Characterizing Autophagy in the Cold Preservation of Small Bowel Grafts

Ph.D. Thesis Summary

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1. Introduction

Intestinal transplant is a lifesaving option for patients with poor intestinal function who have become refractive to other conservative therapies. In the background of intestinal failure is a complete or partial congenital absence, or organ resection along the length of the intestinal tract. If more than 70% of the intestinal length is missing or dysfunctional, it is defined as short bowel syndrome. Short bowel syndrome (irrespective of its aetiology) reduces the absorptive capacity of the intestine. To meet the body's nutritional needs, total parenteral nutrition (TPN) is usually initiated in these patients. However, this conservative treatment of the patients may result in life-threatening complications (e.g., septicaemia, hyperglycaemia, and hepatic dysfunction) in 2-5% of adults and 10-15% of the children. Small bowel transplantation is thus a valid therapeutic option in these patients. Indeed, most potential candidates for intestinal transplant are patients presenting with short-bowel syndrome.
The history of intestinal transplant is marred by persistent lack of success. Majority of the patients died from direct complications of the transplant procedure. However, Recent advancements in immunosuppressive pharmacology, surgical techniques and postoperative care, have contributed greatly to making this procedure a valid therapeutic option. However, complications still exist with intestinal transplant, some of which are essentially caused by the unavoidable ischemia and the ensuing reperfusion of the organ. Minimizing the ischemic damage to the intestinal graft during preservation is therefore crucial. Thus, effective measures should be undertaken to ensure its viability.

Ischemia-reperfusion during Intestinal transplantation.

Ischemia is the resulting complication due to brief or prolonged absence of blood supply to organs. Reperfusion, which put simply, means restoration of blood supply to ischemic tissues, is fundamentally the intervention for ischemia. However, rather paradoxically, this reestablishment of blood supply may by itself also propagate further damage. The pathological cascade activated during ischemia, and the distinct injury resulting from reperfusion are together referred to as Ischemia-reperfusion injury (IRI). During intestinal transplantation, the occurrence of two major types of ischemia, the warm and cold ischemia is possible. Warm ischemia describes the ischemia of cells, tissues, or organs under normothermic situation. The process of intestinal retrieval from the donor, as well as the graft rewarming during implantation are both associated with the warm type of ischemia. Whilst the period of small bowel graft preservation under cold (hypothermic) conditions gives grounds to the development of cold ischemia. During implantation of the intestinal grafts, connection of donor vascular conduits to those of the recipient is undertaken. This restoration of blood supply (reperfusion) to the bowel graft may lead to the upregulation of various detrimental molecular and cellular pathways eventually cumulating in cell death. This body of work is mainly concerned with events that occur during the intestinal preservation phase.

Pathophysiology of cold Ischemia during intestinal preservation

Cold ischemia triggers a cascade of harmful events, which are worsened by restoration of blood supply. The development of ischemia during preservation of the small bowel is an inevitable event, which worsens with increasing storage time. Currently, the acquisition of intestinal grafts involves organ resection, vascular flush, and storage in a cold solution to maintain its viability. However, cold preservation is limited, and can only attenuate but not eliminate the developing ischemic cell and tissue injury.
The primary reasons for graft deterioration during cold storage are the lack of perfusion and circulation, leading to no tissue oxygen supply. Hence, Ischemic preservation leads to the rapid depletion of cellular energy stores (mainly ATP stores). To maintain functional energy (ATP) levels, anoxic cells switch to anaerobic glycolysis, resulting in the increased production of lactate. The accumulation of lactate will subsequently decrease the intracellular pH. The glycolytic pathway is an unsustainable alternative for generating cellular energy. The limitation of this pathway may be due to

substrate unavailability, covalent and allosteric modulation of key enzymes and the developing intracellular acidosis. Consequence of the early failure of the glycolytic pathway is further reduction in ATP generation, which in turn negatively affects numerous processes essential for the optimal cellular structure and function. Damage to key organelles like the mitochondria and endoplasmic reticulum are particularly significant as injury to them facilitates the initiation of many biological cascades such as apoptotic, inflammatory, oxidative and autophagy pathways, which may be either protective or propagate further damage. In addition, advanced injury to these organelles makes energetic recovery impossible upon re-oxygenation (reperfusion). Furthermore, the energy decline present during ischemia also directly affects the mucosal integrity of the intestinal graft. The mucosa of the intestine is made up of a complex network of proteins and associated molecules which serve as a barrier. This barrier is selective for various materials and contributes to the homeostasis of intestinal cells. Their dysfunction leads to the loss of intestinal selective permeability, mucosal and epithelial breakdown, and eventually cell death.

Reperfusion of the intestinal graft after cold ischemic preservation further aggravates the molecular and clinical picture. Introduction of oxygenated blood provokes the rapid generation of excessive reactive oxygen species (ROS). ROS are believed to be largely responsible for the direct-cell damage associated with IRI. The free radicals and their peroxidation products will eventually trigger an inflammatory response in cells via various signalling mechanisms. The subsequent neutrophil recruitment and extravasation ('sterile' inflammation) amplifies the local tissue injury and death. Furthermore, the ongoing sterile inflammation may be contaminated by bacteria migrating through the 'leaky' intestinal barrier. In this regard, the intestine is unique compared to other transplantable organs as its lumen contains a selection of potentially harmful bacteria. These bacteria can gain access through the injured mucosal barrier and may result in limited local infections or widespread bacteraemia and sepsis.

