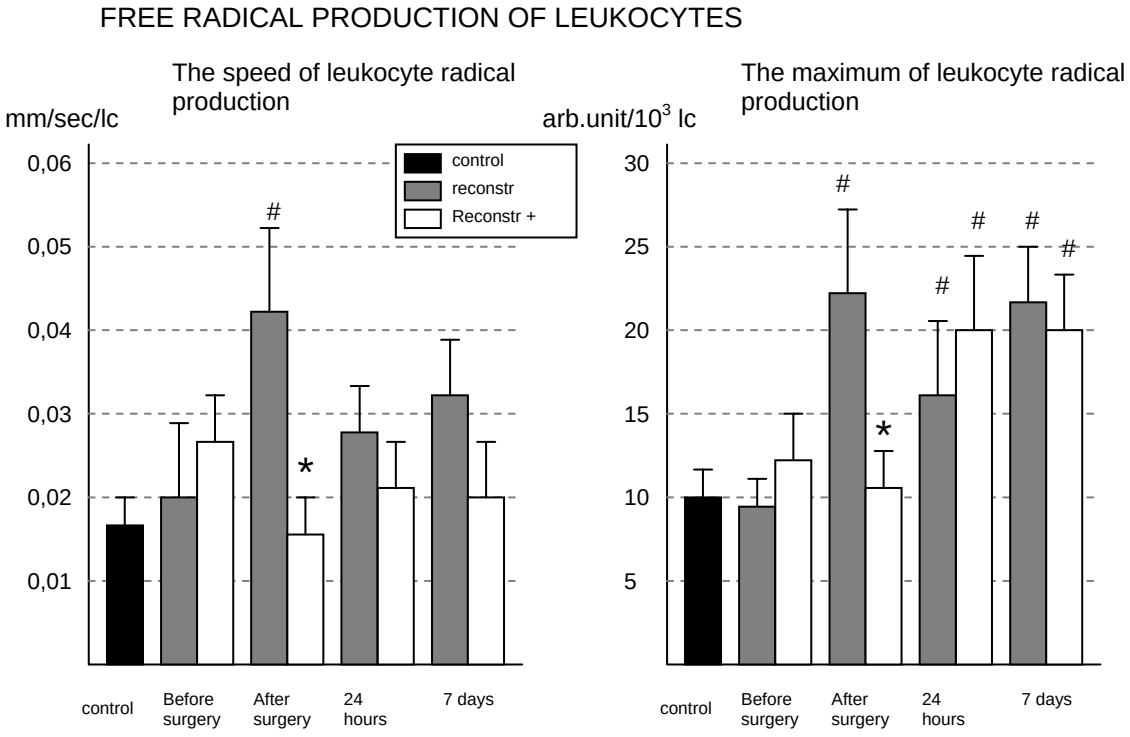


*Figure 13: Changes in the PMA induced free radical production of leukocytes during the perioperative period. We demonstrated the result of the speed and the maximum of radical production. (# p<0.05 vs before surgery; * p<0.05 vs non-conditioned group)*

Leukocyte activation increased significantly immediately after revascularisation surgery in the non-conditioned group, and this elevation could not be observed in the postconditioned group. In the late reperfusion period the maximum of leukocyte-derived free radical production were elevated in both group withouth significant difference between the two groups. (*Figure 13*)

The plasma myeloperoxidase (MPO) concentration was higher in both investigated groups than in healthy control group. We did not observed any significant changes until the 7th day. On the last day of the protocol the plasma MPO concentration elevated significantly in the non-conditioned group, and this elevation was not detectable in the postconditioned group. (*Figure 14*)

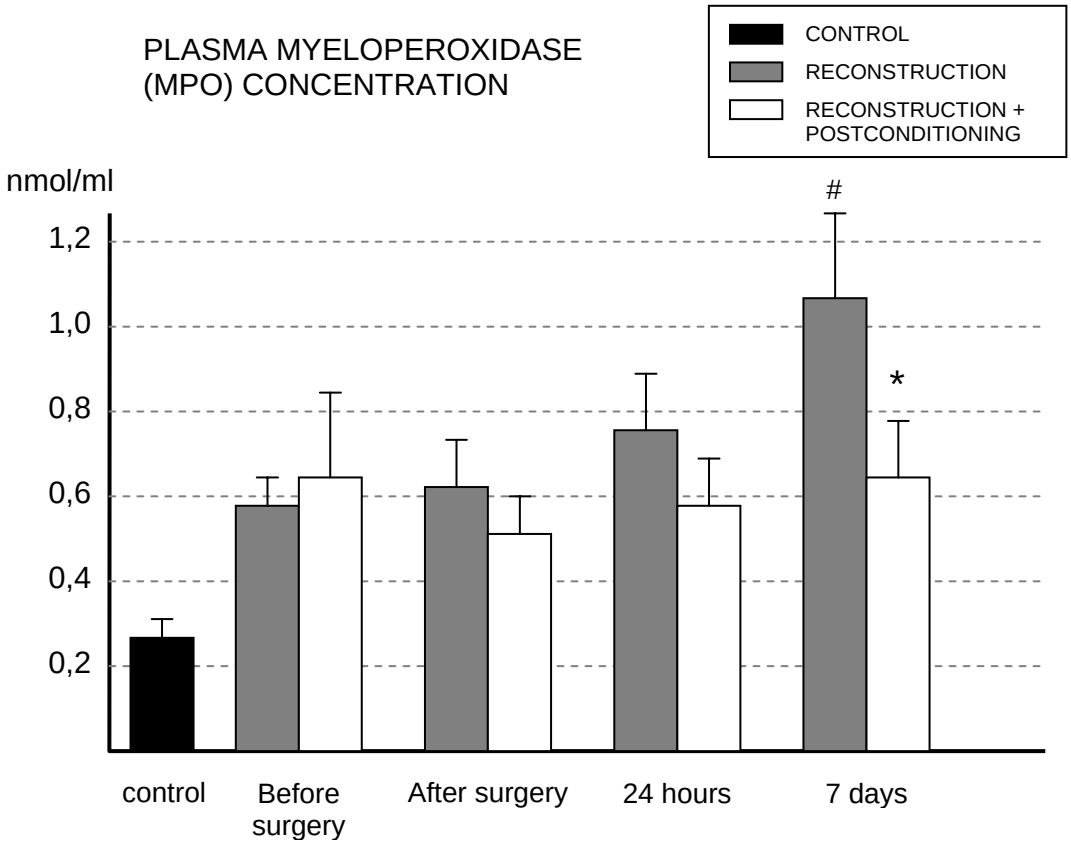


*Figure 14: Changes in plasma myeloperoxidase following operation (# p<0.05 vs before surgery; * p<0.05 vs non-conditioned group)*

Granulocyte surface adhesion molecules were detected by flowcytometer. The detectable expression of CD11a adhesion molecules were significantly lower in the postoperative first samples than before surgery. There was no significant difference at this time between the two groups. After 24 hours in the non-conditioned group a significant expression was observed, which was not detected in the postconditioned group. At the end of the one week period the values reached the starting values.

CD18 showed a significant decrease in the immediate reperfusion period in both groups, and after these changes were the same as the control values. (*Figure 15*)

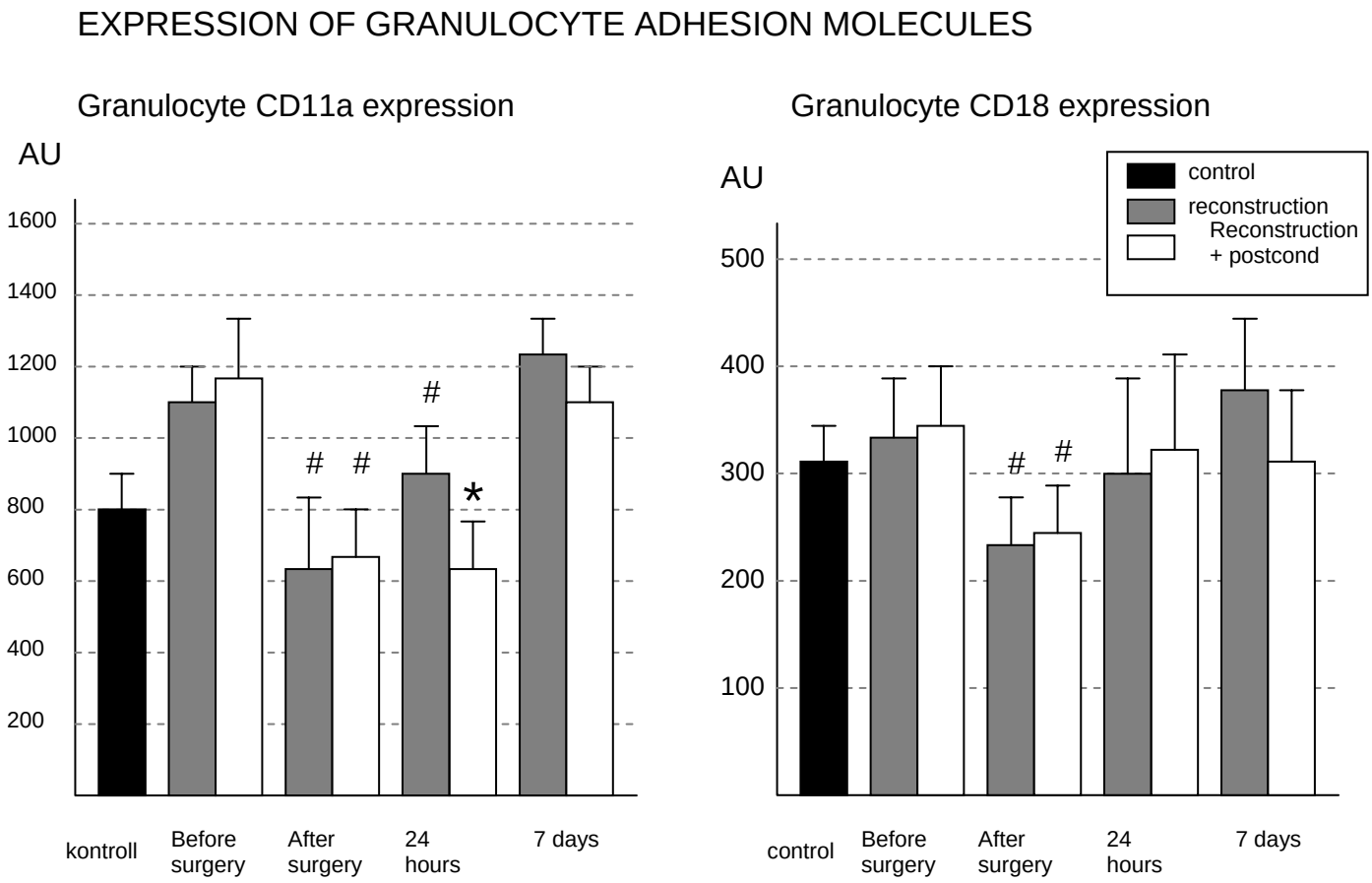


*Figure 15: The graphs show the changes in expression of granulocyte adhesion during the examined perioperative period. (AU= arbitary unit) (# p<0.05 vs before surgery; * p<0.05 vs non-conditioned group)*

In the results of the red blood cell count, white blood cell count, platelet numbers, haemoglobin concentration and haematocrit level we did not detected any difference between the two groups of patient.

4.4. DISCUSSION

In the last 3 years the literature of ischaemic postconditioning exponentially increased in the experimental cardiology. The beneficial effects of the manoeuver has been confirmed in various models, including human results as well, and the cellular and biochemical background is intensively examined. Until now this is the first study to evaluate the effect of ischaemic postconditioning on peripheral tissues in abdominal aortic surgery.

Our results demonstrated that after a prolonged ischaemia, postconditioning can reduce free radical production, TNF-alpha expression and leukocyte activation in the early phase of reperfusion in an animal model of abdominal aortic surgery. In this model we also confirmed that PostC could induce antiapoptotic signaling pathways in the skeletal muscle and in far organs as well.
In our human model we could confirm that ischaemic PostC could decrease in some points the revascularization surgery evoked oxidative stress and inflammatory response.

We have demonstrated that the protective effect of postconditioning is a complex process, involving many cell types, the generation of oxidants, cytokines, and inflammatory pathways, has not only one target, but acts on a diverse site. This complexity, the powerful protective effect and the simplicity in the surgery (lasts for a few minutes) can make the manoeuver really a powerful tool of surgeons.

4.4.1. MECHANISMS INVOLVED IN POSTCONDITIONING

The mechanisms of protection by PostC were initially attributed mainly to improved endothelial function and to the events reducing the detrimental effects of lethal reperfusion injury, such as reduced edema, reduced oxidative stress, reduced mitochondrial calcium accumulation, reduced endothelium damage and reduced inflammation. However, subsequent studies suggest that protection is mediated through the recruitment of signal transduction pathways as in the case of ischaemic preconditioning. Therefore, a distinction in passive and active mechanisms can be proposed. Of course an intricate cross-talk among these events/mechanisms exists, thus this distinction can be useful for a better understanding of the

phenomenon, but we must not forget that a single event/mechanism may not be effective if it occurs alone. (*Figure 16*)

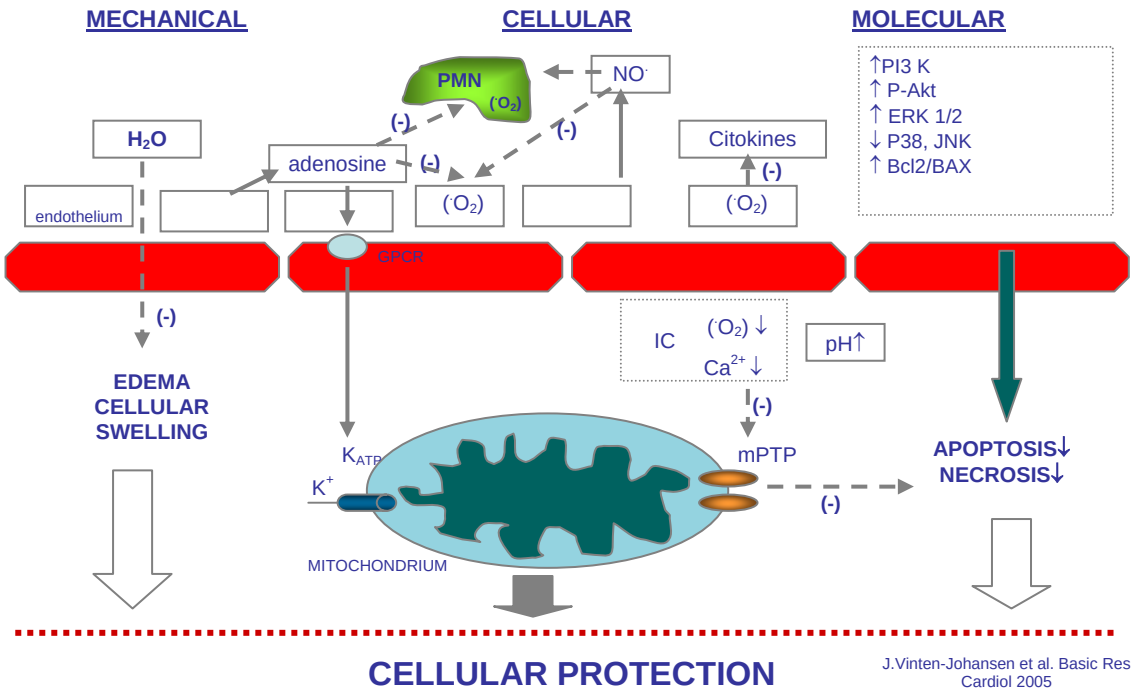


Figure 16: A schematic figure on the mechanisms of ischaemic postconditioning.

4.4.1.1. PASSIVE MECHANISMS

Among passive mechanisms we can consider those strictly related with hydrostatic force – hereafter named as 'Mechanical mechanisms' – and those related with reduced endothelial adhesion of leucocytes and subsequent reduction of inflammatory process that we call 'Cellular mechanisms'.

Mechanical mechanisms

With regard to mechanical or haemodynamic mechanisms, it has been suggested that the stuttering of reperfusion and pressure during PostC manoeuvres may limit the hydrostatic forces in a very important moment, thus limiting early edema and consequent damages. In experiments performed in isolated heart models, the effect of the PostC on the infarct area has been studied perfusing the hearts either with constant pressure or with constant flow. It has been compared the role of these two types in perfusion in affecting the infarct area during

PostC. In the constant pressure model the infarct area was less reduced by PostC than it was with the model of the constant flow reperfusion after PostC[68]. Considering that during the short period of restoration of flow in the PostC manoeuvres the capillary pressure increases less in the constant flow model, than in the constant pressure model (i.e. at the beginning of reperfusion in the constant flow model there is smaller hydrostatic pressure and so smaller transcapillary pressure), it was argued that in the constant-flow model a reduced edema and consequent reduced damages may explain the increased effectiveness of PostC. In other words, in the constant flow model the effectiveness of PostC is greater than in the constant pressure model supports the idea that the reduction of hydrostatic forces during PostC manoeuvres may play an important role in determining the protective effects.

