INTESTINAL PRESERVATION FOR TRANSPLANTATION:

TRANSLATIONAL APPROACHES

John Mackay Søfteland

Department of Transplantation Surgery
Institute of Clinical Sciences
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg, Sweden

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A Doctoral Thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have either already been published or are manuscripts at various stages (in press, submitted, or in manuscript)

Front cover:
Immunofluorescence staining of tight junction proteins ZO-1 and claudin-3 in human intestinal crypts.

Intestinal preservation for transplantation: translational approaches

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To my loving and supportive wife Madiha
and our wonderful children, Enaya and Noura.

In memory of my father,
who really wanted to be here;
To my mother, who always is.

"Don't Panic"

-The hitchhikers guide to the galaxy

INTESTINAL PRESERVATION FOR TRANSPLANTATION: TRANSLATIONAL APPROACHES

John Mackay Søfteland
Department of Transplantation Surgery, Institute of Clinical Sciences
Sahlgrenska Academy at the University of Gothenburg
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ABSTRACT

Background: Intestinal preservation injury (IPI) may result in various degrees of mucosal damage, which may later favor bacterial translocation, post-reperfusion syndrome, and upregulation of alloreactivity. Experimental evidence suggests that combining vascular perfusion and cold storage with luminal interventions using polyethylene glycol (PEG) solutions may mitigate the mucosal damage and extend the safe storage time. During the last years, there has been an increasing trend towards using livers and kidneys from older donors for transplantation, yet the field of intestinal transplantation is far more conservative as the impact of age on the preservation injury is unknown. Clinical translation of various experimental models is hampered by interspecies differences, as little is known about how IPI development differs between rodents, pigs, and humans. The current thesis aimed to explore if the size of the PEG molecule or the donor age has an impact on the development of IPI and whether the development of IPI differs between rats, pigs, and humans. It also examines if luminal preservation (LP) with PEG is safe and efficient in delaying the development of IPI in the human intestine.

Methods: In Paper I, we used small intestines from rats to study the effect of PEG size on the development of IPI. Paper II compared the development of IPI in rat, porcine, and human intestinal specimens. Paper III assessed the effect of donor age on IPI in a rat model. Paper IV studied the effect of LP with a low-sodium PEG solution on human small intestinal specimens. In all studies, we analyzed injury development using histological and molecular biological approaches. We also used Ussing chamber experiments for intestinal functional assessment in Paper I.

Results: The luminal presence of PEG rather than its molecular size appears to reduce and delay the development of IPI when compared with controls undergoing standard cold preservation. Increasing donor age does not appear to accelerate the development of the IPI in rats. LP is effective in all age groups. Pig intestines are more ischemia resistant than human and rat intestines. LP with a low-sodium PEG solution is effective in delaying tissue injury in human specimens and does not cause excess edema.

Conclusions: The development of IPI differs significantly between species, with the rat being a sensitive model when studying IPI. LP is effective in protecting against IPI regardless of the size of the PEG molecule or donor age. LP appears to delay the development of IPI in humans without causing tissue edema and could be introduced in clinical practice.

Keywords: intestinal preservation, luminal preservation, tight junction, apoptosis.

LIST OF PUBLICATIONS

The thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Casselbrant A*, **Søfteland JM***, Hellström M, Malinauskas M, and Oltean M. *Luminal Polyethylene Glycol Alleviates Intestinal Preservation Injury Irrespective of Molecular Size.* J Pharmacol Exp Ther. 2018 Jul; 366(1):29-36. * Shared first authorship

II. **Søfteland JM,** Casselbrant A, Biglarnia A-R, Linders J, Hellström M, Pesce A, Padma AM, Jiga LP, Hoinoiu B, Ionac M, and Oltean M. *Intestinal Preservation Injury: A Comparison Between Rat, Porcine and Human Intestines.* Int. J. Mol. Sci. 2019, 20, 3135.

III. **Søfteland JM**, Casselbrant A, Akyurek L, Hellström M, and Oltean M. *The Impact of Age and Luminal Preservation on the Development of Intestinal Preservation Injury in Rats.* (Transplantation, in press, 2019)

IV. **Søfteland JM**, Padma AM, Casselbrant A, Zhu C, Wang Y, Pesce A, Hellström M, Olausson M, and Oltean M. *Luminal Preservation of the Human Small Bowel Using a Polyethylene-Glycol Based Solution.* (in manuscript)

SUMMARY IN SWEDISH
POPULÄRVETENSKAPLIG SAMMANFATTNING

Tarmen är ett organ som sällan transplanteras; dels då vi har en bra alternativ behandling för tarmsvikt i form av näringsdropp men framför allt mot bakgrund av allvarliga komplikationsrisker som avstötning samt livshotande infektioner. Många av dessa komplikationer uppkommer till följd av skada på tarmslemhinnan. Slemhinnan har två huvudfunktioner; upptag av näringsämnen från tarminnehållet samt dess barriärfunktion. Med barriärfunktion avser vi tarmens kapacitet att förhindra bakterier som normalt huserar i tarmen att ta sig till blodomloppet och därmed ge upphov till livshotande infektioner. En potentiell risk vid tarmtransplantaton är när signifikant slemhinneskada uppkommer under och strax efter preservationstiden, dvs tiden mellan avstängning av tarmcirkulationen i donatorn, tills blodet släpps på genom transplantatet i mottagaren. Under preservationen förvaras tarmen i ett isbad efter att det blivit genomspolat med preservationslösning. Den låga temperaturen bromsar ämnesomsättningen, men kan inte komplett hindra utvecklingen av skador under tiden organet måste förvaras ocirkulerat, fram till transplantationen.

I denna avhandling har vi jämfört två vanligt förekommande, experimentella djurmodeller för tarmtransplantationsforskning med human vävnad. Vi har studerat effekten av donatorns ålder på tarmpreservationskada och undersökt huruvida fyllnad av det blivande tarmtransplantatat medelst polyethyleneglycol (PEG) har någon inverkan på preservationsskadan och sedermera transplantatfunktionen. Metoden består i att fylla tarmen med en lösning som innehåller PEG. Vi har även studerat effekten av olika storlekar av PEG-molekylen på preservationsskadans omfattning. I humana försök har vi utvärderat en kommersiellt tillgänglig tarmrengöringslösning som preservationslösning, tillfört i tarmens lumen. Tillförsel av dylik lösning tycks fördröja utvecklingen av slemhinneskada.