The consequences of intestinal cold ischemia and subsequent reperfusion injury can range from mild to severe. Many studies have correlated the severity of this injury to the length of ischemic time. Among all the transplantable organs, the intestine has one of the least cold ischemic tolerance time. For example, the liver and kidney can stay up to 24-30 hours in optimal conditions, while for the intestine, the cold ischemic tolerance time is restricted to below 9-10 hours. Early empirical evidence has suggested that a cold storage time less than 9 hours results in only slight changes in the human intestine. Furthermore, other studies have shown that the occurrence of bacterial translocation, sepsis, delayed graft function are all related to the length of intestinal preservation. Therefore, most centres strive to limit cold preservation within this narrow time frame. Hence, an ideal future of intestinal transplantation would be one where both extended ischemic tolerance time and reduced consequences of cold IRI in the grafts is achieved.

Preconditioning as an ischemia modifying technique

Insight into the pathophysiological actors during intestinal ischemia-reperfusion injury forms the bedrock of most current interventions. One such intervention is the concept of organ preconditioning. Organ Preconditioning is a process whereby intrinsic protective mechanisms are initiated before the onset of ischemia by brief cycles of ischemia-reperfusion or by administration of various chemicals. Over the years, this technique has been reproduced in many organs in experimental, clinical and transplantation settings. Though not fully expatiated, the logic behind preconditioning seems to be that if an ischemic event is anticipated e.g., during vascular surgery or organ retrieval and preservation, a prior and transient activation or inhibition of certain molecular mediators and pathways may attenuate the ensuing ischemic damages.

The use of chemical agents to achieve organ protection prior to ischemia is referred to as *pre-treatment*. In the cold ischemia of small bowel grafts, several pharmacological agents have also been investigated. In experimental settings, drugs which have potentials to target multiple pathways such as oxidative stress, inflammatory processes, apoptosis, and autophagy are generally favoured.

General remarks on autophagy

Autophagy (from the Ancient Greek “autos and phagos”, meaning "self-devouring”) generally refers to the degradation of cytoplasmic components within lysosomes. During autophagy, cytoplasmic and organelle bound proteins are broken down and their products recycled to the cytosol for reuse. This pathway consists of three variants: Macroautophagy, Microautophagy and Chaperone-mediated autophagy. In Macroautophagy (simply referred to as autophagy henceforth), an isolation membrane (phagophore), forms and encloses around parts of the cytoplasm, maturing into autophagosomes. The autophagosome is a unique feature of autophagy that distinguishes it from other protein degradation processes like the endocytosis-lysosomal and the ubiquitin-proteasome systems. Mature autophagosomes fuse with lysosomes, delivering their contents for degradation. Thereafter the end-products, such as amino acids, are then utilized again by the cell. Accordingly, autophagy allows for the clearance of old or damaged proteins and the reuse of nutrients. It is often rapidly upregulated during cell starvation or stress.

Autophagy as a potential target during cold Ischemia

Studies into autophagy with various models show that this pathway is rapidly upregulated under conditions of ischemia. More recent studies have suggested a role for this pathway in the pathology of intestinal ischemia.
Autophagy has also been investigated during the cold preservation of organs for transplant. In the lung for example, autophagy is activated briefly after cold preservation and gradually approaches basal levels after a peak period. In the kidney, the activation of autophagy has also been reported and a detrimental role may be associated with this activation. Yet, with all these advances in autophagy studies, little to none is known about autophagy’s involvement during the cold ischemia of small bowel grafts. Since cold ischemic preservation of the intestine magnifies cell stress and nutrient deprivation, measuring the autophagy activity during cold storage may yield significant results. More so, previous studies on the intestine have shown that disruption of the epithelial barrier by amino acid deprivation can induce protective autophagy in intestinal epithelial cells. Likewise, a study into intestinal burn stress injuries suggests that autophagy might be protective for the small bowel mucosa and decreases the level of apoptosis and oxidative stress responses. Therefore, the central theme of this body of work, is to explore via different experiments the changes and possible interactions of autophagy during intestinal cold preservation.

2. Aims

Our main aims were the following:

- To establish an easily replicable animal model for simulating cold ischemia damage of the intestine.
- To evaluate Rapamycin as a possible preconditioning agent, and autophagy stimulating agent during cold intestinal preservation.
- To follow using biological means, the possible changes in the autophagy pathway during cold bowel storage.
- To determine the effects of pharmacological modulation of this pathway on cold intestinal ischemia-reperfusion injury.

Based on these four major investigations were carried out.

3. Morphological alterations of the intestinal mucosa during small bowel preservation

Background

Traditionally, the intestines obtained from donors are flushed intravascularly and stored in a preservation solution at cold (0-4 C) temperatures until implantation. However, this method proves insufficient for the intestine, whose mucosa is extremely sensitivity to ischemia. Ischemic damage of the mucosa is manifested by epithelial detachment from the lamina propria at the tips of the villi and extending down to the submucosa and muscular layers. The nature of the ischemic damage during preservation may depend on the type of preservation solution, the length of preservation and the nature of the organ to be transplanted. Indeed, in different preservation solutions, the extent of small bowel damage has been shown to differ. Also the literature suggests that there are notable differences in cold intestinal mucosal injury developing among different animal species. Hence, at the beginning of this project, we set our own benchmark for mucosal injury during cold preservation with IGL-1 solution in our experimental subjects (Wistar rats). We report these microscopical changes as a function of time.

Materials and Methods

Experimental Animals

Healthy male Wistar rats (n=30) weighing between 250-300g were used for this study. They were housed under standard conditions and fed rat chow and water ad libitum. Food was withdrawn 24 hours prior to the experiment. Animals were anesthetized with intraperitoneal (i.p) mixture of ketamine hydrochloride (0.075 mg/g of body weight) and diazepam (0.075 mg/g of body weight). All procedures were performed in accordance with ethical guidelines (BA02/2000-02/2021) to minimize pain and suffering of the animals.