Cellular mechanisms

Among the cellular mechanisms we consider acute inflammatory response. It occurs through the release of cytokines, activation of vascular endothelial cells and leukocytes with expression of cell surface adhesion molecules, and up-regulation of a program of proinflammatory genes. PostC delays the onset and reduces the maintenance of post-ischaemic inflammation[69]. As stated before, whether this is a cause or an effect of PostC protection remains to be elucidated.

4.4.1.2. ACTIVE MECHANISMS (intracellular mechanisms)

Studies have identified a signalling pathway that is recruited at the time of reperfusion and which is similar in ischaemic preconditioning and PostC. This pathway includes the survival kinases phosphatidylinositol 3-kinase (PI3K)-Akt and Erk1/2, the major components of the reperfusion-injury salvage kinase pathway, termed the RISK- pathway, which may influence the mPTP, a non-specific pore of the mitochondrial membrane whose opening in the first few minutes of myocardial reperfusion promotes cell death[70]. Delayed washout of endogenously produced adenosine and activation of the adenosine receptor also seems to be required for PostC protection[71], by activating the survival pathway.

Thus delayed washout of adenosine in the setting of PostC might recruit RISK at the time of reperfusion through the activation of adenosine responsive G-protein-coupled receptors. It seems that adenosine receptors are repopulated during PostC manoeuvres. While in murine hearts adenosine A2a and A3 subtypes [72] have been seen to be involved, in rabbit hearts PostC seems to depend on A2b subtype [73]. An important role of the redox environment has also been observed[74].

Therefore, similar to preconditioning, PostC has been proposed to be triggered by receptor stimulation, mediated by one or more complex and interrelated signal transduction pathways, and, ultimately, achieved via phosphorylation of one or more end-effectors of cardioprotection[75].

4.4.2. Triggers of postconditioning

Ligands, such as adenosine [76] and bradykinine [77] what accumulate during PostC manoeuvres may initiate the cascade that lead to PostC protection. It has been recently reported that inhibition of opioid receptors with opioid antagonists administered 5 min. before reperfusion in the absence or presence of PostC, reversed the infarct sparing effect of PostC in an in vivo rat model[78]. The activation of protein kinase C and G (PKC and PKG) and opening of mitochondrial K_{ATP} channels after PostC (see below) would be consistent with the involvement of BK and endogenous opioids. Nitric oxide and ROS may be included among the triggers. Nitric oxide is demonstrated to act both as a trigger and as a mediator of the preconditioning response in a variety of species. The role of endogenous NO in classic ischaemic preconditioning was controversial. Cohen and Downey's group suggested that exogenously administered NO could trigger the preconditioned state through a free radical-mediated process not shared by endogenous NO. Very recently these authors questioned whether their observation was due to a bias in the experimental model. These authors are now on the opinion that endogenous NO participates in triggering in vivo preconditioning[79]. Among the autocaids released by the ischaemic heart there is BK that may induce nitric oxide release (*Figure 3*). It has been suggested that the mechanism whereby NO protects myocardium includes the activation of guanylate-cyclase[80]. As an inducer of the protection, nitric oxide may also directly open the mitochondrial K_{ATP} channels[81]. Therefore, nitric oxide acting on mitochondria may play a relevant role in protection both through activation of these channels and via modulation of respiratory chain; both mechanisms favor ROS signalling, which can trigger protection[82]. A relevant role of nitric oxide may also be attributed to the endothelial protection brought by this molecule [83] or to its role as antioxidant under certain conditions [84].

The one-electron-reduction product of nitric oxide, HNO/NO– (nitrosyl hydride/nitroxyl anion), has been scarcely studied in an I/R scenario. In our laboratory low doses of Angeli's salt, a donor of HNO/NO–, have been seen to induce early/classical preconditioning against myocardial damages[85]. Intriguingly, the protective effects of HNO/NO– generated by Angeli's salt were more potent than the protective effects induced by equimolar concentration

of the pure nitric oxide donor diethylamine/nitric oxide (DEA/NO). While the HNO/NO–donor seems deleterious in reperfusion[86], there is evidence that NO may also be involved in the cardioprotection by ischaemic PostC. When the nitric oxide synthase (NOS) inhibitor N-omega-nitro-L-arginine methyl esther (LNAME) was given 5 min. before start of reperfusion of *in vivo* rabbit hearts, the infarct limiting effect was abolished[87]. We have shown that nitric oxide participates in PostC, but NOS inhibitors given for the entire reperfusion period only blunted the protective effect of PostC[88] . Paradoxically, the same inhibitor, given only during PostC manoeuvres completely blocked the protective effects[89]. At the moment, we do not have an explanation for this apparent paradox. In a previous study, we argued that nitric oxide may be produced in post-conditioned heart both by NOS and by non-enzymatic mechanisms. Nitric oxide can then activate the guanyl cyclase to produce cyclic guanosine monophosphate (cGMP), which mediates protection [90] (see also below). The infusion of a NOS inhibitor only during PostC manoeuvres may alter the equilibrium between ROS and nitric oxide thus leading to the production of the wrong kind of radical which does not trigger the protective pathway. It can be argued that in the absence of this protection the stronger limitation of nitric oxide production by NOS may be protective during reperfusion. In fact, data have demonstrated that NOS inhibitors can attenuate I/R damage[91]. Also, the different doses of nitric oxide inhibitors applied and the different basal levels of nitric oxide endogenously produced may explain these disparities.

The beneficial and deleterious effects of nitric oxide and nitrite in pathophysiological conditions and contradictory results about the effects of nitric oxide during reperfusion have been reviewed by Bolli in 2001[92], Wink et al. in 2003[93], Pagliaro in 2003[94] and Schulz et al. in 2004 [95]. ROS could also be included among the triggers of PostC. In fact, ROS scavengers such as N-acetylcysteine and 2-mercapto-propionylglycine given during PostC manoeuvres prevent the protective effects[96]. It is possible that the low pH during the PostC cycles prevents mPTP opening, while the intermittent oxygen bursts allow mitochondria to make enough ROS in a moment in which other enzymes, able to produce massive quantity of ROS, are not yet re-activated. Then mitochondrial ROS may activate PKC and put the heart into a protected state. The importance of the role of acidosis in the triggering of PostC protection has been recently confirmed by two independent laboratories[97]. Acidosis may also prevent mPTP opening in the early reperfusion (see below). Recently, it has been reported that redox signaling and a low pH at the time of myocardial reperfusion are also required to mediate the cardioprotection triggered by ischaemic preconditioning[98].

4.4.3. Mediators of postconditioning

We considered ROS among triggers as they are necessary during PostC manoeuvres. Nevertheless, PostC activated the RISK pathway, with increased expression of the phosphorylated form of endothelial nitric oxide synthase (eNOS) as one of the results[99]. Thus it is likely that after NOS activation the cGMP is produced and PKG is activated; then mitochondrial ATP-dependent potassium (mK_{ATP}) channels are opened and ROS produced. Therefore cGMP, PKG, mKATP and ROS may be considered as mediators of PostC protection, which are likely to be upstream to PKC activation. We demonstrated that cGMP production is increased during reperfusion of postconditioned hearts[100]. Moreover we showed that in these hearts mKATP and PKC must also be active (i.e. they should not be blocked) during late reperfusion[101]. Regarding the role of mK_{ATP} channels a couple of papers indicate that the mKATP channel is important for PostC[102]. In these studies two different mK_{ATP} channel blockers (glibenclamide and 5-hydroxidecanoate) abolished the protective effect of PostC [103].

It is interesting that many of the RISK elements (e.g. PI3K/Akt and MEK1/2-ERK) involved in the signaling pathway in preconditioning and protection against reperfusion injury have recently been documented also in PostC[104]. Some differences, however, may exist between pre- and PostC (see also Table 1 and Table 2). Darling et al. [105] showed an increase of phospho-ERK, but not of PI3K/Akt in PostC, while Yang et al.[106] showed that ERK is involved in PostC, but not in preconditioning. These findings may explain a certain degree of additive protection between ischaemic preconditioning and PostC, as observed by Yang et al.[107]. Yet in contrast with Yang et al.[108], Cao et al.[109] reported that ERK is present in preconditioning trigger pathway. The reasons for the differences are not clear. Different species and/or protocols may play a role[110]. Different methods of tissue sampling also be may play a role[111]. Besides protein kinase C, the possible roles for tyrosine kinase, and members of the MAPK family other than ERK1/2 in PostC has been suggested[112]. (*Figure 17*)

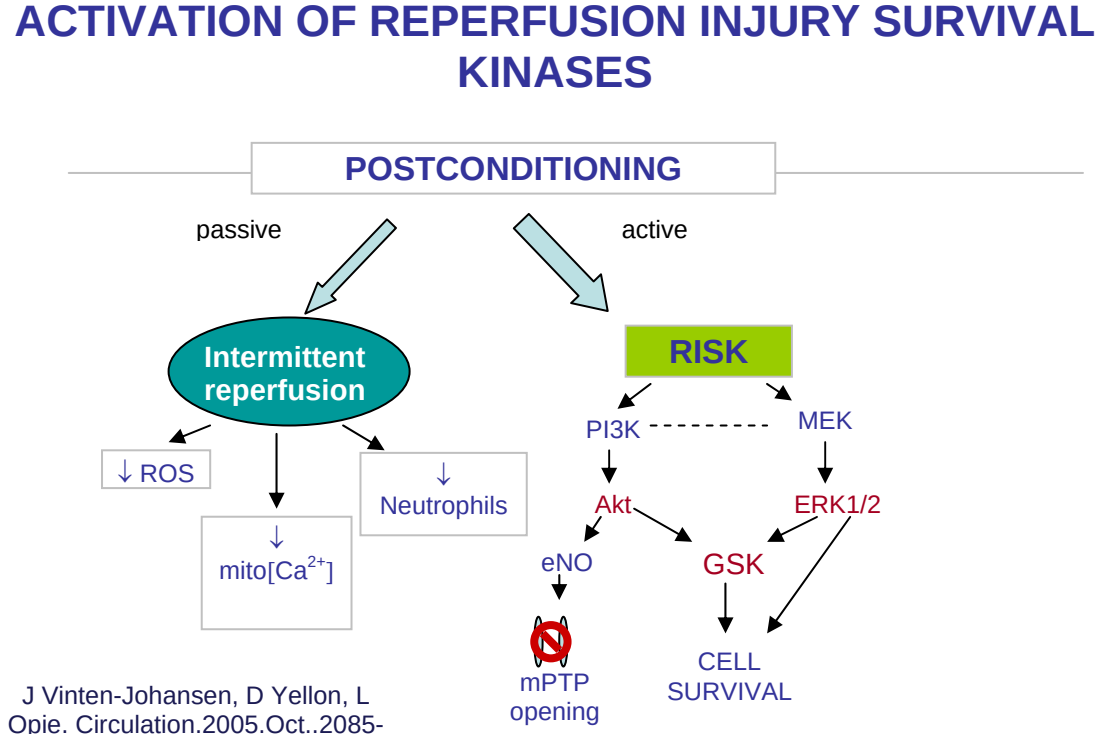


Figure 17.: Possible contexts in the activation of reperfusion injury survival kinases in ischaemic postconditioning

Focal disorganization of gap junction distribution and down-regulation of connexin 43 (Cx43) are typical features of myocardial remodelling [113] and Cx43 – especially Cx43 localized in mitochondria – has been indicated as one key element of the signal transduction cascade of the protection by preconditioning. However, Cx43 does not seem to be important for infarct size reduction by PostC [114]. These results, together with the above reported differences on kinase activation by pre- and PostC, suggest a certain degree of differences between the protective pathways activated by these two procedures.

4.4.4. End-effectors of postconditioning

Mitochondrial PTPs opening represents a fundamental step of reperfusion injury. Among the potential mechanisms responsible for mPTP opening during reperfusion, Ca^{2+}-overload has received particular attention. In particular, mitochondrial Ca^{2+}-overload occurring during ischaemia must bring mitochondria closer to the threshold at which mPTP opening takes place, favouring the occurrence of mPTP opening during reperfusion, a phenomenon described as mitochondrial priming[115]. Additionally, reduced mitochondrial Ca^{2+} overload

during ischaemia has been pointed out as a potentially important mechanism of ischaemic and pharmacological preconditioning[116].

Neonatal rat cardiomyocytes subjected to 3 hrs of hypoxia and 6 hrs of re-oxygenation, "hypoxic PostC" with alternating exposure to three cycles of 5 min. hypoxic and normoxic conditions preceding re-oxygenation reduced intracellular and mitochondrial Ca^{2+} loading compared to non-postconditioned cardiomyocytes. This was associated with a reduction in cardiomyocyte death assessed by propidium iodide and lactate dehydrogenase release[117]. However, the signalling pathways and physiological consequences of this lower intracellular Ca^{2+} by PostC are not known at present, especially in vivo. For instance, it cannot be excluded that reduced mitochondrial Ca^{2+} overload could actually be a consequence of a more preserved Ca^{2+} handling by the sarcoplasmic reticulum in postconditioned cardiomyocytes rather than a cause of protection. It has been reported that PostC reduces calcium-induced opening of the mPTP in mitochondria isolated from the myocardial area at risk[118]. PostC was also associated with a reduction in infarct size after both acute and long-term (72 hrs) reperfusion. Bopassa et al.[119] demonstrated in isolated perfused rat hearts that maintenance of mPTP closure was associated with PI3K activation, which is consistent with the activation of survival kinase pathways described above, but the functional involvement of these pathways and regulation of the mPTP in vivo is not yet clear. It seems that in the PostC scenario the inhibition of GSK3 contributes to the prevention of mPTP opening[120]. Taken together it would appear that the trigger pathway for PostC involves the following sequence of events: occupation of surface receptors (adenosine and NOS and non-enzymatic processes to make nitric oxide, activation of cGMP-dependent kinase (PKG), opening of mK_{ATP}, production of ROS and finally activation of PKC and MAPKs as well as inhibition of GSK3 which put the heart into a protected state. The protect state may include a central role of the prevention of mPTP opening by acidosis in the early phase and by the aforementioned mechanisms in the late reperfusion (*Figures 2 and 3*).

Cardioprotection by pre- and postconditioning is redox-sensitive

It has been already established that preconditioning triggering, that is the period that precedes the index ischaemia, is redox sensitive. This was demonstrated by both avoiding preconditioning with ROS scavengers and inducing preconditioning with ROS generators given before the index ischaemia [121]. Also, several metabolites, including acetylcholine, BK, opioids and phenylephrine, trigger preconditioning-like protection via a mK_{ATP}-ROS dependent mechanism[122]. As stated in the case of reperfusion injury, ROS are also implicated

in the sequel of myocardial reperfusion injury[123]. These studies supported the paradigm that ROS may be protective in pre-ischaemic phase, but are deleterious in the post-ischaemic phase. Thus the main idea was that ROS play an essential, though double-edged, role in cardioprotection: they may participate reperfusion injury or may play a role as signaling elements of protection in pre-ischaemic phase[124]. The importance of ROS signalling (as opposed to excess ROS in the development of injury) has been examined closely in great detail in recent years[125]. Intriguingly, and in contrast to the above-described theory of ROS as an obligatory part of reperfusion induced damage, some studies suggest the possibility that some ROS species at low concentrations could protect ischaemic hearts[126]. Yet, from the above reported mechanisms of PostC, it appears that also ischaemic PostC is a cardioprotective phenomenon that requires the intervention of redox signaling to be protective[127]. Moreover, as mentioned, very recently it has been shown that redox signalling is also required at the time of myocardial reperfusion to mediate the cardioprotection elicited by ischaemic preconditioning[128]. Therefore, the role of ROS in reperfusion may be reconsidered as they are not only deleterious. This fact may help to understand the variability in the results of studies aimed at proving a role of ROS in reperfusion injury. For instance, negative results came from trials in which free radical scavengers such as recombinant human superoxide dismutase or vitamin E were administered to patients with coronary artery disease or risk factors for cardiovascular events[129]. In addition to the dual role of ROS (beneficial versus deleterious), among the reasons why these scavengers did not show any consistent benefit in these human studies may be: (1) the type of ROS generated (e.g. superoxide dismutase only removes the superoxide and not the hydroxyl radical); (2) the site of ROS generation (e.g. most scavengers scarcely enter into the cells) and (3) the rate of reaction between two ROS and/or scavengers. The importance of the rate of reaction can be understood if we consider that, despite a five times lower concentration of nitric oxide with respect to superoxide dismutase, 50% or more of the available superoxide will react with nitric oxide to form ONOO– instead of reacting with superoxide dismutase[130]. Notwithstanding the evidence of a protective role of ROS signalling in reperfusion, we were unable to reproduce cardioprotection with ROS generation by purine/xanthine oxidase given at reperfusion[131].

Since ROS scavengers (N-acetyl-L-cysteine or 2-mercapto-propionylglycine), given at the beginning of reperfusion, abolished both IP- and PostC-induced protection[132] it is likely that the type, the concentration and/or the compartmentalization of ROS may play a pivotal role in

triggering protection at reperfusion time. We are performing studies in the attempt to clarify this issue.

4.4.5. Conclusion

Postconditioning has the advantage of being a way to influence and modify reperfusion injury after it has occurred. This may open a therapeutic alternative in situations of unexpected and uncontrolled ischaemic-reperfusion injury, for instance in the situation where technical complications occur during surgery, making a simple procedure into a complicated one, and making aortic cross-clamping longer than anticipated.

