MODERATION OF OXIDATIVE STRESS DURING EXPERIMENTAL SMALL BOWEL AUTOTRANSPLANTATION

Studying the ischemia-reperfusion injury and ischemic preconditioning of the intestine

Ph.D. Thesis

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Contents

1. INTRODUCTION

The small bowel has long been considered as a "forbidden" organ to transplant and in fact, is the last of the splanchnic viscera to have acceded to the clinical arena. Although, in 2002 the 5-year graft survival of the small bowel transplantation is closed to 65-70 % in some centers, the widespread application of this procedure is still limited by the high rate of complications.

1.1. Historical background

The evolution of the technical procedure first reported in experimental animals by Carrel in 1902. In 1959, Lillehei *et al* first described orthotopic small bowel transplantation of dogs. Monchik and Russell developed heterotopic bowel transplantation in a rat model in 1971. Later, this model has been adopted and modified by a number of investigators. Early clinical attempts to transplant small intestine in humans were made between 1960 and 1980. Firstly in 1988, Grant *et al* performed case of successful cadaveric transplantation of bowel graft. That same year, Deltz described the first case of intestinal transplantation using a living donor. From 1992, introduction of new immunosuppressive regiments has led to dramatic improvements in outcome following transplantation.

1.2. Indications for small bowel transplantation

The majority of potential candidates for intestinal transplantation are patients with short-bowel syndrome. Both adults and children generally develop this syndrome after extensive intestinal resection or intestinal malfunction. These patients are treated with long-term parenteral nutrition, which may be associated with hyperglycaemia and hepatic dysfunction. Thus, transplantation could give an alternative for these patients. Common aetiologies of intestinal failure are vascular diseases (superior mesenteric artery and vein thrombosis or embolic occlusion, vasculitis), intestinal diseases (Crohn's disease, malabsorption disease, radiation enteritis, necrotizing enterocolitis, mid-gut volvulus), neuroendocrine abnormalities (aganglion syndrome, progressive motility disorder), trauma, long-term total parenteral nutrition, congenital abnormalities (intestinal atresia, complicated gastroschisis).

1.3. Role of warm ischemia and reperfusion injury in the small bowel

The susceptibility of a given organ to oxidative stress depends on an overall balance between oxidants and antioxidants. Oxidative lesions result from a disturbance of the sophisticated oxidant-antioxidant balance and occur when oxidants overwhelm the endogenous antioxidant defence mechanisms. This condition is associated with warm ischemia followed by reperfusion (I/R). Cell damage following ischemia is a biphasic process. Ischemia initiates the injury, while the reperfusion exacerbates this damage by triggering an inflammatory reaction involving oxygen free radicals (OFRs) and polymorphonuclear leukocytes (PMNs).

The uniquely susceptibility of the bowel to I/R injury is attributed to the high content of xanthine dehydrogenase (XD). During warm ischemia, XD is converted to xanthine oxidase (XO), a major source of OFRs generated in postischemic tissue. XO is widely distributed among tissues, with the intestinal mucosa of the small bowel being the richest source. Another potential source of OFRs are the PMN leukocytes. PMNs infiltrate the gut mucosa

during the ischemic period, with reperfusion causing a significantly greater increment in tissue PMNs. Activated PMNs can produce OFRs by NADPH oxidase and release variety of proteolytic enzyme. Hence, the actual tissue destruction occurs by two mechanisms: direct cell injury and lipidperoxidation by OFRs, and OFR-associated PMNs activation and infiltration that amplifies cell and microvasculature damage.

Continuous exposure of intestine to prooxidant challenges has endowed mucosa cells with efficient antioxidant systems. Thiol compounds can protect the cell from oxidative stress and reduced glutathione (GSH) is the most abundant low molecular mass cellular thiol. GSH synthesized in liver can undergo transport across membranes. The presence of the specific transports at both intestinal cell poles is capable of supplying GSH to plasma and to other cells. In 1969, McCord and Fridovich first demonstrated, that superoxide dismutase (SOD) is a naturally occurring, highly specific enzyme that plays a central role in protecting cells and tissues against oxidative stress by destroying OFRs.

1.4. Goals of small bowel cold preservation

Small bowel transplantation has progressively improved with modern immunosuppressive strategies. Another major problem hampering bowel transplantation is ischemia-reperfusion injury, which is determined mainly by the quality of organ procurement. Early graft function and recipient survival largely depend on the duration of cold organ ischemia, preservation solution and developed oxidative stress. Besides threatening graft function and survival, oxidative injury potentially contributes to the process of acute and chronic rejection. Many preservation solutions such as Euro Collins (EC), Ringer's lactate (RL), University of Wisconsin (UW) were developed along with research efforts to find appropriate solution for intestine storage. At present, no consensus exists about the method of choice or preservation solution for a small bowel graft, nor is it known what maximal ischemia time is tolerable.

1.5. Ischemic preconditioning of the small intestine

Preconditioning is such an endogenous adaptation of the organ and was first described in the myocardium by Murry *et al*. Ischemic preconditioning (IPC) is the phenomenon of increased adaptive tolerance to a severe ischemic insult that follows short bouts of non-lethal ischemia-reperfusion cycles. IPC induces two phases of protection. Classic preconditioning appears to be an immediate response developing within minutes from the initial ischemic insult and lasting 2-3 hours. The second wave of protection (second window of protection: SWOP) becomes apparent 12-24 hours later and lasts for 3-4 days. The beneficial effects of IPC were first demonstrated in heart, now it seems that preconditioning can induce ischemic tolerance in a variety of organs including intestine. Nevertheless, the exact cellular and molecular mechanisms underlying this phenomenon remain to be deciphered in the small bowel.

Although the generation of OFRs during I/R has generally been viewed as a deleterious phenomenon, mounting evidence indicates that OFRs in small amounts could also serve as important intracellular signalling molecules in IPC cascades. In preconditioned small intestine during the I/R periods the increased OFRs along with other triggers (adenosine, acetylcholin, nitric oxide, endogen opioid peptides) can act as an inducers of phospholipase C, which in turn generates diacylglycerol (DAG). DAG may then act as a second messenger

on various isoenzymes of protein kinase C (PKC). It is believed that PKC activation and translocation in an important denominator in the preconditioning cascade. The activated PKC can lead to downstream opening of mitochondrial ATP-sensitive potassium channels (K_{ATP}) leading to early protection in the small intestine. Yang *et al* reported that K_{ATP} channel opening is the key effector in conferring early protection to the bowel tissue. Another kinase that may be acting either downstream of, or in concert to PKC, is tyrosine kinase (TyK). This pathway is by TyK phosphorylation, and the activation of mitogen activated protein kinases, inducing the activation and translocation of nuclear factor-kappa binding (NF-κB) to the nucleus stimulating specific gene expression and new protein synthesis, leading to delayed protection.

In 1986, Sen and Baltimore characterized the transcriptional activation mediated by NF-κB. NF-κB is a major transcription factor which, when activated, plays a pivotal role in many cellular responses to environmental changes. Both extracellular and intracellular OFRs would favour the activation of NF-κB. In resting cells, NF-κB is kept in the cytoplasm by the inhibitor protein IκB. Appropriate stimuli induce selective IκB phosphorylation, which is then ubiquitinated and targeted for degradation by the proteasome pathway. Free NF-κB migrates to the nucleus by virtue of its nuclear localization signal and induces transcription of multiple κB-dependent genes. Then, NF-κB inactivated by newly synthesized IκB both in the cytoplasm and the nucleus. Many of the genes that are activated in the initiation of apoptosis are target genes of NF-κB.

