INVESTIGATION OF INTESTINAL ISCHEMIA/REPERFUSION AND ITS DEFENCE MECHANISMS ON EXPERIMENTAL ANIMAL MODELS

Ph.D thesis

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Pécs, Hungary
2016

1. Introduction

According to 2013 data of the International Intestinal Transplant Registry from 21 countries of the world in 77 centers approximately 1300 patients per year are carried out isolated or multivisceral small bowel transplantation. In Europe, intestinal transplantation is happening in 25 center, but in our country does not perform this operation et all. The 1 year and 3 years survival of the grafts and recipients are about 75-70%, whereas the 5 year survival is under 60%. The number of operations and survival data are behind from the other abdominal organ transplant statistics.

In the background of the intestinal failure should be complete or partial congenital absence, or organ resection in various lengths. If more than 70% of intestinal length is missing or there is a permanent dysfunction in the tract, it is called short bowel syndrome. In these cases, the working length of the small bowel is less than 2 meters in the adults, which causes disturbance in the electrolyte balance and malabsorption of essential nutrients, and eventually it leads to severe malnutrition and dehydration. The conservative treatment of the patients is the total parenteral nutrition, which should cause a life-threatening complication in 2-5% of the adults and in 10-15% of the children, which requires small bowel transplantation as a curative solution. Many diseases should stand in the background of clinical problem in children and in adults (intestinal poliposis, Hirschsprung disease, gastroschisis, necrotizing enterocolitis, ischemia, trauma, volvulus, Crohn's disease, Gardner's syndrome, radiation enteritis, etc.).

Fundamentally, intestinal transplantation is hampered by two factors. It has significant immunological role, and increased sensitivity to ischemia/reperfusion (I/R) injury. During this especially important immune organ transplantation a large numbers of lymphatic tissue located in the intestinal wall and mesenterium are transplanted also. This is the major reason of graft-versus-host disease, namely organ rejection, and the transplanted immune cells should cause host-versus-graft disease under immunosuppression in patients.

Another factor complicating with small bowel transplantation is the organ increased sensitivity to I/R injury. Its degree is determined by the severity and duration of hypoxia. Reperfusion paradox phenomenon was first described in 1986 by Park and Granger in feline I/R gut model. Accordingly, tissue damage developed during reperfusion greater extent than which is formed alone under ischemia. I/R can generate various cellular interactions that lead to microvascular damage, and to cellular necrosis and/or apoptosis. Occlusion of splanchic arteries and subsequent reperfusion can cause activation of polymorphonuclear (PMN) leukocytes, increased vascular permeability associated with cardiovascular insufficiency, and extreme release of pro-inflammatory factors, nitrogen and oxygen free radicals (OFRs). These cause lipid peroxidation of membranes, damages in proteins and DNA, and finally cell death. I/R lead intestinal motility disorders by damaging muscle tissue, which is due to OFRs and disturbance in calcium homeostasis.

Intestinal I/R through mucosal barrier damage leading to bacterial translocation and systemic inflammatory activation, causing electrolyte and acid-base imbalance. Severe damage will result

sepsis, multiple organ failure and should cause the patient death.

During transplantation to reduce warm ischemic injury the organ must be cooled. For this purpose the first step is the perfusion, when a cold preservation solution is perfused through the graft vessels. After that it is storaged between melting ice in 4 ºC preservation solution for different time. In case of human isolated small intestinal or in multivisceral transplantation usage of University of Wisconsin (UW) solution is the most accepted today. This was the first intracellular osmolarity solution, which was developed in the late 1980s. Osmotic stability of UW solution provides metabolically inert molecules, such as laktobinát and raffinose; hydroxyethyl starch and antioxidant components (glutathione, allopurinol) prevent oedema, and adenosine serves as energy source. Using cold preservation solution can decrease cell metabolic activity and the possible tissue damage, but the preservation process itself entirely unable to defend the organ against cold ischemic injury.

Researches related to I/R and transplantation began several decades ago. Knowing basic studies, the molecular background and defense mechanisms now targeted by the investigations, to which our research team wished to contribute with the investigation of small bowel transplantation in different animal models.

2. Investigation of intestinal injury after warm and cold ischemia/reperfusion

2.1. Aims

In the first series of our research, different durations warm I/R, and cold preservation followed small bowel autotransplantation have been established, and we had the following questions:

1. What level of oxidative stress occurs in reperfused intestinal tissue after warm and cold ischemia?
2. How the cold preservation can reduce oxidative damage in the cells?
3. How change the structure with the intestinal ischemic time increase?
4. Can be detectable the damages by DSC technique? And, if so, whether are there any differences among intestinal layers?

2.2. Materials and methods

2.2.1. Experimental groups and surgical protocols

Adult male Wistar rats (body weight: 250-300 g; $\Sigma n = 35$) were used to our investigations. Preoperatively, animals allowed drinking only water with sugar for 24 hours. Rats were anesthetized with intramuscular ketamine hydrochloride (0.075 mg/g of body weight) and diazepam (0.075 mg/g of body weight; Richter Gedeon, Budapest, Hungary). The Committee on Animal Research of Pécs University has approved the study (BA02/2000–9/2008).

In the warm ischemia groups, after median laparotomy superior mesenteric artery was prepared and ligated with atraumatic clip for 1 hour (Group A1), for 3 hours (Group A2) and for 6 hours (Group A3) to performe whole ischemia. After removal of clips, 3 hours reperfusion is started.

In the cold ischemia groups, after laparotomy small bowel was resected from the Treitz-ligament to the ileocoecal part of the ileum. Intestinal lumen was washed out with antibiotic-free normal saline. Grafts vasculature were perfused with 4 °C UW solution (Via-span, Bristol-Myers Squibb GesmbH, Bécs, Ausztria) and were preservated for 1 hour (Group B1), for 3 hours (Group B2), and for 6 hous (Group B3). After preservation, orthotopical autotransplantations were performed with end-to-end vessel anastomoses with microsurgical technique (8/0, synthetic, monofilament, non-absorbable suture material). Bowel anastomoses were not performed. Reperfusion lasted for 3 hours in each groups. In each groups, jejunal biopsies were collected after laparotomy (control: C) and at the end of the reperfusion periods. From sham operated animals (sham operated: S), tissue biopsies were collected after laparotomy (control) and at the same time of ischemic groups biopsies.

2.2.2. Biochemical examinations

Malondialdehyde (MDA) was determined in bowel tissue homogenates with the use of lipid peroxidation assay kit (Calbiochem-Novabiochem Corp, Darmstadt, Germany), as a colorimetric assay kit specific for MDA (OD=532 nm). Final values were given as micromoles per gram.

Reduced glutathione (GSH) was quantified in bowel tissue homogenates using a 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 micromoles per gram.

Superoxide dismutase (SOD) was measured in bowel tissue homogenates using a superoxide dismutase assay kit (Calbiochem). One reagent of the kit underwent alkaline autooxidation, which was accelerated by SOD. Autooxidation of this reagent yielded a chromophore, which absorbed maximally at 525 nm. The value of the activity of SOD was given in units per gram.

2.2.3. Histological examinations

Bowel tissues were fixed in 10% buffered formalin, embedded, cut to 10 μm, and stained with hematoxylin and eosin. Histologic damage was assessed in a "blind" manner using Park's histologic classification of intestinal injury (Nicon Eclipse 80 Light Microscope, Kingston, England; original magnification ×100). Total mucosa and submucosa thickness, depth of the crypts, and thickness of muscular layer were quantitatively analyzed using the software Scion Image (Scion Corporation, Maryland, USA). The number of square pixels was counted in five fields per sections at ×400 magnification, and the length was given in micrometer.

2.2.4. DSC analysis

The temperature-dependent measurements of the physical and chemical properties of the samples are called thermoanalytical methods. Differential scanning calorimetry (DSC), as thermal analysis was described in the 1960s by Watson and O'Neill mainly for determining physical properties of minerals and other inorganic materials in the USA. Later, this sensitive and validated analytical method used to detect structural changes in a variety of biological macromolecules, and from the 2000s it was used to examining in experimental animal models and in various clinical studies also.