Intestinal Procurement and Grouping

After median laparotomy, the intestine was retrogradely perfused via the aorta at 6 mL/min with ice-cold IGL-1 solution (Institute George Lopez) for 2 min. The portal vein was cut to facilitate venous venting. At the end of the perfusion, small bowel grafts were resected from the ligament of Treitz and stored in the same solution at 4 °C.
Grouping: Rats were randomly divided into 5 groups (n=6/group) based on the length of cold preservation:
O hour (sham control, grafts were not preserved)
3 hours (3hr group)
6 hours (6hr group)
9 hours (9hr group)
12 hours (12hr group)

Evaluation of Mucosal Changes

In this study, mucosal changes were monitored by histological staining of tissues with Hematoxylin-Eosin (HE) staining and Periodic Acid-Schiff (PAS) staining. The following parameters were of interest to us: Mucosal injury score, mucosal length, crypt depth and mucin-containing goblet cell count.
Histology (Hematoxylin-Eosin)
Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. They were cut in 3mm thick sections and stained with hematoxylin and eosin. Slides were digitized with Mirax scanner and photographs were taken with CaseViewer 2.4 software (3DHISTECH Ltd.). Intestinal mucosa damage was evaluated blindly by two individuals. The degree of injury was determined using the Park/Chiu system described by Park et al. (See Table 1.0 below). A minimum of three fields randomly selected

from four quadrants of each intestinal sample were evaluated.
Morphometric analysis of total mucosa thickness and villous depth was analysed using the CaseViewer 2.4 software (3DHISTECH Ltd. Total mucosa thickness was assessed by measuring the distance between the villus tip to the lamina-muscularis mucosae in at least four- axially oriented villi in four quadrants. Crypt depth was determined in at least a total of five axially oriented, open, non -destroyed crypts from three quadrants.

Periodic Acid-Schiff (PAS) staining
Goblet cells are important cells in the intestinal mucosa. Changes to these special group of cells during ischemia have been shown to have detrimental effects on the mucosa integrity. To evaluate the amount of mucus containing goblet cells in the mucosal layer, tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, cut in 3 micrometre thick sections with a rotational microtome and mounted on coated glass microscope slides. After deparaffinization and rehydration, samples were incubated in 1% periodic acid for 20 minutes followed by a 0.5 minute rinse in distilled water. Samples were stained with Schiff reagent for 20 minutes, differentiated in Schiff-rinsing solution for 2 minutes and immersed in tap water for 5 minutes to further evolve colour. Slides were then incubated in Meyer's hematoxylin for 10 minutes, and bluing was performed with tap water for 5 minutes. Samples were dehydrated in alcohol, cleared in xylene and mounted with permanent mounting medium. The amount of blue/purple-stained goblet cells was evaluated by manually counting the number of visible cells in at least three high-power fields (hpf) selected randomly from four different quadrants using the Caseviewer 2.4 software (3DHISTECH Ltd).

Injury grade	Description
0	Normal mucosa
1	Subepithelial space at the tips of the villi
2	Extension of the epithelial spaces
3	Massive epithelial lifting down the sides of the villi
4	Denudation of the villi
5	Loss of villi
6	Crypt layer damage
7	Transmucosal infarction
8	Transmural infarction

Table 1.0. *Histology Injury Grading according to the method described by Park et al.(doi:10.1159/000129130).*

Results and Conclusions

Histology results using HE revealed that the mucosal injury worsened with increasing storage time. After 3 hours, blebs were already visible at the villi tips. At 12 hours of preservation, denudation of villi, total loss of villi material and crypt occurred more frequently. Further morphometric analysis on these slides showed a decline in mucosal thickness as storage time increased. Similarly, the depth of the intestinal crypts also decreased with time.

PAS staining revealed that the number of goblet cells decreased slightly at the start of the preservation. However, the decline became more prominent as the cold storage time increased, with the least goblet cells observed at the end of 12 hours of preservation. These results confirm that the intestinal mucosa is susceptible to hypothermic preservation. Furthermore, they confirm that our model is suitable for studies on intestinal preservation injury, hence can be used for further investigations.

4. Time-dependent effects of Rapamycin pretreatment on the cold Ischemic Injury of small bowel grafts

Background

Instigating protective molecular responses before the onset of ischemia (preconditioning) either by brief cycles of IR or pharmacological agents has been proven effective in different studies. However, clinical adaptation of these methods has so far been marred by the complexity of some of the methods, toxicity, and narrow therapeutic window of some of the compounds. Rapamycin however seems to be an ideal drug for preconditioning because its clinical efficacy as an immunosuppressive and antiproliferative chemical is known. More so by targeting multiple pathways involved in ischemic cascade like apoptosis, oxidative stress and autophagy, Rapamycin has a potential in various ischemia related diseases. Thankfully, this drug is already used in the field of transplantation, hence translation of possible findings might be more realistic.
This study was conducted to establish the effects of giving a single dose of Rapamycin on intestinal mucosa and cellular injury and correlated the findings with time of drug administration. Furthermore, we preliminary evaluated its autophagy inducing effects during small bowel preservation

Materials and Methods

The nature of the experimental animals and the way the intestines were procured and stored are the same as in ***section 3***.
Rats were randomly divided into 5 groups (n=6/group):

1) Sham control: Intestinal samples collected after retrieval, no preservation.
2) Preservation control: Intestinal samples collected after 12 hours of preservation
3) Rapa-0: Rapamycin administered at 0 mins before intestinal retrieval; preservation time: 12 hrs
4) Rapa-30: Rapamycin administered at 30 mins before intestinal retrieval; preservation time: 12 hrs
5) Rapa-60: Rapamycin administered at 60 mins before intestinal retrieval; preservation time: 12 hrs

Drug preparation and dosage

2mg/kg of Rapamycin (Hb2779 Hellobio) was dissolved in 1ml dimethyl sulfoxide solution (DMSO)[54]. The drug was injected intraperitoneally (i.p) at different times (0, 30 and 60) corresponding to the experiment group. The Sham control and Preservation control group received same volume of the solvent (DMSO) used.