Vår konklusion blir således att;

- Den luminala närvaron av PEG snarare än dess molekylstorlek tycks minska och fördröja utvecklingen av preservationsskada, jämfört med kontroller som genomgår standardpreservation.
- Ökande donatorsålder tycks inte påskynda utvecklingen av preservationsskada hos råttor.
- Preservation i tarmens lumen är effektivt i alla åldersgrupper.
- Gristarmar är mer ischemiresistenta än människors och råttetarmar.
- Luminal preservation med en lågnatrium-PEG-lösning är effektiv för att försena vävnadsskada i humana tarmprover och orsakar inte mer ödem.

ABBREVIATIONS

AGR	Autologous gut reconstruction
ATP	Adenosine triphosphate
CIT	Cold ischemia time
CVC	Central venous catheter
DAMP	Damage-associated molecular pattern
FD4	Fluorescein isothiocyanate-dextran
FSS	Fluorescein sodium salt
GALT	Gut-associated lymphoid tissue
GC	Goblet cell
GVHD	Graft versus host disease
H&E	Hematoxylin and eosin
HES	Hydroxyethyl starch
HLA	Human leukocyte antigen
HMGB1	High-mobility group box chromosomal protein 1
HSP	Heat-shock protein
HTK	Histidine-tryptophan-ketoglutarate solution
IEC	Intestinal epithelial cell
Iep	Epithelial ion current
IESC	Intestinal epithelial stem cell
IF	Intestinal failure
IFALD	Intestinal failure-associated liver disease
IGL-1	Institut Georges Lopez solution
IPI	Intestinal preservation injury
IRI	Ischemia-reperfusion injury
ISB	Isolated small bowel
ITR	Intestinal Transplant Registry
ITx	Intestinal transplantation
JNK	c-Jun NH2-terminal kinase
LP	Luminal preservation
LPS	Lipopolysaccharide
LSB	Liver-small bowel
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MMVT	Modified multivisceral transplant
MVT	Multivisceral transplant
NADPH	Nicotinamide adenine dinucleotide phosphate
NEPT	Neuroendocrine pancreas tumor

NF-κB	Nuclear factor-κB
NRP	Normothermic regional perfusion
OPTN	Organ Procurement and Transplantation Network
PD	Potential difference
PEG	Polyethylene glycol
PN	Parenteral nutrition
PRR	Pattern-recognition receptor
PTLD	Post-transplantation lymphoproliferative disorder
Rep	Epithelial electrical resistance
ROS	Reactive oxygen species
SBS	Short bowel syndrome
SCS	Static cold storage
SMA	Superior mesenteric artery
SMV	Superior mesenteric vein
TNF	Tumor necrosis factor
UW	University of Wisconsin preservation solution
ZO-1	Zonula occludens-1

TABLE OF CONTENTS

INTRODUCTION

Intestinal transplantation (ITx) has become a viable therapeutic choice for patients with complicated intestinal failure (IF).[1] The results of ITx have continuously improved over the last three decades since the procedure became clinically feasible with the introduction of tacrolimus (a potent immunosuppressive drug) in the late 1980s.[2-4] However, the post-transplant course is still frequently hampered by a broad range of complications, some of them directly related to ischemia-reperfusion injury (IRI). The intestine tolerates a shorter cold ischemia time (CIT) compared to other abdominal organs. The complexity and variable length of the recipient surgery, coupled with the short CIT, create challenges for the surgical procurement and transplant teams, and the transplant coordinators. As a transplantable organ, the intestine is unique due to its contaminated content. Bacteria may translocate in the event of mucosal barrier damage secondary to IRI, leading to sepsis.[5] The severity of the IRI is determined both by donor factors in the pre-donation phase and the length and quality of the preservation period in the post-donation phase.[6] The transplant team can influence only the latter. Since logistical constraints largely determine the length of the preservation period, further research should be aimed at improving the quality of preservation techniques to alleviate IRI.

The main aim of this thesis was to further evaluate luminal preservation (LP) as a strategy that may be able to reduce damage to the intestinal graft, thus improving the results of ITx. This thesis also explored animal and human models in the context of intestinal preservation, evaluated the effect of various sizes of PEG molecules in LP solutions, and examined if donor age affects intestinal preservation injury (IPI) in a rodent model.

CLINICAL INTESTINAL TRANSPLANTATION

History

Alexis Carrel and Emerich Ullman are frequently credited as the pioneers of intestinal transplantation in the early 1900s. In 1959, Richard Lillehei provided the first description of intestinal transplantation with a vascular pedicle in a canine model, with a focus on the surgical technique and graft preservation.[7] With surgical principles established and the availability of early immunosuppressants, the first clinical attempts were made in the late 1960s. Unfortunately, albeit in retrospect predictably, these attempts were followed by severe rejection, sepsis, and patient death. Seven such transplants were carried out by 1970, with the most prolonged survival being 76 days, and the field was all but abandoned. The introduction of cyclosporine in the 1980s and its remarkable effectiveness in renal transplantation rekindled some interest in the field. But of the 15 isolated bowel transplants done between 1985 and 1990 under cyclosporine immunosuppression, most grafts failed early due to rejection.[8] The first intestinal transplants achieving long-term survival were done in 1989 by Eberhard Deltz in Germany, David Grant in Canada, and Olivier Goulet in France.[9] These initial successes were expanded upon and consolidated at the University of Pittsburgh when a clinical intestinal transplant program was started in 1990 using tacrolimus as the primary immunosuppressant. Their success was dramatic for that era, with 59 patients transplanted in the first three years with a one-year survival of around 60%.[10]

The first two intestinal transplantations in the Nordic countries were performed in 1990 in Stockholm and Uppsala, but both recipients, unfortunately, had short survival.[11] The first successful multivisceral transplantation was performed in 1998 in Gothenburg by Michael Olausson[12], while Gustaf Herlenius performed the first successful transplantation of an isolated intestinal graft in 2007. Both these patients were children at the time of their transplants, and both are currently alive.