We think, that many more examinations are needed to describe and understand in details the mechanism of ischaemic PostC. We are sure, that this manoeuver is easy to perform, quick, and does not any expensive instruments, so it may have a place in the therapeutic arsenal of vascular surgeons.

5. THE EFFECT OF CONTROLLED REPERFUSION ON REPERFUSION INJURY USING A SIMPLIFIED PERFUSION SYSTEM

5.1. INTRODUCTION

Persistent and acute ischaemia of the extremity, including neurologic dysfunction of the compromised leg, is associated with high morbidity and mortality. The most common reasons for acute limb ischaemia are embolism of cardiac or arterial origin and in situ thrombosis of arteriosclerotic vessels.[133] Since the late 1960s, surgical revascularization with the use of Fogarty catheter primarily has been the therapeutic gold standard. It must be emphasized that the results of surgical therapy have not improved over the decades[134]. Even the introduction of new interventional treatment options such as intra-arterial thrombolysis did not reduce the high rates of mortality and amputation[135]. Crucial in treating acute lower-limb ischaemia is that restoration of arterial blood flow, essential for limb salvage, can further damage ischemic tissue in a phenomenon known as reperfusion injury[136]. Reperfusion injury and its systemic effects on remote organs can cause severe local and systemic complications such as renal and pulmonary failure [137]. There is experimental evidence that modifying the initial perfusion modalities, especially perfusion pressure and composition of the initial perfusate, can reduce reperfusion injury [138]. The therapeutic principle named "controlled reperfusion" was first used to treat myocardial ischaemia[139]. Reduction of the initial reperfusion pressure is aimed at reducing edema development, the modification of the initial reperfusate is aimed at counteracting the known biochemical changes that occur with ischaemia-reperfusion, such as the breakdown of aerobic metabolism, metabolic acidosis, an increase in intracellular calcium, and the development of oxygen-derived free radicals with the onset of reperfusion. In an animal model of acute lower-limb ischaemia, local and systemic complications were reduced by the use of controlled reperfusion[140]. The concept of controlled reperfusion has been successfully used in clinical practice for treating patients with severe, prolonged lower-limb ischaemia[141].

All techniques described so far have required the use of a heart-lung machine or roller pumps. Beyersdorf et al. have developed a new blood bag reperfusion system that allows the application of controlled reperfusion on acute ischemic limbs with minimal technical effort[142].

5.2. AIMS

We hypothesized that controlled reperfusion using a simple blood bag perfusion system reduces reperfusion injury and thus facilitates the return of normal function. We used an animal model with infrarenal aortic occlusion and reperfusion and aimed to figure the effect of the controlled reperfusion on the oxidative stress, and inflammatory pathways. Furthermore, we aimed to examine the protective effect of controlled reperfusion in the reperfusion injury sensitive organs. With histological examinations we would to detect the pathological changes in the lung, kidney, heart, and liver to see the beneficial effect of our method.

5.3. MATERIALS AND METHODS

5.3.1. Study protocol.

We used ten Yorkshire pigs for the animal model. Five of these animals undervent a 4-hour infrarenal aortic occlusion followed by continuous reperfusion without any therapy. (Control group)

Five of these animals were treated with controlled reperfusion. In these cases after a 4-hour aortic occlusion we made the controlled reperfusion for 30 minutes and after that we started the continuous reperfusion with normal blood flow.

5.3.2. Surgical Preparation

These protocols were approved by the Institutional Animal Care and Use Committee of the Uniformed Services University of the Health Sciences and conformed to the standards in the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH Publications No. 85-23, Revised 1996).

Yorkshire pigs of either sex, weighing between 18 and 22 kg and free of clinically evident disease, were entered to this study. Pigs were sedated with an intramuscular injection of 15 mg/kg ketamine hydrochloride (Vetalar, Fort Dodge) and anesthetized with pentobarbital sodium (30 mg/kg, Sigma; St. Louis, MO). Pigs were placed on a homeothermic blanket control unit (Harvard Apparatus; Holliston, MA) designed to maintain a core body temperature of at least 37°C, as measured by a thermistor probe placed in the rectum.

Interanimal variation in temperature was minimized with careful monitoring.

An intratracheal intubation was performed, and pigs were mechanically ventilated (Harvard) with room air supplemented with oxygen. A saline filled catheter was placed in the right external jugular vein for drug administration and fluid infusion. A 9-Fr catheter introducer (Catheter Sheath Introducer System, Cordis; Miami, FL) was placed in the right carotid artery. Through this introducer catheter, a Mikro-Tip dualpressure transducer catheter (model SPC-780C, Millar) was inserted to measure aortic and ventricular pressure and to permit simultaneous electronic differentiation to yield the change in pressure change over time (dP/d*t*). End-tidal CO2 was monitored continuously (Hewlett- Packard, model 78356A; Palo Alto, CA), arterial blood gases were measured periodically, and ventilatory parameters were adjusted as needed to maintain blood gases within physiological ranges. Slow intravenous infusion of normal saline maintained hydration throughout the surgery, and additional anesthetic was administered as needed.

After these preparation a median laparatomy was made, and with gentle retraction of the bowels we isolated the infrarenal aorta. We administered heparin to prevent thrombostisation. We occluded the aorta with DeBakey clamps. After the ischaemic period we removed the clamps and checked the restoration of continuous blood flow with the pulsation of the iliac arteries.

5.3.3. Management of controlled reperfusion

The perfusion set for applying controlled limb reperfusion consisted of two blood bags (each capable of holding 1 liter), crystalloid solution, a blood line, and a reperfusion line (both made of 0.25-inch polyester tubes). Controlled limb reperfusion was performed as follows: After the aortic occlusion and before restoration of blood flow, a 10-Charrier (CH) (1 CH is equivalent to .33 mm) cannula was inserted proximally into the aorta, another 10-CH cannula was inserted distally. The proximal cannula was connected to the blood line, and oxygenated blood was drawn into the first blood bag where it was mixed with the crystalloid reperfusion solution (blood:reperfusion solution ratio, 1:1). The composition of the crystalloid solution is given in table 2 . According to the hemodynamic status of the animals, either 200 mL or 300 ml of blood was taken every cycle. After the blood-reperfusion solution had been transferred to the blood bag, the reperfusion line was connected to this bag. After all air had been expelled from the reperfusion line, controlled reperfusion was initiated via the distal cannula. A 12-gauge cannula was inserted into the aorta distal to the reperfusion cannula for continuous pressure control. Perfusion pressure was kept strictly 60 Hgmm. In most cases, the

blood reperfusion solution was returned to the leg by gravity alone. If necessary, a pressure-cuffed bag was put around the reperfusion bag to achieve a perfusion pressure close to 60 mm Hg. Perfusion pressure was varied by changing the height of the blood bag. The procedure was repeated for 30 minutes. The number of cycles performed depended on the flow that could be achieved. After removal of the cannulas, the arteriotomy was closed with direct suture, and normal blood flow was re-established.

Table 2. Composition of the crystalloid reperfusion solution

NaCl 27.27 mmol/L	
Glutamate - H_2O	34.27 mmol/L
Aspartate - H_2O	34.0 mmol/L
Citric acid -H_2O	2.41 mmol/L
Na citrate - H_2O	13.41 mmol/L
NaH2PO4 - H_2O	2.40 mmol/L
Glucose - H_2O	157.96 mmol/L
Allopurinol	7.35 mmol/L

Blood samples were collected :

1. before the intervention	peripheral venous blood from jugular vein
2. on the end of ischaemia	central venous blood from inferior caval vein
3. 15 min of reperfusion	central venous blood from inferior caval vein
4. 60 min of reperfusion	peripheral venous blood from jugular vein
5. 24 h of reperfusion	peripheral venous blood from ear vein
6. 7^{th} day of reperfusion	peripheral venous blood from ear vein

5.3.4. Oxidative stress parameters and the leukocyte activation measurements

The methods are described in the previous capture.

5.3.5. Histological examinations

The animals both from treated and control groups were anesthetized one week after terminating ligation and biopsy was taken from quadriceps muscle and large parenchymal organs (liver, kidney, lungs, heart) as well as large and small intestines.

The fragments of muscle did not contain well-identified fascia. The definite aim of the biopsy was to register the quantitative and qualitative differences in changes between the two animal groups, firstly the transformations in the striated muscular tissue. No aim of recent study to

make statements on pathogenesis or other aspects. We examined all available organs from each animal, including transversal and longitudinal cut biopsies from striated muscular tissue. The biopsy concentrated first of all on striated muscular tissue, 5-6 paraffin-embedded blocks were made from striated muscle-pieces, and from each block several sample slices were prepared staining by hematoxylin and eosin, we also performed immunohistochemical examinations as well.

The biopsies were made with the following method:
The fresh tissue was fixed in 10% neutral buffered formalin. Sample preparation was performed with a tissue processor equipment (Thermo Shandon Path centre, Thermo Fisher Scientific Inc., Waltham, MA, USA). Sectioning was performed with a sledge microtome (5 µm, Reichert Optische Werke AG, Vienne, Austria) from the paraffin-embedded blocks, and staining was carried out with a carousel-type slide stainer (Thermo Varistain 24-4, Thermo Fisher Scientific Inc., Waltham, MA, USA) with hematoxylin and eosin, at the County Hospital of Baranya, Department of Pathology, Pécs, Hungary.

The process of immunohistochemical examinations made on paraffin-embedded blocks:
Immunohistochemical marking:

1. Deparaffinization
2. Distilled-water washing, for 3 minutes
3. Microwave treatment: in pH 6.0, 0.01 M citrate puffer for 25 minutes
4. Washing in pH 7.6 Tris puffer fluid for 5 minutes
5. Endogen- inhibition in 3%- H202 fluid for 10 minutes
6. Washing in Tris puffer 2x
7. Incubation with the following antibodies (1 hour):
   Desmin (clone D33, DAKO, cat: M760, dilution/ dil: 1:100)
   Actin (clone 1A4, NeoMarkers, cat: MS 113, dil.:1:400)
   Calponin, (clone CaLP, NeoMarkers, cat: MS 1168, dil.:1:100)
8. Washing in Tris puffer 3x
9. Secondary PO-s antibody 40 minutes Novocastra Impress Universal KIT (MP 7500)
10. Washing in Tris puffer 3x
11. Developing with Aminoethil-carbazol for 15-20 minutes Novocastra Substrate KIT (SK 4200)

12. Washing in distilled water 2x
13. Nuclear staining with Hematoxylin 10 seconds
14. Covering with glycerin-gelatin from water

We completed the immunohistochemical staining for actin, desmin and calponin antibodies available in trade, produced against human antigens, expecting results first of all from actin and desmin antibodies. In the given animal tissues i.e. in striated muscular tissues the desmin reaction proved to be valuable, it gave strong reaction in the striated muscular tissue. The desmin immunoreactions also showed well-valuable marking in the heart muscle. The process with the actin antibody did not show reaction either in the striated muscular tissue or in the heart muscle or non-striated muscle tissues of vascular wall, but considerable reaction was observed in the positive control application. The calponin reaction indicated very slight uncertain marking at the same conditions, so we did not use it for the evaluation. We note, that the calponin immunoreactions showed moderately strong but well- valued marking in the heart muscle. To determine the quantitative data showing the rate of normal and degenerative fibers, we performed our applications in blocks of the fiber cross sections and areas showing fibers definite staining changes.

Desmin is a protein taking part to from the intermediter filament of muscle tissue in polymerized form, i.e. type III intermediter filament found near the Z line in sacromeres. Desmin is expressed only in vertebrates, however homologous proteins are found in many organisms. Desmin class-III intermediate filaments are found in muscle cells. In adult striated muscle they form a fibrous network connecting myofibrils to each other and to the plasma membrane from the periphery of the Z-line structures. It is a 53kD protein, which is considered as a subunit of intermediate filaments in skeletal muscle tissue, smooth muscle tissue, and cardiac muscle tissue. Desmin also plays an important role in muscle cell architecture and structure since it connects many components of the cytoplasm. In case of muscle fiber degeneration, its involvement and damage is predictable.

5.3.6. Statistical analysis.

All numeric data are presented as mean ± SE. Numeric data were compared using unpaired Student's t-test, and the 2 test was used to compare binomial data. $p < 0.05$ was considered statistically significant.

5.4. RESULTS

5.4.1. Hemodynamic Data

Hemodynamic data are summarized in Table 3. Baseline heart rate, systolic and diastolic pressure were not significantly different among the various groups, and controlled reperfusion did not differ from control at any measurement period.

*Table 3. Hemodynamic variables and body temperature at baseline. Values are mean ± SE. No group was significantly different from control in any measurement period. (IR= ischaemia-reperfusion; CR= controlled reperfusion) *p < 0.05, statistical significance of difference from baseline.*

	Baseline	Occlusion	Reperfusion
Heart rate, beats/min			
IR	123±5	128±9	130±7
CR	128±5	123±4	127±4
Systolic blood pressure, mmHg			
IR	104±3	95±4	97±5
CR	110±4	100±3	89±3*
Diastolic blood pressure, mmHg			
IR	80±5	76±4	77±5
CR	84±3	77±3	68±3*
Temperature, °C			
IR	37.5±0.2	38.0±0.2*	38.1±0.3*
CR	37.9±0.2	38.2±0.2	38.2±0.3

5.4.2. Oxidative stress parameters

The plasma MDA concentration elevated in the full reperfusion group right after the beginning of reperfusion and decreased for the 24th hour of reperfusion. This elevation was much milder in controlled reperfusion group, we measured significant difference between the two groups. On the end of the investigated period we detected a recurrent elevation in the reperfusion group, but lower than in the early reperfusion period. (*Figure 18*)

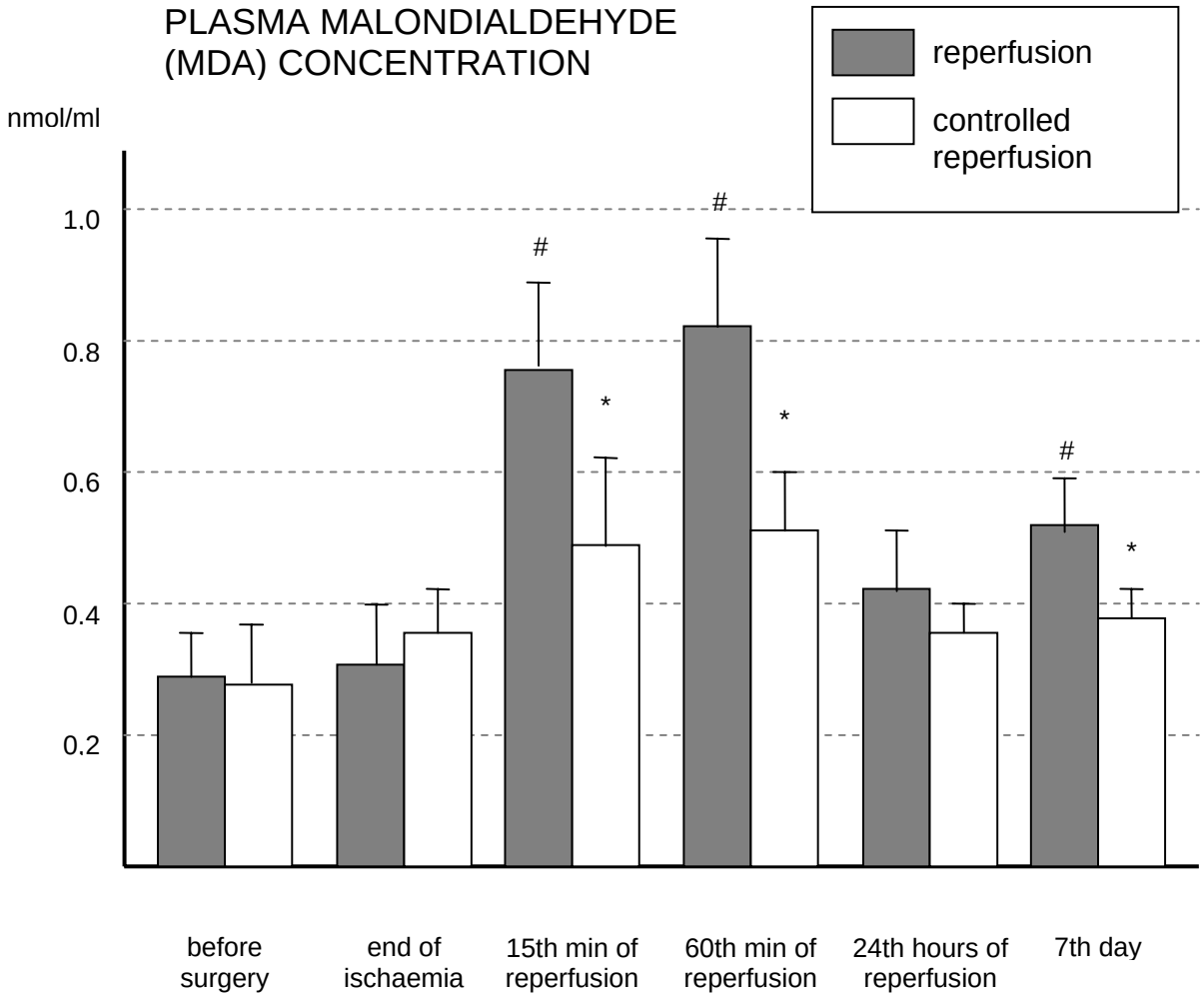


*Figure 18: Changes in plasma malondialdehyde concentration in the experimental groups during the examined perioperative period. (# p<0.05 vs before surgery; * p<0.05 vs reperfusion group)*