Preservation injury (Cold ischemic injury)

In this study cold ischemic injury was described by the extent of mucosa changes and cellular injury. Mucosa changes were evaluated from HE stained histology slides using the method of Park et al. which was described earlier (*see Table 1.0 above*). Biochemical analysis of the preservation fluid was carried out to determine the extent of release of two well established markers of cellular injury namely Lactate and LDH.

Biochemical analysis
At different time points during preservation and reperfusion, fluid samples were obtained and analysed for the presence of Lactate and Lactate dehydrogenase enzyme. After centrifugation (10 min,

room temperature, 1,500 rcf), both parameters were quantified using the Cobas integra 400 plus Analyzer (Roche Diagnostics, GmbH, Mannheim, Germany) following the manufacturer's instructions.

Autophagy proteins

The autophagy pathway was assessed using two known protein markers namely Beclin-1 and microtubule-associated proteins 1A/1B light chain 3B (LC3B). Beclin-1 is a protein which is associated with the initiation of the autophagy pathway while LC3B is important for the elongation of the autophagosome. These proteins were determined by making use of immunohistochemistry.

Immunohistochemistry
Intestinal tissues fixed in 10% neutral buffered formalin and embedded in paraffin, were cut in serial 3mm thick sections. After deparaffinization and rehydration, samples were pretreated with heat induced epitope retrieval method in 1 mM (pH=6.0) citrate buffer (Histopathology Ltd.) in a microwave oven for 15 minutes at 750W. After cooling at room temperature, tissues were washed in TRIS buffered saline solution (TBS) (pH=7.6). For immunohistochemistry, samples were incubated in Beclin-1 antibody (Cat. Nr. bs-1353R, Bioss Antibodies Inc., 1:2000, 1 hour at room temperature) and LC3B antibody (NB100-2220 Novus Biologicals, 1:400, 1 hour at room temperature). Sections were washed in TBS and incubated with HISTOLS-R anti- rabbit HRP labelled detection system (Cat. Nr. 30011.R500, Histopathology Ltd., 30 minutes at room temperature,). After washing in TBS, the reaction was developed with HISTOLS Resistant AEC Chromogen/substrate System (Cat. Nr. 30015, Histopathology Ltd.) while controlling the intensity of the staining under microscope. Sections were counterstained with hematoxylin solution, and bluing was performed with tap water. Samples were dehydrated in alcohol, cleared in xylene and mounted with permanent mounting medium. Slides were digitized with Mirax scanner and photographs were taken with CaseViewer 2.4 software (3DHISTECH Ltd.).
Analysis of stained tissues for both proteins was performed with the help of the IHC profiler plug-in of the Image J software, and the optical density (OD) was scored according to the following method previously described. Briefly, The image processing software showed a percentage of stained areas in the slide as High Positive (HP), Positive (P); Low Positive (LP); and Negative (N). From these numerical values, the Optical density score is calculated using the recommended algebraic formula: (HP × 4 +P × 3 +LP × 2 +N × 1)/100.

Results and Conclusions

Histology results indicate that compared to the preservation control group (PC group), both Rapa-30 and the Rapa-60 group displayed less mucosa injury and histo-morphometric damage. In contrast, the Rapa-0 group showed no significant difference between the mucosa injury and histomorphometry when compared to the PC group. The summary of the microscopic results from this study, suggests that that Rapamycin is an effective preconditioning agent capable of attenuating mucosal injury especially if administered 30 minutes before organ retrieval.

The results of the *biochemical analysis of cell injury markers* closely resemble those of the histology. Here as well grafts of the Rapa-30 and the Rapa-60 showed significantly less release of LDH when compared to the PC group. However, whilst the levels of Lactate were significantly lower in the Rapa-30 group vs the PC group, the levels in the Rapa-60 group were lower but not statistically significant. There was no significant difference in the level of the LDH and Lactate between the PC group and the Rapa-0 group. Taken together, the results from the cell injury markers confirm that intestinal preservation injury can be attenuated by Rapamycin, especially when administered 30 minutes before organ retrieval.

Immunohistochemistry evaluation of the autophagy markers Beclin-1 and LC3B reveals that Rapamycin

pretreatment at 30mins (Rapa-30) and 60 mins (Rapa-60) respectively had significant increase in both proteins compared to the PC group. Here as well, the grafts of Rapa-0 and PC groups were statistically identical. These results when evaluated suggests that Rapamycin promotes autophagy especially at the 30 minute pretreatment mark.

An interesting pattern from this study was that the groups which were treated with Rapamycin and had an elevated amount of autophagy proteins also showed the least mucosa damage. An initial appraisal of these findings might suggest that the autophagy pathway plays a role in the beneficial effects of Rapamycin on intestinal grafts being preserved. More studies into autophagy would be needed to further expatiate on these results.