To understandings of the fundamental methodology of DSC the basic factors are the follows. Of certain substances, such as biological samples depending on the temperature in different chemical compounds, or in crystal structure are present in the cells or tissues. In case of a living organism, this temperature range is relatively small, usually around 36-37 °C. Changing the temperature can reach a point where the chemical form of the crystal structure or physical condition of each substance is changed. These transformations are specific to the materials. Moreover, after various harmful effects changes in the molecules or samples can give specific picture from them. This transformation is accompanied by thermal phenomenon. Detecting the transition or transformation temperature (T_{m1}, T_{m2}, T_{m3}, …, T_{mx}), and meanwhile absorb or release thermal energy can be identified the chemical structure of sustances, if it is compared with known, pure chemical substances has been measured its thermal phenomena.

The DSC device contains two chambers, one of which contains the sample and other contains the reference. The latter must be inert, which means during measurement it does not suffer any

structural changes. This method measures that electric power, which is necessary to hold the sample and the reference materials in same and standard temperature during both heating and cooling (ΔT=0).

When evaluating the DSC-thermogram we get a response how changed the heat flow after temperature increase, i.e. heat flow per unit of time over the material. At a given temperature (maximum conversion/denaturation temperature: T_m, the peak of the curve) 50% of the total amount of the macromolecules are denatured. After that, there will be more denatured protein and less and less is converted per unit of time, so you need less and less overplus energy feed into the sample. In a closed system at constant pressure the invested compensation heat flux converted to electrical power is the enthalpy changes (ΔH), which is necessary to maintain the required heat balance between the sample and the reference.

During DSC measurement the thermal unfolding of the total intestinal wall, its mucosa and muscle components were monitored separately by Setaram Micro DSC-II calorimeter. All experiments were conducted between 0 and 100 °C. The heating rate was 0.3 K min^{-1} in all cases. Conventional Hastelloy batch vessels were used during the denaturation experiments with 850 µL sample volume (samples plus buffer) in average. Typical sample wet masses for calorimetric experiments were between 100-150 mg. In warm ischemic experiments the reference sample was normal saline (0.9 % NaCl), while is cold I/R groups UW solution was used as a reference. The sample and reference vessels were equilibrated with a precision of ± 0.1 mg. There was no need to do any correction from the point of view of heat capacity between sample and reference vessels. The repeated scan of denatured sample was used as baseline reference, which was subtracted from the original DSC curve. Calorimetric enthalpy was calculated from the area under the heat absorption curve by using two-point setting Setaram peak integration. Graphical representation of data was performed after ASCII conversion with Origin program (ver. 6.0, Microcal Software Inc, Northampton, USA).

2.2.5. Statistical analysis

Results are expressed as mean values±SEM. Data were analyzed with one-way analysis of variance. The level of significance was set at $P<0.05$. The MicroCal Origin (ver. 6.0) program (Microcal Software, Northampton, MA, USA) was used for data evaluation.

2.3. Results

2.3.1. Biochemical results

The extent of lipid peroxidation was determined by measuring the tissue concentration of its byproduct, malondialdehyde. Concentration of MDA increased in each warm ischemia groups at the end of the reperfusion periods. The elevation was significant after 3 and 6 hours ischemia (Group A2 and Group A3 vs. C; $p<0.01$; $p<0.001$). Cold ischemia ameliorated tissue lipidperoxidation, but after 3 and 6 hours elevation was detectable also (Group B2 and Group B3 vs. C; $p<0.05$). Comparing to the warm ischemia groups, cold preservation decreased intestinal lipidperoxidation, which was a significant difference in 6 hours group (Group B3 vs. Group A3; $p<0.05$).

The content of the endogenous scavenger GSH decreased at the end of reperfusion followed both 3 and 6 hours warm ischemic periods (Group A2 and Group A3 vs. C; $p<0.05$; $p<0.001$). The same cold ischemia time ameliorated the decrease in GSH concentration. While the GSH level significantly decreased in 6 hours samples compared to control, until then this was significantly higher compare to its warm ischemia values (Group B3 vs. C; Group B3 vs. Group A3; $p<0.05$).

In contrast, SOD activity showed significant decrease, which was comparable to increase of the warm ischemic time (Group A1, Group A2 and Group A3 vs. C; $p<0.05$; $p<0.01$; $p<0.001$). Its activity deacresed after cold ischemia also, but its numeric value was higher (Group B1, Group B2 and Group B3 vs. C; $p<0.05$). After 3 and 6 hours cold storage, better preservation of SOD activity was observed (Group B2 vs. Group A2, Group B3 vs. Group A3; $p<0.05$; $p<0.01$).

2.3.2. Histological results

According to Park's classification, both in the control samples and in the sham group showed an injury grade 0, corresponding to normal bowel structure. At the end of reperfusion followed 1 hour warm ischemia, histological findings were corresponding to an injury grade 2, showing minor clefting with the villus epithelium adjacent to the crypts intact. Until then, in 3 hours ischemia group the structural injury was grade 3, characterized by massive epithelial lifting. After 6 hours warm ischemia, the injury showed denuded villi and decellularization of lamina propria and crypt corresponding to a grade 5 injury. By contrast, cold preservation caused smaller intestinal structural injury, exeption to this 3 hours group, where the injury was the same like in warm ischemia group.

By Scion Image quantitative analysis, the histological injury showed good correlation to the warm ischemia time. During reperfusion followed by 3 and 6 hours warm ischemia the mucosal, submucosal and muscle thickness, and depth of crypts decreased significantly compare to control and sham operated tissues (Group A2 and Group A3 vs. C and S; $p<0.05$). In the cold preserved intestinal tissues were measured a reduction in the mentioned layers, but comparing to the warm I/R groups it was significantly higher after 3 and 6 hours cold storage (Group B1 vs. Group A1; Group B2 vs. Group A2; Group B3 vs. Group A3; $p<0.05$). Tissue injury correlation to the ischemia time was significant mainly in the mucosal and submucosal layers; in the case of the crypts it was less visible, while changes in muscle layer were the least conspicuous.

2.3.3. DSC results

According to DSC results the mucosa has undergone significant changes in a time-dependent manner during different type and duration of ischemia. The control sample has an exotherm with T_ms 53.6 °C and with a total calorimetric enthalpy changed to -4.1 ± 0.22 J/g. Applying warm ischemia more severe trend could be seen except of the case of 6 hours intervention, where ΔH is greater than in case of 3 hours warm ischemia. The 3 hours treatment resulted different transition temperatures and calorimetric enthalpies: in case of warm ischemia the T_m and the enthalpy decreased to 48.7 ± 0.2 °C

and 1.7 ± 0.2 J/g. An exception is the effect of 6 hours warm ischemia on mucosa, where extremely high enthalpy has been seen, surpassing the the control and 1 and 3 hours of warm ischemia values.

In case of the smooth muscle compound of intestinal wall the control exhibited a denaturation transition with T_ms 52,8 (myosin head), and 59.9 °C (myosin tail + actin) together with a 0.58 J/g calorimetric enthalpy. In case of 1 hour warm ischemia the contribution of myosin can not be resolved, because the main denaturation peak shifted to the conventional actin denaturation range (62 °C). It means that in the actomyosin complex the contribution of myosin tails can not be resolved, that is it exhibits more significant structural effect with smaller ΔH (1.4 J/g). During 3 hours warm ischemia the muscle exhibited a further increase in T_m and decrease in the calorimetric enthalpy. The effect of 6 hours ischemia was the most striking one. As a result of muscle components DSC shows that 6 hours of warm ischemia the T_m was 52 °C and 59 °C, and ΔH is elevated to extremely high value (3.1 J/g).