In the normal intestinal mucosa the apoptotic process is a possible role in physiological cell renewal. Normal physiological processes and pathological stimuli induce apoptosis via different signal transduction cascades. The proapoptotic pathway may become activated either by external signals that trigger receptors on the plasma membrane or by intracellular alteration, such as OFRs in bowel tissue.

2. OVERALL AIM OF THE PROJECTS

Extensive research over the years in the field of small bowel transplantation has greatly extended our understanding of the underlying mechanism of intestinal failure to warm and cold oxidative injury. Although, many questions are yet to be elucidated, especially with regard to the ischemic tolerance of the bowel, it remains one of the most powerful experimental tools in protection that may one day translate into a clinical reality.

In our first series of small bowel investigations, we aimed to determine changes of OFRs mediated reactions, above all marker of lipidperoxidation malondialdehyde, endogen antioxidant scavenger reduced glutathione and endogen antioxidant enzyme superoxide dismutase during intestinal warm ischemia and reperfusion.

In our second series of investigations, we set upon examining the optimal and tolerable cold ischemia time and preservation solution for small bowel tissue. Furthermore we examined the relationship between prolongation of cold preservation time and reperfusion induced oxidative stress by biochemical monitoring. In this series we demonstrated the OFRs production of polymorphonuclear leukocytes and detected internucleosomal DNA strand breaks generated during apoptosis after cold preservation of the intestine.

In our third series of investigations, we aimed to investigate changes beyond the protective effect of cold preservation the influence of ischemic preconditioning prior to autotransplantation. The purposes were to estimate how ischemic preconditioning can influence oxidative stress parameters in small bowel tissue, and to demonstrate the role of PMNs in OFRs production. In addition, we attempted to prove the presence and activation of NF-κB in preconditioned bowels, and to detect DNA strand breaks generated during apoptosis in preconditioned and autotransplanted small intestinal tissue.

3. THE EFFECTS OF INTESTINAL WARM ISCHEMIA AND REPERFUSION
(First series of investigations)

3.1. Insight into this study

The observation that reperfusion following warm intestinal ischemia intestine leads to bowel tissue dysfunction or injury led to the concept that reperfusion injury mediated by the formation of reactive oxygen metabolites. These OFRs are highly reactive with a variety of cellular components. Membrane-associated polyunsaturated fatty acids are readily attacked by OFRs results in the peroxidation of lipids. Measurement of malondialdehyde is frequently used as an index of lipid peroxidation. Intestinal cells possess elaborate defence mechanisms to detoxify OFRs. The presence of glutathione in the intestinal mucosal cells has been reported and indicated an important detoxifying function for GSH in the intestinal epithelium. As previously mentioned one of the key enzymatic defence mechanism is that SOD catalyses the dismutation of the superoxide anion. The aim of this study was to monitor oxidative stress during different time of warm ischemia and reperfusion periods. We examined the marker of lipidperoxidation malondialdehyde, the endogenous antioxidant scavenger reduced glutathione, and endogenous antioxidant enzyme superoxide dismutase in animal model.

3.2. Materials and methods

3.2.1. Animals
This study was performed with adult mongrel dogs of either sex (n=20) weighting between 21 and 25 kg. All experiments were in accordance with rules and regulations regarding the use of animals in medical research. The Committee on Animal Research of Pécs University has approved the study (1301-7/1999).

3.2.2. Anaesthesia
Animals were fasted for 24 hours prior to the experiments and were premedicated with Droperidol (1.5 mg/kg), Fentanyl (0.03 mg/kg) and Atropin (1 mg). Anaesthesia was induced by intravenous administration of Thiopentone-sodium (5-10 mg/kg). General anaesthesia and ventilation were maintained using a gaseous mixture of nitrous oxide (70%) and oxygen (30%) in addition to 1.0-1.5% isoflurane. Under sterile conditions the right femoral artery and vein were prepared and cannulated for systemic arterial pressure monitoring and venous blood withdrawal respectively. Throughout the procedure standard limb ECG and systemic blood pressure were monitored continuously. Arterial blood pH, pO_2, pCO_2 were maintained within the physiologic range.

3.2.3. Surgical procedures
The abdomen was opened through a mid-line incision. After Na-heparin administration (200 units/kg) the superior mesenteric artery was ligated (warm ischemia) for different times. Following the clamp removal reperfusion was allowed to test the bowel viability.

3.2.4. Experimental protocol
Two warm ischemia groups (group I: GI, group II: GII) were designed to determine the oxidative injury in blood and bowel tissue samples (Table 1).

Table 1. Warm ischemia groups

Groups	Number of cases	Warm ischemia time	Reperfusion time
GI	n=10	1 hour	3 hours
GII	n=10	3 hours	1 hour

3.2.5. Sampling procedure

To measure the oxidative stress we collected venous blood samples and small bowel tissue samples according to the same protocol: first after laparotomy (control), then at the end of warm ischemia, and at the 5th minute (early reperfusion) and also at the 60th minute (late reperfusion) of the reperfusion periods.

3.2.6. Biochemical assays

Malondialdehyde has been determined in blood samples and in bowel tissue homogenates using Lipid Peroxidation Assay Kit (Calbiochem). This is a colorimetric assay kit, which is specific for MDA. Final values were given as μM and μM/g wet tissue. Reduced glutathione has been determined in blood samples and bowel tissue homogenates using Glutathione Assay Kit (Calbiochem). This method allows transforming GSH into a chromophoric thione with a maximal absorbance at 400 nm. Values of glutathione were expressed in μM and μM/g wet tissue. Superoxide dismutase has been determined in blood samples and bowel tissue homogenates using Superoxide Dismutase Assay Kit (Calbiochem). One reagent of the kit underwent alkaline autoxidation, which was accelerated by SOD. Autoxidation of this reagent yielded a chromophore, which absorbed maximally at 525 nm. The value of the activity of SOD was given in IU/ml and IU/g wet tissue.

3.2.7. Statistical analysis

All results are expressed as Mean values ± SEM. Data were analysed with one-way analysis of variance (ANOVA). The level of significance was set at $P< 0.05$. Micro Cal Origin (Ver 4.10) (Microcal Software Inc., Northampton, USA) program was used for data evaluation.

3.3. Results

3.3.1. Changes of oxidative stress markers in blood samples

Value of lipidperoxidation was increased both in 1 hour and 3 hours warm ischemia groups in blood samples compare to control. The MDA level was increased more at early phase of reperfusion, while these changes were decreased in the 60th minutes of reperfusion. Furthermore, the content of endogenous scavenger GSH was increased continuously during 1 hour and 3 hours warm ischemia and subsequent reperfusion period compare to control values. The endogenous antioxidant enzyme, SOD activity was increased following warm ischemia and reperfusion in peripheral blood samples.