5. Characterising autophagy changes during small bowel preservation

Background

In the recent years, autophagy, a molecular process present in almost all cells has been gaining traction in different pathologies. Even though autophagy is primarily regarded as a cell survival pathway, some studies have also linked it with cellular death. This seemingly dual nature of autophagy makes it a rather interesting area of research in multiple disease states.
Recent papers suggest a role this biological pathway during intestinal ischemia. However, these studies were mostly performed in the warm ischemic setting, and there is therefore a paucity of studies into autophagy changes during hypothermic states of the intestine. In contrast, studies exist for other solid organs like the kidney and lungs, which have described of this complex biological cascade during cold preservation.
Our previous experiment in *section 4* showed that Rapamycin could induce autophagy when we detected changes in two important markers, Beclin-1 and LC3B. Therefore, in this third study, we follow the changes in these two proteins during the cold preservation of intestinal grafts.

Materials and Methods

The nature of the experimental animals and the way the intestines were procured and stored are the same as in ***section 3***.
Rats were randomly divided into 5 groups (n=6/group) based on the length of cold preservation.

O hour (sham control, no preservation)
3 hours (3hr group)
6 hours (6hr group)
9 hours (9hr group)
12 hours (12hr group)

Autophagy

Autophagy was monitored by following the proteins Beclin-1 and LC3BII. LC3BII is formed by the conjugation of LC3B to phosphatidylethanolamine (PE) on surface of autophagosomes. We detected changes in these proteins by means of western blot and immunohistochemistry. We also observed the presence of autophagosomes using transmission electron microscopy (TEM).

Immunohistochemistry
Immunohistochemistry methods are the same as described in ***section 4.***

Western blot
Intestinal tissue samples were frozen in liquid nitrogen, then manually pulverized in mortar and dissolved in ice-cold lysis buffer (containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mm EGTA, 1 mM Na_3VO_4, 100 mM NaF, 5 µM $ZnCl_2$, 10% glycerol, and 1% Triton X-100 plus 10 µg/ml of the protease inhibitor aprotinin). Lysates were subjected to centrifugation at 40 000 x g at 4 °C for 30 minutes, then the protein concentration of the supernatants was determined using Protein Assay Dye Reagent Concentration (Bio-Rad) and light absorption measurement at 595 nm. Samples containing 30 ug of denatured total protein have been prepared and loaded onto 10% polyacrylamide gels. Proteins separated based on size have been electro- blotted for half an hour onto PVDF membranes using the Trans-Blot Turbo semi-dry system (Bio-Rad), then blocked in 3% BSA dissolved in Tris-buffered saline

containing 0.2% Tween 20. Probing of the membranes with the primary antibodies (Beclin-1, LC3 (Cat. Nr. 2775 and Cat. Nr.4108 Cell Signaling Technology), diluted 1:1000 in the blocking solution followed at 4 °C overnight. Binding of the antibodies to the membrane was detected by a secondary anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz) diluted 1:10,000. The enhanced chemiluminescent signal was visualized using a Gbox gel documentation system (Syngene). All membranes were then stripped from the antibodies and detected again as above for possible loading differences using a primary antibody against GAPDH (Cell Signaling Technology) at a dilution rate of 1:3000. Analysis of band densities was performed using the Image J software. Each of the densities was further quantified in relation to GAPDH.

Transmission electron microscopy (TEM)
The intestines were cut into large blocks approximately 1mm³ and fixed overnight at 4°C in 4% paraformaldehyde with 2.5% glutaraldehyde in phosphate buffer (PB). These blocks were then fixed in 1% osmium tetroxide in 0.1M PB for 35 minutes. Serial dehydration was performed with increasing ethanol concentration. At the 70% ethanol stage, uranyl acerate (1%) was included to increase contrast. Blocks were transferred to propylene oxide before being placed into aluminium-foil boats containing Durcupan resin (Sigma) and then embedded in gelatin capsules containing the same resin. Semithin sections were cut with a Leica ultramicrotome, and mounted either on mesh, or on Collodion-coated (Parlodion, Electron Microscopy Sciences, Fort Washington, PA) single-slot, copper grids. Additional contrast was provided to these sections with uranyl acetate and lead citrate, and they were examined in JEM-1400 Flash transmission electron microscope. Qualitative observation for the presence of autophagosomes was undertaken in a blind manner from samples obtained from the 0hr, 6hr and 12 hr groups.

Results and Conclusion

Western blot results for Beclin-1 and LC3BII illustrate a significant increase in these proteins in the 3 and 6 hr groups compared to the sham control. The peak protein expression for both Beclin-1 and LC3BII was 3 hours from the start of preservation. Thereafter, while the both 9hr groups show an increase in autophagy proteins, only the LC3BII was significant compared to the sham control. In both proteins the 12 hr group showed a slight but significant decline compared to the baseline sham group.

Immunohistochemistry results follow a similar trend for Beclin-1 protein. This method stains the cytosolic presence of Beclin-1. A darker stain signifies more prominence of the protein and can be quantified by measuring the optical density (OD). Compared to the control, average OD scores were significantly increased in the 3hr group and significantly decreased in the 12hr group respectively.

TEM observation for autophagosomes in the cytoplasm of intestinal epithelial cells with microvilli revealed that when compared, more autophagosome could be observed in the 6hr group, than the 0hr or 12hr group respectively.

Taken together these various biological methods suggest that the autophagy pathway is activated during cold ischemic stress of intestinal grafts. Furthermore, it follows a pattern of an initial increase, with a peak at 3 hours during storage, thereafter a steady decline. Of note, after 12 hours a decline from the basal expression seen in the sham group is observed. We offer two possible explanations for this. First, it is likely that the baseline used for our study (0 hour, sham group) is not the true basal expression of autophagy in intestinal cells. Our experimental animals were starved for 24 hours as part of the protocol, and since starvation is a strong moderator of autophagy, we suspect that the autophagy process was already upregulated in the intestine before we retrieved the organ. It could therefore be argued that the baseline we adopted is a good one for monitoring changes in autophagy within a limit but should be interpreted cautiously. Secondly, in some instances, prolonged periods of ischemia (starvation) impair autophagy. The consumption and depletion of essential components for

autophagy after sustained starvation, and the inhibition of key regulators such as Beclin-1 by caspases is said to be behind this phenomenon. The exact reason for this occurrence in our results was not investigated further. These time-based differences in the expression of autophagy proteins, do however suggest a role for autophagy in cold intestinal preservation.