Intestinal failure

Intestinal failure (IF) is defined as the reduction in gut function below the minimum necessary for the absorption of macronutrients and/or water and electrolytes, such that intravenous supplementation is required to maintain health and/or growth.[13]

Parenteral nutrition

Parenteral nutrition (PN) remains the treatment of choice for chronic intestinal failure. It may be administered to cover all the patient's nutritional requirements or as an adjunct to an insufficient enteral intake/uptake. Reducing the ratio of parenteral/enteral nutrition is one of the main goals of conservative strategies for treating IF,

such as autologous gut reconstruction (AGR) and pharmacological treatment. The two main complications of PN are loss of vascular access[1] and liver failure[14]. Long-term central venous catheters (CVC) cause strictures and thromboses in central veins after long term use.[15] They also predispose patients to infections. A referral for ITx is warranted after the loss of two or more access sites or if the patient has had repeated septic episodes.[1]

Intestinal failure-associated liver disease (IFALD)

Parenteral nutrition can affect the liver adversely, leading to IFALD. This is a serious condition often becoming irreversible and requiring a liver transplant in addition to an intestinal transplant. It is also one of the leading causes of death in this patient population. IFALD is, in part, caused by the composition of parenteral nutrition formulations, specifically its lipid component.[16] Sepsis is a major risk factor for IFALD, and recurring infectious episodes are associated with a 30% increase in risk.[14]

Autologous gut reconstruction (AGR)

The purpose of AGR procedures is to make a larger area of the native bowel available to nutrients for an appropriate amount of transit time. Common examples of AGR strategies are converting dilated bowel segments into longer ones by making perpendicular incisions in a contralateral stepwise fashion or ameliorating a rapid transit time by the reversal of a short segment. AGR procedures can improve transit times or increase the available mucosal area. The goal is to boost nutrient uptake, which in turn could lead to improved nutritional autonomy.[17,18] These procedures avoid the complications and lifelong immunosuppression associated with ITx and should be employed where possible. Data from the Intestinal Transplant Registry (ITR) shows a recent decline in the number of ITx performed annually, and this may reflect a trend towards performing more AGR procedures instead. AGR seems to be taking up an increasing amount of space in the middle ground between TPN and ITx.[17]

Medical treatment for intestinal failure

Teduglutide is a glucagon-like peptide-2 analog that enhances the absorption of nutrients and fluids. A randomized controlled trial found a 63% response (defined as a 20% decrease in PN requirements) in the treatment arm compared to a 30% response in the placebo group.[19] Cost-effectiveness may be an issue.

Summary: treatment for intestinal failure

The treatment for chronic IF is a combination of PN, AGR, ITx, and in the future, possibly pharmaceuticals. Their respective places in the treatment of ITx is somewhat dynamic and in constant flux. Any future improvement of any of these modalities will likely increase its place in the treatment armamentarium. For example, reducing the

problem of central vein thromboses and infections associated with central lines will increase the place for PN (e.g., by using AV-fistulae for PN delivery[20]). Improvements in AGR techniques or pharmaceuticals could increase enteral uptake. The result of this would be to reduce the need for parenteral nutrition, thus decreasing the risk of IFALD and delaying or eliminating the need for ITx in a subset of patients. Improvements in PN formulations may also reduce the incidence of IFALD.[14] A reduction in the price of Teduglutide may justify its use. Advances in graft preservation and immunosuppression, leading to better graft/patient survival, could increase the role of ITx.

Current indications for intestinal transplantation

Due to the severe complications that can arise after ITx, long-term use of immunosuppressive therapy, and only moderately good long-term results, strict eligibility criteria exist to ensure appropriate patient selection.

ITx is offered to patients who have one of the following problems: complications of parenteral nutrition, inability to adapt to the quality of life limitations posed by IF, or high risk of death if the native gut is not removed. Examples of the latter may be unresectable mesenteric tumors or chronic intestinal obstruction. The most common reason for performing ITx in both the adult and pediatric population is short bowel syndrome (SBS).[1,21] The conditions leading to ITx differ between the pediatric population and adults. In children, SBS leading to ITx is most commonly caused by surgical resection following gastroschisis, volvulus, necrotizing enterocolitis, and intestinal atresia. Other common causes in children are malabsorption due to microvillus inclusion, motility disorders due to pseudoobstruction and Hirschsprung's disease, and retransplantations. In adults, SBS leading to ITx is most commonly caused by surgical resection following ischemia, Crohn's disease, trauma, and volvulus. Other common causes in adults are tumors, pseudoobstruction, and retransplantations.[2] Intraabdominal desmoid tumors represent the majority of intraabdominal tumors leading to ITx. Although histologically benign, desmoid tumors may cause entrapment of the mesenteric vasculature. Medical treatment is unsatisfactory, necessitating surgical resection that often results in SBS.[22]

Multivisceral transplants have been used to treat complications of portal hypertension when extensive splanchnic venous thrombosis precludes restoration of hepatopetal portal blood flow during a standard liver transplantation.[23] An alternative procedure is a liver transplant with a portocaval hemitransposition. Patients with unresectable neuroendocrine pancreas tumors (NEPT) with liver metastases may be treated with a multivisceral graft. While the results are quite good, the primary disease often recurs, and the evidence supporting this method is based on a small number of patients and heterogeneous study designs.[24]