3.3.2. Changes of oxidative stress parameters in bowel tissue samples

Concentration of tissue MDA elevated slightly in GI, and the elevation was significant by the end of the reperfusion (GI: control 104.28±5.96 μM/g wet tissue, late reperfusion 127.22±8.70 μM/g wet tissue, $P< 0.05$ vs. control). Moreover, severe tissue lipidperoxidation was measurable in GII. During warm ischemia and reperfusion periods MDA level was increased significantly in all samples compare to control (GII: control 107.18±4.41 μM/g wet tissue, late reperfusion 171.48±7.32 μM/g wet tissue, $P< 0.01$ vs.

control). The content of tissue GSH decreased slightly following warm ischemia and reperfusion. However, there was not a significant fall in samples compared to control (GI: control 341.12±15.35 μM/g wet tissue; late reperfusion 317.18±15.26 μM/g wet tissue; GII: control 338.82±14.70 μM/g wet tissue, late reperfusion 319.19±13.25 μM/g wet tissue). In contrast, the endogenous antioxidant enzyme, SOD activity decreased significantly in all samples in the same period (GI: control 265.33±11.68 IU/g wet tissue, late reperfusion 26.65±10.31 IU/g wet tissue, $P< 0.001$; GII: control 273.66±10.45 IU/g wet tissue, late reperfusion 21.86±13.67 IU/g wet tissue, $P< 0.01$).

3.4. Conclusions from this study

We demonstrated, that oxidative stress parameters, such as lipid peroxidation and endogenous antioxidant compound are reliable markings to monitor the ischemic intestinal damages. Total warm ischemia and subsequent reperfusion generated severe peroxidation of tissue lipids also in peripheral blood and bowel tissue.

Reduced glutathione, an essential component of the cellular defence mechanisms against OFRs mediated tissue injury, has been used as indicator of oxidative stress in the process of ischemia-reperfusion. Results from our work indicated that GSH level decreased especially following 1 and 3 hours of warm ischemia and not decreased more during reperfusion. We suggested that reperfusion per se did not cause a further decrease in the GSH content of the intestine beyond that observed during ischemia.

It was observed in our model that both 1 and 3 hours of warm ischemia significantly decreased the endogenous SOD activity during reperfusion in the intestine. These observations, along with those described earlier, suggest that OFRs mediate reperfusion-induced lipidperoxidation and oxidative stress in the small intestine.

4. ROLE OF COLD PRESERVATION BEFORE INTESTINAL AUTOTRANSPLANTATION

(Second series of investigation)

4.1. Insight into this study

The principles of organ preservation are flushing, cooling, and pharmacological intervention. The most common procedure for solid organ storage is the perfusion with cold preservation solutions followed by simple cold storage. Some investigators suggested that not any commercially available solution could be used for small bowel preservation. Thus, the creation of a more optimal preservation solution for the small intestine still seems desirable. Next to the preservation solution used, among other factors the cold ischemia time may be value to minimize preservation and reperfusion injury of the small bowel grafts. For the lack of excellent preservation techniques, small bowel grafts are transplanted in the clinical settings as soon as possible after harvest. Recent findings suggest that oxidative stress and intracellular OFRs are involved in the induction of apoptosis. The apoptotic process in epithelial cells were associated with decreased level of GSH due to increased efflux of this antioxidant from the cells, and significant decrease of SOD activity. Moreover, data showed that low activity of SOD in cells might be another contributing factor in apoptosis. Therefore, in this second study we focused on the preservation solutions (EC and UW) and different cold ischemia (preservation) times to monitor induced oxidative stress. Furthermore, we demonstrated the OFRs production by PMN leukocytes and detected DNA strand breaks generated during apoptosis after cold preservation of the intestine.

4.2. Materials and methods

4.2.1. Animals

The study was performed with adult mongrel dogs of either sex (n=40, average body weight: 22.3±2.4 kg). All experiments were in accordance with rules and regulations regarding the use of animals in medical research. The Committee on Animal Research of Pécs University has been approved the study (1301-7/1999).

4.2.2. Anaesthesia

Animals were anaesthetized as described above (3.2.2.).

4.2.3. Surgical procedures

Total orthotopic intestinal autotransplantation was performed in mongrel dogs. Laparotomy was performed by a midline incision. After Na-heparin administration (200 units/kg) grafts were resected from the angle of Treitz to descending colon, and flushed the lumen of the bowel with 1500 ml normal saline. Grafts were perfused by the superior mesenteric artery and stored in 4ºC EC or UW solution for different times. After preservation was performed end-to-end anastomosis between the stumps of mesenteric vessels. Reperfusion periods took 1 hour in all grafts.

4.2.4. Experimental protocol

Four cold ischemia groups (group I: GI, group II: GII, group III: GIII, group IV: GIV) were established according to different preservation times and preservation solutions (Table 2).

Table 2. Cold ischemia groups

Groups	Number of cases	Preservation solutions	Cold ischemia time (preservation)	Reperfusion time
GI	n=10	Euro Collins	2 hours	1 hour
GII	n=10	Euro Collins	3 hours	1 hour
GIII	n=10	Euro Collins	6 hours	1 hour
GIV	n=10	University of Wisconsin	3 hours	1 hour

4.2.5. Sampling procedure

To measure the oxidative stress we collected venous blood samples and small bowel tissue samples according to the same protocol: first after laparotomy (control), then at the end of cold ischemia (preserved), and at the 5th minute (early reperfusion) and also at the 60th minute (late reperfusion) of the reperfusion periods.

4.2.6. Biochemical assays

We determined concentration of MDA, GSH and activity of SOD in blood samples and bowel tissue homogenates as described above (3.2.).

4.2.7. Demonstration of OFRs production in PMNs isolated from blood

In GII and GIV venous blood samples were collected from the mesenteric vein after laparotomy (control) and at the end of the reperfusion period (reperfused). Leukocytes were harvested from blood. Large drops of blood were immediately placed on microscopy slides and incubated in a moist chamber at 36 °C for 2 min. The resulting coagulum was removed and the slides were rinsed free of nonadherent cells. More than 90% of the adherent cells were PMNs. The cells were incubated for 10 min in a cerium-trichloride ($CeCl_3$) solution (20 mmol/L $CeCl_3$ in lactated Ringer solution), and then the cytochemical reaction was stopped by methyl alcohol fixation for 2 minutes. The nuclei were stained with propidium iodide (PI, Sigma, 50 μg/ml), and after being washed the preparation was mounted in glycerol gelatine (Sigma). Negative control preparations included PMNs without Ce treatment. Positive control cells were activated for 10 min with 1 μmol/L phorbol-12-myristate-13-acetate (PMA, Sigma) and $CeCl_3$ (20 mmol/L).

4.2.8. Confocal Laser Scanning Microscopy (CLSM)

The imaging and semiquantification of leukocyte OFRs production, represented by laser reflectance signals of Ce-perhydroxide deposits, was performed with a Nikon Eclipse TE-300 inverted microscope attached to an MRC-1024ES confocal system (Bio-Rad, Hertfordshire, UK) using multichannel detection. The sample was illuminated at 488 and 457 nm with an argon ion laser. Ce-precipitates were detected using reflectance mode. Detecting fluorescence above 550 nm imaged the PI-stained nuclei. For morphological studies, high-resolution (magnification x2500, 100x N.A.: 1.4 oil immersion objective) images were taken (9 optical sections/cell), followed by pseudo-3D object volume reconstruction (Laser Sharp software, Bio-Rad) and digital superposition to give a 3-layer composite image using Confocal Assistant TM 4.02 software (Todd Clark Brelje, USA).

