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Ischaemic Postconditioning Reduces Reperfusion Injury After Aortic Revascularization Surgery

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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.

2. 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.

2.1 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 necrosis². Apoptosis can be

caused by both prolonged ischaemia/hypoxia and by reperfusion³. 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 role⁴. 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 inflammation⁵. 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 apoptosis⁶. It reacts with the nitric oxide, forming peroxynitrite ($ONOO^-$). Therefore, $ONOO^-$, represents a sign of a reduced availability of nitric oxide⁷ and it participates with O_2^- in the lesion of tissues⁸. 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^\cdot), 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 mitochondria⁹. 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 necrosis¹⁰.

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 reperfusion¹¹.

Intriguingly, the nuclear factor kappa B (NF κ B) plays a double-edge sword role in tissue-protection. Activation of NF κ B is essential for late preconditioning, in which NF κ B is involved in the up-regulation of iNOS and COX-2 genes. However, in the longer time the role NF κ B is also important in reperfusion injury. It contributes to the exacerbation of the tissues lesions sustaining inflammatory reactions. The activation of NF κ B is induced from several agents included hydrogen peroxide¹². NF κ B 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 cells¹³. Moreover, the reduced nitric oxide availability determined by I/R participates to the activation of transcription codifying for molecules of cellular adhesion¹⁴. 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 NF κ B. The nitric oxide deficiency can also cause vasoconstriction and formation of micro-thrombi into the lumen of the small vessels¹⁵. 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 NF κ B, which lead to the augmented expression of molecules of cellular adhesion, leukocyte infiltration and no-reflow phenomenon.

3. 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*)

4. Definition of postconditioning

The concept of 'Ischaemic PostC' was first described by Vinten-Johansen's group¹⁷. 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.

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.

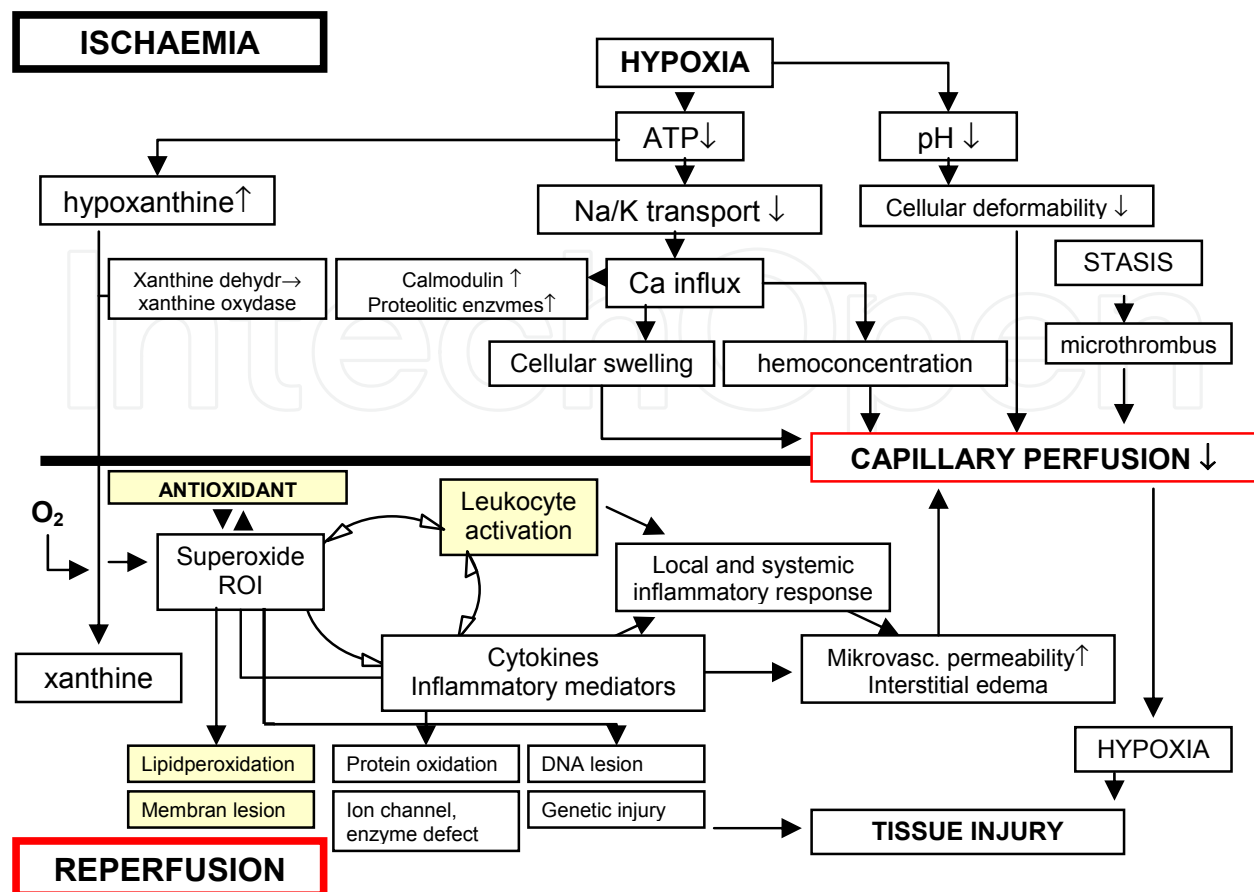


Fig. 1. Simplified presentation of the mechanism of ischaemic-reperfusion injury. Emphasizing, that the engine of reperfusion injury is the ROI - cytokine - leukocyte positive feedback circle.