The effect of ischemia in case of total intestinal wall has shown a big variety. The thermal contribution of mucosa can be seen clearly in this latter case (23 °C). During warm treatment the contribution of myosin and actin compounds separated more significantly with increased main melting temperature (50.5 °C; 60.2 °C), where the myosin rod contribution could not be separated from actin. The effect of 3 hours treatment was more definite in mucosa. In the cases of muscle proteins the 3 hours warm ischemia exhibited only one main transition (59.8 °C). Moreover, in the total intestinal wall the 6 hours warm ischemia caused the biggest calorimetric enthalpy in the actomyosin system.

After 1 hour cold ischemia a lower denaturation appeared with a T_m= 30.4 ± 0.2°C and the higher transition shifted to 59.3 ± 0.2 °C with a total calorimetric enthalpy of -5.94 ± 0.4 J/g. In contrast, 3 hours cold storage increased the first transition by 2.2 °C compared to the first transition, while the enthalpy is decreased (-2.67 J/g). After 6 hours ischemia the enthalpy is decreased further.

Cold ischemia caused smaller structural changes in the muscle layer (T_m=53.5 °C, 56 °C; ΔH=2.21 J/g) compared to the same time warm ischemia (ΔH=1.4 J/g). One hour cold preservation also indicates a significant structural change mainly in myosin thermal domains as well as in the actin-myosin interaction. While 3 hours warm ischemia caused increase in T_m and decrease in ΔH, the cold treatment revealed an decrease in denaturation temperatures and increase in calorimetric enthalpy (T_m=53.6 °C, 58.3 °C; ΔH=3.4 J/g). DSC curves of muscle components after 6 hours preservation showed supprising similar thermal parameters compared to following 6 hours warm ischemia.

The effect of cold ischemia in case of total intestinal wall has shown a big variety also. Applying 1 hour cold ischemia caused a double thermal transition (T_m=53.5 °C, 56 °C). After 3 hours storage it was significant. In the cases of muscle proteins the 3 hours preservation caused the biggest structural changes with 3 thermal transition (T_m=53 °C, 55 °C, 59.6 °C). Next to these results 6 hours cold ischemia had effect to the calorimetric appearance of the mucosa, and caused significant changes in the acto-myosin complex.

2.4. Discussion

Researches associated with intestinal warm and cold I/R injury started several decades ago, which serve as a good basis to compare our results obtained from first series of experiments. According to our experimental warm I/R results a time-dependent oxidative injury was observed in the reperfused bowel tissue. After 1 hour warm ischemia the OFRs-induced oxidative stress has performed with lipid peroxidation and activation of endogenous scavenger and antioxidant enzymes. The process is spread as a chain reaction, 3 and 6 hours ischemia generates more and more radicals causing greater and greater tissue damade. Importance of our measurements is in the fact, that after warm ischemia changes in the physical-chemical properties of cell membranes destabilize receptor-ligand interactions and changes cell contact with the extracellular matrix. These results demonstrated the role of lipid peroxidation in tissue damage and in bringing cell dysfunction.

In several ischemic animal models described both systemic and local elevation of MDA levels as an indicator of lipeidperoxidation. MDA concentration both in intestinal mucosa, as well as in blood plasma increased to 3-4-fold during the early (5 minutes) reperfusion and its concentration reach the maximum about to the third hour of reperfusion. However, no consensus in the literature, how long that warm ischemic period when the intestinal lesions considered to reversible. Gisele et at showed in rat model that the small intestinal critical warm ischemic time is 40 minutes. If the warm ischemia time was longer hemorrhagic enteropathy occurred in all cases. In the researches of Slavikova this time was 45 minutes, this was 60 minutes in Beuk's research, and 90 minutes in Park'experiments, while Schweizer determined in 2 hours the critical warm ischemic period of the small intestine.

Endogenous scavenger GSH serve as the basic defenses of the cells, its concentration was significantly decreased in our study after 3 and 6 hours ischemia. Sola et al described tissue GSH halving in bowel samples after 90 minutes of ischemia followed by 30 minute reperfusion. Activity of endogenous antioxidant SOD significantly decreased in all three groups. The reason of this that the huge number of OFRs produced during reperfusion is not able to dismute by endogenous SOD. Deshmukh also showed that SOD activity significantly reduced during small bowel ischemia. This is due to several factors: on the one hand the activity of enzyme decreases, on the other hand the amount of SOD reduces, and thirdly, synthesis of SOD reduces also.

Our experimental results showed that cold preservation in UW solution has reduced oxidative damage in intestinal tissue compared with same warm ischemia caused injury. Tissue lipid peroxidation in the cold I/R groups could be measured, but the degree of damage was milder. The gut suffered about the same lipid peroxidation damage during 3 hours cold preservation than after 1 hour warm ischemia. Moreover, tissue MDA levels were significantly lower in reperfused samples after 6 hours cold ischemia than in the warm ischemic results. Similarly, results reported by De Oca et al, that lipid peroxidation is also rising in the preserved gut, but there was no significant increase in MDA concentration when the intestinal storage was shorter than 40 minutes.

Cold ischemia time mitigated the decrease in GSH concentration. While in 6 hour samples GSH levels were significantly decreased compared to controls, but it remained significantly higher

than warm ischemic values. SOD activity of intestinal samples significantly decreased following cold ischemia, but regarding activity its value remained higher. Due to 3 and 6 hours cold preservation the enzyme activity is better preserved than in the same warm ischemic groups. Similar findings reported by Zhang et al, who compared intestinal graft damages after 12 hours storage in UW, in EC and in Ringer Lactate solutions. Overall, we can conclude that small intestine can tolerate about 3 hours cold preservation without severe oxidative tissue damage.

Warm and cold intestinal ischemic injury is frequently occurring problem in the clinical practice. Routine diagnostics of these diseases are based on standard hematoxylin and eosin-stained histological methods, and for evaluation different histological staging is applied. In our experiments tissue structural changes were determined by quantitatively using Park's classification and by qualitatively using Scion Image Software. At the end of reperfusion followed 1 hour warm ischemia, histological findings showed minor clefting with the villus epithelium adjacent to the crypts intact. Until then, in 3 hours ischemia group the structural injury was characterized by massive epithelial lifting. After 6 hours warm ischemia the highest grade lesions developed with denuded villi, and decellularization of lamina propria and crypt, eventually with the total disintegration of intestinal structure. By contrast, cold preservation caused smaller intestinal structural injury, exeption to this 3 hours group, where the injury was the same like in warm ischemia group. According to the quantitative analysis, the histological injury showed good correlation to the warm ischemia time. In the cold preserved intestinal tissues were measured a reduction in the mentioned layers, but comparing to the warm I/R groups it was significantly higher after 3 and 6 hours cold storage. Tissue injury correlation to the ischemia time was significant mainly in the mucosal and submucosal layers; in the case of the crypts it was less visible, while changes in muscle layer were the least conspicuous.

Most of the different histological staging methods are qualitative or semi-quantitative methods, which causes difficulty in comparing the results. Disadvantages of histologic examination are that (1) does not always give precise value for each of structural damages, (2) it is a time-consuming process, (3) a qualitative assessment of the evaluation largely depends on the pathologist, (4) do not give an exact picture from the progression of the process alone, and (5) there is no consensus in the literature regarding the applied grading systems.

In this study, we detected correlation between the results of conventional histology and DSC results both in warm ischemia and cold preserved intestinal tissue. Based on our conclusions, the DSC data clearly reflected the graids of Park's classification with respect of mucosa, but the DSC analysis gave detailed information from smooth muscle tissue damage. In this study using DSC analysis we compared the effect of 1, 3 and 6 hours of warm or cold ischaemia to structural damage on the individual layers of the small intestine (mucosa, muscle) and on the total gut wall. Our results showed that the structure of the mucosa depending on the type and duration of ischemia significantly damaged. Based on the changes of calorimetric enthalpy the extent of damage is increased by the end of warm ischemic periods. Intestinal mucosa stored in cold UW solution preserved its "normal physiological structure" comparing to both control and warm ischemic samples. The changes can be seen most

prominently in the separate layers, but in the total intestinal wall curves can be found also. Calorimetric results are correlated with a number of experimental and clinical observations: increasing ischemic time more and more morphological changes are detectable both macro- and microscopically.