4.2.9. In situ detection of nuclear fragmentation by Terminal deoxynucleotidyl Transferase (TdT) -Mediated dUTP Nick End-Labelling (TUNEL)
In GII and GIV bowel tissue sections were prepared after laparotomy (control) and at the end of the reperfusion period (reperfused). Apoptotic cell death was determined using In Situ Cell Death Detection Kit, POD (Roche, Mannheim, Germany) according to the manufacturer's protocol. Activation of the apoptosis-associated endonuclease results in extensive DNA cleavage, which generates a large number of DNA strand breaks. The presence of free 3'-OH termini of the strand breaks were detected by labelling with modified nucleotides (fluorescein-dUTP) in a reaction catalyzed by TdT. Incorporated fluorescein was incubated by anti-fluorescein antibody conjugated with horse-radish peroxidase (POD). Finally, the sections were incubated with diaminobenzidine-substrate solution, stained with Methyl Green (Riedel-de Haën, Hannover, Germany) and analysed under light-microscope (magnification x40). In negative control sections the TdT was omitted.

4.2.10. Hemodynamic examinations
Mesenteric flow was monitored with Electromagnetic Flowmeter (Nycotron, Norway) and intestinal mucosal capillary perfusion with Laser Doppler Flowmeter (Moor Instruments Ltd, Devon, UK) after laparotomy and during reperfusion period.

4.2.11. Statistical analysis
All results are expressed as Mean values ± SEM. Data were analysed with one-way analysis of variance (ANOVA). The level of significance was set at $P< 0.05$. Micro Cal Origin (Ver 4.10) program was used for data evaluation.

4.3. Results

4.3.1. Changes of oxidative stress markers in blood samples
During cold preservation and reperfusion the MDA level was increased moderately in all blood samples compare to control. The elevation was the largest at the early phase of reperfusion in every group. There was not significantly difference between four groups using EC, UW solutions and different preservation time. Cold preservation moderated the elevation of peripheral blood GSH concentration compare to control in cold ischemia groups. The changes were the highest during early phase of reperfusion and then reduced in all preserved groups. Mild elevation of SOD activity was observed in blood samples of groups.

4.3.2. Changes of oxidative stress parameters in bowel tissue samples
Cold preservation in EC and UW solutions moderated bowel tissue lipidperoxidation in cold ischemia groups (GI: control 103.47±3.98 μM/g wet tissue, late reperfusion 110.12±5.51 μM/g wet tissue; GII: control 101.46±4.99 μM/g wet tissue, late reperfusion 113.33±2.65 μM/g wet tissue, $P< 0.01$; GIII: control 109.01±8.32 μM/g wet tissue, late reperfusion 115.17±4.97 μM/g wet tissue; GIV: control 105.16±4.52 μM/g wet tissue, late reperfusion 119.56±9.04 μM/g wet tissue). The elevation in EC preserved grafts was significantly by the end of the reperfusion. The content of GSH increased in preserved grafts by the end of the reperfusion period (GI: control 349.67±17.02 μM/g wet tissue, late reperfusion 387.52±10.72 μM/g wet tissue; GII: control 347.01±19.46 μM/g wet tissue, late reperfusion 402.57±20.83 μM/g wet tissue; GIII: control 357.64±18.88 μM/g wet tissue, late reperfusion 400.52±15.03

μM/g wet tissue; GIV: control 363.75±12.79 μM/g wet tissue, late reperfusion 385.03±15.54 μM/g wet tissue). However, this elevation was not significant compare to control. Tissue SOD activity decreased significantly during reperfusion in every preserved graft (GI: control 280.84±9.44 IU/g wet tissue, late reperfusion 98.59±8.18 IU/g wet tissue, P< 0.01; GII: control 284.28±10.61 IU/g wet tissue, late reperfusion 60.28±9.49 IU/g wet tissue, P< 0.001; GIII: control 271.77±11.67 IU/g wet tissue, late reperfusion 47.39±8.87 IU/g wet tissue, P< 0.001; GIV: control 270.22±11.87 IU/g wet tissue, late reperfusion 86.09±10.56 IU/g wet tissue, P< 0.001). This reduction was the largest in tissue preserved for 6 hours.

4.3.3. Visualization of OFRs production by circulating PMNs
Native images of normal living PMNs showed barely detectable reflectance, whereas PMA-induced OFR production resulted in an increase of reflectance signals of cerium perhydroxide deposits (9 optical sections of cell). Across the groups small amount of intracellular OFRs were detected even in the control samples. The qualitative imaging of reflectance signals of Ce-perhydroxide deposits showed more dramatically increase during reperfusion both in EC and UW preserved groups. An apparent release of OFRs was observed, resulting in surprisingly large reflectant deposits around PMNs.

4.3.4. Detection of nuclear fragmentation by TUNEL technique
Both groups preserved in EC and UW, beside normal cells a few DNA-damaged cells were found in the mucosal layer even in control samples in the small intestine. The number of cells suffering DNA strand breaks slightly elevated by the end of the reperfusion period in cold ischemia groups.

4.3.5. Hemodynamic parameters
Control mesenteric flow was 138.52±6.69 ml/minute, and not decreased less than 129.73±4.01 ml/minute during reperfusion in cold ischemia groups (GI, GII, GIII, GIV). The intestinal mucosal capillary perfusion was similarly sufficient after laparotomy and during reperfusion period.

4.4. Conclusions from this study

In our second study we have conclusively demonstrated the role of cold preservation of the canine small bowel prior to autotransplantation. The success of storage we defined by the development of oxidative stress. We observed that cold ischemia could moderate oxidative stress parameters both in blood and intestinal tissue in our model. It means that lipidperoxidation was increased slightly, meanwhile the endogen antioxidant scavenger capacity, namely GSH content and SOD activity elevated mildly in blood samples. Furthermore, cold preservation mitigated the biochemical changes also in intestinal tissue. Thus, our results suggested that small bowel was able to tolerate 2 and 3 hours cold preservation without serious oxidative injury. In contrast, 6 hours preservation caused more serious oxidative damage for small bowel, which indicated the largest decrease of SOD activity.

In respect of oxidative stress parameters we didn't find considerable difference between EC and UW solutions in this models. As a result of other authors, appeared no advantage for intestinal graft function with the use of the UW solution. Previous studies have indicated that

although UW solution contains OFRs scavengers (glutathione and allopurinol), the stability and effectiveness of these agents have been questioned. Consequently, EC or UW solution might be sub-optimal for small bowel preservation.

Direct in vivo histological detection of OFRs is not fully resolved. In our experiments we applied cerium histochemistry combined with reflectance Confocal Laser Scanning Microscopy: in which capture of OFRs by cerium atom results in laser-reflectant cerium-perhydroxide precipitates. This method is a sensitive, reproducible, and spectacular test for detection of oxidizing species in biological samples. Isolated PMN leukocytes -collected from mesenteric vein- generated small amount of OFRs even in control specimen. Furthermore, by the end of 1 hour reperfusion large amount of cerium-perhydroxide deposits were observed both in intracellular and in extracellular areas of PMNs. We supposed these radicals were produced during 3 hours cold ischemia and subsequent reperfusion. As reperfusion commences after cold ischemic intervals OFRs are generated in increasing amounts in the mucosa, and simultaneously the mucosa undergoes infiltration by PMNs.