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 catalysed - 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.¹⁸ 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.¹⁹ (ROI - reactive oxygen intermediers; ATP - adenosine triphosphate; DNA - deoxyribonucleic acid)

6. Reduction of necrosis

In their seminal study Vinten-Johansen and coworkers²⁰ 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 well²¹. As mentioned, in multiple

species and models, PostC reduces infarct size by ~20–70% versus matched controls with matched risk areas²². Studies from Przyklenk's laboratory and other laboratories confirmed the infarct size reduction in rat isolated heart model²³. 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 pressure²⁴.

7. Reduction of apoptosis

Apoptosis is a genetically programmed cell death that occurs in reperfusion injury²⁵. 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.²⁶ 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 peripheral PostC model.

8. Reduction of endothelial dysfunction

The endothelial cell dysfunction is a common characteristic of various heart pathologies²⁷. In their seminal study Zhao et al.²⁸ 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.

9. 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 risk²⁹. A reduction in superoxide anion generation in the perivascular area has also been observed in the proximity of risk area of postconditioned hearts³⁰. 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)³¹.

10. Possibilities of postconditioning

It has been reported that PostC-induced necrosis reduction persists up to 72 hrs³². 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.³³ 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.³⁴ and by us³⁵ in isolated perfused rat hearts. However, Yang et al.³⁶ 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 rats³⁷. It remains to be 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. ischaemia³⁸. 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 protection³⁹.

In clinical practice ischaemic postconditioning seems even as effective as ischaemic preconditioning. Furthermore, PostC could be used after ischaemia, thus it could be used in acute ischaemia as well. There 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.

11. Effects of ischaemic postconditioning in human vascular surgery

11.1 Aims

Ischaemic postconditioning was found effective to reduce reperfusion injury not only in experimental animal models, but in humans as well in cardiac interventions. In 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.

11.2 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.

12. Aorto-bifemoral bypass surgery

In general anaesthesia median laparotomy 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.

13. 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.

14. The measurement of oxidative stress parameters

Measurement of malondialdehyde (MDA):

Malondialdehyde was determined in anticoagulated whole blood, by photometric method⁴⁰.

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 Lindsay⁴¹.

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 activity⁴².

15. Measurement of inflammatory response, leukocyte activation

Determination of free radical production from whole blood:

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

16. Serum myeloperoxidase assay

Anticoagulated blood was centrifuged with 2000g, and 200 μ l plasma was mixed with 1 ml working solution (0,1 M sodium-citrate 10,9 ml, 0,05% Triton-X 100 5 μ l, 1mM H₂O₂ 1 ml, 0,1% o-dianisidine 100 μ l). The mixture was incubated at 37 °C 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 analyser (Diatron Ltd, Budapest, Hungary).

17. Leukocyte adhesion molecule measurement

The leukocytes were marked with fluorescein isothiocyanide (FITC) labeled antibodies for adhesion molecules (CD11, CD11b, CD18, CD49d, és CD97) (Becton Dickinson Biosciences, Pharmingen, USA), and measurements were performed on BD FACS Calibur (Becton Dickinson Biosciences, Pharmingen, USA) flowcytometer.^{43 44}

18. Hematology test

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

19. Statistical analysis

Data are expressed as mean \pm 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 $p < 0.05$.

20. 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 2)