Under physiological conditions, mucosal cell renewal time approximately 3-4 days, and after they died by apoptosis and new mucosa cells will produce from basal membrane. In the literature a number of warm ischemic models have been described, wherein according to the qualitative scales the warm ischemic tolerance time is varied between 15 and 90 minutes. There is no consensus on the duration of the cold preservation time either. The small intestine has to be transplant as soon as possible, but it is proposed to transplant within 6-8 hours. Presently, calorimetry data indicates that serious mucosal damage occurs after 3 hours warm ischemia and 6 hours cold preservation.

Based on our previous studies showed that muscle thickness decreased slightly due to both warm and cold ischemia, but significant difference was not found. Several studies have confirmed that significant differences in the muscle layer with routine histological method are not visible. In contrast, comparative DSC data indicated that after warm ischemia the myosin tail can not dissolve in the actomyosin complex, indicating a significant structural alteration with a lower enthalpy changes. Localisation of actin and myosin is not clearly visible, the heat capacity changes (the baseline shift between the native and denatured states), and the elevated melting temperature showed the greater loose of the intermolecular bound water, therefore the protein system is becoming a more compact and rigid state. During warm ischemia the accumulating OFRs are in the background, which can damage the myosin catalytic center. This modification depends on the time, the concentration and the chemical structure of OFRs. On the other hand, oxidative stress disconnects the ATP hydrolysis. Thereby, ischemia results structural and functional changes in the muscular layer. Due to cold ischemia a various muscles components distinct on well-defined manner, indicating that preservation in UW solution has reduced the structural damages. The most significant change can be observed in the actomyosin complex interactions. Pathophysiological studies measured on smooth muscle tissue have been shown warm ischemia reduces the numbers of myenteric ganglion cells which are unable to regenerate in the postoperative period. This is the main reason of intestinal motility changes in the postoperative and in the postischemic periods. In the background of these functional disorders are standing known processes: oxidative damage of the macromolecules, disturbance in cells calcium balance, and activation of inflammatory cascades.

Thermal behaviour of total intestinal wall is showed great diversity, which basically comes from a layered structure of intestine. But, previously mentioned thermal characteristics of the separated smooth muscle and mucosa can be observed also, which confirms that the DSC analysis is reflected in the total intestinal tissue structural differences. In summary, our research team firstly examined bowel tissue structural injury in different warm and cold I/R models by differential scanning calorimetry method. This thermoanalytical technique allows a quantitative, objective, and precise determination of complex structural damages in biological systems, and thus allows its methodological application both in experimental and clinical research.

3. Ischemic postconditioning of the small bowel

3.1. Introduction

Intensive research results of cytoprotection against I/R injury, Murry et al in 1986 described the concept of ischemic preconditioning (IPC). Usage of short I/R periods which do not cause organ damage prior to long ischemic period should triggering the activation of the endogenous defense mechanism, and thus the organ will be more resistant to ischemic insult. In 2003, Vinten-Johansen et al introduced the ischemic postconditioning (IPO) theory. At the beginning of reperfusion using several, few second I/R cycles, which allow blood flow with fractional reperfusion to the ischemic area reducing reperfusion injury. If the restoration of blood flow is happening fully and suddenly, it will result cells calcium overload and oxidative stress, causing rapid pH neutralization, which play a role in the opening of mitochondrial channels leading to ATP depletion and ultimately to cell death. Due to IPO the cellular calcium homeostasis will be more sustainable providing protection against severe intracellular oxidative stress, it has a role in delayed pH correction and in mitochondria protection, which serves cells structural and functional defence. The first area of IPO research was in cardiology, but following introduction of the concept a number of studies have been published its protective effect on intestinal I/R models also. Application of IPC - despite its protective effect - is limited, because its usage is not possible if ischemia already existing. In contrast, clinical applicability of IPO should represent a breakthrough in preventing and treating injuries during organ I/R.

3.2. Aims

In the second part of our research, application of ischemic postconditioning in warm I/R and small bowel autotransplantation models the following questions were arised:

1. How schange the oxidative stress after IPO application in warm I/R and autotransplanted intestine?
2. How can IPO decrease the oxidative injury of the cells?
3. How changes the intestinal structure after IPO application in our models?

3.3. Materials and methods

3.3.1. Experimental groups and surgical protocols

Experiments were performed on white male domestic pigs (body weight: 25-30 kg, $\Sigma n = 16$). Preoperatively, animals allowed drinking only water with sugar for 24 hours. Pigs were premedicated with intramuscular azaperone (Stresnil, 0.4 mg/kg of body weight, Jannsen Animal Health, Belgium) and thiopenthal (0.1 mg/kg of body weight, Biochemie Gmb, Austria). Operations were performed under general anesthesia with mixture of isoflurane, N_2O and O_2 gases. All experiments were approved by our Committee on Animal Research (BA02/2000-20/2006).

In warm ischemia groups, after median laparotomy superior mesenteric artery was prepared and occluded with atraumatic vessel clip for 1 (Group A1), for 3 (Group A2) and for 6 hours (Group A3). After removal of clips, 3 hours reperfusion is started.

In the cold ischemia groups, after laparotomy small bowel was resected and intestinal lumen was washed out with antibiotic-free normal saline solution. Grafts vasculature were perfused with 4 °C UW solution and were preservated for 1 hour (Group B1), for 3 hours (Group B2), and for 6 hours (Group B3). After preservation, orthotopical autotransplantations were performed with end-to-end vessel anastomoses using 6/0, synthetic, monofilament, non-absorbable suture material. Bowel anastomoses were not performed. Reperfusion lasted for 3 hours in each groups.

Classical IPC protocol was performed at the end of the ischemic and immediately before reperfusion periods in warm ischemia (Group A4, Group A5, and Group A6) and in cold ischemic (Group B4, Group B5, and Group B6) groups. IPO was applied with 3 cycles, 30 seconds ischemia and 30 seconds reperfusion per cycles. In each groups, jejunal biopsies were collected after laparotomy (control: C) and at the end of the reperfusion periods. From sham operated animals (S) biopsies were collected after laparotomy (control) and at the same time of ischemic group biopsies.

3.3.2. Biochemical examinations

Measurements were performed as described in 2.2.2. section previously.

3.3.3. Histological examinations

Intestinal samples were prepared and examined as described in 2.2.3. section above.

3.3.4. Statistical analysis

Assessment of results was performed as described in 2.2.5. section.

3.4. Results

3.4.1. Biochemical results

Warm ischemia increased the concentration of tissue MDA in each group compared to control and sham group. This elevation showed closed correlation to the duration of ischemia time (Group A2 and Group A3 vs. C; $p<0.05$). The highest concentration was measured after 6 hours warm ischemia in grafts by the end of the reperfusion period. IPO significantly decreased the lipid peroxidation at the end of the reperfusion (Group A4 and Group A5 vs. C; Group A6 vs. Group A3; $p<0.05$). Cold preservation increased the concentration of tissue MDA in each group compared to control and sham (Group B2 and Group B3 vs. C; $p<0.05$). This elevation showed closed correlation to the duration of preservation time. The highest concentration was measured after 6 hours grafts by the end of the reperfusion period. IPO significantly decreased the reperfusion-ended lipidperoxidation's value (Group B3 vs. Group A3; Group B6 vs. Group B3; $p<0.05$).

The content of the endogenous scavenger GSH decreased after 3 and 6 hours ischemia compared to control and sham values (Group A2 and Group A3 vs. C; $p<0.05$). Moreover, the concentration of GSH was elevated significantly in IPO groups compared to control and sham level (Group A5 and Group A6 vs. C; Group A5 vs. Group A2; Group A6 vs. Group A3; $p<0.05$). Its capacity was significantly higher after cold tissue preservation (Group B3 vs. C; $p<0.05$). The decrease was significantly lower in cold storage bowel (Group B3 vs. Group A3; $p<0.05$). Moreover,

the concentration of GSH was elevated in IPO groups. Its capacity was significantly higher mainly in grafts preserved for 6 hours (Group B6 vs. C vs. Group B3 and vs. Group A6; $p<0.05$).