Types of transplant grafts

An intestine-containing graft may be limited to the intestine only or contain other abdominal viscera as well. Currently, the general principle is to choose the smallest graft functionally and anatomically suitable for a given patient.[3,17] For a patient with intestinal failure without IFALD, an isolated small bowel (ISB) transplant is suitable (Figure 1). When IFALD is present, a liver-small bowel (LSB) transplant is necessary. In patients with pseudo-obstruction, portomesenteric-thrombosis, metastasized NEPT, or anatomical defects (fistulae, trauma) affecting multiple foregut structures, a multivisceral transplant (MVT) may be indicated. Depending on which recipient viscera are possible to salvage, the MVT graft may be modified to include less donor viscera (MMVT). Liver-containing grafts are more tolerogenic than the ISB and MMVT grafts without the liver.[3] Current one-year graft survival rates favor isolated ITx, but after five years, LSB grafts have superior survival.[3,4] Waiting list mortality for isolated ITx is lower than for MVT, which probably reflects earlier listing for these smaller grafts and patients in an earlier stage of their disease. Also, waiting times are generally shorter for ISB grafts compared to LSB grafts.[21] Failure of an ISB graft is, in theory, reversible, as it can be explanted in the case of graft failure, severe graft versus host disease (GVHD), post-transplantation lymphoproliferative disorder (PTLD), or infectious complications. After this, immunosuppression can be withdrawn, and the patient can revert to PN. A liver-containing graft cannot be explanted without a simultaneous retransplantation. This precludes the withdrawal of immunosuppression to combat GVHD, PTLD, or complicated and severe infections. Another consideration is the relative scarcity of liver grafts compared to intestinal grafts. Donor colon and ileocecal valve may be included in any of the aforementioned graft types, without additional risk.[25]

Donor criteria

Several criteria for donor selection have been suggested, but the evidence supporting them is generally weak.[26-28] Donor selection is primarily guided by experience in matching an appropriate donor to a planned recipient. Age is considered important, and donors older than 50 years are avoided.[27] The size of the donor (height, weight, and body-mass index) is a critical consideration since many of the recipients have restricted abdominal space.[29] Blood-group should be compatible.[27] The donor's cause of death, history of cardiac arrest or circulatory instability, and length of cardiopulmonary resuscitation (CPR) also need to be considered.[30,31] Infectious concerns and possible tumors in the donor may be a contraindication to donation. The use of multiple or high doses of vasopressors may lead to significant mucosal damage.[32] When death is caused by anoxic events, such as drowning or hanging, the bowel may also be adversely affected.

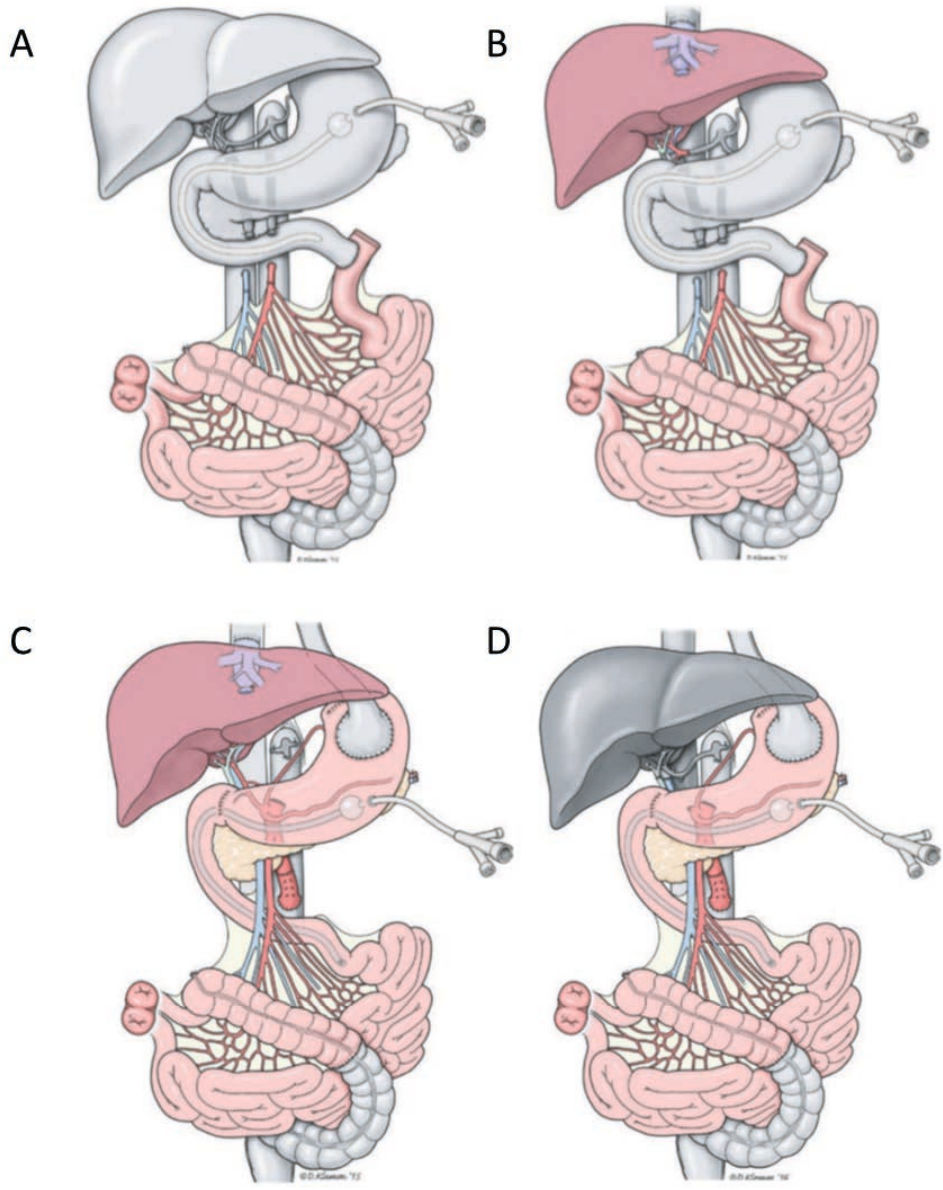

Figure 1. Types of intestine-containing grafts with the graft in color and the native viscera in gray. **(A)** Isolated small bowel (ISB) graft with colon. **(B)** Liver–small bowel (LSB) graft with colon. **(C)** Multivisceral graft (MVT) with en bloc liver, stomach, duodenum, pancreas, jejunoileum with colon. **(D)** Modified multivisceral graft (MMVT) with en bloc stomach, duodenum, pancreas, jejunoileum with colon. Adapted from *Hawksworth et al.*[21]

Donor biochemistry is an important consideration. High plasma sodium level at the time of donation is associated with primary nonfunction and bowel swelling.[32] Poor liver function tests, amylase, creatinine, and especially lactate may be viewed as surrogate markers for ischemic injury.[27] On arterial blood gas samples, it can be valuable to assess the first sample taken upon hospital arrival as a low pH may give an estimate of the degree of circulatory downtime and the resulting ischemia. Crossmatching, real or virtual, has been advocated to reduce the risk of humoral rejection in the long term. It may guide donor selection, immunosuppressive strategies post-transplant, or both.[33]