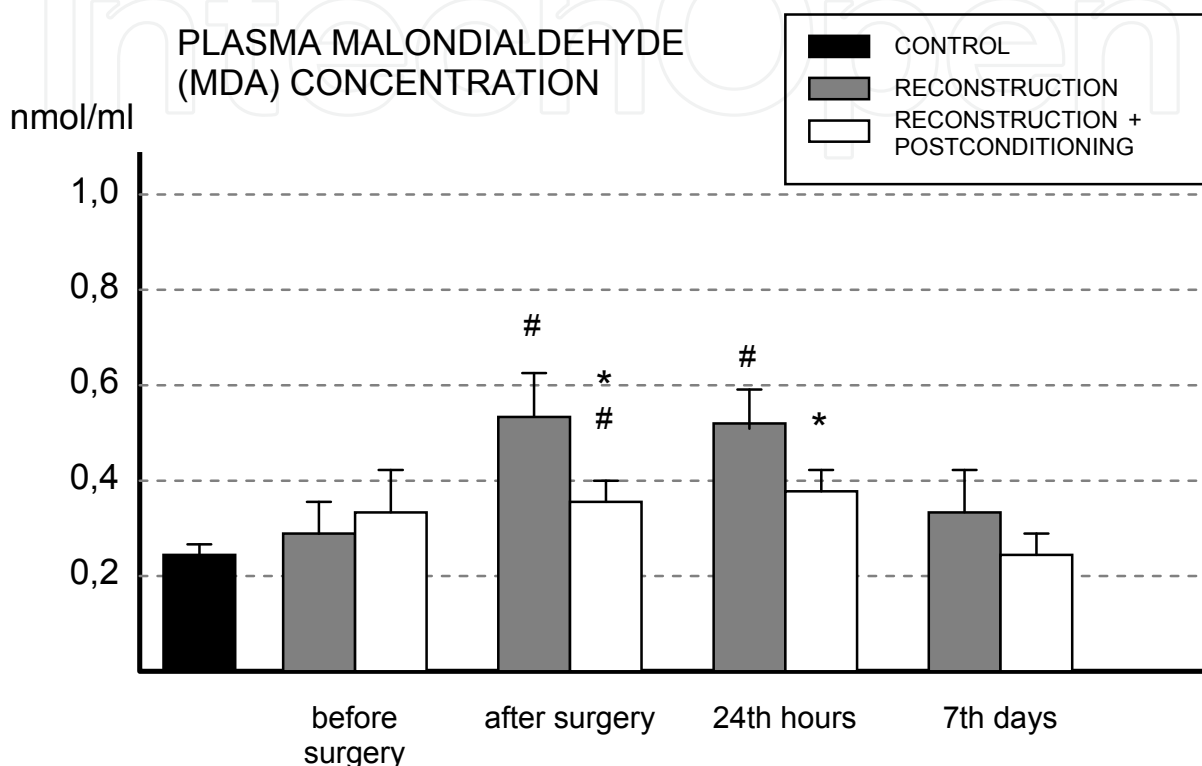


Fig. 2. Changes in plasma malondialdehyde concentration in the patients following the operations. (# $p < 0.05$ vs before surgery; * $p < 0.05$ vs non-conditioned group)

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 leukocytes⁴⁵).

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 3)

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 4)