Warm ischemia decreased significantly the tissue SOD activity in a time-dependent manner (Group A1 and Group A2 and Group A3 vs. C; $p<0.05$). SOD activity decreased in IPO groups also, but remained significantly higher compared to groups without IPO (Group A5 and Group A6 vs. C; Group A5 vs. Group A2; Group A6 vs. Group A3; $p<0.05$). During cold preservation tissue SOD activity decreased in a time-dependent manner. It was nearly half of the control level after 6 hours preservation. SOD activity decreased in IPO groups also, but remained significantly higher compared to groups without IPO (Group B6 vs. C and Group B3 and Group A6; $p<0.05$).

3.4.2. Histological results

According to Park's classification, at the end of reperfusion followed 1 hour warm ischemia, histological findings were corresponding to an injury grade 2, showing minor clefting with the villus epithelium adjacent to the crypts intact. Until then, in 3 hours ischemia group the structural injury was grade 3, characterized by massive epithelial lifting. After 6 hours warm ischemia, the injury showed denuded villi and decellularization of lamina propria and crypt corresponding to a grade 5 injury. By contrast, cold preservation caused smaller intestinal structural injury, exeption to this 3 hours group, where the injury was the same like in warm ischemia group. The IPO was deterioration in the group stages milder than groups without IPO. Among them it is highlighted crypts better conservation of the end of the reperfusion. The qualitative assessment shows that the cold preservation alone had an impact on preserving tissue structure, which is further enhanced in the IPO.

According to Scion Image quantitative analysis, tissue injury is increased by the duration of warm ischemia time. Mucosal and submucosal thickness, and depth of the crypts decreased significantly after 3 and 6 hours ischemia (Group A2 and Group A3 vs. C; $p<0.05$). IPO significantly decreased this injury in each group (Group A5 vs. Group A2; Group A6 vs. Group A3; $p<0.05$). Quantitative analysis showed that tissue injury increased by the duration of preservation periods. IPO significantly decreased this injury in each group (Group B5 vs. Group B2; Group B6 vs. Group B3; $p<0.05$). Muscle thickness showed mild decrease in each group, but these changes were not significantly different by the end of the reperfusion periods.

3.5. Discussion

Continuous research efforts have been directed toward how else to develop protective factors or methods against I/R. This study investigated the effect of IPO manoeuvre to I/R injury following intestinal warm ischemia. The quality of damage was defined by the development of oxidative stress and tissue injury by the end of the reperfusion periods. We employed the classical IPO protocol with three cycles of 30 seconds reperfusion followed by 30 seconds re-occlusion of SMA during the initial moments of reperfusion. Our results showed that IPO decreased the level of tissue lipidperoxidation in each group compared to groups without IPO. Furthermore, IPO caused an elevation of tissue GSH

concentration, indicating the activation of the endogenous scavenger capacity. Moreover, while the warm ischemia decreased SOD activity dramatically, its activity decreased in IPO groups also, but remained significantly higher. These changes were confirmed by some studies. They described, that the protection was associated with attenuation of oxidative stress and inflammatory responses as well as neuthrophil activation. Despite the fact that the pathways of the IPO are complex and poorly understood, it is widely accepted that it has interrelated passive and active components in its underlying cellular protective mechanisms.

I/R injury is a complex phenomenon that greatly contributes to the mortality and morbidity rates in clinical transplantation of small intestine. Although the introduction of better preservation solutions has reduced the severity of ischemic lesion, I/R injury still remains a major problem. Cold preservation increased the concentration of tissue MDA in each group compared to control and sham. This elevation showed closed correlation to the duration of preservation time. In contrast, IPO significantly decreased the reperfusion-ended lipidperoxidation's value. Moreover, the concentration of GSH was elevated in IPO groups, and its capacity was significantly higher mainly in grafts preserved for 6 hours. SOD activity decreased in IPO groups also, but remained significantly higher compared to groups without IPO. Previous studies demonstrated that the IPC and the IPO together have a synergistic effect, and the important fact that the late (3 minutes after reperfusion) executed IPO ineffective because the SOD activity can no longer be retained, and does not reduce the tissue lipid peroxidation. In addition, the IPO was ineffective if it was applied with four cycles of 30 seconds ischemia and 30 seconds reperfusion protocol. The contradictory results arise from different ischemic times and different IPO protocols as well.

In the present study, we also demonstrated that warm ischemia caused tissue injury is correlated to the duration of time, with the highest destruction observed following 6 hours warm ischemia. Quantitative analysis demonstrated that IPO ameliorated tissue injury induced by prior to reperfusion periods. Mucosal and submucosal thickness and depth of crypts were better preserved in each IPO groups by the end of the reperfusion. In this study, we also demonstrated that autotransplantation caused tissue injury is correlated to the duration of preservation time, with the highest destruction observed following 6 hours cold storage. Quantitative analysis demonstrated that IPO ameliorated preservated intestinal injury induced by prior to reperfusion periods. Mucosal and submucosal thickness and depth of crypts were better preserved in each IPO groups by the end of the reperfusion.The exact mechanism of the protective effect of IPO in the present model is not yet known. Few studies show, however, that IPO decreased small bowel injury in the tissue layers. Despite the fact that during the IPO-activated signaling pathways are extremely complex relationship, and many of them are not yet known, widely accepted that the IPO of the cell's natural defense mechanisms of passive and active factors can be related.

In summary, given the fact that IPO method can be utilized in clinical practice in the future, so in order to clarify and answer to many questions arised it should be important in all current and future research.

4. Effects of PACAP on small bowel autotransplantation models

4.1. Introduction

Small bowel is a highly sensitive tissue to I/R injury in the body. Intestinal I/R injury is caused by many clinical conditions, including small bowel transplantation. Both clinical and experimental data demonstrate that transplant I/R injury has deleterious short- and longterm effects, manifesting as increased episodes of acute rejection and chronic allograft dysfunction. Graft viability prior to implantation is a key factor in the outcome after organ transplantation. Along with surgical manipulation, I/R injury and preservation damage are some of the many essential factors that affect the quality of intestinal graft and its multiple functions. The current standard in organ preservation with UW solution was developed for kidney/liver preservation and it is suboptimal for the intestinal graft despite good results for other organs. The benefit of the UW solution for the preservation of other intraabdominal organs remains unclear and the maximum storage time for small bowel remains relatively brief (6-8 h). Thus, no general agreement exists about optimal preservation solution for intestinal grafts so far. Recently, there are continuous research efforts to modify the commercially available solutions (adding more components, high-energy intermediates, and nutrients) or to develop new preservation solutions.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a widespread neuropeptide with diverse effects not only in the nervous system but also in the cardiovascular system and peripheral organs including endocrine glands, respiratory organs, and gastrointestinal tract. The polypeptide exists in two forms, with 38 and 27 amino acid residues, named PACAP-38 and PACAP-27. Endogenous PACAP-38 and PACAP-27 were demonstrated in all parts of the gastrointestinal tract with high levels detected in the jejunum and ileum. PACAP-38 and PACAP-27-immunoreactivities have been shown in the cell bodies and nerve fibers throughout the gastrointestinal tract. PACAP-38 and PACAP-27 act through the specific PAC1 receptor and VPAC1 and VPAC2 receptors that bind VIP and PACAP with equal affinity. All three types of PACAP receptors have been shown in the intestinal system: in the mucosa and myenteric neurons, in neuroendocrine cells, blood vessels, and smooth muscle.

Endogenous PACAP-38 has been implicated in protection against harmful stimuli; the peptide has anti-apoptotic, anti-inflammatory, and anti-oxidant actions in numerous different in vivo and in vitro models. Although the exact mechanisms, by which intestinal I/R and gut injury contribute to the systemic inflammatory response, are not completely known, cytokines play a key role in these actions. There is also evidence that intestinal injury resulting from I/R can lead to the bowel becoming a cytokine-generating organ. The anti-inflammatory actions of PACAP in several inflammatory models are partially mediated through its suppressing effect on cytokine/chemokine production. However, there are no data in the literature about changes of intestinal PACAP-38 or PACAP27 and tissue cytokine levels using PACAP-38-containing preservation solution in stored and transplanted small bowel grafts.