Table 1. Organ Procurement and Transplantation Network (OPTN) donor acceptance criteria for intestinal grafts. From *Roskott et al.*[26]

OPTN Criteria
Donation after brain death
Cold ischemia time < 9h
Donor age <50 y
Other organs retrieved from the same donor
ASAT and ALAT <500 u/L
Last serum sodium <170 meq/L
Serum creatinine <2 (if donor >1 y)/<1 mg/dL (if donor <1 y)
Negative virology (HIV, HBsAg/cAB, HCV AB)
Maximal 2 inotropes at recovery
Resuscitation <15 min if cardiac arrest after the declaration of brain death

ASAT = aspartate aminotransferase; ALAT = alanine aminotransferase; HIV = human immunodeficiency virus; HBsAg/cAB = hepatitis B serum antigen/core antibody; HCV AB = hepatitis C viral antibody.

Donor operation

The donor procedure is usually conducted through a long midline incision. After a Cattell-Braasch maneuver (medialization of the right colon with an extended Kocher maneuver), access is gained to the large retroperitoneal vessels which may then be cannulated for cold perfusion. The liver and small bowel, with or without the colon and additional foregut structures, may be removed separately or taken out as a combined graft with their central vascular structures in continuity. Further anatomical dissection and tailoring may be carried out in situ or on back-table dependent on the type of recipient operation that is planned. Decontamination of the donor bowel lumen with antibiotics may be carried out, but this has not been found to confer any advantage.[34]

Recipient operation

The intestine may be transplanted using three different approaches depending on the indication and graft type. An isolated intestinal segment may be transplanted alone (ISB) in the case of intestinal failure without IFALD. If the liver is irreversibly damaged, the intestine may be transplanted along with the liver (LSB). In the latter situation and in the case of mesenteric thrombosis or tumors, the intestine may be transplanted as part of a composite graft that may contain other viscera (MVT and MMVT).[21] The arterial inflow to an isolated intestinal graft is achieved by anastomosing the graft's superior mesenteric artery (SMA) to the recipient aorta. The venous drainage can be done systemically, with an end to side anastomosis between the donor superior mesenteric vein (SMV) and the recipient inferior vena cava or portally with the donor SMV anastomosed to the recipient SMV or portal vein.

In the case of MVT, the continuity of the portal axis is maintained, and the hepato-intestinal graft is drained through the liver veins like a standard liver transplant.[35] The graft may be transplanted with or without the ileocecal valve and the first part of the colon. The right colon down to the level of the mid transverse colon is vascularized through the SMA via its ileocolic, right colic, and middle colic branches, forming the most distal part of the splanchnic organ cluster. The inclusion of the colon in the graft is becoming increasingly common.[4,36] There is also some debate concerning the preservation of the recipient's native structures like the spleen and the pancreaticojejunal complex in order to retain as much native tissue as possible in the recipient.[17] These strategies add to the complexity of the surgeries and may only be suitable at high-volume centers. At the end of the intestinal transplant procedure, an ileostomy is performed in order to allow easier access to the small intestine for the purpose of rejection monitoring.[35]

Complications

A broad spectrum of surgical, infectious, and immunological complications are associated with intestine-containing transplant procedures (ISB, LSB, MVT, and MMVT). This is due to the complexity of the surgeries, the potentially contaminated content of the graft, the frequently compromised nutritional status of the recipients, and intensive immunosuppression. Ischemic injury to the graft predisposes to bacterial translocation and may increase the risk of rejection by priming the recipient's immune system to donor antigens. Local or systemic infections (bacterial, viral, or fungal) are almost universal after intestinal transplantation. This is due to the aforementioned translocation, as well as intraoperative contamination, leaks, indwelling catheters, or prolonged stay in the intensive care unit.[34,37,38] Viral infections (cytomegalovirus, adenovirus, or norovirus) are more frequent than in other types of transplants and mandate repeated screening for prompt identification and management as they may severely affect the intestinal allograft.[39] The immunological complications in

the form of acute rejection or GVHD are more common after ITx than other solid organ transplants. Rejection may progress rapidly with severe acute rejection being observed only within days from a histologically normal biopsy.[40] Intestinal acute rejection combines the involvement of T-cell-mediated injury targeting the epithelial cells (i.e., enterocytes, Paneth cells), and antibody-mediated acute injury primarily directed against the microvascular endothelium. Early rejection is the most significant risk factor for graft loss.[3] Most of these complications occur during the first months after transplantation. However, significant long-term complications are increasingly being reported, such as solid tumors, PTLD, GVHD, impaired renal function, chronic rejection, and protracted psychological strain.[11,41]

Results of intestinal transplantation

As of 2019, over 4100 transplants have been reported to the Intestinal Transplant Registry (ITR) from around the world. According to the ITR, current patient survival rates for ISB in adults are 77% and 53% at 1 and 5 years, respectively. While one-year graft survival has improved, long term results have not improved substantially over the last 20 years (Figure 2). The leading causes of death are infections and graft failure secondary to rejection.[41] Grafts that include a colon segment have a better function with higher rates of fluid and nutritional autonomy.[25] ITR data shows that the amount of grafts now being transplanted with a colon segment has increased from less than 10% in 2001 to over 50% in 2018. Patient survival after LSB transplantation is 73% and 60%, MMVT 72% and 48%, and MVT for all indications is 60% and 35% at 1 and 5 years, respectively. A recent review of MVT for metastatic NEPT gives patient survival figures of 81% and 40% at 1 and 5 years.[24] Graft survival for adult and pediatric patients divided by transplant era and transplant type are seen in Figure 2.