PLASMA LEVELS OF ANTIOXIDANT COMPOUNDS

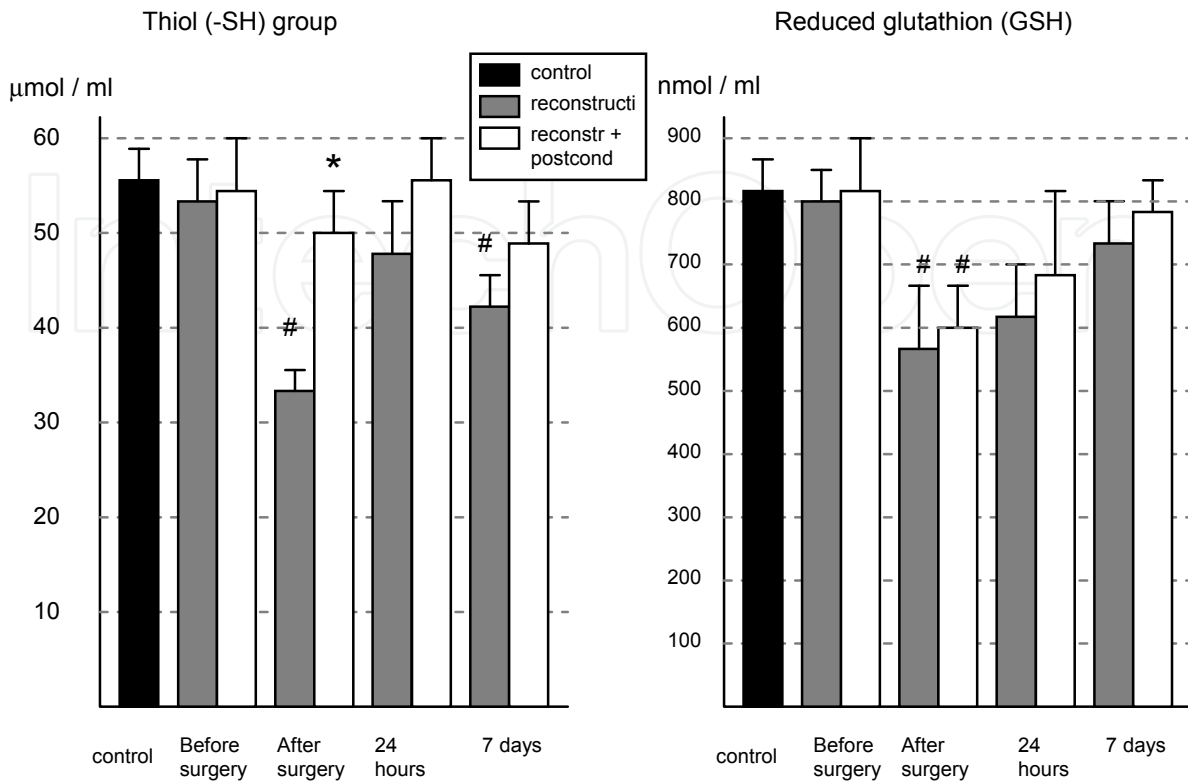


Fig. 3. Changes in antioxidant compounds (thiol group, reduced glutathion) plasma levels during the examined perioperative period. (# p < 0.05 vs before surgery; * p < 0.05 vs non-conditioned group)

ACTIVITY OF SUPEROXIDE DISMUTASE (SOD)

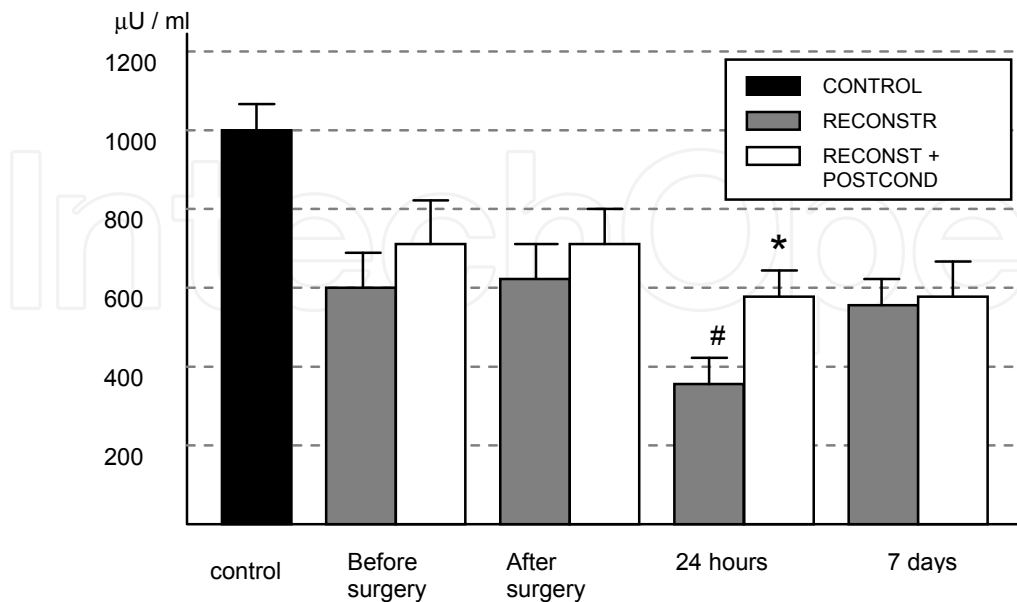


Fig. 4. Changes in blood superoxide dismutase activity during the examined perioperative period. (# p < 0.05 vs before surgery; * p < 0.05 vs non-conditioned group)