4.2. Aims

In the third part of our research in various warm I/R and in small bowel autotransplantation models the following questions were arised:

1. How change the endogenous PACAP-38-like immunoreactivity in warm I/R bowel tissue?
2. How change the oxidative tissue damage in PACAP-38 KO and in wild-type mice?
3. How change the oxidative tissue injury after PACAP-38 containing UW preservation, and how change the PACAP-38-like and PACAP-27like immunoreactivities in the preserved bowel tissue?
4. How change the cytokine expression in this model?

4.3. Materilas and methods

4.3.1. Experimental groups and surgical protocols

In the Ist and IIIrd investigations adult male Wistar rats (body weight: 250-300 g; Σn=56, n=8/group), in the IInd study generation by a gene targeting technique, maintenance and backcrossing of CD1 PACAP-/- and PACAP-38 wild type mice (body weight: 25-30 g; Σn=40, n=20/group) were used. Animals were housed under pathogen-free conditions and were fasted for 24 hours preoperatively, but had free access to water with sugar. Animals were anesthetized with intramuscular ketamine hydrochloride (0.075 mg/g of body weight) and diazepam (0.075 mg/g of body weight) (Richter Gedeon). All procedures were performed in accordance with the ethical guidelines of National Institute of Health and guidelines approved by the University of Pécs (BA02/2000-20/2006, BA02/2000-9/2008) to minimize pain and suffering of the animals.

In the Ist series of investigation warm ischemia was applied with occlusion of superior mesenteric artery for 1 hour (Group A1), for 3 hours (Group A2) and for 6 hours (Group A3). After clip removal 3 hours reperfusion was began.

In the IInd study cold ischemic groups were created. Small intestine was resected from Treitz-ligament to the end of ileal bowel. Intestinal lumen was flushed with antibioticum-free normal saline. Grafts were prefunded with 4 °C UW solution for 1 hour (Group PVB1, Group KO.B1), for 3 hours (Group PVB2, Group KO.B2) and for 6 hours (Group PVB3, Group KO.B3).

In the IIIrd part of this study cold ischemic groups were performed. After perfusion with 100 ml 4°C UW solution grafts were stored for 1 hour (Group B1), for 3 hours (Group B2) and for 6 hours (Group B3). Further grafts were preserved in 100 ml UW solution containing 100 μg PACAP-38 for 1 hour (Group PB1), for 3 hours (Group PB2) and for 6 hours (Group PB3). Orthotopic autotransplantation was performed with end-to-end vessel anastomosises (10/0, synthetic, monofilament, non-absorbable suture materilas) without bowel anastomoses. Reperfusion lasted for 3 hours in each groups. Bowel biopsies were collected after laparotomy (C) and at the end of the reperfusion periods. From sham operated animals C and S biopsies were collected.

4.3.2. Biochemical examinations

Measurements were performed as described in 2.2.2. section previously.

4.3.3. Histological examinations

Intestinal samples were prepared and examined as described in 2.2.3. section above.

4.3.4. Measurement of PACAP-38 and PACAP-27 by radioimmunassay (RIA)

Intestinal tissue samples (600 mg) were homogenized in icecold distilled water. After centrifugation at 12000 rpm/4 °C for 30 min, the supernate was further processed for RIA analysis of PACAP-38- and PACAP-27-like immunoreactivity. Briefly, the antiserum for PACAP-38 was "88111-3" and for PACAP-27 was "88123". The tracer was mono-125I-labeled ovine PACAP-38 and mono-125I-labeled ovine PACAP27 (5000 cpm/tube). Assays were prepared in 1 ml phosphate buffer (0.05 mol/l, pH 7.4) containing 0.1 M NaCl, 0.05 % NaN3, and 0.25 % bovine serum albumin. The assay procedures include 100 µl antiserums (1:10000; 1:45000), 100 µl RIA tracers, and 100 µl standards or unknown samples were measured. Following centrifugation the tubes were decanted, and the radioactivity of the precipitates was measured in a gamma counter. PACAP-38 and PACAP-27 concentrations of the unknown samples were read from the appropriate calibration curves. Results of PACAP-38- and PACAP-27-like immunoreactivities are given as femtomoles per milligram tissue.

4.3.5. Mesurement of intestinal citokine activity

Citokin Array

Intestinal tissues from control sample, from tissue exposed to 6 hours cold storage in UW, from 6 hours cold preservation in PACAP-38-containing UW solution, and subsequent 3 hours reperfusion period were measured. Briefly, cytokine array from tissue homogenates was performed using rat cytokine array (Panel A Array kit from R&D Systems, Biomedica Hung., Budapest, Hungary). Small bowel samples were excised then homogenized in PBS with protease inhibitors. Triton X-100 was added to the final concentrations of 1 %. After blocking the array membranes for 1 hour and adding the reconstituted Detection Antibody Cocktail for another 1 hour at room temperature, the membranes were incubated with 1 ml of tissue homogenates at 2-8 °C over night on a rocking platform. After washing with buffer for three times and addition of horse radish peroxidase-conjugated streptavidin to each membrane, we exposed them to a chemiluminescent detection reagent (Amersham Biosciences, Hungary) then side up to an X-ray film cassette.

Luminex Multiplex Immunassay

The levels of three host markers (soluble intercellular adhesion molecule-1 (sICAM1), L-selectin, and metalloproteinase-1 (TIMP-1)) were determined in the selected bowel samples using customized Flurokine MAP Rat Base Kit (R&D Systems). All analyte levels in the quality control reagents of the kits were within the expected ranges. Standard curve for sICAM-1 is 17–12500 pg/ml, for L-selectin is 100–73000 pg/ml, and for TIMP-1 is 55-40600 pg/ml. Measurements were done with Luminex100 instrument, and Luminex100 IS software was used for the analysis of bead median fluorescence intensity. The R&D Systems Rat Base kit assay was carried out according to the

manufacturer's instructions. Briefly, an eightpoint standard curve was generated by performing serial dilutions of the reconstituted normalized standard (lot#1279612). Bowel samples were homogenized with RPMI-1640 (GIBCO) containing 1 % protease inhibitor cocktail. In order to assess recovery, bowel samples were used in 20 mg/ml concentrations. A 50-µl volume of each sample, control, or standard was added to a 96-well plate containing 50 µl of antibody-coated fluorescent beads. Biotinylated secondary and streptavidin-PE antibodies were added to the plate with alternate incubation and washing steps. After the last wash step, 100 µl of wash buffer was added to the wells; the plate was incubated and read on the Luminex100 array reader, using a four-PL regression curve to plot the standard curve. Data were subsequently analyzed using the Luminex100 manager software.

4.3.6. Statistical analysis

Assessment of results was performed as described in 2.2.5. section.

4.4. Results

4.4.1. Biochemical results

In the IInd experience, in grafts preserved in UW solution increased the concentration of tissue MDA in each group in a time dependent manner compared to the control and Sham groups. The highest concentration was measured in PACAP-38 deficient tissues 6 hours cold storage. Moreover, significant difference of MDA value was between wild-type and PACAP-38 deficient mice following 3 hours cold ischemia (Group KO.B2 vs. Group PVB2; $p<0.05$), and 6 hours preservation (Group KO.B3 vs. Group PVB3; $p<0.05$). The content of the endogenous scavenger GSH and SOD activity decreased significantly in PACAP-38 deficient groups after 3 and 6 hours storage wild type animals (Group KO.B2 vs. Group PVB2; Group KO.B3 vs. Group PVB3; $p<0.05$).