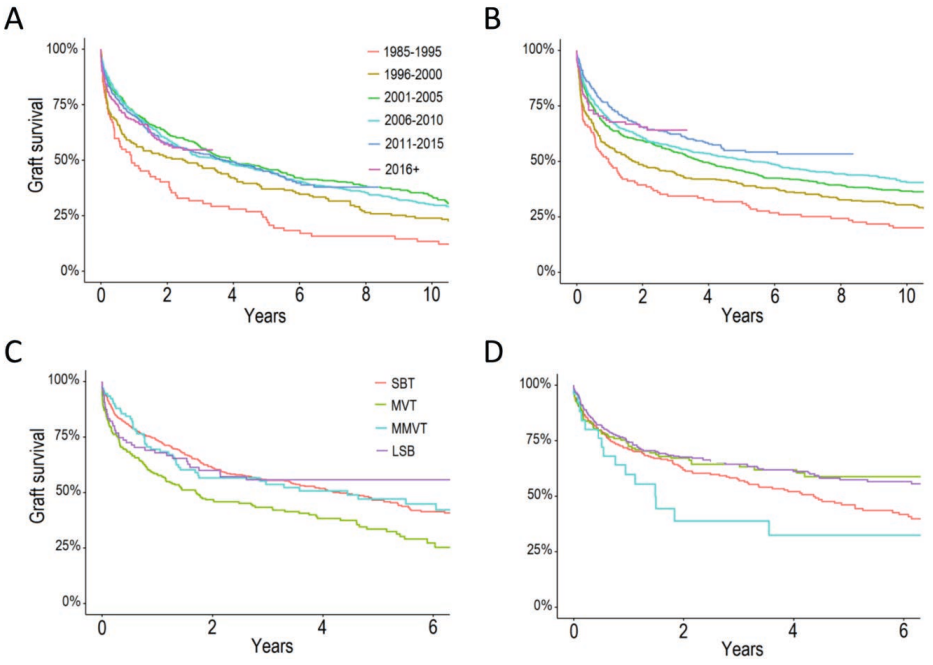

Figure 2. Graft survival data from the Intestinal Transplant Registry – May 2019 **(A)** Adult graft survival by transplant era. **(B)** Pediatric graft survival by transplant era. **(C)** Adult graft survival by transplant type 2009-2018. **(D)** Pediatric graft survival by transplant type 2009-2018.

INTESTINAL HISTOLOGY AND PHYSIOLOGY

Normal intestinal histology and physiology

The intestine is a hollow, tubular organ, lined on its luminal side by a monolayer of intestinal epithelial cells (IECs) with selective permeability. IECs are polar cells exhibiting different functions on their luminal and basolateral aspects. These serve as a barrier between the host and luminal microbes while allowing functions like absorption of nutrients and water. They sense both harmful and useful microbes and can induce and modulate immune responses.[42] The IEC layer consists of specialized cells that can be divided by functions: the absorptive enterocytes and the secretory cells, namely, Goblet cells (GCs), Paneth cells, and enteroendocrine cells (Figure 3).[43] These cells maintain the barrier and control crosstalk between the microbiota and the underlying gut-associated lymphoid tissue (GALT). The GALT consists of collections of B-cells, T-cells, plasma cells, macrophages and other antigen-presenting cells.[44]

The various cell types making up the epithelium have a rapid turnover. Intestinal epithelial stem cells (IESC) in the crypts provide renewal of the epithelial cell population, which migrates towards, and is shed from, the villus tip during their lifecycle. Paneth cells are also located in the crypts and produce bactericidal proteins that are secreted into the mucus layer.[42] The Paneth cells, along with mesenchymal cells, produce ISC stimulatory factors. Their function is essential for the regenerative capacity of the intestinal epithelium.[45]

Lamina propria

The lamina propria consists of loose connective tissue, which lies beneath the epithelium, and together with the epithelium and basement membrane constitutes the mucosa. It is rich in cells such as fibroblasts, lymphocytes, plasma cells, macrophages, eosinophilic leukocytes, and mast cells. It contains an extensive network of subepithelial capillaries and a central lacteal. Myofibroblasts in the lamina propria contribute to inflammation and wound healing responses. Myofibroblasts are capable of releasing cytokines and chemokines, and their contractile capacity pulls the epithelial sheets together when damaged. The lamina propria also has a strong osmolality gradient from the base to the tip, which facilitates the absorption of water especially in the apical parts of the villi.

The capillary 'hairpin' and the countercurrent exchange

An important morphological feature relevant to the development of ischemic injury, particularly during circulatory instability, is the hairpin configuration of the capillaries inside the villus. The afferent and efferent limbs of the capillary loop run in

close proximity along the length of the villus. This proximity allows a countercurrent exchange mechanism for oxygen[47] and electrolytes[48].

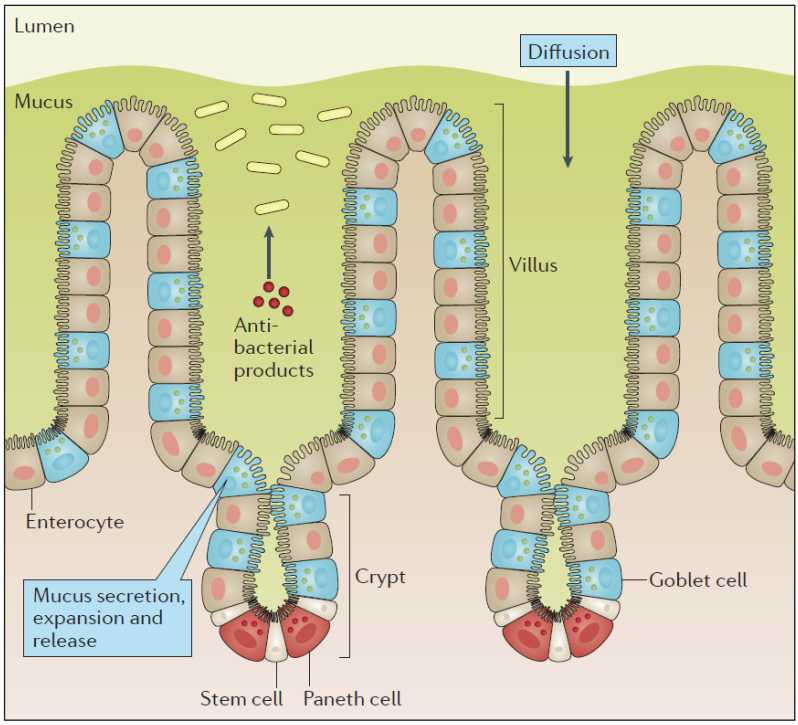

Figure 3. A general overview of the mucus layer and the most important cellular components of the intestinal barrier. Adapted *from Johansson et al.*[46]

Relative hypoxia is a normal physiological finding at the villus tip, compared to the more oxygen-rich environment at the villus base and crypts.[47] This provides some level of protection to the IESC-containing crypts when blood flow is rerouted from the splanchnic circulation in physiologic situations such as exercise, or in pathophysiologic situations such as hypotensive shock.