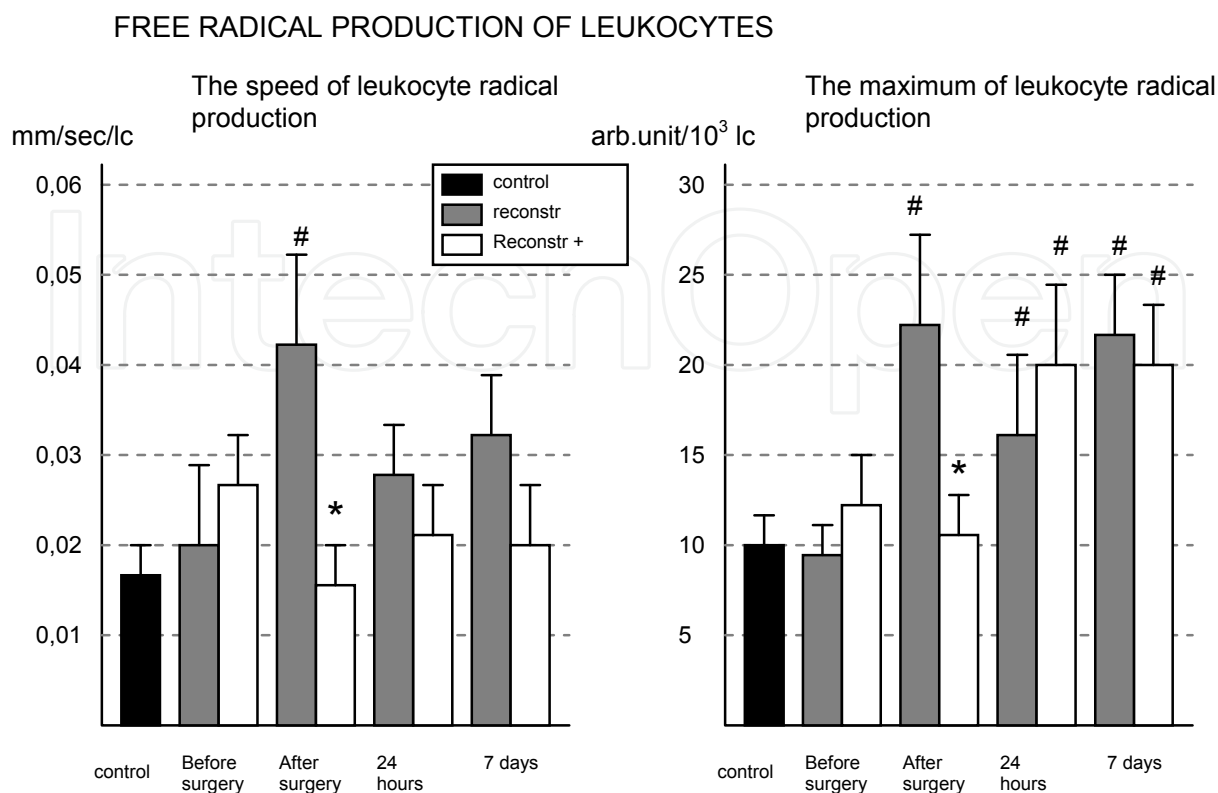


Fig. 5. Changes in the PMA induced free radical production of leukocytes during the perioperative period. We demonstrated the result of the speed and the maximum of radical production. (# $p < 0.05$ vs before surgery; * $p < 0.05$ vs non-conditioned group)

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 without significant difference between the two groups. (Figure 5)

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 6)

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 7)

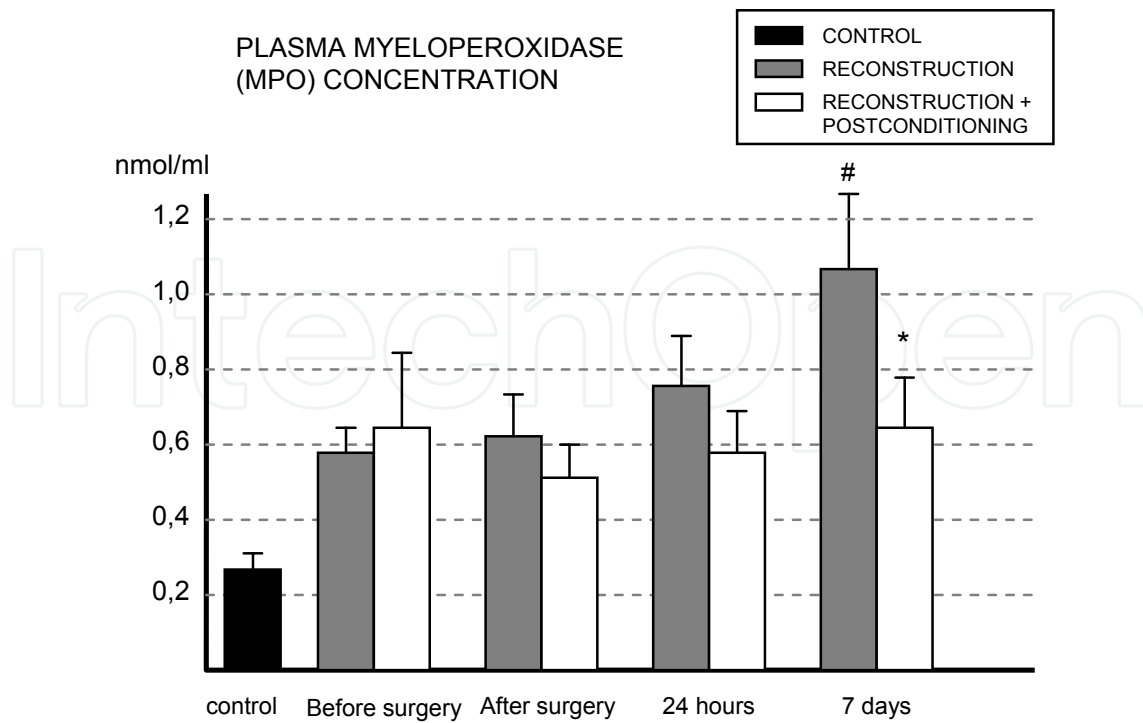


Fig. 6. Changes in plasma myeloperoxidase following operation (# p<0.05 vs before surgery; * p<0.05 vs non-conditioned group)