This study has identified apoptosis as the principle mode of cell death after I/R in cold-storaged small intestine. Spontaneous apoptotic cells were observed in control mucosa layer, but numbers of TUNEL positive cells slightly elevated by the 1-hour of the reperfusion period. There was no difference between preservation solutions according to degree of programmed cell death. Other investigators have been characterized the role of apoptosis in intestinal I/R, where small bowel grafts were stored in cold saline or modified UW (mUW) solution for 12 hours. Similarly, they have demonstrated the induction of apoptosis by intestinal I/R injury, which begins within an hour of reperfusion.

5. SMALL BOWEL ISCHEMIC PRECONDITIONING PRIOR TO AUTOTRANSPLANTATION

(Third series of investigations)

5.1. Insight into this study

Oxygen free radicals are generated every time there is a bout of warm and cold ischemia followed by reperfusion. Although, better known for OFRs toxicity, when in large quantities they overwhelm the endogenous antioxidant systems, recently it has been suggested that at low concentrations they can modulate functions within the cell. Ischemic preconditioning refers to a phenomenon in which a tissue rendered resistant to the deleterious effects of prolonged ischemia and reperfusion by prior exposure to brief periods of vascular occlusion. This protective effect has been also recently described in the intestine. Firstly, Tsuruma *et al* described small bowel ischemic preconditioning. They applied 30 minutes first ischemia period and after 4 or 7 days it was followed 30 minutes second ischemia time. By now, this IPC protocol not acceptable for bowel. In our study we used generally accepted IPC protocol, thus we preconditioned the intestine occlusion of mesenteric artery by 4 cycles of 5 minutes of ischemia with intermittent 10 minutes reperfusion (4 x 5 IPC).

In the third series of study we examined the effect of ischemic preconditioning prior to autotransplantation. The purposes were to estimate how ischemic preconditioning could influence oxidative stress parameters in small bowel tissue, and to visualize production of OFRs by PMNs. We attempted to prove the presence and activation of NF-κB in preconditioned bowel, and to detect DNA strand breaks generated during apoptosis in small intestine.

5.2. Materials and methods

5.2.1. Animals

This study was performed with adult mongrel dogs of either sex (n=30, average body weight: 25.1±1.4 kg). All experiments were in accordance with rules and regulations regarding the use of animals in medical research. The Committee on Animal Research of Pécs University has been approved the study (1301-7/1999).

5.2.2. Anaesthesia

Animals were anaesthetized as described above (3.2.2.).

5.2.3. Surgical procedures

Total orthotopic intestinal autotransplantation was performed as described above (4.2.3.).

5.2.4. Ischemic preconditioning protocol

After Na-heparin administration (200 units/kg) we preconditioned the bowel occlusion of superior mesenteric artery by 4 cycles (1 cycle= 5 min ischemia+10 min reperfusion).

5.2.5. Experimental protocol

Three preconditioned groups (group I: GI, group II: GII, group III: GIII) were established (Table 3).

Table 3. Preconditioned groups

Groups	Number of cases	Preconditioning	Preservation solutions	Cold ischemia time (preservation)	Reperfusion time
GI	n=10	4 cycles	without preservation and autotransplantation		3 hours
GII	n=10	4 cycles	Euro Collins	3 hours	1 hour
GIII	n=10	4 cycles	University of Wisconsin	3 hours	1 hour

5.2.6. Sampling procedure

To measure the oxidative stress we collected venous blood samples and small bowel tissue samples according to the same protocol: first after laparotomy (control: in GI after laparotomy; in GII and GIII prior to IPC), then at the end of cold ischemia (preserved), and at the 5^{th} minute (early reperfusion) and also at the 60^{th} minute (late reperfusion) of the reperfusion periods

5.2.7. Biochemical assays

We determined concentration of MDA, GSH and activity of SOD in blood samples and bowel tissue homogenates as described above (3.2.2.).

5.2.8. Demonstration of OFRs production in PMNs isolated from blood

We determined OFRs production in GII, GIII as described above (4.2.7. and 4.2.8.).

5.2.9. Gel Electrophoretic Mobility Shift Assay (EMSA)

In GI mucosal layer was separated from bowel after laparotomy (control), 15, 30 min and 1, 2, 3 hours after preconditioning. 100 mg tissues were homogenized in 1 ml TE buffer containing 10 μM PMSF. Nuclei were separated from cytosol by centrifugation, and this separation procedure was repeated for 3 times. The last pellet was resuspended in 2 volumes of buffer containing 20 mM HEPES, 0.5 mM DTT, protease inhibitors (Complete Mini, Boehringer Mannheim, Germany) and placed on ice for 20 min. After 10 sec centrifugation the supernatants were saved, alliquoted and stored. Protein concentration was determined with the Bio-Rad Protein Assay kit. 5'-end labelling of oligonucleotides was performed using [γ-^{32}P]-ATP and T4 polynucleotide kinase (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's protocol. 20 μg nuclear proteins were mixed with 1 μg poly(dl-dC), 100 ng non-specific single-stranded oligonucleotide and 4 μl buffer containing 10 mM HEPES pH 7.5, 10% glycerol, 1 mM EDTA, 100 mM NaCl. After 15 min incubation at room temperature the mixtures was completed with 2 μl, approximately 100 000 cpm of ^{32}P oligonucleotide and then incubated for another 30 min. DNA-protein complexes were electrophoresed in a 5% non-denaturating polyacrylamide the gel using a Tris Base, Borate, EDTA buffer system for 2.5 h at 200 V. Gel was dried and analysed by a Cyclone Phosphorlmager System (Packard Instrument Co., Meriden, USA). The specificity of binding interactions was assessed by competition with an excess of unlabelled double-stranded NF-κB binding oligonucleotide, and excess of unlabelled cAMP responsive element (CRE) oligonucleotide.

5.2.10. TUNEL
We determined DNA strand breaks in GII and GIII as described above (4.2.9.).

5.2.11. Hemodynamic examinations
Mesenteric flow and intestinal mucosal capillary perfusion were monitored after laparotomy and during reperfusion period as described above (4.2.10.).

5.2.12. Statistical analysis
Results are expressed as Mean values ± SEM. Data were analysed with one-way analysis of variance (ANOVA). The level of significance was set at $P< 0.05$. Micro Cal Origin (Ver 4.10) program (Microcal Software Inc., Northampton, USA) was used for data evaluation.

5.3. Results

5.3.1. Changes of oxidative stress markers in blood samples
Ischemic preconditioning more reduced the elevation of tissue lipidperoxidation by the end of the reperfusion. Preconditioning and cold preservation commonly reduced the elevation of peripheral blood GSH level during the same period in GII and GIII. During reperfusion peripheral blood SOD activity increased slightly in samples of preconditioned groups compare to control.