4.4.2. Histological results

According to Park's classification, the highest grade of injury was observed in PACAP-38 deficient tissue after 6 hours cold storage (Group KO.B3, Grade 4), whereas the lowest grade of injury was found in wild-type tissue following 1 hour preservation (Group PVB1, Grade 1). The sham group showed an injury grade 0, corresponding to normal bowel structure. After 1 hour cold preservation in wild-type tissue, the histological findings were corresponding to an injury grade 1, showing minor clefting with the villus epithelium adjacent to the crypts intact. In contrast, in PACAP-38 deficient tissue (Group KO.B1) the structural injury was grade 2, with subepithelial space at villus tip and more villus clefting. Three hours cold ischemia signs were more severe in PACAP-38 deficient tissue, where the histological analyses showed an injury of grade 3, characterized by massive epithelial lifting and villus tip denudation (Group KO.B2). In wild-type tissue, 6 hours cold preservation resulted in injury of grade 3 (Group PVB3). In PACAP-38 deficient mice, 6 hours cold storage caused injury showed severely injured crypts and denuded villi corresponding to a grade 4 injury (Group KO.B3).

By Scion Image quantitative analysis, mucosal thickness decreased significantly in PACAP-38 wild type animals after 6 hours preservation compared to control (Group PVB3 vs. C; $p<0.05$). In PACAP-38 deficient grafts the decrease was significant after 1, 3 and 6 hours cold storage compared to control and sham operated (Group KO.B1, Group KO.B2 and Group KO.B3 vs. C and S; $p<0.05$). At these layers received significantly worst results in PACAP-38 KO samples compared to PACAP-38 wild type grafts (Group KO.B2, Group KO.B3 vs. Group PVB2 and Group PB.B3; $p<0.05$). Muscle thickness showed mild decrease in each group compared to controls, but these changes were not significantly different by the end of the preservation periods.

4.4.3. Changes of intestinal PACAP-38 and PACAP-27 immunreactivity

In the Ist series of investigation intestinal endogenous PACAP-38 content were measured in warm I/R groups by RIA. Endogenous PACAP-38-like immunoreactivity decreased in each I/R small bowel compared to control and sham operated group (Group A1, Group A2 and Group A3 vs. C and S; $p<0.05$). In the 6 hours warm ischemic groups, in the ischemia-end samples the endogenous PACAP-38-like immunoreactivity significantly decreased compared to control and sham operated group (Group A3 ischemia vs. C; $p<0.01$; Group A3 reperfusion vs. C; $p<0.001$).

In the IIIrd part of this study level of intestinal PACAP-38-like immunoreactivity (LI) was 55.1±2.5 fmol/mg in sham-operated group and it was 57.32± 3.5 fmol/mg in control samples. After 1 hour cold storage, intestinal PACAP-38-LI was 50.4±3.5 fmol/mg (Group B1), and after 3 hours preservation it decreased to 40.1±5.5 fmol/mg (Group B2). These changes were significant following 6 hours cold storage (Group B3: 32,6±3,0 fmol/mg; $p<0.05$) compared to control or sham values. Levels remained significantly higher in grafts stored in PACAP-38-containing UW solution. After 3 and 6 hours cold storage in PACAP-38-containing preservation solution, the PACAP-38-LI levels were 55.6±4.2 fmol/mg and 48.9± 3.2 fmol/mg. These resulted significantly higher compared to preservation only in UW without PACAP-38.

Tissue PACAP-27-LI level also decreased during cold storage and autotransplantation procedure compared to the control value (4.2±0.2 fmol/mg). This decrease was significant in the 1 hour (Group B1: 2±0.2 fmol/mg; $p<0.05$), 3 hours (Group B2: 1.6± 0.3 fmol/mg; $p<0.05$), and 6 hours (Group B3: 0.9±0.2 fmol/mg; $p<0.01$) groups. Levels of PACAP-27-LI remained significantly higher in grafts stored in PACAP-38-containing UW solution (Group PB4: 3.5±0.3 fmol/mg; Group PB5: 3.0±0.2 fmol/mg; Group PB6: 2.6±0.15 fmol/mg; $p<0.05$).

4.4.4. Changes of intestinal citokine expression

Among several cytokines, according to cytokine array, the expression of the sICAM-1 (CD54) and L-selectin (CD62L/LECAM-1) regulated upon activation was detectable in control bowel samples. The expression did not changed after 6 hours cold preservation in UW and subsequent reperfusion period in Group B3. Both 6 hours cold storage in PACAP-38-containing UW solution and 3 hours reperfusion caused a strong reduction in the activation of these cytokines in Group PB6. The

RANTES (CCL5) levels were high in all groups and did not change, as could be observed in the PACAP-treated groups. We found no activation of the tissue inhibitor of TIMP-1 in the control samples, but strong activation was detected in 6 hours preserved grafts without PACAP-38 (Group B3). PACAP-38-containing cold storage could decrease its activation in Group PB4.

Measurement of cytokines levels by Luminex Immunoassay confirmed these results. sICAM and L-selectin were expressed at similar levels in the control and ischemic groups, while both were significantly reduced in the PACAP-treated groups. TIMP, on the other hand, was expressed at detection limit in the control group, and it was markedly increased upon ischemia. The elevated TIMP levels were significantly attenuated by PACAP treatment.

4.5. Discussion

In this study, firstly, we determined the intestinal tissue oxidative injury and structural changes in PACAP-38 wild type and in PACAP-38 KO mice during experimental intestinal autotransplantation. Secondly, we examined the intestinal endogenous PACAP-38-like immunoreactivity in warm I/R rat model. Thirdly, PACAP-38-like and PACAP-27-like immunoreactivity and tissue cytokine expression were determined using PACAP 38-containing UW preservation solution during small bowel autotransplantation.

The effect of cold preservation is defined by the development of oxidative stress in small bowel tissue. Our results with homogenates showed that value of lipid peroxidation elevated in a time-dependent manner, but it was significantly higher in PACAP-38 deficient than in wild-type animals. Tissue levels of endogenous antioxidant scavenger GSH decreased both in wild-type and PACAP-38 deficient mice during cold storage, its concentration significantly decreased in PACAP-38 deficient intestine after 3 and 6 hours. The activity of endogenous antioxidant SOD decreased significantly following 3 and 6 hours cold storage both in wild-type and in PACAP-38 deficient tissues. Moreover, in wild-type intestine, the activity remained to a greater extent, and it was significantly higher than in PACAP-38 deficient tissues. There are no data indicating the exact mechanism of the protective effect of PACAP-38 in the early phase in ischemic small bowel.

Several studies show, however, that the antioxidant, antiapoptotic, and anti-inflammatory effects of the peptide are important in its cytoprotective effects. PACAP has mild direct scavenger activity, but it is suggested that PACAP rather acts indirectly, via stimulating antioxidant enzyme activity/synthesis and via influencing the oxidative stress-induced pathways. As far as the oxidative stress-induced signaling pathways are concerned, it has been shown to counteract the alteration caused by oxidative stress at several levels of the downstream pathway in various cells of different origin.

In the present study we also demonstrated that cold preservation caused tissue injury in the small bowel. This damage correlated with the duration of preservation time, with the highest destruction observed in tissue following 6 hours storage in PACAP-38 deficient mice. Both qualitative and quantitative analyses in our study demonstrated that PACAP-38 ameliorated tissue injury induced by cold storage. Mucosal and submucosal thickness, and depth of crypts were better preserved in

tissues containing PACAP-38 in wild-type animals. The exact mechanism of the protective effect of PACAP-38 in the present model is not yet known. Our present study is also in accordance with the numerous reports that demonstrate different abnormalities in PACAP-38 deficient mice. Among others, it has been shown that mice lacking PACAP-38 display altered cerebellar development and abnormal axonal arborization. Interestingly, in peripheral tissues, which have been studied in PACAP-38 deficient mice, intact tissues show little or no alteration compared to wild-type animals. This has been also described in the large intestine. However, when tissues of different origin are exposed to harmful stimuli, PACAP-38 deficient mice react with a significantly worse outcome in most tested models. This has been found in dextran-induced colitis model, in cerebellar granule cells and kidney cell culture exposed to oxidative stress, in cerebral ischemia, and in axonal regeneration. We also found that intact small intestinal structure was not different between wild-type and PACAP-38 deficient mice, but these latter animals reacted to injury with a worse outcome.