6. Effects of autophagy modulation on apoptosis and cold preservation injury of small bowel grafts.

Background

The function of autophagy during small bowel preservation and reperfusion remains unclear. Recent evidence suggests that the intestinal epithelium and autophagy may coordinate to maintain gut homeostasis in porcine intestinal epithelial cell. More so, autophagy which is constantly involved in cellular degradation- regeneration, has been implicated in the intestinal mucosa changes developing during warm ischemia-reperfusion injury of the small bowel. Therefore, the objective of this present study was to determine the effect of manipulating autophagy on intestinal preservation injury and apoptosis after cold storage.

Materials and Methods

The nature of the experimental animals and the way the intestines were procured and stored are the same as in ***section 3***.

Rats were randomly divided into 5 groups (n=6/group):

(a) 0hr (sham control)

(b) Preservation control (PC): Preservation time of 12 hrs

(c) Rapamycin group (Rapa): Rapamycin administered at 30 minutes before intestinal retrieval; preservation time:12 hrs

(d) 3-methyladenine group (3-MA): 3-MA administered at 30 minutes before intestinal retrieval; preservation time: 12 hrs

(e) Drug Control group (DC): Both Rapa and 3-MA given to animals 30 minutes before intestinal retrieval; preservation time: 12 hrs

Drug preparation and dosage

2mg/kg of an inhibitor of autophagy, 3-methyladenine (3-MA) (Hb2267 Hellobio) was dissolved in 1ml double distilled water (ddH20). 2mg/kg of a promoter of autophagy, Rapamycin (Hb2779 Hellobio) was dissolved in 1ml dimethyl sulfoxide solution (DMSO). The respective drugs corresponding to the groups were injected intraperitoneally (i.p) at 30 minutes prior to organ retrieval. The Sham control and Preservation control group received same volume of the solvents (DMSO and ddH2O) used.

Reperfusion

The total time of preservation was 12 hours. After cold storage, grafts were perfused ex-vivo using oxygenated Krebs Henslet buffer solution (KHBS) for 60 minutes.

Preservation and reperfusion injury

Samples of the intestine were obtained right after retrieval (0hr), 6hrs and 12hrs after preservation, and at the end of reperfusion.

Preservation injury was defined by mucosal changes and changes in LDH and lactate. Mucosal changes were determined by simple histology of the intestine as described in ***section 3*** and PAS staining for goblet cells as described in ***section 3***. Biochemical analysis for LDH and lactate was performed as described in ***section 4.***

Reperfusion injury was determined by simple histology and presence of LDH in the perfusate after the first minute and at the end of the reperfusion phase.

Autophagy

In this study, autophagy changes were monitored using the Beclin-1, LC3II, and p62/SQSTMI protein at 0hr, 6hr and at the end of the preservation (12hr).
Immunohistochemistry (IHC) and Western Blot (Wb) were performed for LC3 and Beclin-1 according to the method described in ***sections 4 and 5,*** respectively.
We introduced another protein used to monitor autophagy the p62/SQSTMI protein or simply p62. This protein is one of the known substrates of the autophagy machinery. The level of p62 protein inversely correlates with the autophagic activity. Immunohistochemistry (IHC) was also performed for the p62 protein using the previously described methods ***(section 4)***.
Analysis of both Beclin-1 and p62 immunostaining was performed using the IHC profiler plug-in of the Image J software, and the optical density (OD) was scored according to the method described previously ***(section 4)***.
Analysis of western blot band densities was performed using the Image J software. Each of the densities were further quantified in relation to GAPDH.

Apoptosis

Apoptosis was determined using the marker protein cleaved caspase-3 (Cat. Nr. 9661, Cell Signaling Technology, Inc.) at 0hr, 6hrs and 12hrs.
Immunohistochemistry technique according to the method in ***section 4*** and Western blot techniques according to the method in ***section 5,*** were used to visualize changes in this protein.
Analysis of the cleaved caspase-3 stained cells (from IHC) was achieved by manually counting the visibly stained apoptotic cells in at least four high-power fields (hpf) randomly selected from four different quadrants using the CaseViewer 2.4 software. Analysis of western blot band densities was performed using the Image J software. Each of the densities were further quantified in relation to GAPDH.

Results and Conclusion

Changes in autophagy activity

Immunostaining immediately after organ retrieval revealed no observable differences amongst the groups for Beclin-1 and p62 proteins.
After both 6 and 12 hours of preservation, immunostaining for p62 in the Rapa group had significantly less optical density (OD) scores than the preservation control (PC) group. In contrast, the OD scores for p62 immunostaining in the 3-MA group were significantly higher at both 6 and 12hours time points respectively.
Beclin-1 staining on the other hand revealed that the Rapa group scores were significantly higher than the PC group after both 6 and 12 hours of preservation. The 3-MA group showed the opposite trend.
Western blot analysis at the end of cold storage indicates that autophagy was enhanced in the group treated with Rapamycin, as evidenced by significant increased LC3II expression and significant increased Beclin-1 expression when compared to the preservation control (PC group). By contrast, the autophagy was inhibited in the group of animals treated with 3-methyladenine. We noted significant LC3II and Beclin-1 decrease, compared to the PC group.
Taken together, these results confirm that Rapamycin enhances autophagy activity, while 3-MA inhibits autophagy in the intestinal grafts.