5.3.2. Changes of oxidative stress parameters in bowel tissue samples
Ischemic preconditioning prior to cold preservation reduced markedly tissue lipidperoxidation in all samples (GII: control 102.33±4.36 μM/g wet tissue, late reperfusion 98.74±6.17 μM/g wet tissue; GIII: control 104.52±3.82 μM/g wet tissue, late reperfusion 99.12±9.69 μM/g wet tissue). Concentration of MDA was similar to control during cold preservation and reperfusion periods. However, there was a significant GSH elevation in all samples compared to control in preconditioned groups, which indicated the activation of endogenous antioxidant protective system (GII: control 354.45±11.88 μM/g wet tissue, late reperfusion 495.73±12.38 μM/g wet tissue, $P< 0.001$; GIII: control 361.12±9.15 μM/g wet tissue, late reperfusion 489.04±15.18 μM/g wet tissue, $P< 0.001$). Furthermore, we observed a better preservation of SOD activity in preconditioned groups (GII: control 282.14±12.95 IU/g wet tissue, late reperfusion 168.45±15.03 IU/g wet tissue, $P< 0.05$; GIII: control 275.85±10.99 IU/g wet tissue, late reperfusion 192.62±14.36 IU/g wet tissue, $P< 0.05$).

5.3.3. Visualization of OFRs production by circulating PMNs
Small amount of intracellular OFRs was detected in the control samples. The qualitative imaging of reflectance signals of Ce-perhydroxide deposits slightly increased inside of PMNs by the end of reperfusion in preconditioned groups.

5.3.4. Activation of NF-κB after small bowel preconditioning
We observed that PC induced a time-dependent increase in NF-κB DNA binding activity. Both in the control and the 15 and 30 minutes following preconditioning a low NF-κB activation could be observed in samples. The NF-κB activation peaked at the 1-hour time-point, and decreased by the third hour in mucosal cells after small bowel PC.

5.3.5. Detection of nuclear fragmentation by TUNEL technique
In each preconditioned group a few DNA-damaged cells were found in the mucosal layer in control samples in the small intestine. Interestingly, several TUNEL positive cells in preconditioned groups are indicative of more generalized programmed-cell death by the end of the reperfusion period.

5.3.6. Hemodynamic parameters
Control mesenteric flow was 135.69±8.48 ml/minute, and not decreased less than 130.20±6.33 ml/minute during reperfusion in preconditioned groups (GI, GII, GIII). The intestinal mucosal capillary perfusion was similarly sufficient after laparotomy and during reperfusion period.

5.4. Conclusions from this study

In the third series of our study investigated the effect of ischemic preconditioning prior to autotransplantation. The 4 x 5 IPC stimuli conveyed protection against tissue oxidative injury in the intestine. With respect to MDA, we demonstrated that IPC and cold preservation commonly reduced tissue lipidperoxidation. Meanwhile, the tissue GSH concentration elevated, to indicate the activation of endogenous antioxidant protective system. In the case of preconditioned and later preserved grafts the SOD activity remained to a greater extent, although it was found to be lower than the control activity. There are no data indicating the exact mechanism of the maintained activity in the early phase. It can be due to a lower amount of OFRs produced or to an increase in GSH transport or synthesis. Other investigators confirmed our results regarding to the IPC groups. Sola *et al* have also reported the modification of oxidative stress after IPC with 10 minutes of ischemia and 10 minutes of reperfusion. Namely, in their models IPC prevented elevation of tissue MDA and significantly increased concentration of GSH.

The present results are believed to be the first to visualize OFRs production of PMNs after small bowel ischemic preconditioning. Our results confirmed that IPC can decrease OFRs release during reperfusion, but low amount of OFRs are necessary for the inductive effect to begin IPC cascade. Similarly, Jaberansari *et al* reported the role of OFRs to induce IPC cascade in pig myocardium.

Accumulating evidence has revealed that NF-κB is involved in the control of the transcription of a variety of cellular genes that regulate numerous responses in cells. The involvement of NF-κB is especially of interest as it is activated by oxidative stress and antioxidant compounds can modulate its activation. Our finding showed that 15 and 30 minutes following IPC stimuli a low NF-κB activation could be observed. But it peaked at the 1-hour time-point and decreased by the third hour in preconditioned mucosa.

In the present study we demonstrated the connection between IPC prior to bowel autotransplantation and programmed cell death. Few DNA-damaged cells were found in the mucosa specimens before preconditioning. Interestingly, several cell suffered DNA-damage were observed after preconditioned and autotransplanted grafts. The role of NF-κB in the intestinal epithelial cells apoptotic process remains to be established, since this transcription factor also seems to play a proapoptotic role in cells.

6. DISCUSSION

The past three decades have seen increasing interest in the role played by oxygen derived free radicals and oxidants in human disease. Gerschman *et al* proposed, "that oxygen poisoning and radiation injury have at least one common basis of action, possibly the formation of oxidizing free radicals". This pioneering idea soon began to capture the imaginations of scientists, thus the role of free radical reactions in human disease, biology and toxicology has become an area of intense interest.

In Hungary, the interest in researches of OFRs has been begun in the early 1980 years yet. Investigations based on the experience of the role of OFRs in the pathomechanism of oxidative damage and the antioxidant protection owing to the endeavours of Fehér, Blázovics, Lakatos, Matkovics, Mózsik, Rőth.

In the living organisms there are three main source of the generation of OFRs: mitochondria, xanthine oxidase system, and polymorphonuclear leukocytes. Under physiological respiration in mitochondria of all cells the electron transport chain is generally tightly regulated, but some leakage (1-2 %) occurs leading to the formation of OFRs. Normally the intracellular antioxidant enzyme can neutralize mainly $O_2^{\cdot-}$ radicals as the side-product of terminal oxidant inhibiting the increased lipid peroxidation in mitochondria.

It is generally considered that endothelium, particularly in the microvasculature, is the primary cellular sources of OFRs. Levels of XO are normally low in human tissues, but they may increase after I/R. In the intestinal tract XO presences not only in the microvascular endothelial cells but intestinal epithelium also contains large amounts of the enzyme. During ischemia the XD converted to the XO. At the same time, there is a progressive breakdown of ATP. This in turn leads to accumulation of the purine metabolites xanthine and hypoxanthine. XO converts hypoxanthine to xanthine and xanthine to urate, O_2 being simultaneously reduced to both $O_2^{\cdot-}$ and H_2O_2. High level of H_2O_2 can attack several cellular energy-producing systems. H_2O_2 also forms the $OH^{\cdot}$ in the presence of transition metal ions and O_2 can facilitate this reaction.

PMNs have the function of recognizing foreign particles such as bacteria. An oxidase enzyme that seems to have specifically evolved to make $O_2^{\cdot-}$ exists in the plasma membrane of PMNs. Although normally inactive, this enzyme is activated when PMNs encounter a foreign particle. The enzyme takes NADPH from the PMN cytosol, oxidizes it to $NADP^+$ and passes electron onto O_2, hence reducing O_2 to $O_2^{\cdot-}$ outside the cell. $O_2^{\cdot-}$ production is activated when foreign particles touch the PMN surface and some $O_2^{\cdot-}$ escapes into the extracellular fluid.

While a large number of cellular constituents are potentially subject to OFRs attack, a frequent target is the lipid component of membranes. Peroxyl radicals are formed during lipid oxidation chain reactions, such as the oxidation of polyunsaturated fats and unsaturated fatty acids. Oxidation of membrane lipids can impair the function of internal and cell surface membranes and the enzymes and receptors associated with them. Peroxidation of lipids causes cleavage of double bonds, resulting in the formation of aldehydes, such as malondialdehyde. These aldehydic products are diffusible cytotoxins.