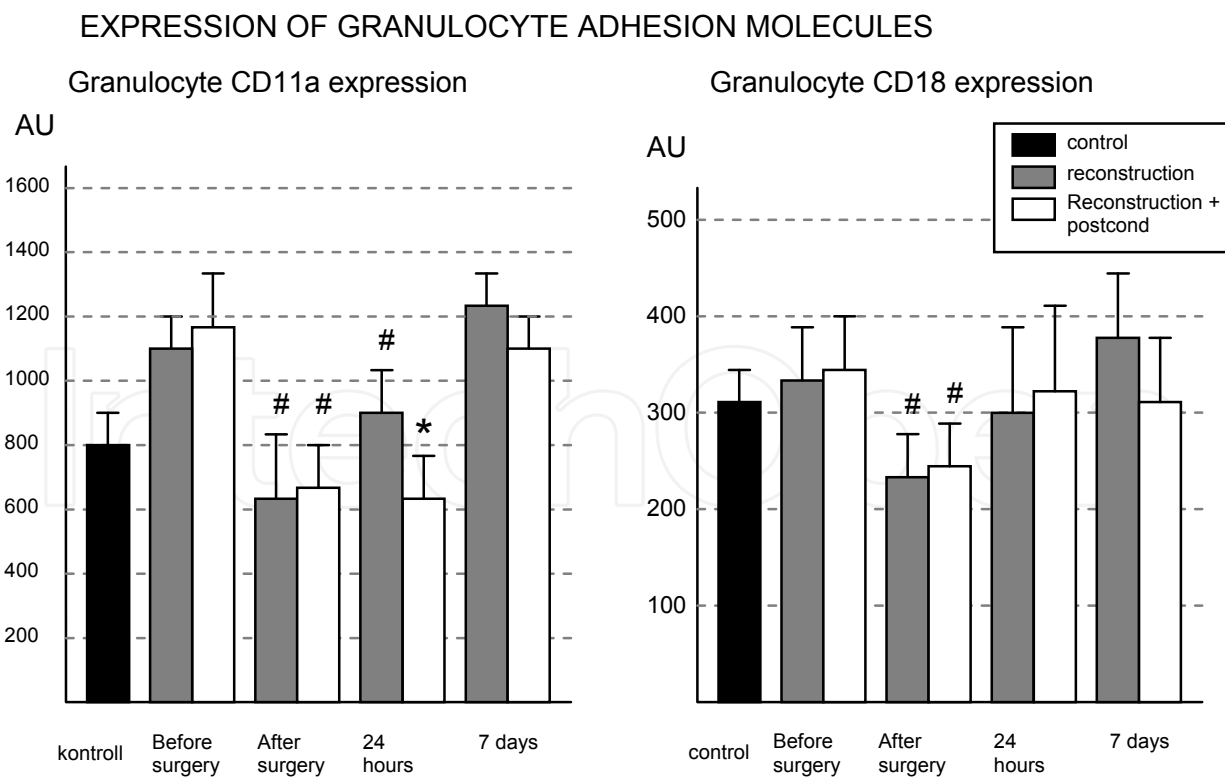


Fig. 7. The graphs show the changes in expression of granulocyte adhesion during the examined perioperative period. (AU= arbitrary unit) (# p<0.05 vs before surgery; * p<0.05 vs non-conditioned group)

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

21. Discussion

In the last 3 years the literature of ischaemic postconditioning exponentially increased in the experimental cardiology. The beneficial effects of the manoeuvre 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 manoeuvre really a powerful tool of surgeons.

22. 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 8)

23. 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’.