We observed changes in the –SH group in the first hour of reperfusion. There was a significant decrease in the regulary reperfused group, but this change can not be seen in the controlled reperfusion group. The measured values in the 24th hour were similar to the values before reperfusion. A second decrease was detected on the 7th day in the normal reperfusion group. (*Figure 19*)

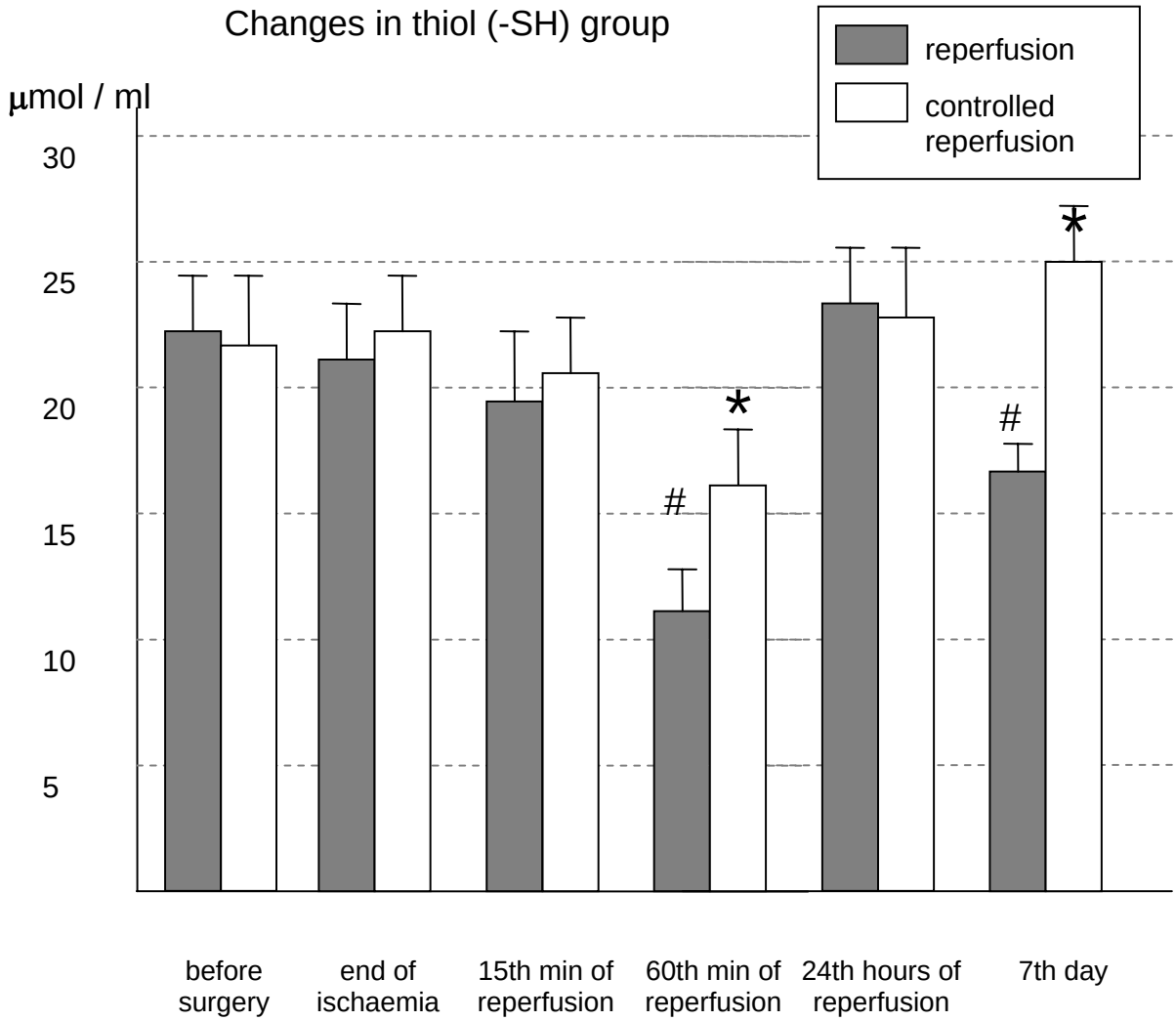


*Figure 19: Changes in antioxidant group (thiol group) plasma levels in the experimental groups during the examined perioperative period. (# p<0.05 vs before surgery; * p<0.05 vs reperfusion group)*

GSH plasmalevels decreased significantly in the early reperfusion period until the 24th hour. We did not detected differences between the two groups. On the 7th day of reperfusion the measured values in both group reached the start values. (*Figure 20*)

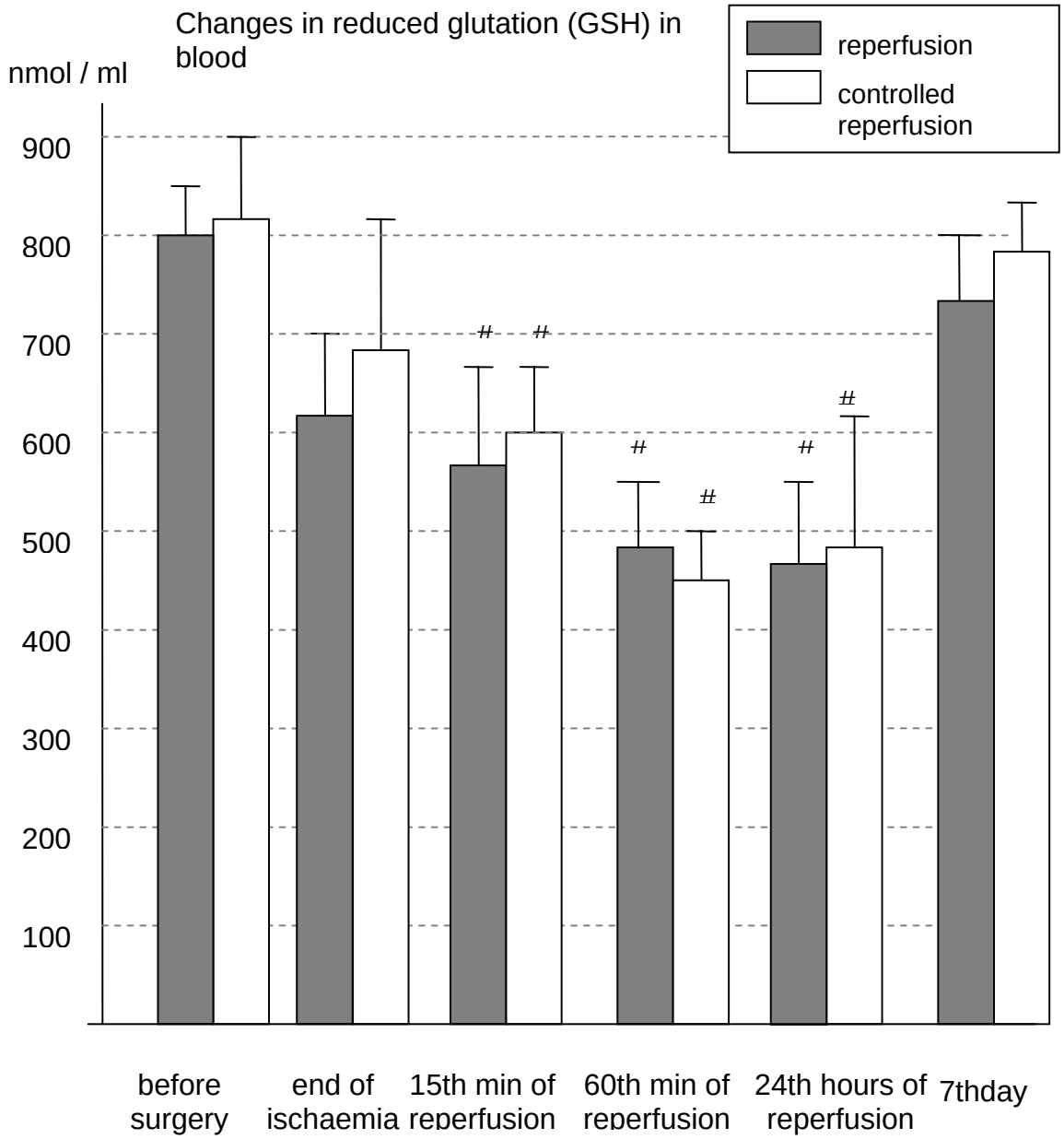


*Figure 20: Changes in reduced glutahtion plasma levels in the groups of animals during the examined perioperative period. (# p<0.05 vs before surgery; * p<0.05 vs reperfusion group)*

We did not measure changes in SOD activity during the ischaemia and in the early reperfusion. A significant decrease in SOD activity was detected in the 24th hour of reperfusion in the normal reperfusion group. This decrease could not be detected in the controlled reperfusion group. On the last measure time both groups showed the same values as before the ischaemia, and there was no any difference between them. (*Figure 21*)

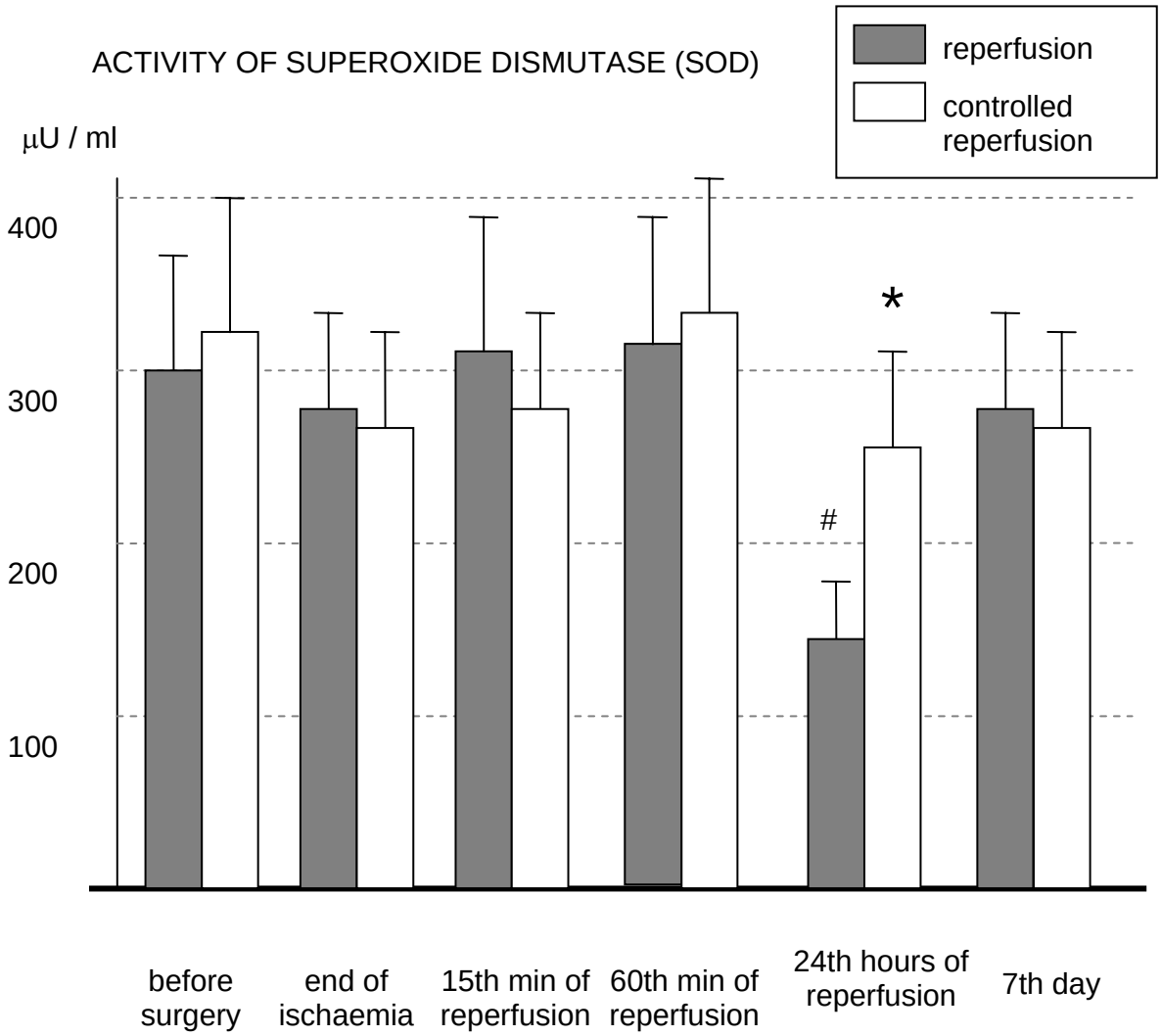


*Figure 21: Changes in plasma superoxide dismutase activity in the experimental groups during the examined perioperative period. (# $p<0.05$ vs before surgery; * $p<0.05$ vs reperfusion group)*

We detected significant increase in the speed of leukocyte radical production in the early period – in the 15th and in the 60th minutes – in the totally reperfused group. These changes could not be observed in the controlled reperfusion group. In the 24th hour we measured physiological values in both group. (*Figure 22*)

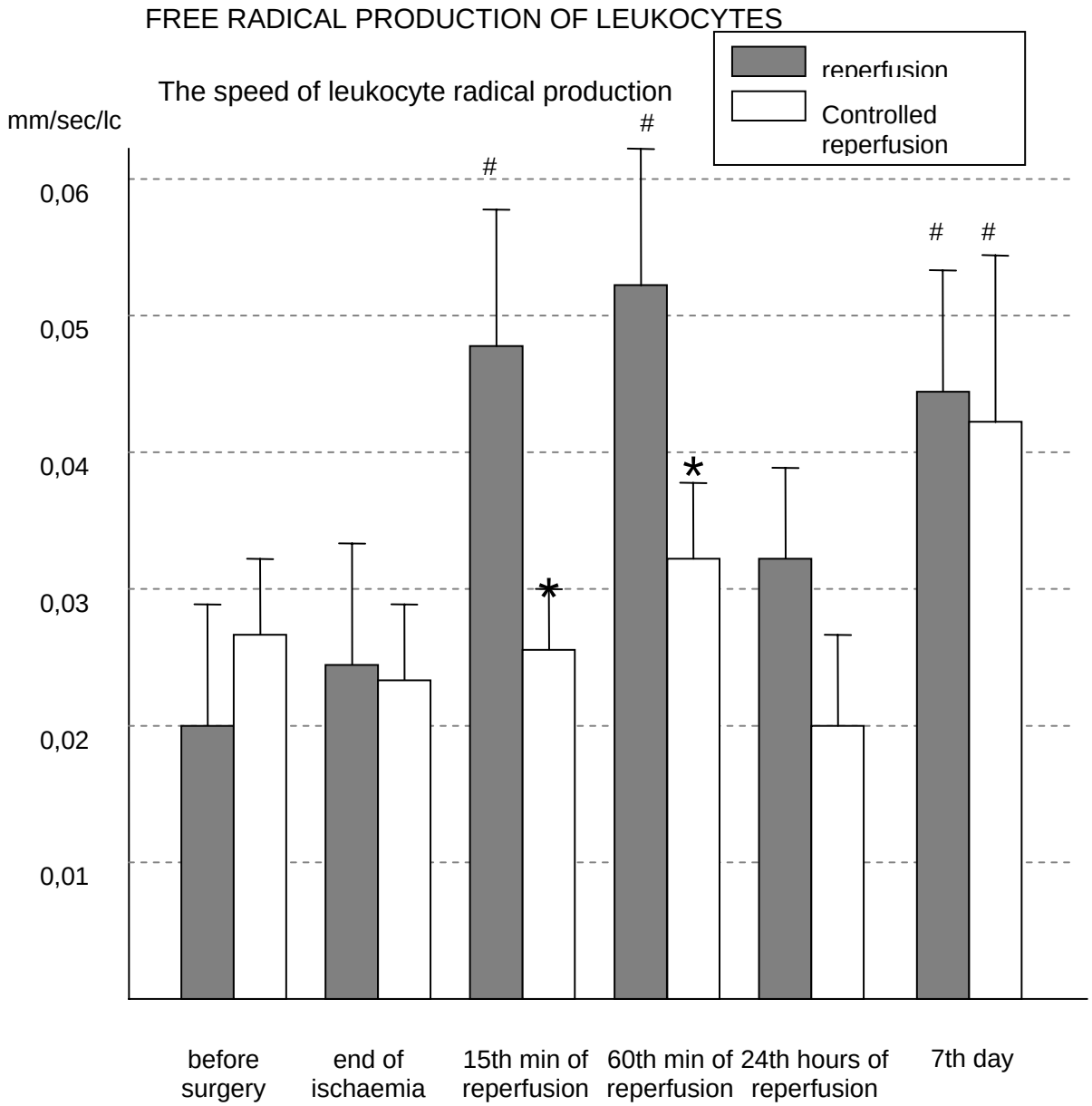


*Figure 22: Changes in the speed of PMA induced free radical production of leukocytes in the experimental groups during the examined perioperative period. (# p<0.05 vs before surgery; * p<0.05 vs reperfusion group)*