All cells of organisms are constantly exposed to endogenous or exogenous OFRs. Organisms have evolved an array of defence endogenous enzymatic and non-enzymatic mechanisms. In the late 1960', McCord and Fridovich recognized that SOD was the first example of the enzyme that uses OFRs as a substrate, catalysing the dismutation of $O_2^{\cdot-}$ into H_2O_2 and O_2 at diffusion-controlled rates. SOD have come to be recognized as being nearly ubiquitous in living organisms, an essential component of the cellular defence against toxic OFRs generated during the normal biological reduction of O_2. SOD is one of three enzymes, each named for its active site metal ions and cellular location. Dimeric CuZnSOD (SOD1) is found in the cytoplasm, a homotetrameric MnSOD (SOD2) is located in the mitochondrial matrix, and a homotetrameric glycosylated CuZnSOD (SOD3) is confined to the extracellular space.

Cellular thiols, because of their relative abundance, stability, and ease of forming reversible disulfide bounds, play a very important role in the biochemistry of metals and in the maintenance of cellular redox homeostasis. Among the most critical sources of cellular reducing power and redox stabilization are cellular thiols, particularly glutathione. The compartmentalization and the transport of GSH (L-γ-glutamyl-L-cysteinyl-glycine) are self-regulated and serve to regulate the transport of other molecules. Glutathione peroxidase catalyses the reduction of H_2O_2 and the formation of oxidized GSH (GSSG). Glutathione reductase uses NADPH and H^+ to reduce the GSSG back to GSH. The relative redox status of different cellular compartments can be modulated though variations in the ratio of GSH:GSSG. The balance of reduced versus oxidized GSH plays a key role in maintaining active, reduced cellular proteins and is an important determining factor in metal-induced alterations of the cellular redox status.

Oxidative stress is the term referring to the imbalance between generation of OFRs and the activity of the antioxidant defences. Clearly this can result either from depletion of the latter or from excessive formation of OFRs. Cells can often adapt to increased oxidative stress up to a certain point. However, it is also entirely possible that the oxidative stress becomes overwhelming, leading to irreversible damage and to cell death. The importance of oxidative stress injury is dependent on the molecular target, the severity of the stress and the mechanism by which the oxidative stress is imposed.

Early reperfusion is an absolute prerequisite for the survival of ischemic tissue. However, reperfusion has been referred to as the 'double edged sword' because abundant evidence suggests that reperfusing ischemic tissues carries with it a component of injury known as 'reperfusion injury'. In the intestinal tissue reperfusion results in OFRs formation via the endothelial and epithelial cell using mainly the XO pathway. The accumulated hypoxanthine at the tissue of ischemia is quickly oxidized xanthine yielding superoxide anion. OFRs then potentiate villus injury with necrosis of the villus tip, leading to increased capillary permeability, reduced absorption of nutrients, protein leak into the lumen and translocation of bacteria, endotoxin and bowel enzymes into the portal circulation. OFRs, in particular the H_2O_2 coming from activated PMNs, may also stimulate splanchnic vasoconstriction, which exacerbates the I/R injury. Intestinal injury following I/R occurs in a hierarchical manner. The initial injury is merely functional, but as the extent and duration of I/R increases, morphologically detectable injury, ranging from superficial changes at the tip of the villi to transmural infarction. One of the sensitive indicators of mild injury to the intestine is an increase in lipid peroxides and a decrease in defence mechanisms.

Our findings confirmed that physiological steady-state balance between the production of lipidperoxides and their neutralization by antioxidants upset following ischemia and reperfusion. In this study we observed, that OFRs were generated during warm ischemia and subsequent reperfusion caused a significant elevation in tissue MDA. Meanwhile, the endogen antioxidant defence systems such as GSH concentration slightly, but not significantly decreased. Disparately another organs (heart, kidney, liver), principle answer of bowel tissue to this imbalance was to put into action SOD. The great extent in the reduction of SOD activity can be explained by the exhaustion of the SOD enzyme carrying out neutralization as a result of increased O_2·- production because of large amount of XO. Meanwhile, SOD could not to control these processes oxidative injury has been explained. These mechanisms reflect to susceptibility and specific manner of the intestine against oxidative stress.

Transplantation has become a well-accepted treatment of end-stage disease in many organs including small intestine. Development of surgical techniques as well as management strategies for immunological rejection and recurrence of the native disease has occupied researchers for the past several decades. The emergence of I/R injury as a topic crucial to outcome of transplant surgery has been not recent problem. The problems of damage by intense cold ischemia on organs are unique to transplantation. Organs are routinely flushed with cold preserve solution and quickly cooled. Like warm ischemia, cold ischemia can disrupt membrane electrical gradients, inhibits Na^+-K^+-ATP pump activity, leading to intracellular accumulation of Na^+ and Ca^{2+} and causing increased cellular oedema. In addition to metabolic derangements, structural abnormalities occur in the setting of cold storage. Activation of PMNs is another well-recognized mechanism whereby cellular injury results from cold I/R. In small bowel transplantation, 'rolling and sticking' of leukocytes was significantly increased in the submucosal post-capillary venules following reperfusion.

The possibility of reducing I/R injury by improving the cold preservation is currently considered being one of the ways to prevent oxidative stress and to restore the morphology and functionality of the small bowel grafts. Successful bowel transplantation may require optimal preservation conditions and minimal reperfusion injury to decrease postoperative complications. Because most of the studies on the ischemic susceptibility of intestine are based on definition the cold ischemia time and optimum preservation fluid for bowel grafts. Zhang *et al* compared the efficacy of UW, EC and RL solutions in preserving canine bowel. They suggested that, the degree of the changes varied among the different groups, with the worst change occurring in the lactated Ringer's solution group, moderated changes in the EC solution group, and minimal changes in the UW solution group. Three days after transplantation some of the EC grafts and most of the UW grafts showed mucosal re-epithelialisation with near-normal villus height. In paradox, Hamamoto and colleagues reported that EC the best preservation fluid for the canine bowel, as compared to UW or RL. Although, UW solution contains glutathione as OFRs scavenger, Wiconm and Collins first noted that glutathione was unstable and spontaneously converted to oxidized form during storage of the solution. The role of allopurinol as a XO inhibitor in UW solution and whether this agent is beneficial in an organ-specific manner are uncertain.

In our investigations, using different solutions of which the two most widely accepted ones were used can perform cold preservation. Regarding to oxidative stress parameters we didn't

find significant difference between EC and UW solutions. We have found that, cold preservation slightly moderated tissue lipid peroxidation. However it was surprised that SOD activity dramatically decreased in spite of low temperature confirmed the key role of XD/XO conversion and abundant $O_2^{\cdot-}$ production, which couldn't be beneficially modified by cold preservation. By now, it seems that any commercially available solution could be not used for small bowel storage. The creation of a more optimal preservation solution for the small intestine still seems desirable.

The question arises how else the protection against I/R injuries could be increased. Ischemic preconditioning of tissues has become a successfully and intensely cultivated research area in the past 15 years. Research has brought a lot of success in the case of myocardium resulting in clinical application by now, but the results of experiments on IPC of other tissues (liver, brain, skeletal muscles) are also well known. IPC is the stress response that occurs during repeated episodes of brief ischemia and reperfusion, and can render the organ more tolerant to subsequent potential lethal ischemic injury. Ischemic adaptation occurs in two different steps: an early or immediate effect (classic IPC) and a late effect (SWOP). A number of factors, which were found to regulate early adaptation, have also been found to play a crucial role in the delayed adaptation to ischemia. The three principal players are adenosine, ATP-sensitive potassium channels, and protein kinase C. The delayed adaptive protection has mediated by gene expression, transcriptional regulation and subsequent protein synthesis. These genes include those encoding for proto-ontogenesis (c-fos, c-myc, c-jun), the heat shock proteins (HSP72, HSP70), and antioxidants (SOD, catalase), cytokines, transcription factors, immunoreceptors and growth factors.