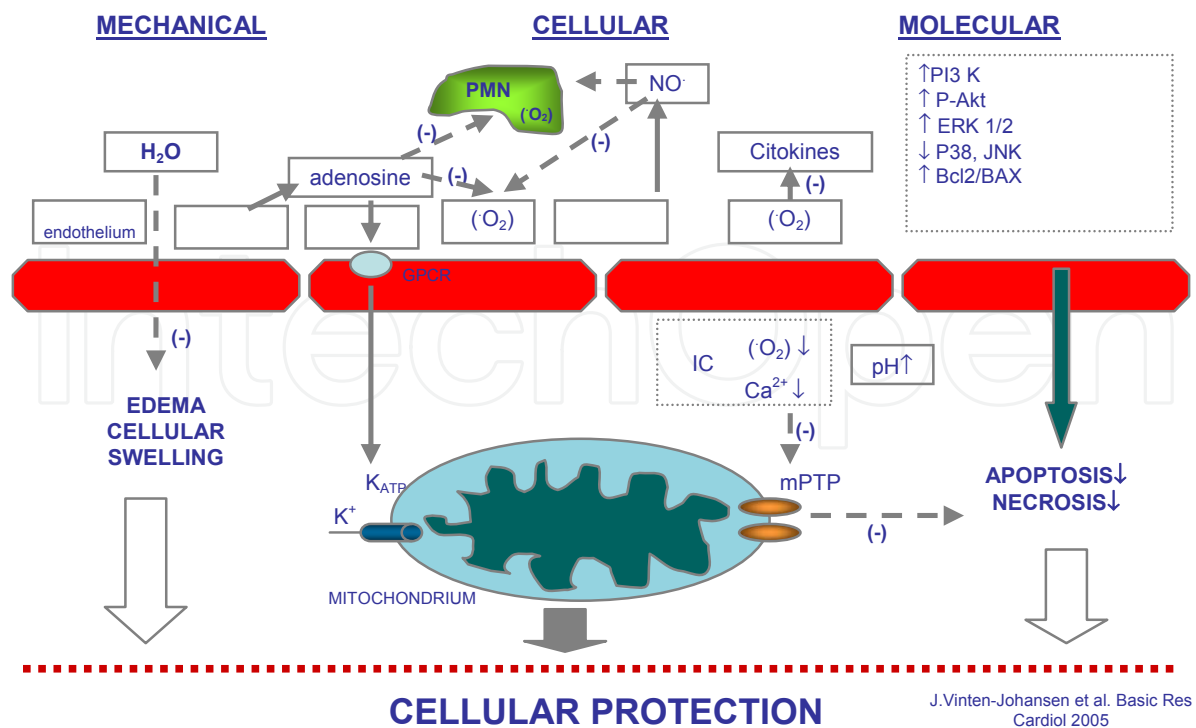


Fig. 8. A schematic figure on the mechanisms of ischaemic postconditioning.

24. 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 PostC⁴⁶. 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.

25. 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 inflammation⁴⁷. As stated before, whether this is a cause or an effect of PostC protection remains to be elucidated.

26. 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 death⁴⁸. Delayed washout of endogenously produced adenosine and activation of the adenosine receptor also seems to be required for PostC protection⁴⁹, 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 subtypes⁵⁰ have been seen to be involved, in rabbit hearts PostC seems to depend on A2b subtype⁵¹. An important role of the redox environment has also been observed⁵².

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 cardioprotection⁵³.

27. Triggers of postconditioning

Ligands, such as adenosine⁵⁴ and bradykinine⁵⁵ 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 model⁵⁶. 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 preconditioning⁵⁷. 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-cyclase⁵⁸. As an inducer of the protection, nitric oxide may also directly open the mitochondrial K_{ATP}

channels⁵⁹. 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 protection⁶⁰. A relevant role of nitric oxide may also be attributed to the endothelial protection brought by this molecule⁶¹ or to its role as antioxidant under certain conditions⁶².

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 damages⁶³. 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 reperfusion⁶⁴, 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 ester (LNAME) was given 5 min. before start of reperfusion of *in vivo* rabbit hearts, the infarct limiting effect was abolished⁶⁵. We have shown that nitric oxide participates in PostC, but NOS inhibitors given for the entire reperfusion period only blunted the protective effect of PostC⁶⁶. Paradoxically, the same inhibitor, given only during PostC manoeuvres completely blocked the protective effects⁶⁷. 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 protection⁶⁸ (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 damage⁶⁹. 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 2001⁷⁰, Wink et al. in 2003⁷¹, Pagliaro in 2003⁷² and Schulz et al. in 2004⁷³. 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 effects⁷⁴. 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 laboratories⁷⁵. 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 preconditioning⁷⁶.

28. 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 results⁷⁷. 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, mK_{ATP} 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 hearts⁷⁸. Moreover we showed that in these hearts mK_{ATP} and PKC must also be active (i.e. they should not be blocked) during late reperfusion⁷⁹. Regarding the role of mK_{ATP} channels a couple of papers indicate that the mK_{ATP} channel is important for PostC⁸⁰. In these studies two different mK_{ATP} channel blockers (glibenclamide and 5-hydroxidecanoate) abolished the protective effect of PostC⁸¹.

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 PostC⁸². Some differences, however, may exist between pre- and PostC (see also Table 1 and Table 2). Darling et al.⁸³ showed an increase of phospho-ERK, but not of PI3K/Akt in PostC, while Yang et al.⁸⁴ 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.⁸⁵. Yet in contrast with Yang et al.⁸⁶, Cao et al.⁸⁷ 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 role⁸⁸. Different methods of tissue sampling also may play a role⁸⁹. Besides protein kinase C, the possible roles for tyrosine kinase, and members of the MAPK family other than ERK1/2 in PostC has been suggested⁹⁰. (Figure 9)

Focal disorganization of gap junction distribution and down-regulation of connexin 43 (Cx43) are typical features of myocardial remodelling⁹¹ 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 PostC⁹². 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.

29. End-effectors of postconditioning

Mitochondrial PTPs opening represents a fundamental step of reperfusion injury. Among the potential mechanisms responsible for mPTP opening during reperfusion, Ca²⁺-overload has received particular attention. In particular, mitochondrial Ca²⁺-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 priming⁹³. Additionally, reduced mitochondrial Ca²⁺ overload during ischaemia has been pointed out as a potentially important mechanism of ischaemic and pharmacological preconditioning⁹⁴.