The maximum of free radical production first elevated in the reperfusion group in the 15th min of reperfusion and this elevation could be detected until the examined period. Significant difference between the groups could be measured in the first hour of reperfusion. On the seventh day we measured increased values in both groups. (*Figure 23*)

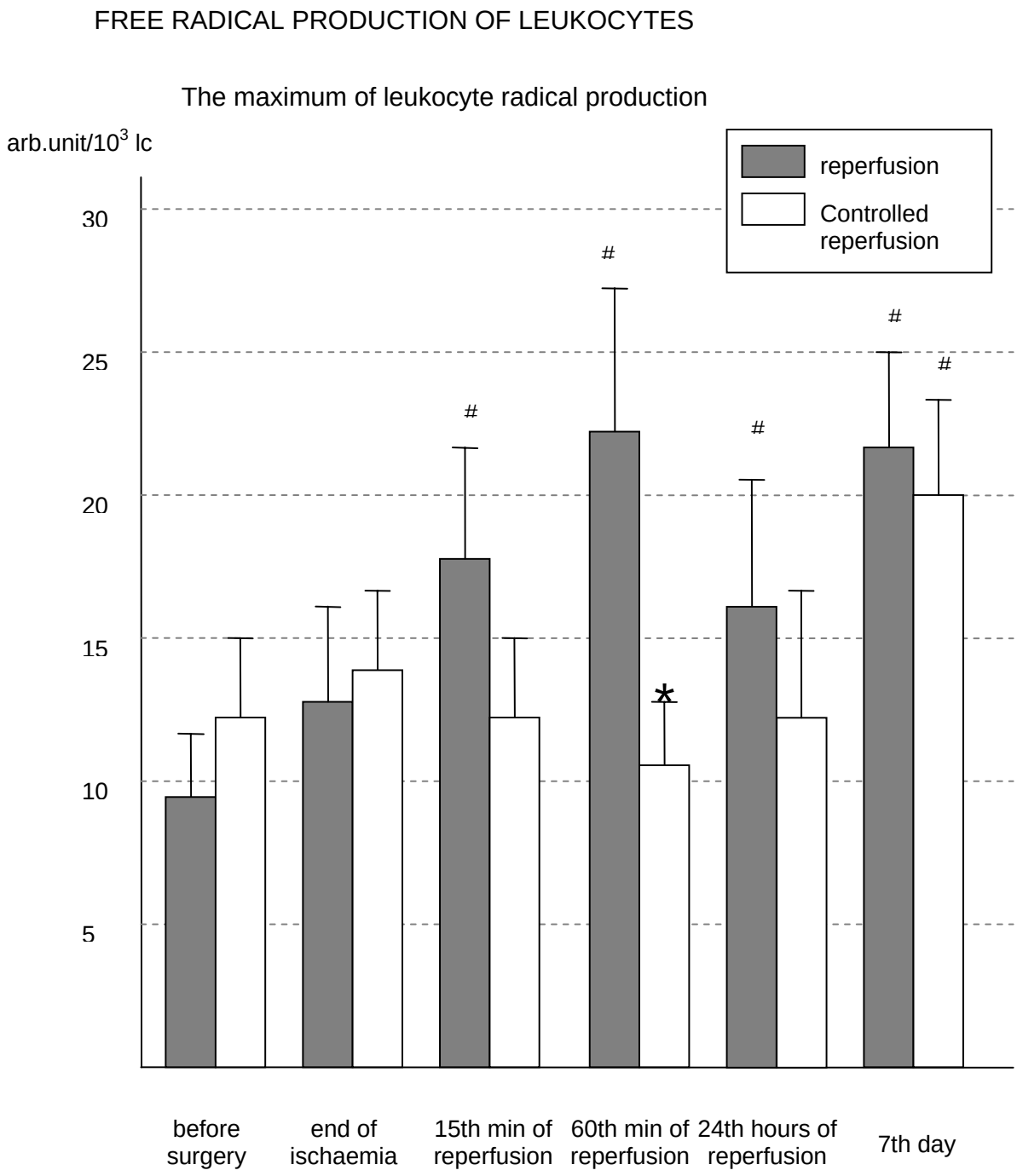


*Figure 23: Changes in the maximum of PMA induced free radical production of leukocytes in the experimental groups during the examined perioperative period. (# p<0.05 vs before surgery; * p<0.05 vs non-conditioned group)*

We detected a significant increase in the reperfusion group in the 24th hour. At this time there was no elevation in the controlled reperfusion group, and a significant difference could be seen between them. We measured still elevated values at the end of the experimental period. (*Figure 24*)

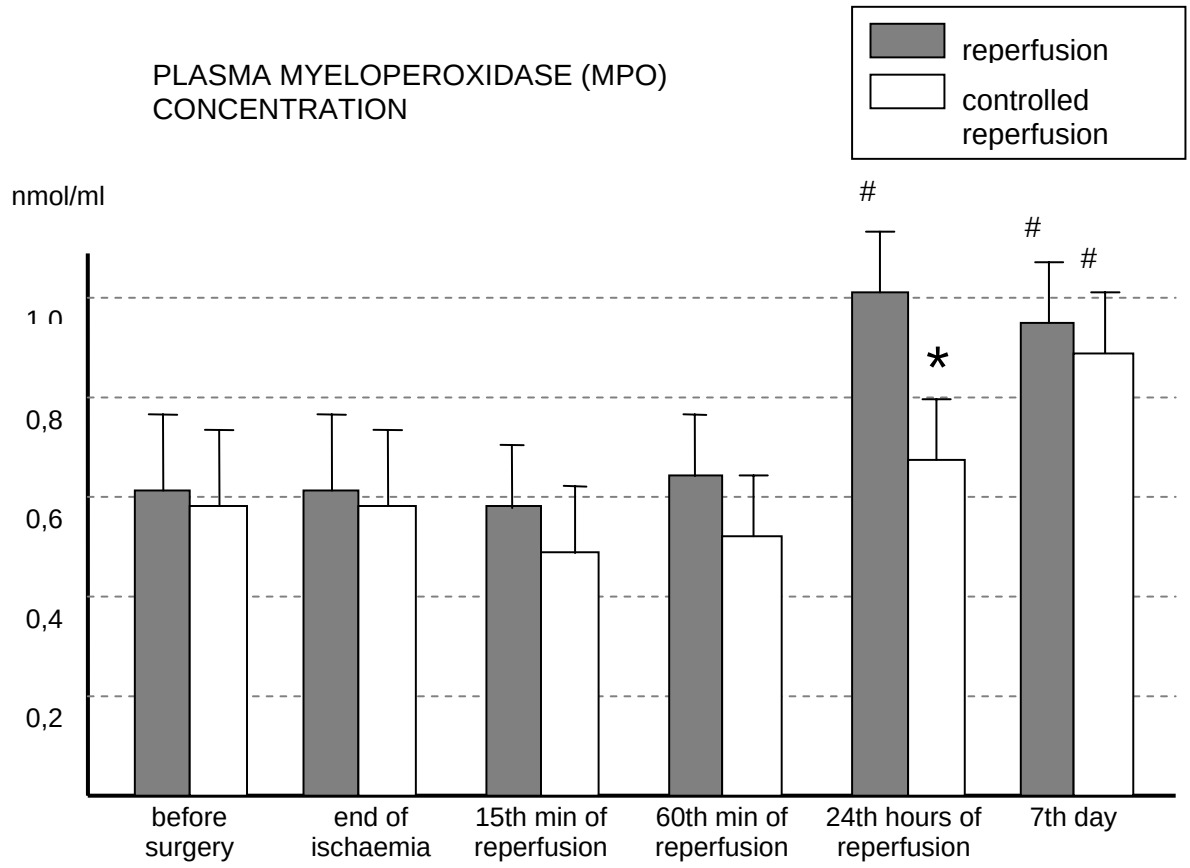


*Figure 24: Changes in plasma myeloperoxidase concentration during the examined perioperative period. (# p<0.05 vs before surgery; * p<0.05 vs reperfusion group)*

5.4.3. The results of histological investigations

Transformations detailed below were well-observable in the H.E. stained sections, but the desmin immunohistochemical reaction divided the healthy and degenerative muscle fibers more unambiguously, showing sharper differences. The differences were the most prominent, the most identifiable in sections representing the cross-section of fibers. In section showing the fibers in longitudinal cut, the regenerative inflammatory symptoms appeared well, but they –as seemed, were not present characteristically in the picture.

Because of it the comparison happened in 10 times larger-magnification views (400x) healthy and damaged fibers, comparing their defined number on cross-section material with desmin immunohistochemical marking. During the histological evaluation mostly the same tissue changes were shown mainly characterized by degeneration in both animal groups, that is in the complete reperfusion group immediately after ischaemia and in group treated with controlled reperfusion after ischaemia, but there changes appeared concerning bigger areas even without quantity definition in the first animal group, and more moderately in the second.
In the first group of animals the basic tissue structure is mainly kept in the striated muscle tissue, there is no fibrosis and necrosis cannot be defined with absolute certainty and neither significant inflammation cannot be observed. At the same time the muscle tissues show well-observed size and shape differences. Regular-morphology was seen, normal staining muscle fibers, where the desmin immunohistochemical reaction is strong, smooth and fully covers the area of fibers. At the same time the other fibers are swelled, irregular-shaped and the interstitial space between the fibers is pressed, decreased. The fibers staining are paler, less even and slightly basophile- shade, in places the sacroplasma seems tattered and the nucleuses are fewer. In smaller areas the replacement of nucleus can be observed from the edge of fibers to their central area, and same round-celled inflammative infiltration and regenerative signs also appear, the last can indicate to necrosis occasionally. But the last two deformations occurred very sporadically, almost accidentally, and quantity evaluation was not possible. The Desmin immunohistochemical reaction is uneven, dim and it falls out in some fibers. The described transformations occur in uneven frequency but local characteristics cannot be shown in connection of more defined or less concerned areas. We found kept basic structural conditions in the second animal group. The described degenerative features were observed also here, but they referred to smaller areas, and signs of inflammation and regeneration observed only in one section.
The transformations also happened in uneven distribution without any traceable localization feature.
The process was completed in cross- sectional material, examining in large magnification field, counting 1000 muscle fibers.

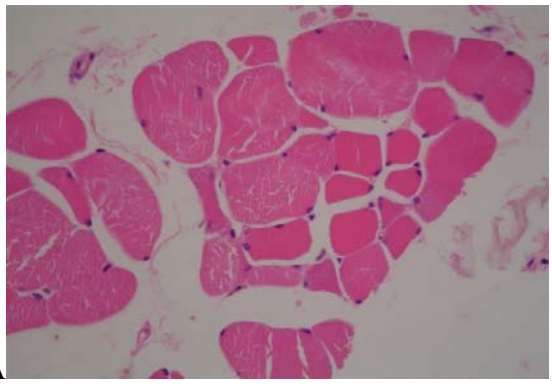
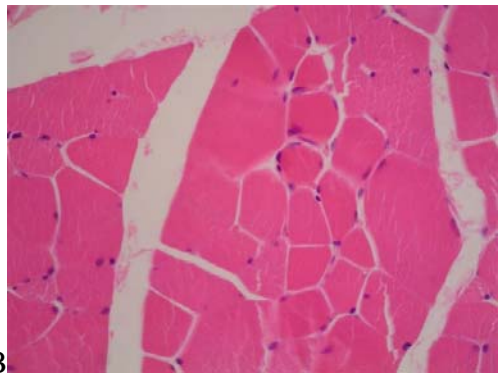
A   B

Fig 25: A: Untreated sceletal muscle H.E. 400x. fiber degeneration (cross section). B: treated sceletal muscle,H.E., moderate fiber degeneration (cross section)

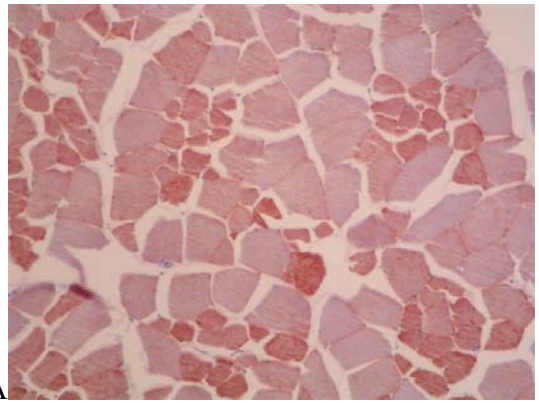
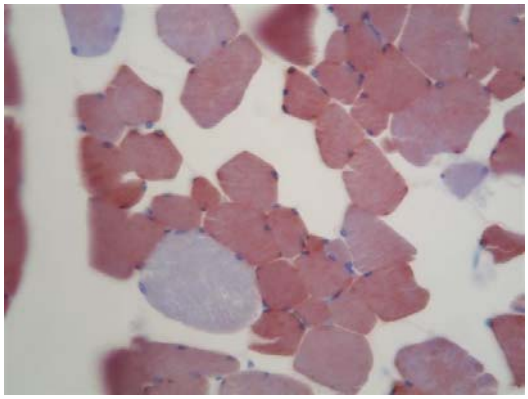
A   B

Fig 26: A: Untreated sceletal muscle Desmin I.H. 200x B: Treated sceletal muscle, Desmin I.H. 400x, moderate degeneration.

The rate of affected muscle fibers by degeneration was 61.7% in the first-untreated-animal group (617:383), and it exceeds 50% in every areas. The rate of damaged fibers is 42.4% (424:576) in the second treated group, and it stays under 50% in every area. (figure 25 and 26)

We examined the large parenchymal organs in both groups and the small and the large intestines as well. Transformations called consistent could be observed only in the kidneys. Here the smaller veins were definitely diluted in many places, sometimes in groups in one or two sections, and in other places concerning only one vein. It was shown in each individual of both animal groups in the cortex and in only one animal of the first group in the marrow and around the collective tubes. We could not demonstrate any other consistent changes. But it was remarkable, that the smaller veins were diluted in places in the lungs, liver and heart, it was observed in both groups, but not in every individual and its occurrence can be said

randomized. Smaller necrotic patches could also be observed in the liver sporadically partly around the portal areas and partly in the centrolobular region accompanied with reactive inflammatory process. But these appeared in small numbers and randomly. We could discover fibrinoid necrosis in a central vessel in heart specimen in one individual of the second animal group and in only one place altogether. There was smaller heart muscle necrosis around it.
But all these transformations do not give basis to draw any conclusions in course of recent studies. At best, they indicate that it does worth to do further examinations in bigger number of animals so that to make clear it's occidental or regular character. (figure 27-33)

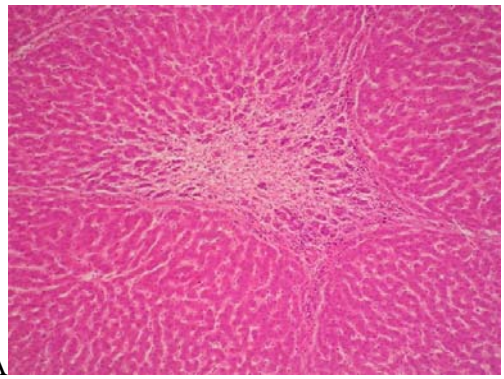
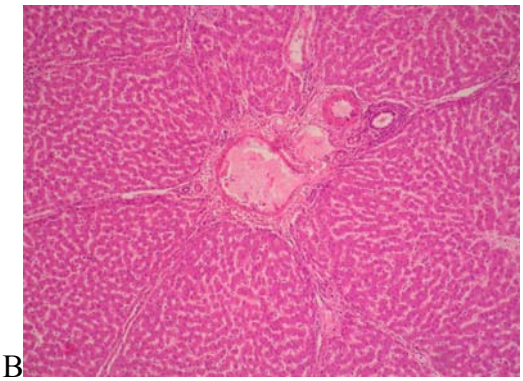
A  B 

Fig 27.: A: Untreated (IR) liver HE 200x, focal necrosis. B: Treated (IR) liver HE 200x.

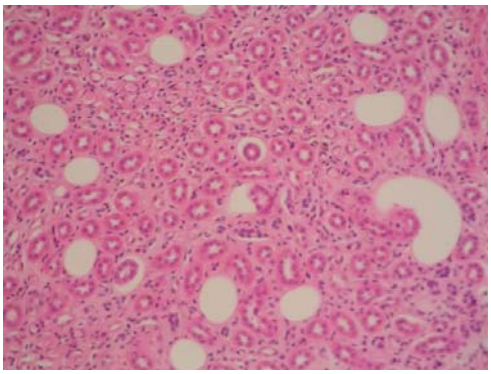
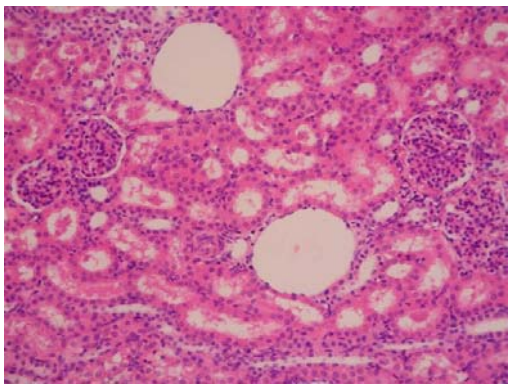
A  B 

Fig 28.: A: Untreated kidney H.E.,200x. B: Treated kidney H.E.400x.