Changes in apoptosis

Immunostaining for active caspase-3, showed that at onset of preservation (0 hour), there was no significant difference between the Rapa and 3-MA groups versus the PC group. At 6 hours of

preservation, there were significantly more caspase positive stained cells in the 3-MA group than the PC group. While in the Rapa group, there were significantly fewer positive cells present. After 12 hours, the 3-MA group still had a significantly higher amount of positively stained apoptotic cells compared to the preservation control, while in the Rapa group, we observed less apoptotic cells.
Consistently, *western blot analysis for the expression of cleaved caspase-3* at the end of the 12-hour long preservation period showed a significant increase in the 3-MA group and decrease in the Rapa group when juxtaposed with the PC group.
These results altogether suggest that enhancing autophagy attenuated apoptotic damage, while inhibiting this autophagy process accelerated apoptotic injury

Effects on intestinal mucosa

Mucosal examination was not remarkable between the treated groups (Rapa and 3-MA) and the control group (PC) at the onset of preservation. Injury scores, mucosal thickness, crypt depth and goblet cell density showed no significant difference between the groups.
After 6 hours of preservation however, the 3-MA group showed higher injury scores, characterized by areas of epithelial breakdown and some denuded villi compared to the PC group. While the Rapa group displayed only minimal injury in comparison, with some regions containing subepithelial blebbing at the tip of the villus. Similarly, mucosal thickness and crypt depth decreased in the 3-MA group significantly when compared to the PC group. In the Rapa group, the values of mucosal thickness were significantly superior to the PC group. While the values for crypt depth were higher, but not significant enough in comparison. Goblet cells count was significantly higher in the Rapa group compared to the PC. In the 3-MA group, the number of goblet cells reduced significantly.
At the end of the preservation period (12 hours), the Rapa group still displayed significant lesser tissue injury, superior mucosal thickness and crypt depth, compared to the control. Similarly, the goblet cell count was significantly higher. In contrast, the 3-MA group had worse injury scores characterized by more regions of crypt damage compared to the control. The crypt depth was also significantly reduced, while the mucosal thickness was reduced but not significant enough. Furthermore, the amount of goblet cells was less in the 3-MA group.
These results from the intestinal mucosa collectively suggest that enhancing autophagy attenuated preservation induced mucosal changes while inhibiting this process proved detrimental for the graft mucosa.

Effects on LDH and Lactate

After 6 hours of preservation, there was less release of LDH and Lactate into the preservation solution in the Rapa group compared to the PC group. In contrast, in the 3-MA there was significantly more LDH and Lactate in the preservation solution. Similarly, after 12 hours of preservation, increased concentration of LDH and lactate was measured from the preservation fluid in the 3-MA group when juxtaposed with the PC group. In the Rapa group, both the concentration of LDH and lactate were significantly less.
These results indicate that Rapamycin-enhanced autophagy attenuates while inhibiting autophagy exacerbates cellular injury during intestinal preservation.

Reperfusion injury

Compared to the PC group, the grafts with enhanced autophagy (Rapa group) had significantly less mucosal damage. In contrast, the 3-MA group exhibited worse average injury scores but not significant. Similarly, the amount of LDH released in the first minute and 60th minute was significantly less in the Rapa compared to the PC group; while in the 3-MA group, LDH was significantly increased at both times.
These results illustrate that enhancing autophagy limits the extent of reperfusion injury while inhibiting

autophagy aggravates this injury.

The protective effects of Rapa-enhanced autophagy are attenuated by 3-MA

Comparison of Park scores, LDH, lactate and apoptotic changes of the Rapa group to the drug control (DC) group was undertaken after preservation (12hrs) to determine if Rapamycin acted through autophagy.
Park/Chiu injury scores were significantly higher in the DC group compared to the Rapa group. Similarly, there were also significantly higher concentrations of LDH in the DC group than in the Rapa group.
With regards to apoptosis, the protective role of Rapamycin was still due to its pro-autophagy effect. Immunostaining for cleaved caspase-3 revealed more positive cells in the DC group than in the Rapa group. Similarly, the blot analysis for cleaved caspase-3 showed significant increase in the DC group in comparison to the Rapa group.
Taken together, these results indicate that Rapamycin acts significantly through the autophagy pathway to attenuate small bowel preservation injury.

The highlights from this 4th experiment is the following: Our results show that Rapamycin is an effective enhancer of autophagy in small bowel tissue. This enhanced autophagy correlated with lesser tissue injury and decreased apoptosis after cold preservation of the graft. Furthermore, our results suggest that Rapamycin and autophagy work hand in hand for the benefit of the intestinal tissue, because when we gave the inhibitor of autophagy 3-methyladenine to animals who received Rapamycin, the positive effects of Rapamycin were diminished.

7. Discussion

Despite significant progress in the field of intestinal transplantation, the procedure is still hampered by the quality of graft preservation. The gold standard for the intestinal preservation has not changed over the years, it still remains the cold static storage. In this method the organ is flushed with a cold preservation solution and thereafter stored in it for varying periods of time. This method has been proven not to be effective for the small bowel, with increasing time of storage the grafts deteriorate significantly. Our **first study** depicted this quite clearly. After 3 hours of preservation damage could already be seen in the mucosa of the intestinal grafts, by the 12hour mark, the damage was considerably worsened.