ACTIVATION OF REPERFUSION INJURY SURVIVAL KINASES

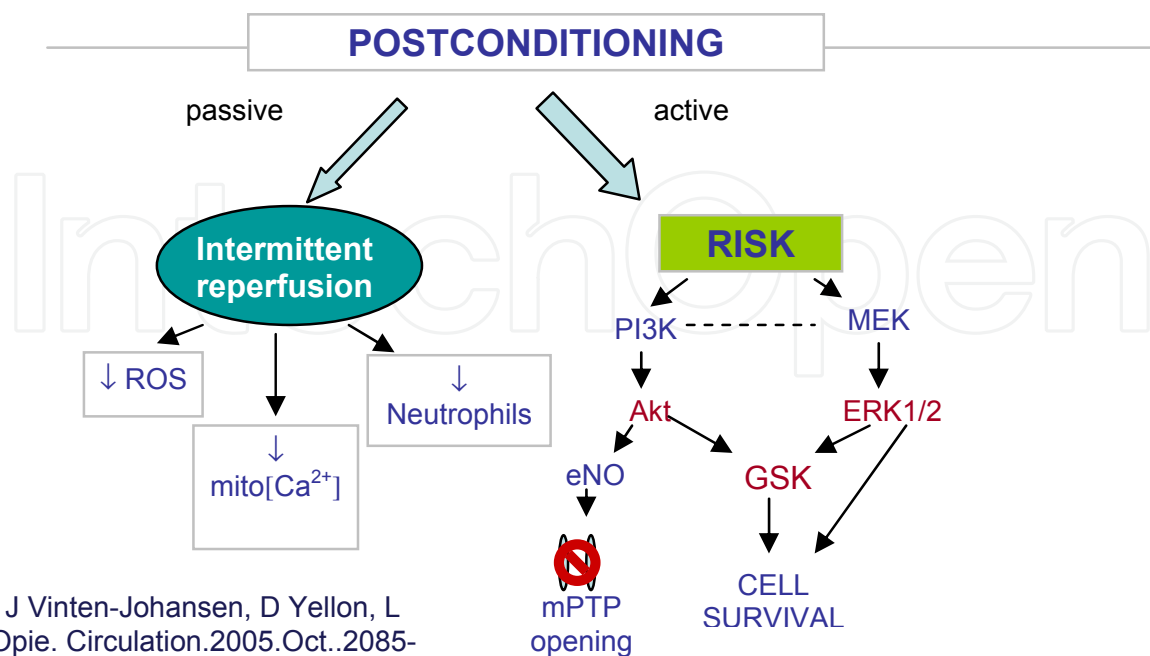


Fig. 9. Possible contexts in the activation of reperfusion injury survival kinases in ischaemic postconditioning

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 release⁹⁵. 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 risk⁹⁶. PostC was also associated with a reduction in infarct size after both acute and long-term (72 hrs) reperfusion. Bopassa et al.⁹⁷ 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 opening⁹⁸. 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*).

30. Cytoprotection 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 ischaemia⁹⁹. Also, several metabolites, including acetylcholine, BK, opioids and phenylephrine, trigger preconditioning-like protection via a mK_{ATP} -ROS dependent mechanism¹⁰⁰. As stated in the case of reperfusion injury, ROS are also implicated in the sequel of myocardial reperfusion injury¹⁰¹. 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 phase¹⁰². The importance of ROS signalling (as opposed to excess ROS in the development of injury) has been examined closely in great detail in recent years¹⁰³. 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 hearts¹⁰⁴. 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 protective¹⁰⁵. 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 preconditioning¹⁰⁶. 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 events¹⁰⁷. 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 dismutase¹⁰⁸. 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 reperfusion¹⁰⁹.

Since ROS scavengers (N-acetyl-L-cysteine or 2-mercapto-propionylglycine), given at the beginning of reperfusion, abolished both IP- and PostC-induced protection¹¹⁰ 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.

31. 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 manoeuvre is easy to perform, quick, and does not any expensive instruments, so it may have a place in the therapeutic arsenal of vascular surgeons.

32. References

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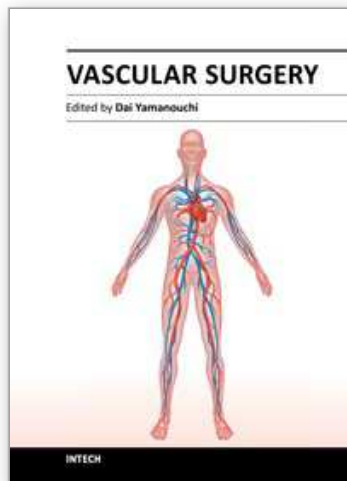
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This book aims to provide a brief overview of conventional open vascular surgery, endovascular surgery and pre- and post-operative management of vascular patients. The collections of contributions from outstanding vascular surgeons and scientists from around the world present detailed and precious information about the important topics of the current vascular surgery practice and research. I hope this book will be used worldwide by young vascular surgeons and medical students enhancing their knowledge and stimulating the advancement of this field.

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