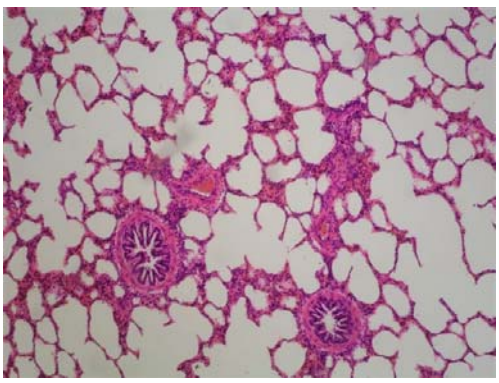
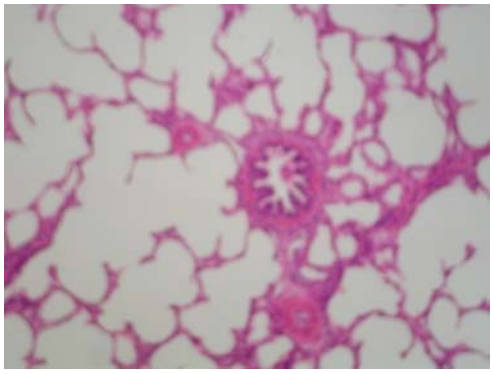
A  B 

Fig 29.: A: Untreated lung, H.E. 200x. B: Treated lung, H.E. 200x.

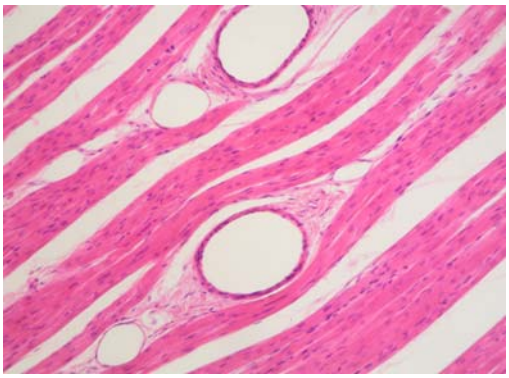
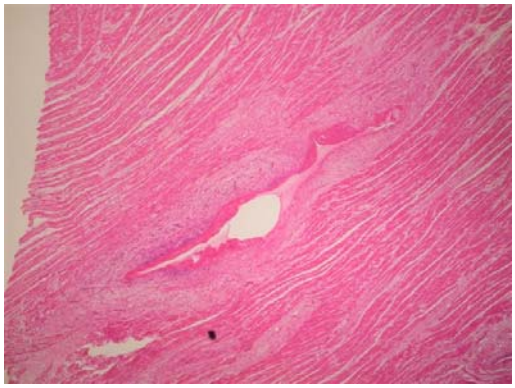
A  B 

Fig 30.: A: Untreated heart H.E.400x. B: Treated heart H.E. 200x.

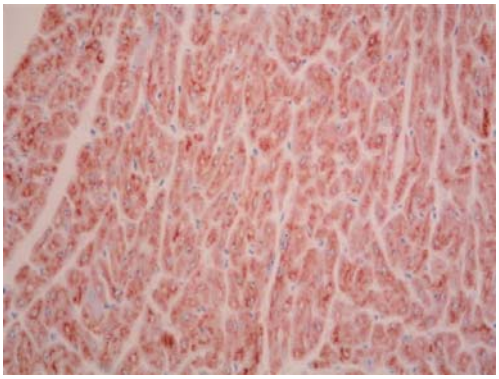
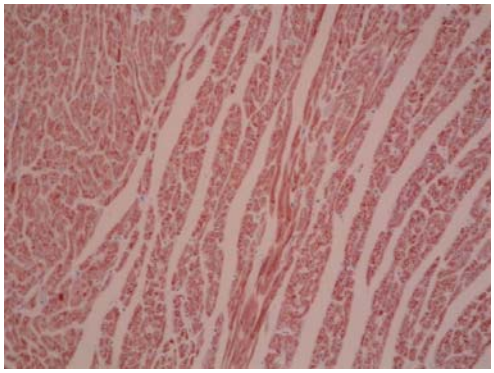
A  B 

Fig 31.: A: Untreated heart desmin I:H..,400x. B: Treated heart desmin I:H..,200x .

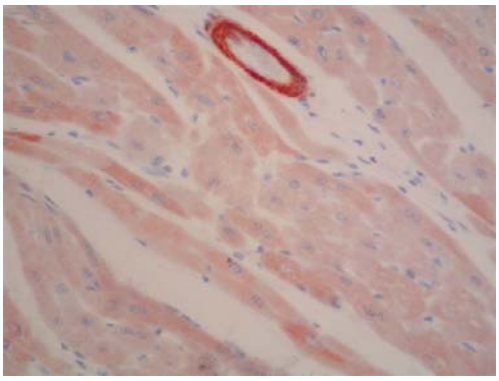
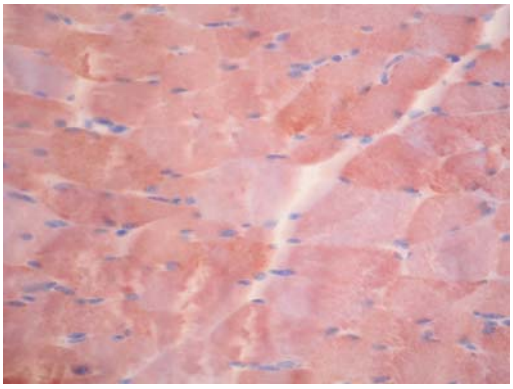
A  B 

Fig 32.: A: Untreated heart calponin I:H..,200x . B: Treated heart calponin I:H..,400x

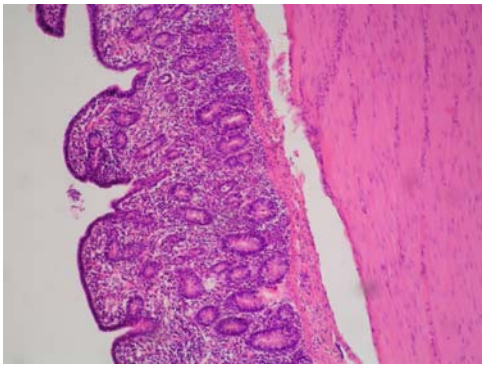
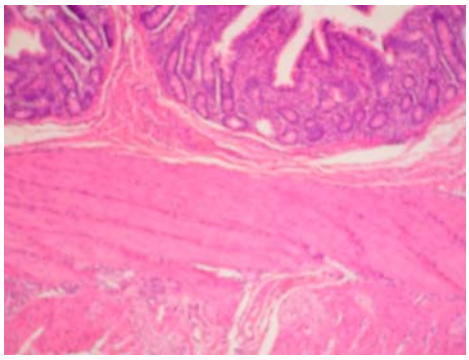
A  B 

Fig 33.: Untreated small bowel HE. 200x B.: Treated small bowel HE 400x

5.5. DISCUSSION

Acute lower-limb ischaemia is probably the most common reason for emergency admission to a vascular surgery unit. The predominant reasons for acute limb ischaemia are embolism of cardiac or arterial (eg, aortic aneurysm with adherent thrombus) origin and arterial in situ thrombosis of arteriosclerotic vessels. Re-establishment of arterial blood flow to the compromised leg is essential for limb salvage. With the introduction of Fogarty catheter in the early 1960s, a simple surgical technique for the revascularization of occluded vessels was introduced into clinical practice. In combination with other surgical techniques for revascularization, such as local thromboendarterectomy and bypass-procedures, vascular surgeons today have a variety of therapeutic options for revascularization of acute ischemic limbs. Through the last decades, interventional treatment options such as thrombolytic therapy have become another therapeutic option in treating acute limb ischaemia.

Despite improvements in revascularization techniques, the results of surgical and interventional treatments have remained unsatisfactory, with high amputation rates and high mortality[143]. The poor results of revascularization therapy alone may be mainly due to additional reperfusion injury[144]. Reperfusion injury is descriptive of the fact that the reperfusion of ischemic tissue, which is absolutely necessary for tissue salvage, causes further tissue damage that in turn can result in tissue apoptosis and necrosis. However, a prerequisite for evaluating different reperfusion protocols is the achievement of a complete revascularization. Even controlled reperfusion will result in amputation if revascularization cannot be achieved because of obliteration of distal vessels. The therapeutic principle of controlled reperfusion has been used successfully in treating myocardial ischaemia and has been shown to improve clinical outcome[145]. Experimental studies on isolated rat hindlimbs have shown that cellular integrity and biochemical function is preserved after 4 hours of warm ischaemia. The severe changes occur after the onset of uncontrolled reperfusion[146]. Reduction of initial reperfusion pressure alone resulted in improved functional and metabolic recovery in an animal model of myocardial ischaemia[147]. In an animal model of skeletal muscle ischaemia, a reduction of reperfusion blood flow was shown to reduce edema generation and muscle injury[148].

Controlled reperfusion, with reduced reperfusion pressure and modification of the initial reperfusate, can reduce the local consequences of reperfusion injury such as depletion of high-energy phosphates and local swelling. This is accompanied by improvement in the return of contractile function[149]. The systemic complications of reperfusion, such as release of

muscle proteins and potassium into the systemic circulation, could be reduced in an animal model of acute lower-limb ischaemia[150].

By using a simple blood bag reperfusion system, as we did in this study, two main principles of controlled limb reperfusion—a reduction of initial reperfusion pressure and modification of the composition of the initial perfusate— can be achieved in clinical practice with minimal technical effort. As there is evidence that most additional tissue injury caused by uncontrolled reperfusion occurs during the first 20 to 30 minutes, a 30-minute interval for controlled reperfusion was chosen[151]. Several well-known biochemical changes occur during ischaemia and reperfusion. With the onset of ischaemia, aerobic metabolism is suspended and anaerobic metabolism is activated. This leads to a breakdown in high-energy phosphates, an increase in intracellular lactate, and intracelluar acidosis[152]. The Krebs cycle loses intermediates[153]. With the start of reperfusion, the washout of lactate and protons from the ischemic tissue leads to an increase in intracellular calcium. An oxidative burst with the generation of oxygen-derived free radicals occurs with the return of oxidative metabolism[154].

The chrystalloid reperfusion solution is aimed to counter these changes. Glucose is added to provide a substrate for anaerobic metabolism and as a hyperosmolar substance to reduce edema generation. With glutamate and aspartate, amino acid precursors of the Krebs-cycle are added to ensure more effective oxidative metabolism with the onset of reperfusion. Allopurinol is added to reduce the generation of oxygen-derived free radicals. Sodium citrate is added to reduce intracellular calcium. The results of our study shows that controlled reperfusion using this simplified reperfusion system can be safely performed in any operating room. Controlled reperfusion has been used before with good results. All the techniques described so far required the use of a heart-lung machine or roller pumps[155]. This required an enormous technical effort and limited the use of controlled reperfusion to large vascular surgery centers.

Most patients with acute lower-limb ischaemia will not present at centers capable of providing these techniques.

After a period of severe ischaemia, reperfusion of the involved leg results in the washout of large amounts of muscular waste products into the systemic circulation. This has been described as a part of the *reperfusion syndrome* and is associated with *multiorgan failure* and death[156]. Experimental studies on isolated rat hindlimbs showed that controlled reperfusion significantly reduces the amount of metabolic waste products[157]. The duration of ischaemia in

the animals included in this study was quite long. Clinical studies on controlled reperfusion for severe lower-limb ischaemia have showed good results[158].

5.6. CONCLUSION

Our results strongly support the hypothesis that controlled reperfusion can improve outcome after acute severe lower-limb ischaemia even though this study was limited by the small number of animals. We have seen that controlled reperfusion significantly reduced the postischaemic oxidative stress and inflammatory responses in the early reperfusion period.

Our pathohistological results confirm, that controlled reperfusion has real beneficial effect not only on the ischaemic skeletal muscle, but also protects against reperfusion syndrome in the lung, kidney and liver.

Our results confirm, that controlled reperfusion might be also a potential therapeutic approach in vascular surgery against reperfusion injury in acute limb ischaemia.

6. NOVEL FINDINGS

In the first series of our investigations we observed the protective effects of **ischaemic postconditioning** after aortic occlusion-induced reperfusion injury in an experimental animal (rat) model.

We have three important observations in the study.

1. Our results demonstrated that ischaemic PostC significantly reduced the reperfusion induced early oxidative stress and the inflammatory responses (leukocyte activation, cytokine expression, myeloperoxidase elevation) after a sustained skeletal muscle ischaemia-reperfusion.

2. In our molecular biology investigations we observed that skeletal muscle ischaemia-reperfusion could activate both pro-, and anti-apoptotic pathways in myocardial, lung, kidney and liver. Thus peripheral ischaemia-reperfusion simultaneously induces survival and death signalisation in central organs that were not suffered from ischaemia-reperfusion.

3. Ischaemic postconditioning after peripheral muscle ischaemia-reperfusion showed significant reduction of proapoptotic signal activation and significant increase in antiapoptotic (protective) signal activation in central organs. Thus these results suggest that ischaemic PostC could reduce not only the local reperfusion injury but also can protect the other parenchymal organs in reperfusion injury.

The second series of our investigations we described the beneficial protective effect of **ischaemic PostC in human** vascular surgery interventions. Our results showed that ischaemic PostC after aortic occlusion in aorto-bifemoral bypass surgery could significantly reduce the oxidative stress in the early phase of reperfusion, preserves the endogenous antioxidant capacity, and depress the local and systemic inflammatory pathways (leukocyte activation, surface adhesion molecule expression).

PostC seems to be a beneficial and simple surgical method in cases of relatively long ischaemia affecting a mass of tissue to lower the surgical complications of revascularization.

In the third series we described the effect of **controlled reperfusion** on aortic occlusion-induced lower limb skeletal muscle reperfusion injuy in a pig model. We confirmed that with chrystalloid solution-diluted low pressure blood reperfusion significantly reduced the reperfusion induced oxidative stress and leukocyte activation and preserved the plasma antioxidant capacity.

The histological investigations showed reduced cellular necrosis and intracellular edema in the skeletal muscle treated with controlled reperfusion.
Controlled reperfusion also reduced lung, kidney, liver and myocardial injury after a long aortic occlusion.
Controlled reperfusion seems to be an effective method in long-lasting ischaemia – critical lower limb ischaemia in vascular surgery to reduce local and systemic reperfusion injury.

7. ACKNOWLEDGEMENT

I would like to take this opportunity to express my thanks for the overwhelming support I have received from my supervisor Prof Erzsébet Rőth, and Gábor Jancsó in completing this work.

I would thank the scientific support of my Chief Prof Lajos Kollár, and the help and patiente of my collegues on the Department of Surgery.

I would also like to acknowledge the help and assistance of Mária Kürthy, János Lantos and of all the staff at the Department of Surgical Research and Technique of Pécs University to carrying out the investigations and giving me the inward support over the years.

I would thank the indispensable help in the molecular biology methods to Krisztina Kovács, Alíz Kiss and Prof Balázs Sümegi in the Department of Medical Biochemistry of Pécs University.

Special thanks to Géza Hegedűs for giving me the chance to carry out the pathological analysis in the laboratory of the Department of Pathology in the Baranya County Hospital.

REFERENCES

[1] Piper HM, Meuter K, Schafer C. Cellular mechanisms of ischaemia-reperfusion injury. Ann Thorac Surg. 2003; 75: S644–8.

[2] Takemura G, Fujiwara H. Morphological aspects of apoptosis in heart diseases. J Cell Mol Med. 2006; 10: 56–75.

[3] Van Cruchten S, Van Den Broeck W. Morphological and biochemical aspects of apoptosis, oncosis and necrosis. Anat Histol Embryol. 2002; 31: 214–23.

[4] Tritto I, Ambrosio G. Role of oxidants in the signaling pathway of preconditioning. Antioxid Redox Signal. 2001; 3: 3–10.

[5] Zhao ZQ, Vinten-Johansen J. Myocardial apoptosis and ischemic preconditioning. Cardiovasc Res. 2002; 55: 438–55.

[6] Ambrosio G, Flaherty JT, Duilio C,Tritto I, Santoro G, Elia PP, Condorelli M, Chiariello M. Oxygen radicals generated at reflow induce peroxidation of membrane lipids in reperfused hearts. J Clin Invest. 1991; 87: 2056–66.

[7] Kaeffer N, Richard V, Thuillez C. Delayed coronary endothelial protection 24 hours after preconditioning: role of free radicals. Circulation. 1997; 96: 2311–6

[8] Lefer AM, Lefer DJ. Endothelial dysfunction in myocardial ischaemia and reperfusion: role of oxygenderived free radicals. Basic Res Cardiol. 1991; 86: 109–16.

[9] Zhao ZQ. Oxidative stress-elicited myocardial apoptosis during reperfusion. Curr Opin Pharmacol. 2004; 4: 159–65.