Our results showed that endogenous PACAP-38 concentration decreased during ischemia and subsequent reperfusion in the small intestinal tissue. These changes were significant following 6 hours ischemia and 3 hours reperfusion. The PACAP-38 levels measured in our sham group were in the concentration range as previously demonstrated by Hannibal et al. The reason for the decreased PACAP levels after ischemia may be due to either excessive uptake by ischemic cells or decreased synthesis. Similar observations have been made by others in an experimental ulcer model, where an acute decrease in PACAP immunoreactivity was observed.

Small bowel transplantation is increasingly performed in recent years, yet, clinically, there are still many obstacles to improve patient and graft survival. For most grafts, the preservation solution plays a fundamental role in minimizing the detrimental effects of ischemia during cold storage and subsequent reperfusion periods. The current clinical standard for small bowel consists of a vascular flush with cold UW solution. This solution has many advantages in the preservation of liver and kidney; however, for small bowel storage it still is unclear whether UW is optimal. Several research efforts have been directed towards methods to protect against I/R injury, using modified composition of the commercially available solutions or adding new components appropriate for intestinal storage.

In the present study, we demonstrated that intestinal tissue PACAP-38 and PACAP-27 levels decreased in a time-dependent manner after 1 and 3 hours cold preservation procedure. These changes were significant following 6 hours cold storage. The reason for the decreased PACAP-38 levels after cold ischemia may be due to either excessive uptake by ischemic cells or decreased synthesis/increased degradation paralleling tissue degeneration. Similar observations have been made by others in an experimental ulcer model, where an acute decrease in PACAP immunoreactivity was observed. Values remained significantly higher in grafts stored in PACAP-38-containing UW solution. Interestingly, PACAP-38 levels increased above control values following 1 hour preservation. Three and 6 hours cold storage in PACAP-38-containing preservation solution resulted in significantly higher PACAP-38-LI and PACAP-27-LI in bowel tissue homogenates compared to only in UW-preserved grafts without PACAP-38. There are no data indicating the exact mechanism of the elevated

values at the end of the reperfusion periods. It could be due to the decrease in intracellular cyclic adenosine monophosphate (cAMP) through the reduction of adenylate cyclase activity induced by hypoxia in endothelial cells in vitro. These changes were confirmed in an in vivo small intestine preservation study. Among these mechanisms, the cellular cAMP signal may represent a major determinant of the intestinal integrity after global ischemic preservation. Studies confirmed that administration of PACAP-38 enhancing the cAMP level exerted tissue protection against I/R injury. Moreover, after extrinsic denervation, which is an indispensable procedure during intestinal transplantation, PACAP-38 concentration decreased in the stomach, but not in the small intestine. These findings suggested a dual intrinsic and extrinsic origin of the PACAP-containing nerve fibers in the small intestine. Another explanation of the present result is that PACAP-38 intake from the preservation solution and attached to the specific receptors could result in the anti-oxidant and protective effect to the bowel structure as described in our previous studies.

I/R injury is one of the main factors affecting the function and structure of the small intestine, by generation of proinflammatory mediators including cytokines. The generated inflammatory cascade may activate leukocytes and endothelial cells, which ultimately lead to tissue inflammation, multiple organ dysfunction, and death. Following I/R in small bowel transplantation, the gut turns into a cytokine producing organ, threatening graft and patient survival.

In the present study, we found that the expression of the sICAM-1 (CD54) and L-selectin (CD62L/LECAM-1) regulated upon activation was detectable in control bowel samples, and those after 6 hours cold preservation in UW and subsequent reperfusion period. In contrast, 6 hours cold storage in PACAP-38-containing UW solution caused strong reduction in the activation of these cytokines. Increased expression of sICAM-1 and L-selectin was also observed after renal I/R, and it was decreased in PACAP-treated groups in renal model. In fact, these adhesion molecules, involved in the distinct cellular crosstalk between leukocytes, platelets, T cells, and endothelial cells, can cause microvascular dysfunction and reperfusion damage. The RANTES (CCL5) chemokine is not constitutively expressed; it is released during inflammation. In our model, the RANTES (CCL5) levels were increased in all groups, but slight reduction was observed in PACAP-treated groups. During inflammatory events, the transcription of matrix metalloproteinase-9 and its endogenous inhibitor TIMP-1 is induced by pro-inflammatory mediators. In our experiment, TIMP-1 showed a strong activation in 6 hous preserved grafts without PACAP-38. PACAP-38-containing cold storage could decrease its activation. The anti-inflammatory actions of PACAP in several inflammatory models are partially mediated through its suppressing effect on cytokine/chemokine production.

5. New results

1. We observed that the oxidative injury in small bowel tissue was proportional to ischemic time in different warm I/R and in cold preserved and autotransplanted intestinal grafts.

2. According to our measurements, the structural damage of the intestine was consistent with this, which was detected by qualitatively and quantitatively also.

3. Our research group was the first examined the effect of warm and cold ischemia on the structural injury of intestinal layers by differential scanning calorimetry method. Overall, we can say that the small intestine can tolerate about 3 hours cold preservation without serious oxidative stress or structural damage.

4. Usage of classical IPO protocol [3 x (30 seconds ischemia + 30 seconds reperfusion)] the tissue lipid peroxidation decreased, improved the endogenous antioxidants capacity and reduced the structural damage in warm I/R or in autotransplanted small intestinal tissue.

5. The endogenous intestinal PACAP-38-like immunoreactivity is decreased in a time-dependent manner following warm I/R.

6. In intestinal warm I/R and in autotransplanted models we demonstrated that significantly lower tissue oxidative stress and structural damage were in PACAP-38 wild type mice compared to PACAP-38 deficient animal results.

7. PACAP-38-like and PACAP-27-like immunoreactivity levels were increased in autotransplanted intestinal tissue preserved in PACAP-38-containing UW solution.

8. After preservation in PACAP-38-containing UW solution from inflammatory cytokines the sICAM, L-Selectin and TIMP-1 expression significantly decreased.

6. Publications

Publications related to the thesis:

1. **Nedvig K**, Ferencz A, Rőth E, Lőrinczy D. DSC examination of intestinal tissue following warm ischemia and reperfusion injury. J Therm Anal Calorim 2009;95:775-9. IF: 1,587

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3. **Nedvig K,** Zapf I, Fekecs T. A vékonybél meleg és hideg ischaemiás károsodásának kimutatása differenciál pásztázó kalorimetriás vizsgálattal. Magy Seb 2011;64:207-12.

4. **Nedvig K,** Völgyi E, Wéber G, Rőth E, Ferencz A. Az ischaemiás posztkondícionálás hatása az oxidatív stresszre és a szöveti struktúrára vékonybél meleg ischaemiás és autotranszplantációs modellekben. Magy Seb 2011;64:294-300.

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6. **Nedvig K,** Szabó Gy, Csukás D, Sándor J, Németh J, Kovács K, Reglődi D, Kemény A, Wéber G, Ferencz A. A PACAP-38 citoprotektív és antiinflammatórikus hatásának vizsgálata vékonybél-autotranszplantációs modellben. Magy Seb 2013;66:250-5.

ΣIF: 6,377

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ACKNOWLEDGEMENT

I would like to take this opportunity to thank for the overwhelming support I have received from my supervisor Dr. Andrea Ferencz (Semmelweis University, Department of Surgical Research and Techniques) in completing this work. Her guidance and leadership has been a blessing.

Special thanks to Professor Dénes Lőrinczy (University of Pécs, Department of Biophysics) to my supervisor, who established scientific intellectual and infrastructural background for my DSC work.

I would like to thank Professor Dóra Reglődi (University of Pécs, Department of Anatomy) that I was able to participate in PACAP research team.

Also thanks to Professor Elizabeth Rőth and Dr. Gábor Jancsó that they supported my research work. And last, but not least, I would also like to acknowledge the help and kind assistance of all the staff at the Department of Surgical Research and Techniques of University of Pécs.