Electrolyte countercurrent exchange is thought to take place in villi, allowing isotonic blood to leave the villi regardless of the intraluminal osmolality. This results in an essential feature of the interstitium of the villi, namely a significant osmolality gradient from the villus tip to the base. [48]

The intestinal barrier

Several lines of defense achieve protection against the contaminated contents of the intestinal tract.

At the epithelial-luminal junction, a layer of mucus forms a physical and chemical barrier. It is mostly composed of expanded and hydrated mucin polymers secreted by GCs but also contains IgA antibodies, and antibacterial peptides secreted by Paneth cells. Mucus is secreted both continuously, at a slow rate, and rapidly in response to external stressors.[49] It prevents the adherence of microorganisms to the epithelium and thus impedes potential translocation.[50] The loose mucus found in the small intestine allows nutrient uptake while at the same time protecting the IECs against luminal aggression such as autodigestion by pancreatic enzymes[51], but it is easily displaced[52].

The intestinal epithelial lining provides the next line of defense. The enterocytes are connected by tight junctions that seal the apical part of the paracellular spaces and provide another physical barrier.[44] Maintenance of the intestinal epithelial lining is of major importance since the loss of this barrier facilitates the translocation of bacteria, causing inflammation and infection.[49]

The third line of defense is provided by the multiple types of immune cells contained in the intestinal tissue (GALT).[44]

The junctional complex and cytoskeleton

Enterocytes are held together by the junctional complex, which is connected to the contractile microfilaments of the cytoskeleton. This complex can be subdivided into three parts from the lumen facing apex to the base: the tight junction (TJ), the adherens junction, and the desmosome (Figure 4).[53]

The TJ is a complex multi-protein structure located at the boundary of the apical and lateral membrane surfaces of adjacent epithelial cells. It provides both intercellular adhesion and a paracellular seal. More than twenty different TJ-associated proteins have been described. The TJ protein ZO-1 controls not only intercellular contacts but also the actin polymerization machinery and contractility apparatus of the apically situated actin and myosin. Occludin is essential for providing tight junction stability and barrier function. Tricellulin is an occludin-like molecule responsible for sealing the TJ at tricellular contacts. The claudins can be divided into two main groups: those mediating permeability (claudin-2,-7,-12) and those increasing epithelial barrier properties (claudin-1,-3,-4,-5,-8). The TJs have a "gate function," which is to regulate the intercellular flux of ions, solutes, and water. They also have a "fence function," which is to maintain cell polarity and keep apical membrane proteins at the lumen-facing domain of the epithelial cell.[54]

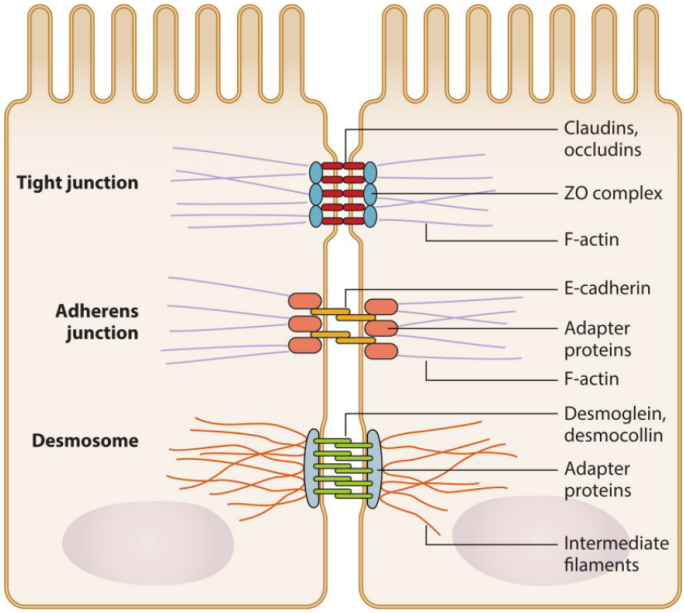

Figure 4. Epithelial cell junctional complex. Junctional complexes hold together adjacent epithelial cells. Tight junctions at the apical end of junctional complexes are composed of occludin and claudin proteins that span the intercellular space and bind intracellular adapter proteins, such as zonula occludens complex proteins. Adherens junctions are composed of E-cadherins and adapter proteins. Desmosomes are formed of desmoglein and desmocollin proteins that bind internal adapter proteins. Adapter proteins associated with tight junctions, adherens junctions, and desmosomes in turn bind components of the cytoskeleton, including F-actin or intermediate filaments. From *Hudson et al.*[55]

The adherens junctions are located on the basolateral membrane closer to the base and further connect the cells as well as the cells and the basal membrane. Both TJs and adherens junctions are connected to contractile fibers that help regulate barrier function.

Desmosomes are adhesive structures formed from dense protein plaques of two adjacent cells.[44]

Intestinal permeability

Intestinal permeability occurs through the transcellular and the paracellular pathways. The transcellular pathway is based on transmembrane channels, transporter proteins, and endocytosis. It mediates the transport of nutrients varying greatly in chemical structure and size. The paracellular pathway is involved in the transport of small molecules (<600 Da), ions, and solutes between epithelial cells. As long as the TJs are intact, they maintain the integrity of the paracellular pathway and prevent large molecules from passing. Water is absorbed via both pathways.

PATHOPHYSIOLOGY OF GRAFT INJURY

Mechanisms of injury to the intestinal graft

The process of brain death, circulatory instability, and finally, the interruption of blood flow to an organ leads to complex molecular and cellular alterations. Depending on their cumulative severity, these alterations can cause irreversible damage and loss of function. Paradoxically, the restoration of blood flow could further aggravate the injury.