[10] Hoffman JW Jr, Gilbert TB, Poston RS, Silldorff EP. Myocardial reperfusion injury: etiology, mechanisms, and therapies. J Extra Corpor Technol. 2004; 36: 391–411.

[11] Gateau-Roesch O, Argaud L, Ovize M. Mitochondrial permeability transition pore and postconditioning. Cardiovasc Res. 2006; 70: 264–73.

[12] Schreck R, Albermann K, Baeuerle PA. Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukariotic cells. Free Radical Res Commun. 1992; 17: 221–37.

[13] Baldwin AS. The transcription factor NFkB and human disease. J Clin Invest. 2001; 107: 3–6.

[14] Lefer AM, Lefer DJ. The role of nitric oxide and cell adhesion molecules on the microcirculation in ischaemia-reperfusion. Cardiovasc Res. 1996; 32: 743–51.

[15] Radomski MW, Palmer RM, Moncada S. Comparative pharmacology of endothelium-derived relaxing factor, nitric oxide and prostacyclin in platelets. Br J Pharmacol. 1987; 92: 181–7.

[16] Reffelmann T, Kloner RA. The “no-reflow” phenomenon: basic science and clinical correlates. Heart. 2002; 87: 162–8.

[17] LB Becker , New concepts in reactive oxygen species and cardiovascular reperfusion physiology, Cardiovasc Res, 15 (2004), 461-70.

[18] KA Kaminski, Bonda TA, Korecki J, Musial WJ, Oxidative stress and neutrophil activation-the two keystones of ischaemia/reperfusion injury, Int J Cardiol, 86 (2002), 41-59. Review.

[19] Zhao ZQ, Corvera J, Halkos ME, Kerendi F,Wang NP, Guyton RA, Vinten-Johansen J. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning.

Am J Physiol Heart Circ Physiol. 2003; 285: H579–88.

[20] Vinten-Johansen J, Zhao ZQ, Zatta AJ, Kin H, Halkos ME, Kerendi F. Postconditioning A new link in nature's armor against myocardial ischaemiareperfusion injury. Basic Res Cardiol. 2005; 100: 295–310.

[21] Dow J, Kloner RA. Postconditioning does not reduce myocardial infarct size in an in vivo regional ischaemia rodent model. J Cardiovasc Pharmacol Ther. 2007; 12: 153–63.

[22] Schwartz LM, Lagranha CJ. Ischemic postconditioning during reperfusion activates Akt and ERK without protecting against lethal myocardial ischaemia-reperfusion injury in pigs. Am J Physiol Heart Circ Physiol. 2006; 290: H1011–8.

[23] Iliodromitis EK, Georgiadis M, Cohen MV, Downey JM, Bofilis E, Kremastinos DT. Protection from post-conditioning depends on the number of short ischemic insults in anesthetized pigs. Basic Res Cardiol. 2006; 101: 502–7.

[24] Manintveld OC, Te Lintel Hekkert M, van den Bos EJ, Suurenbroek GM, Dekkers DH, Verdouw PD, Lamers JM, Duncker DJ. Cardiac effects of postconditioning depend critically on the duration of index ischaemia. Am J Physiol Heart Circ Physiol. 2007; 292: 1551–60.

[25] Laskey WK. Brief repetitive balloon occlusions enhance reperfusion during percutaneous coronary intervention for acute myocardial infarction: a pilot study. Catheter Cardiovas Interv. 2005; 65: 361–7.

[26] Pagliaro P, Rastaldo R, Penna C, Mancardi D, Cappello S, Losano G. Nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) pathway is involved in ischemic postconditioning in the isolated rat heart. Circulation. 2004; 110: III 136.

[27] Philipp S,Yang XM, Cui L, Davis AM, Downey JM, Cohen MV. Postconditioning protects rabbit hearts through a protein kinase C-adenosine A2b receptor cascade. Cardiovasc Res. 2006; 70: 308–14.

[28] Zhao ZQ, Corvera J, Halkos ME, Kerendi F,Wang NP, Guyton RA, Vinten-Johansen J. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol. 2003; 285: H579–88.

[29] Darling CE, Jiang R, Maynard M, Whittaker P, Vinten-Johansen J, Przyklenk K. 'Postconditioning' via stuttering reperfusion limits myocardial infarct size in rabbit hearts: role of ERK 1/2. Am J Physiol Heart Circ Physiol. 2005; 289: H1618–26.

[30] Yang XM, Philipp S, Downey JM, Cohen MV. Postconditioning's protection is not dependent on circulating blood factors or cells but involves adenosine receptors and requires PI3-kinase and guanylyl cyclase activation. Basic Res Cardiol. 2005; 100: 57–63.

[31] Kerendi F, Kin H, Halkos ME, Jiang R, Zatta AJ, Zhao ZQ, Guyton RA, Vinten-Johansen J. Remote postconditioning. Brief renal ischaemia and reperfusion applied before coronary artery reperfusion reduces myocardial infarct size via endogenous activation of adenosine receptors. Basic Res Cardiol. 2005; 100: 404–12.

[32] Penna C, Cappello S, Mancardi D, Raimondo S, Rastaldo R, Gattullo D, Losano G, Pagliaro P. Post-conditioning reduces infarct size in the isolated rat heart: role of coronary flow and pressure and the nitric oxide/cGMP pathway. Basic Res Cardiol. 2006; 101: 168–79.

[33] Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. J Clin Invest. 1994; 94: 1621–8.

[34] Sun HY, Wang NP, Halkos M, Kerendi F, Kin H, Guyton RA, Vinten-Johansen J, Zhao ZQ. Postconditioning attenuates cardiomyocyte apoptosis via inhibition of JNK and p38 mitogen-activated protein kinase signaling pathways. Apoptosis. 2006; 11: 1583–93.

[35] Heltianu C, Costache G, Gafencu A, Diaconu M, Bodeanu M, Cristea C, Azibi K, Poenaru L,

Simionescu M. Relationship of eNOS gene variants to diseases that have in common an endothelial cell dysfunction. J Cell Mol Med. 2005; 9: 135–42.

[36] Zhao ZQ, Corvera J, Halkos ME, Kerendi F,Wang NP, Guyton RA, Vinten-Johansen J. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol. 2003; 285: H579–88.

[37] Zhao ZQ, Corvera J, Halkos ME, Kerendi F,Wang NP, Guyton RA, Vinten-Johansen J. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol. 2003; 285: H579–88.

[38] Schwartz LM, Lagranha CJ. Ischemic postconditioning during reperfusion activates Akt and ERK without protecting against lethal myocardial ischaemia-reperfusion injury in pigs. Am J Physiol Heart Circ Physiol. 2006; 290: H1011–8.

[39] Sun HY, Wang NP, Kerendi F, Halkos M, Kin H, Guyton RA, Vinten-Johansen J, Zhao ZQ. Hypoxic postconditioning reduces cardiomyocyte loss by inhibiting ROS generation and intracellular Ca2+ overload. Am J Physiol Heart Circ Physiol. 2005; 288: H1900–8.

[40] Sivaraman V, Mudalgiri NR, Di Salvo C, Kolvekar S, Hayward M, Yap J, Keogh B, Hausenloy DJ, Yellon DM. Postconditioning protects human atrial muscle through the activation of the RISK pathway. Basic Res Cardiol. 2007; 102: 453–9.

[41] Penna C, Cappello S, Mancardi D, Raimondo S, Rastaldo R, Gattullo D, Losano G, Pagliaro P. Post-conditioning reduces infarct size in the isolated rat heart: role of coronary flow and pressure and the nitric oxide/cGMP pathway. Basic Res Cardiol. 2006; 101: 168–79.

[42] Couvreur N, Lucats L, Tissier R, Bize A, Berdeaux A, Ghaleb B. Differential effects of postconditioning on myocardial stunning and infarction: a study in conscious dogs and anesthetized rabbits. Am J Physiol Heart Circ Physiol. 2006; 291: H1345–50.

[43] Sivaraman V, Mudalgiri NR, Di Salvo C, Kolvekar S, Hayward M, Yap J, Keogh B, Hausenloy DJ, Yellon DM. Postconditioning protects human atrial muscle through the activation of the RISK pathway. Basic Res Cardiol. 2007; 102: 453–9.

[44] Galagudza M, Kurapeev D, Minasian S, Valen G, Vaage J. Ischemic postconditioning: brief ischaemia during reperfusion converts persistent ventricular fibrillation into regular rhythm. Eur J Cardiothorac Surg. 2004; 25: 1006–10.

[45] Grech ED, Ramsdale DR. Termination of reperfusion arrhythmia by coronary artery occlusion. Br Heart J. 1994; 72: 94–5.

[46] Iliodromitis EK, Georgiadis M, Cohen MV, Downey JM, Bofilis E, Kremastinos DT. Protection from post-conditioning depends on the number of short ischemic insults in anesthetized pigs. Basic Res Cardiol. 2006; 101: 502–7.

[47] Kloner RA, Dow J, Bhandari A. Postconditioning markedly attenuates ventricular arrhythmias after ischaemia-reperfusion. J Cardiovasc Pharmacol Ther. 2006; 11: 55–63.

[48] Vanagt WY, Cornelussen RN, Baynham TC, Van Hunnik A, Poulina QP, Babiker F, Spinelli J, Delhaas T, Prinzen FW. Pacing-induced dyssynchrony during early reperfusion reduces infarct size. J Am Coll Cardiol. 2007; 49: 1813–9.

[49] Mykytenko J, Kerendi F, Reeves JG, Kin H, Zatta AJ, Jiang R, Guyton RA, Vinten-Johansen J, Zhao ZQ. Long-term inhibition of myocardial infarction by postconditioning during reperfusion. Basic Res Cardiol. 2007; 102: 90–100.

[50] Halkos ME, Kerendi F, Corvera JS,Wang NP, Kin H, Payne CS, Sun HY, Guyton RA,Vinten-Johansen J, Zhao ZQ. Myocardial protection with postconditioning is not enhanced by ischemic preconditioning.Ann

Thorac Surg. 2004; 78: 961–9.

[51] Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM. Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. Circ Res. 2004; 95: 230–2.

[52] Penna C, Cappello S, Mancardi D, Raimondo S, Rastaldo R, Gattullo D, Losano G, Pagliaro P. Post-conditioning reduces infarct size in the isolated rat heart: role of coronary flow and pressure and the nitric oxide/cGMP pathway. Basic Res Cardiol. 2006; 101: 168–79.

[53] Yang XM, Proctor JB, Cui L, Krieg T, Downey JM, Cohen MV. Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. J Am Coll Cardiol. 2004; 44: 1103–10.

[54] Sato H, Bolli R, Rokosh GD, Bi Q, Dai S, Shirk G, Tang XL. The cardioprotection of the late phase of ischemic preconditioning is enhanced by postconditioning via a COX-2-mediated mechanism in conscious rats. Am J Physiol Heart Circ Physiol. 2007; 293: H2557–64.

[55] Crisostomo PR,Wang M,Wairiuko GM,Terrell AM, Meldrum DR. Postconditioning in females depends on injury severity. J Surg Res. 2006; 134: 342–7.

[56] Boengler K, Buechert A, Heinen Y, Roeskes C, Hilfiker-Kleiner D, Heusch G, Schulz R. Cardioprotection by ischemic postconditioning is lost in aged and STAT3-deficient mice. Circ Res. 2008; 102: 131–5.

[57] Y Itoh, Takaoka R, Ohira M, Abe T, Tanahashi N, Suzuki N. Reactive oxygen species generated by mitochondrial injury in human brain microvessel endothelial cells. Clin Hemorheol Microcirc. 2006;34(1-2):163-8.

[58] E Arató, M Kürthy, G Jancsó, H Merkli, E Pál, L Kollár, E Rőth, The revascularization syndrome of the lower limbs, Perfusion, 5 (2005), 168-176.

[59] T Gori, Di Stolfo G, Sicuro S, Dragoni S, Parker JD, Forconi S, The effect of ischaemia and reperfusion on microvascular function: a human in vivo comparative study with conduit arteries. Clin Hemorheol Microcirc. 2006;35(1-2):169-73.

[60]T Gori, Lisi M, Forconi S. Ischaemia and reperfusion: the endothelial perspective. A radical view, Clin Hemorheol Microcirc, 35 (2006), 31-4. Review.

[61] Kin H, Zatta AJ, Lofye MT, Amerson BS, Halkos ME, Kerendi F, Zhao ZQ, Guyton RA, Headrick JP, Vinten-Johansen J. Postconditioning reduces infarct size via adenosine receptor activation by endogenous adenosine. Cardiovasc Res. 2005; 67: 124–33.

[62] Ohakawa HN, Okishi N, Yagi K: Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem, 1979; 95: 351-8

[63] Sedlak J, Lindsay RH: Estimation of total protein-bound and non-protein sulphydryl groups in tissue with Ellman'sreagent, Anal Biochem, 1968; 25: 192-205

[64] Misra HP, Fridovich I. The role of superoxide anion in the autooxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 1972. 247: 3170-3175.

[65] Albelda SM, Smith CW, Ward PA. Adhesion molecules and inflammatory injury. FASEB J. 1994, 8: 504-512.

[66] Menger MD, Vollmar B. Adhesion molecules as determinations disease: from molecular biology to surgical research. BR. J. Surg. 1996, 83: 588-601.

[67] Arató E. PhD Thesis 2006 Univ of Pécs

[68] Penna C, Cappello S, Mancardi D, Raimondo S, Rastaldo R, Gattullo D, Losano G, Pagliaro P. Post-conditioning reduces infarct size in the isolated rat heart: role of coronary flow and pressure and the nitric oxide/cGMP pathway. Basic Res Cardiol. 2006; 101: 168–79.

[69] Zhao ZQ, Corvera J, Halkos ME, Kerendi F,Wang NP, Guyton RA, Vinten-Johansen J. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol. 2003; 285: H579–88.

[70] Hausenloy DJ, Yellon DM. The mitochondrial permeability transition pore: its fundamental role in mediating cell death during ischaemia and reperfusion. J Mol Cell Cardiol. 2003; 35: 339–41.

[71] Y Itoh, Takaoka R, Ohira M, Abe T, Tanahashi N, Suzuki N. Reactive oxygen species generated by mitochondrial injury in human brain microvessel endothelial cells. Clin Hemorheol Microcirc. 2006;34(1-2):163-8.

[72] Kin H, Zatta AJ, Lofye MT, Amerson BS, Halkos ME, Kerendi F, Zhao ZQ, Guyton RA, Headrick JP, Vinten-Johansen J. Postconditioning reduces infarct size via adenosine receptor activation by endogenous adenosine. Cardiovasc Res. 2005; 67: 124–33.

[73] Philipp S,Yang XM, Cui L, Davis AM, Downey JM, Cohen MV. Postconditioning protects rabbit hearts through a protein kinase C-adenosine A2b receptor cascade. Cardiovasc Res. 2006; 70: 308–14.

[74] Penna C, Rastaldo R, Mancardi D, Raimondo S, Cappello S, Gattullo D, Losano G, Pagliaro P. Postconditioning induced cardioprotection requires signalling through a redox-sensitive mechanism, mitochondrial ATP-sensitive K+ channel and protein kinase C activation. Basic Res Cardiol. 2006; 101: 180–9.

[75] Hausenloy DJ, Tsang A, Yellon, DM. The reperfusion injury salvage kinase pathway: a common target for both ischemic preconditioning and postconditioning. Trends Cardiovasc Med. 2005; 15: 69–75.

[76] Philipp S,Yang XM, Cui L, Davis AM, Downey JM, Cohen MV. Postconditioning protects rabbit hearts through a protein kinase C-adenosine A2b receptor cascade. Cardiovasc Res. 2006; 70: 308–14.

[77] Penna C, Mancardi D, Rastaldo R, Losano G, Pagliaro P. Intermittent activation of bradykinin B2 receptors and mitochondrial KATP channels trigger cardiac postconditioning through redox signaling. Cardiovasc Res. 2007; 75: 168–77.

[78] Kin H, Zatta AJ, Jiang R, Reeves JG. Activation of opioid mediates the infarct size reduction by postconditioning. J Mol Cell Cardiol. 2005; 38: 827.

[79] Cohen MV, Yang XM, Downey JM. Nitric oxide is a preconditioning mimetic and cardioprotectant and is the basis of many available infarct-sparing strategies. Cardiovasc Res. 2006; 70: 231–9.

[80] Dawn B, Bolli R. Role of nitric oxide in myocardial preconditioning. Ann N Y Acad Sci. 2002; 962: 18–41.
[81] Sasaki N, Sato T, Ohler A, O'Rourke B, Marban E. Activation of mitochondrial ATP-dependent potassium channels by nitric oxide. Circulation. 2000; 101: 439–45.

[82] Moncada S, Erusalimsky JD. Does nitric oxide modulate mitochondrial energy generation and apoptosis? Nat Rev Mol Cell Biol. 2002; 3: 214–20.

[83] Gattullo D, Linden RJ, Losano G, Pagliaro P, Westerhof N. Ischaemic preconditioning changes the pattern of coronary reactive hyperaemia in the goat: role of adenosine and nitric oxide. Cardiovasc Res. 1999; 42: 57–64.