Initial studies on the small intestine revealed that IPC achieved by a 30-min occlusion of the mesenteric artery could reduce the ischemic sensitivity of the bowel. Actually, only a few studies have demonstrated the importance of preconditioning prior to cold preservation of the intestine. Inducing endogenous adaptation may reduce the development of oxidative stress following cold preservation and it increases viability of the bowel tissue making it more suitable for a subsequent transplantation.

Our results obtained in the bowel homogenates showed that, IPC carried out in 4 cycles prior to preservation protected the bowel tissue against increased of lipidperoxidation diminishing characteristic reduction of antioxidant capacity. The tissue level of GSH has significantly increased in bowel tissue, which refers to protection against oxidative stress of the cells. Moreover, we observed the best preservation of SOD activity in the preconditioned and later preserved grafts. There is evidence in the literature that IPC reduces the conversion of XD in to XO, while the amounts of released OFRs also decrease.

Until recently, most studies aimed to demonstrate a link between transcriptional factor NF-κB activation and OFRs. Despite numerous mechanisms of transduction, most inducers of NF-κB share the ability to induce redox changes. Increased OFRs level can act as chemical inducers of the expression of specific genes involved in protecting cells against oxidative damage. Recent results have demonstrated that H_2O_2 and lipidperoxides regulate gene expression. This is important for two reasons, firstly, IPC can significantly decrease the amount of OFRs released during reperfusion, secondly, the extent of this decrease is only as small as necessary for the inductive effect to begin IPC cascade. Experiments clearly showed

that the overexpression of SOD stimulated the activation of NF-κB. The addition of N-acetylcysteine, a powerful neutralizer of OFRs inhibits the activation of NF-κB by H_2O_2. Recent studies have revealed that the overexpression of detoxifying enzymes such as catalase or glutathione peroxidase inhibited the activation of NF-κB. Hence, this confirms that the activation of NF-κB is redox modulated.

Based on our results is can be explained that OFRs generating during IPC may induce cellular signal cascade to activate NF-κB in mucosal cells. Following preconditioning stimuli a time-dependent activation was observed. These findings is believed to be the first to demonstrate the activation of transcription factor NF-κB in preconditioned intestinal tissue causing better preservation of endogenous scavenger capacity of the small bowel mucosa.

The strategy of prevention and treatment of I/R injury includes prevention of cell death. Cell death accompanies all the most significant periods in the life, including embryogenesis and development, the adult state, aging and death. Based on the morphology of dying cells, Kerr *et al* advanced the concept that there exist two distinct types of cell death, pathological cell death as necrosis and physiological cell death as apoptosis. Necrosis occurs as a catastrophic accident to whole cell, swelling of the cytoplasm and organelles including mitochondria due to the loss of selective permeability of the cell membrane. This results inducing an inflammatory reaction in the adjacent viable tissue in response to the released cell debris. Apoptosis is ultrastucturally seen to involve the chromatin condensation and margination, cell shrinkage, blebbing, and nuclear fragmentation. The cell breaks up into several apoptotic bodies, which are phagocytosed by nearby cells without inflammation.

Although data in the literature suggest that the role of NF-κB in programmed cell death is ambivalent, as it has both proapoptotic and antiapoptotic functions, depending on the type of the cell and the death stimulus. Our findings suggest that NF-κB has a proapoptotic effect during the reperfusion of tissues undergoing IPC prior to cold preservation. It seems that IPC stimuli increased apoptosis regarding it has beneficial effect against tissue necrosis. We hypothesize that in the delayed phase of preconditioning by new protein synthesis the member of apoptotic cells will be decreased. These results can be considered as novel as no other results describing the apoptosis of preconditioned and subsequently preserved bowel tissue have been reported so far. However, the precise factors, that determines the ability of NF-κB to regulate apoptotic pathways during I/R period or after ischemic preconditioning in the intestine is unknown.

7. NOVEL FINDINGS

The small bowel was the last splanchnic organ to be transplanted. The difficulty stems from the fact that small intestine is strongly immunogenic and it is one of the most sensitive tissues to ischemia-reperfusion injury. To eliminate the immunological complications we applied several experimental models for studying effects of warm and cold ischemia-reperfusion, and the influence of ischemic preconditioning prior to cold preservation and autotransplantation.

We demonstrated, that oxidative stress parameters, such as lipid peroxidation and endogenous antioxidant compound are reliable indicators to monitor the ischemic intestinal damages.

We determined, that commonly applied cold preservation solutions are usable to eliminate the development of oxidative injury following reperfusion in our autotransplantation model.

We detected, that following warm and cold ischemia changes of superoxide dismutase activity is the most sensitive indicator in the intestinal reperfusion injury.

First we evaluated the effect of ischemic preconditioning before cold preservation in small bowel autotransplantation model. Our investigations revealed the protective effect of ischemic preconditioning on the oxidative stress parameters.

Demonstrating induction of the NF-κB transcription factor in the preconditioned bowel mucosa we confirmed that IPC stimuli could activate the intracellular signal transduction.

This present study is believed to be the first to demonstrate the early protective effects of ischemic preconditioning prior to small bowel preservation and autotransplantation. Ischemic preconditioning of the small intestine may open a new perspective for reducing I/R injury.

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9. PRESENTATIONS AND PUBLICATIONS

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1. **Ferencz A**, Tavakoli A, Kalmár-Nagy K, Szántó Z, Rőth E, Horváth ŐP. Az oxidatív stressz monitorozása kísérletesen létrehozott vékonybél autotranszplantációt követően. Magy Seb 2001; 54: 60-4.

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10. ABBREVIATIONS

$CeCl_3$	Cerium-trichloride
CLSM:	Confocal Laser Scanning Microscopy
CRE:	cAMP Responsive Element
EC:	Euro Collins
EMSA:	Gel Electrophoretic Mobility Shift Assay
DAG:	Diacylglycerol
HSP:	Heat Shock Proteins
I/R:	Ischemia and Reperfusion
IPC:	Ischemic Preconditioning
GSH:	Reduced Glutathione
GSSG:	Oxidized Glutathione
K_{ATP}	ATP-sensitive potassium channels
MDA:	Malondialdehyde
NF-κB	Nuclear Factor kappa Binding
OFRs:	Oxygen Free Radicals
PI:	Propidium Iodide
PKC:	Protein Kinase C
PMA:	Phorbol-12-myristate-13-acetate
PMNs:	Polymorphonuclear leukocytes
POD:	Peroxidase
RL:	Ringer's lactate
SOD:	Superoxide Dismutase
SWOP:	Second Window of Protection
TdT:	Terminal deoxynucleotidyl Transferase
TUNEL:	TdT -Mediated dUTP Nick End-Labelling
TyK:	Tyrosine Kinase
UW:	University of Wisconsin
XD:	Xanthine Dehydrogenase
XO:	Xanthine Oxidase