In a bid to address the problem of preservation ischemic injury, a method of prior administration of drugs or chemical to initiate protective mechanisms before the onset of ischemia has been suggested. It is generally known as preconditioning. Following this school of thought, we used Rapamycin, a well-known drug with great immunosuppressive, and antiproliferative properties, to precondition the intestines before retrieval and storage. The results from this **second study** were promising, as the groups which received Rapamycin at least 30 minutes before organ retrieval were better preserved compared to the control group. Rapamycin is also known to activate a cell degradation-regeneration pathway known as autophagy. Autophagy has been implicated in ischemia states, more recently even in the warm ischemia of the intestine. We therefore measured the markers of this protein from our samples and noticed that the groups which showed enhanced autophagy also had better mucosa integrity. This made investigating autophagy a focal point of the rest of the experimental series.

Autophagy can be beneficial or detrimental. However, if this pathway is even activated during intestinal preservation was yet unexplored. We therefore did a time series investigation of markers of this pathway using various methods. The conclusion was that this pathway is upregulated initially during cold intestinal ischemia, followed by a short peak and a steady decline. In fact, our **third study** showed that prolonging the cold storage of the bowels resulted in autophagy falling below the baseline level from our samples. This type of finding has been reported in the literature before. It might be that excessive ischemia and the detrimental pathways such as apoptosis that go hand in hand with it, can indeed lead to an inhibition of the autophagy mechanism.

Even though we saw an upregulation of the autophagy mechanism during preservation, it still did not give any indication if this pathway was upregulated for good or bad in our small bowel samples being preserved. The **fourth experiment** provided insight into this. Rapamycin-enhanced autophagy here not only attenuated ischemic changes of the small bowel, but also reduced the level of apoptotic activity in our samples. This effect was diminished by the addition of a blocker of autophagy known as 3-Methyladenine. With this we conclude that from our study autophagy has a positive role during the cold preservation of intestinal grafts, thus enhancing this pathway may prove beneficial in the field of bowel transplantation. However, like every budding scientific finding, more studies are needed to expatiate further on this concept and provide more proof.

List of Important Findings

1. In the first experiment we described the mucosal changes occurring during small bowel storage in Institute George Lopez (IGL-1) solution at different time points namely 3,6,9 and 12 hours. To the best of our knowledge, studies showing time-dependent changes of the intestinal mucosa preserved in IGL-1 solution, have not been published elsewhere.

2. In the second experiment, we have shown Rapamycin as a viable preconditioning agent. Rapamycin is already in use in the transplant clinics. This may make the translation of this drug into clinical practice less complicated.

3. In the third experiment, we have characterized the changes in the autophagy markers at different experiment times (3,6,9 and 12 hours). To the best of our knowledge, studies on autophagy's behaviour during small bowel preservation have not been published elsewhere.

4. In the fourth experiment, we have shown that enhanced autophagy was able to attenuate the ischemic and apoptotic changes in the small bowel during preservation. Given that in the last decade autophagy has gained a lot of traction, this may serve as the base of future research in this direction.

List of Publications and Presentations

Publications and Presentations directly linked to the thesis topic:

i) **Caleb, I**.; Erlitz, L., Telek, V.; Vecsernyés, M., Sétáló, G. Jr., Hardi, P., Takács, I., Jancsó, G., & Nagy, T . Characterizing Autophagy in the Cold Ischemic Injury of Small Bowel Grafts: Evidence from Rat Jejunum. *Metabolites*. 2021;11(6):396. Published 2021 Jun 17.doi:10.3390/metabo11060396. ***I.F = 4.932.***

ii) **Caleb, I**., Kasza, B., Erlitz, L., Semjén, D., Hardi, P., Makszin, L., Rendeki, S., Takács, I., Nagy, T., Jancsó, G. The Effects of Rapamycin on the Intestinal Graft in a Rat Model of Cold Ischemia Perfusion and Preservation. *Metabolites* 2022;12(9):794. Published 2022 Aug 25. doi:/10.3390/metabo12090794. ***I.F = 5.581***

iii) **Caleb, I**., Nagy, T. Characterizing autophagy in the cold ischemic injury of small bowel grafts: evidence from rat jejunum. A Magyar Haemorheologiai Társaság XXVII. Kongresszusa; 23 April, 2021.

iv) **Caleb, I.**, Kasza, B., Hardi, P., Takács, I., Jancsó, G., Nagy, T. INSIGHTS INTO AUTOPHAGY, A CYTOPROTECTIVE PATHWAY IN SMALL BOWEL PRESERVATION: AN OVERVIEW OF OUR EXPERIMENTAL DATA. MAGYAR SEBÉSZ TÁRSASÁG KÍSÉRLETES SEBÉSZETI SZEKCIÓ XXIX. KONGRESSZUSA; 8-10 September, 2022.

Publications linked to the topic of cold preservation:

i) Telek, V., Erlitz, L., **Caleb, I.,** Nagy, T., Vecsernyés, M., Balogh, B., Sétáló, G., Hardi, P., Jancsó, G., & Takács, I. (2021). Effect of Pioglitazone on endoplasmic reticulum stress regarding in situ perfusion rat model. *Clinical hemorheology and microcirculation*, *79*(2),311–325.https://doi.org/10.3233/CH-211163. ***I.F = 2.375***

ii) Erlitz, L., **Ibitamuno, C.,** Kasza, B., Telek, V., Hardi, P., Sétáló, G., Vecsernyés, M., Takács, I., & Jancsó, G. (2022). Subnormothermic isolated organ perfusion with Nicorandil increased

cold ischemic tolerance of liver in experimental model. *Clinical hemorheology and microcirculation*, *81*(1), 1–12. https://doi.org/10.3233/Ch-211263. ***I.F = 2.375***

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