[84] Ridnour LA, Thomas DD, Mancardi D, Espey MG, Miranda KM, Paolocci N, Feelisch M, Fukuto J, Wink DA. The chemistry of nitrosative stress induced by nitric oxide and reactive nitrogen oxide species. Putting perspective on stressful biological situations. Biol Chem. 2004; 385: 1–10.

[85] Pagliaro P, Mancardi D, Rastaldo R, Penna C, Gattullo D, Miranda KM, Feelisch M, Wink DA,

Kass DA, Paolocci N. Nitroxyl affords thiol-sensitive myocardial protective effects akin to early preconditioning. Free Radic Biol Med. 2003; 34: 33–43.

[86] Ma XL, Gao F, Liu GL, Lopez BL, Christopher TA, Fukuto JM, Wink DA, Feelisch M. Opposite effects of nitric oxide and nitroxyl on postischemic myocardial injury. Proc Natl Acad Sci USA. 1999; 96: 14617–22.

[87] Yang XM, Proctor JB, Cui L, Krieg T, Downey JM, Cohen MV. Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. J Am Coll Cardiol. 2004; 44: 1103–10.

[88] Penna C, Cappello S, Mancardi D, Raimondo S, Rastaldo R, Gattullo D, Losano G, Pagliaro P. Post-conditioning reduces infarct size in the isolated rat heart: role of coronary flow and pressure and the nitric oxide/cGMP pathway. Basic Res Cardiol. 2006; 101: 168–79.

[89] Penna C, Mancardi D, Rastaldo R, Losano G, Pagliaro P. Intermittent activation of bradykinin B2 receptors and mitochondrial KATP channels trigger cardiac postconditioning through redox signaling. Cardiovasc Res. 2007; 75: 168–77.

[90] Pagliaro P, Rastaldo R, Penna C, Mancardi D, Cappello S, Losano G. Nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) pathway is involved in ischemic postconditioning in the isolated rat heart. Circulation. 2004; 110: III 136.

[91] Patel VC,Yellon DM, Singh KJ, Neild GH,Woolfson RG. Inhibition of nitric oxide limits infarct size in the in situ rabbit heart. Biochem Biophys Res Commun. 1993; 194: 234–8.

[92] Bolli R. Cardioprotective function of inducible nitric oxide synthase and role of nitric oxide in myocardial ischaemia and preconditioning: an overview of a decade of research. J Mol Cell Cardiol. 2001; 33: 1897–918.

[93] Wink DA, Miranda KM, Katori T, Mancardi D, Thomas DD, Ridnour L, Espey MG, Feelisch M, Colton CA, Fukuto JM, Pagliaro P, Kass DA, Paolocci N. Orthogonal properties of the redox siblings nitroxyl and nitric oxide in the cardiovascular system: a novel redox paradigm. Am J Physiol Heart Circ Physiol. 2003; 285: H2264–76.

[94] Pagliaro P. Differential biological effects of products of nitric oxide (NO) synthase: it is not enough to say NO. Life Sci. 2003; 73: 2137–49.

[95] Schulz R, Kelm M, Heusch G. Nitric oxide in myocardial ischaemia/reperfusion injury. Cardiovasc Res. 2004; 61: 402–13.

[96] Downey JM, Cohen MV. A really radical observation–a comment on Penna et al. in Basic Res Cardiol (2006) 101:180–189. Basic Res Cardiol. 2006; 101: 190–1.

[97] Cohen MV,Yang XM, Downey JM. The pH hypothesis of postconditioning: staccato reperfusion reintroduces oxygen and perpetuates myocardial acidosis. Circulation. 2007; 115: 1895–903.

[98] Hausenloy DJ, Wynne AM, Yellon DM. Ischemic preconditioning targets the reperfusion phase. Basic Res Cardiol. 2007; 102: 445–52.

[99] Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM. Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. Circ Res. 2004; 95: 230–2.

[100] Pagliaro P, Rastaldo R, Penna C, Mancardi D, Cappello S, Losano G. Nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) pathway is involved in ischemic postconditioning in the isolated rat heart. Circulation. 2004; 110: III 136.

[101] Penna C, Rastaldo R, Mancardi D, Raimondo S, Cappello S, Gattullo D, Losano G, Pagliaro P. Postconditioning induced cardioprotection requires signalling through a redox-sensitive mechanism, mitochondrial ATP-sensitive K+ channel and protein kinase C activation. Basic Res Cardiol. 2006; 101: 180–9.

[102] Yang XM, Philipp S, Downey JM, Cohen MV. Postconditioning's protection is not dependent on circulating blood factors or cells but involves adenosine receptors and requires PI3-kinase and guanylyl cyclase activation. Basic Res Cardiol. 2005; 100: 57–63.

[103] Penna C, Rastaldo R, Mancardi D, Raimondo S, Cappello S, Gattullo D, Losano G, Pagliaro P. Postconditioning induced cardioprotection requires signalling through a redox-sensitive mechanism, mitochondrial ATP-sensitive K+ channel and protein kinase C activation. Basic Res Cardiol. 2006; 101: 180–9.

[104] Hausenloy DJ, Tsang A, Yellon, DM. The reperfusion injury salvage kinase pathway: a common target for both ischemic preconditioning and postconditioning. Trends Cardiovasc Med. 2005; 15: 69–75.

[105] Darling CE, Jiang R, Maynard M, Whittaker P, Vinten-Johansen J, Przyklenk K. 'Postconditioning' via stuttering reperfusion limits myocardial infarct size in rabbit hearts: role of ERK 1/2. Am J Physiol Heart Circ Physiol. 2005; 289: H1618–26.

[106] Yang XM, Philipp S, Downey JM, Cohen MV. Postconditioning's protection is not dependent on circulating blood factors or cells but involves adenosine receptors and requires PI3-kinase and guanylyl cyclase activation. Basic Res Cardiol. 2005; 100: 57–63.

[107] Yang XM, Philipp S, Downey JM, Cohen MV. Postconditioning's protection is not dependent on circulating blood factors or cells but involves adenosine receptors and requires PI3-kinase and guanylyl cyclase activation. Basic Res Cardiol. 2005; 100: 57–63.

[108] Yang XM, Philipp S, Downey JM, Cohen MV. Postconditioning's protection is not dependent on circulating blood factors or cells but involves adenosine receptors and requires PI3-kinase and guanylyl cyclase activation. Basic Res Cardiol. 2005; 100: 57–63.

[109] Cao Z, Liu L, Van Winkle DM. Met5-enkephalininduced cardioprotection occurs via transactivation of EGFR and activation of PI3K. Am J Physiol Heart Circ Physiol. 2005; 288: 1955–64.

[110] Vinten-Johansen J, Zhao ZQ, Zatta AJ, Kin H, Halkos ME, Kerendi F. Postconditioning A new link in nature's armor against myocardial ischaemiareperfusion injury. Basic Res Cardiol. 2005; 100: 295–310.

[111] Darling CE, Jiang R, Maynard M, Whittaker P, Vinten-Johansen J, Przyklenk K. 'Postconditioning' via stuttering reperfusion limits myocardial infarct size in rabbit hearts: role of ERK 1/2. Am J Physiol Heart Circ Physiol. 2005; 289: H1618–26.

[112] Zhao ZQ, Vinten-Johansen J. Postconditioning: reduction of reperfusion-induced injury. Cardiovasc Res. 2006; 70: 200–11.

[113] Kostin S. Zonula occludens-1 and connexin 43 expression in the failing human heart. J Cell Mol Med. 2007; 11: 892–5.

[114] Heusch G, Büchert A, Feldhaus S, Schulz R. No loss of cardioprotection by postconditioning in connexin 43-deficient mice. Basic Res Cardiol. 2006; 101: 354–6.

[115] Weiss JN, Korge P, Honda HM, Ping P. Role of the mitochondrial permeability transition in myocardial disease. Circ Res. 2003; 93: 292–301.

[116] Murata M, Akao M, O'Rourke B, Marban E. Mitochondrial ATP-sensitive potassium channels attenuate matrix Ca(2+) overload during simulated ischaemia and reperfusion: possible mechanism of cardioprotection. Circ Res. 2001; 89: 891–8.

[117] Sun HY, Wang NP, Kerendi F, Halkos M, Kin H, Guyton RA, Vinten-Johansen J, Zhao ZQ. Hypoxic postconditioning reduces cardiomyocyte loss by inhibiting ROS generation and intracellular Ca2+ overload. Am J Physiol Heart Circ Physiol. 2005; 288: H1900–8.

[118] Argaud L, Gateau-Roesch O, Raisky O, Loufouat J, Robert D, Ovize M. Postconditioning inhibits

mitochondrial permeability transition. Circulation. 2005; 111: 194–7.

[119] Bopassa JC, Ferrera R, Gateau-Roesch O, Couture-Lepetit E, Ovize M. PI 3-kinase regulates the mitochondrial transition pore in controlled reperfusion and postconditioning. Cardiovas Res. 2006; 69: 178–85.

[120] Gateau-Roesch O, Argaud L, Ovize M. Mitochondrial permeability transition pore and postconditioning. Cardiovasc Res. 2006; 70: 264–73.

[121] Tritto I, Ambrosio G. Role of oxidants in the signaling pathway of preconditioning. Antioxid Redox Signal. 2001; 3: 3–10.

[122] Zhao ZQ. Oxidative stress-elicited myocardial apoptosis during reperfusion. Curr Opin Pharmacol. 2004; 4: 159–65.

[123] Pagliaro P. Differential biological effects of products NO. Life Sci. 2003; 73: 2137–49.

[124] Zhao ZQ. Oxidative stress-elicited myocardial apoptosis during reperfusion. Curr Opin Pharmacol. 2004; 4: 159–65.

[125] Yao Z, Tong J, Tan X, Li C, Shao Z, Kim WC, vanden Hoek TL, Becker LB, Head CA, Schumacker PT. Role of reactive oxygen species in acetylcholineinduced preconditioning in cardiomyocytes. Am J Physiol Heart Circ Physiol. 1999; 277: H2504–9.

[126] Urschel WC. Cardiovascular effects of hydrogen peroxide: current status. Dis Chest. 1967; 51: 180–92.

[127] Tsutsumi YM, Yokoyama T, Horikawa Y, Roth DM, Patel HH. Reactive oxygen species trigger ischemic and pharmacological postconditioning: In vivo and in vitro characterization. Life Sci. 2007; 81: 1223–7.

[128] Hausenloy DJ, Wynne AM, Yellon DM. Ischemic preconditioning targets the reperfusion phase. Basic Res Cardiol. 2007; 102: 445–52.

[129] Flaherty JT, Pitt B, Gruber JW, Heuser RR, Rothbaum DA, Burwell LR, George BS, Kereiakes DJ, Deitchman D, Gustafson N. Recombinant human superoxide dismutase (h-SOD) fails to improve recovery of ventricular function in patients undergoing coronary angioplasty for acute myocardial infarction. Circulation. 1999; 89: 1982–91.

[130] Kloner RA, Jennings RB. Consequences of brief ischaemia: stunning, preconditioning and their clinical implications. Circulation. 2001; 104: 2981–9.

[131] Penna C, Mancardi D, Rastaldo R, Losano G, Pagliaro P. Intermittent activation of bradykinin B2 receptors and mitochondrial KATP channels trigger cardiac postconditioning through redox signaling. Cardiovasc Res. 2007; 75: 168–77.

[132] Hausenloy DJ, Wynne AM, Yellon DM. Ischemic preconditioning targets the reperfusion phase. Basic Res Cardiol. 2007; 102: 445–52.

[133] Earnshaw J. Demography and etiology of acute leg ischaemia. Semin Vasc Surg 2001;14:86-92.

[134] Ljungmann C, Holmber L, Bergquist D, Bergström R, Adami H. Amputation risk and survival after embolectomy for acute arterial ischaemia. Time trends in a defined Swedish population. Eur J Vasc Endovas Surg 1996;11:176-82.

[135] Ouriel K, Veith FJ, Sasahara AA. A comparison of recombinant urokinase with vascular surgery as initial treatment for acute arterial occlusion of the legs. N Engl J Med 1998;338:1105-11.

[136] Blaisdell FW. The pathophysiology of skeletal muscle ischaemia and the reperfusion syndrome: a review. Cardiovasc Surg 2002;10:620-30.

[137] Rubin BB, Walker PM. Pathophysiology of acute skeletal muscle ischaemia: adenine nucleotide metabolism in ischemic reperfused muscle. 1992;5:11-14.

[138] Follette DM, Fey K, Buckberg GD, Helly JJ Jr, Steed DL, Foglia RP et al. Reducing post-ischemic damage by temporary modification of reperfusate calcium, potassium, pH and osmolarity. J Thorac Cardiovasc Surg 1981;82:221-38.

[139] Allen BS, Buckberg GD, Fontan FM, Kirsh MM, Popoff G, Beyersdorf F et al. Superiority of controlled surgical reperfusion versus percutaneous transluminal coronary angioplasty in acute coronary occlusion. J Thorac Cardiovasc Surg 1993;105:864-84.

[140] Mitrev Z, Beyersdorf F, Hallmann R, Polokczek Y, Ihnken K, Herold H, et al. Eperfusion injury in sceletal muscle: controlled limb reperfusion reduces local and systemic complications after prolonged ischaemia. Cardiovas Surg 1994;2:737-48.

[141] Beyersdorf F, Mitev Z, Ihnken K, Schmiedt W, Sarai K, Eckel L, et al. Controlled limb reperfusion in patients having cardiac operations.J Thorac Cardiovasc Surg 1996;111:873-81.

[142] Schlensak C, Doenst T, Bitu-Moreno J, Beyersdorf F. Controlled limb reperfusion with a simplified perfusion system. Thorac Cardiov Surg 2000;48:274-8.

[143] Pemberton M, Varty K, Nydahl S, Bell PR. The surgical management of acute limb ischaemia due to native vessel occlusion. Eur J Vasc Endovasc Surg 1999;17:72-6.

[144] Blaisdell FW. The pathophysiology of skeletal muscle ischaemia and the reperfusion syndrome: a review. Cardiovasc Surg 2002;10:620-30.

[145] Allen BS, Okamoto F, Buckberg GD, Bugyi H, Young H, Leaf J, et al. Studies on controlled reperfusion after ischaemia. XV. Immediate functional recovery after 6 hours of regional ischaemia by careful control of conditions of reperfusion and composition of reperfusate. J Thorac Cardiovasc Surg 1986;92:621-35.

[146] Beyersdorf F, Unger A, Wildhirt A, Kretzer U, Deutschlander N, Krüger S, et al. Studies of reperfusion injury in sceletal muscle: preserved cellular viability after extended periods of warm ischaemia. J Cardiovasc Surg (Torino) 1991;32:664-76.

[147] Swanson DK, Myerowitz PD. Effect of reperfusion temperature and pressure on the functional and metabolic recovery of preserved hearts. J Thorac Cardiovasc Surg 1983;86:242-51.

[148] Wright JG, Fox D, Kerr JC, Valeri CR, Hobson RW II. Rate of reperfusion blood flow modulates reperfusion injury in skeletal muscle. J Surg Res 1988:44:754-63.

[149] Beyersdorf F, Matheis G, Krüger S, Hanselmann A, Freisleben HG, Zimmer P, et al. Avoiding reperfusion injury after limb revascularisation: experimental observations and recommendations for clinical application. J Vasc Surg 1989;9:757-66.

[150] Mitrev Z, Beyersdorf F, Hallmann R, Polokczek Y, Ihnken K, Herold H, et al. Eperfusion injury in sceletal muscle: controlled limb reperfusion reduces local and systemic complications after prolonged ischaemia. Cardiovas Surg 1994;2:737-48.

[151] Gidlöff A, Larsson J, Lewis D, Hammersen F. Capillary endothelial alterations affecting reperfusion after ischaemia in human skeletal muscle. Bibl Anat 1981;20:572-7.

[152] Levitsky S, Feinberg H. Biochemical changes of ischaemia. Ann Thorac Surg 1975;20:21-29.

[153] Rosenkranz ER, Okamoto F, Buckberg GD, Robertson JM, Vinten- Johanson J, Bugyi H. Safety of prolonged aortic clamping with blood cardioplegia. III. Aspartate enrichment of glutamate-blood cardioplegia in energy-depleted hearts after ischemic and reperfusion injury. J Thorac Surg 1986;91:428-35.

[154] Mc Cord JM. Oxygen derived free radicals in postischemic tissue injury. N Engl J Med 1985;312:159-62.
[155] Vogt PR, Lutz Hj, Akintürk HI, Roth P, Schönburg M, Menon AK, et al. Prevention of postischemic tissue injury by controlled reperfusion: a preliminary study Int J Angiol 2003;12:172-177.

[156] Blaisdell FW. The pathophysiology of skeletal muscle ischaemia and the reperfusion syndrome: a review. Cardiovasc Surg 2002;10:620-30.

[157] Beyersdorf F, Matheis G, Krüger S, Hanselmann A, Freisleben HG, Zimmer P, et al. Avoiding reperfusion injury after limb revascularisation:experimental observations and recommendations for clinical application. J Vasc Surg 1989;9:757-66.

[158] Defraigne JO, Pincemail J, Laroche C, Blaffart F, Limet R. Successful controlled limb reperfusion after severe prolonged ischaemia. J Vasc Surg
1997;26:346-50.