Injury to the graft can be divided into three stages:

1. Injury in the donor (brain death, hypotension, cardiac arrest)
2. Cold ischemic damage
3. Ischemia-reperfusion injury

Injury to the intestine in the donor

Brain death causes several changes in the cardiovascular, pulmonary, endocrine, and immunological systems that need to be managed skillfully in the intensive care unit. An upregulation of cytokines such as interleukin-6 and tumor necrosis factor-α (TNF-α) initiates a systemic inflammatory response[56], which is detrimental to the intestine[57]. Circulatory instability, cardiac arrest, and vasoconstrictive drugs impart ischemic insults to the intestine.

Cold ischemic injury

Molecular and cellular events

Under normothermic conditions, metabolically active cells maintain constant adenosine triphosphate (ATP) levels. When the circulation is interrupted, ATP levels rapidly decline. Maintaining the viability of a graft during its transport from donor to recipient is mainly based on hypothermia. Hypothermia reduces the metabolic rate but does not stop metabolism. In the uncirculated graft, this residual level of metabolic activity creates an imbalance between energetic supply and demand.

During hypothermic ischemia, the cells react in the following ways:

1. Hypoxia causes ATP depletion by the suppression of oxidative phosphorylation.
2. ATP depletion causes the failure of ATP-dependent ion pumps and secondary

active ion transporters. Decreased activity of membrane Na^+/K^+ ATPase contributes to cell edema by allowing a net influx of Na^+ and water.

3. Hypoxia favors anaerobic glycolysis. This method of energy generation, both yields less ATP than oxidative phosphorylation, and leads to increased lactic acid levels and intracellular acidosis. This causes an influx of Na^+ via the Na^+/H^+ exchanger.
4. Formation of membrane pores that allow an additional influx of Na^+ are caused by an agglomeration of membrane proteins.[58]
5. The inhibition of endoplasmic reticulum Ca^{2+} ATPase, caused by a lack of ATP, exacerbates intracellular Ca^{2+} accumulation.
6. Increased intracellular Na^+ concentration inhibits the Na^+/Ca^{2+} antiporter due to the decreased Na^+ gradient. The increase in cytosolic Ca^{2+} levels results in mitochondrial Ca^{2+} overload. This affects mitochondrial membrane permeability leading to mitochondrial swelling and pore formation, which is associated with proapoptotic events.[58,59]
7. Hypothermia causes an increase in the cellular chelatable, redox-active iron pool capable of producing highly reactive oxygen species. The target of this iron is mitochondria, contributing, along with Ca^{2+} overload, to the triggering of the mitochondrial permeability transition.
8. The mitochondrial permeability transition is initiated by the opening of a pore in the mitochondrial membrane leading to mitochondrial swelling, eventually causing the rupture of the outer membrane. The pore destroys the proton gradient over the inner membrane allowing cytosolic ATP to enter the mitochondrial matrix. ATP is cleaved by ATP-synthase, which has reversed direction in the absence of a proton gradient, thereby consuming what little cellular ATP was left. Furthermore, the mitochondrial pore allows the release of proapoptotic mitochondrial inner-membrane proteins, such as cytochrome c.
9. Cytochrome c forms a complex with various cytosolic proteins resulting in an "apoptosome," which leads to the activation of caspase 9, thus initiating the intrinsic apoptosis pathway.
10. Hypoxia inhibits the formation of reactive oxygen species (ROS) despite the increase in chelatable iron. Notably, this inhibition lasts only until reperfusion.[58]
11. Increased levels of intracellular phosphate ions promote additional Ca^{2+} and Na^+ ion intake, which worsens the osmotic state of the cell, further deteriorating mitochondrial structure and lysosomal integrity.
12. Hypoxia and ATP depletion cause an accumulation of hypoxanthine, which, together with xanthine oxidase, is a source of ROS after reperfusion.
13. Hypoxia impairs endothelial cell barrier functions by increasing vascular permeability and leakage. This is due to a decrease in adenylate cyclase activity,

causing lower cyclic adenosine monophosphate (cAMP) levels.[60]

14. Hypothermia causes cytoskeletal degradation. The structure of microfilaments becomes disorganized, and microtubules depolymerize. Intermediate filaments are less affected.[61]
15. Cell damage causes extracellular ATP release. This induces a procoagulant phenotype in microvascular endothelial cells. It also stimulates platelet aggregation.[62]
16. Cell death signaling pathways are activated. The residual intracellular ATP level, which is related to the duration of the ischemia, functions as a "switch" between apoptosis and necrosis. After prolonged ischemia, the apoptotic signal is blocked, and necrosis develops. Cells undergoing necrosis release various mediators, which further stimulate the inflammatory response.[43,63]

Cold ischemic events specific to intestinal endothelial cells and villi

During ischemia, the microcirculatory countercurrent mechanism of the villus is interrupted, and a decrease in the villus-tip hyperosmolality is observed.[64] This decrease in osmolality is likely due to an influx of water through impaired paracellular channels leading to a decrease in the osmolality of the villus. Fluid collects in the space between the IEC layer and the hyperosmolar lamina propria. The lack of circulation in the subepithelial capillaries combined with a reduction in the activity of energy-dependent ion pumps makes it impossible to clear this excess fluid away. It accumulates most rapidly in the distal villus tip, due to this location having the most substantial osmotic gradient, and then gradually engages the rest of the villus in a proximal direction.

Due to its location between the base of the IEC and the lamina propria, this excess of fluid affects the basal aspects of the IECs first. Sodium and water passively enter the base of the IEC "in reverse." The cells are unable to rid themselves of this excess sodium with its attendant water since the Na^+/K^+ ATPase, which usually transports sodium out of the basal aspect of the cell and towards the subepithelial capillaries, is inhibited. This leads to swelling and ultimately rupture of the basal part of the enterocyte.[65]

Hypothermia substantially reduces metabolic activity, thus increasing the resistance of organs and tissues to lack of oxygen and delays many of the changes noted above.

The role of preservation solutions is to intervene in as many of these changes as possible, but they achieve this imperfectly. The inflammatory reaction caused by reperfusion is triggered by the early hypoxia- and cold-induced injuries.

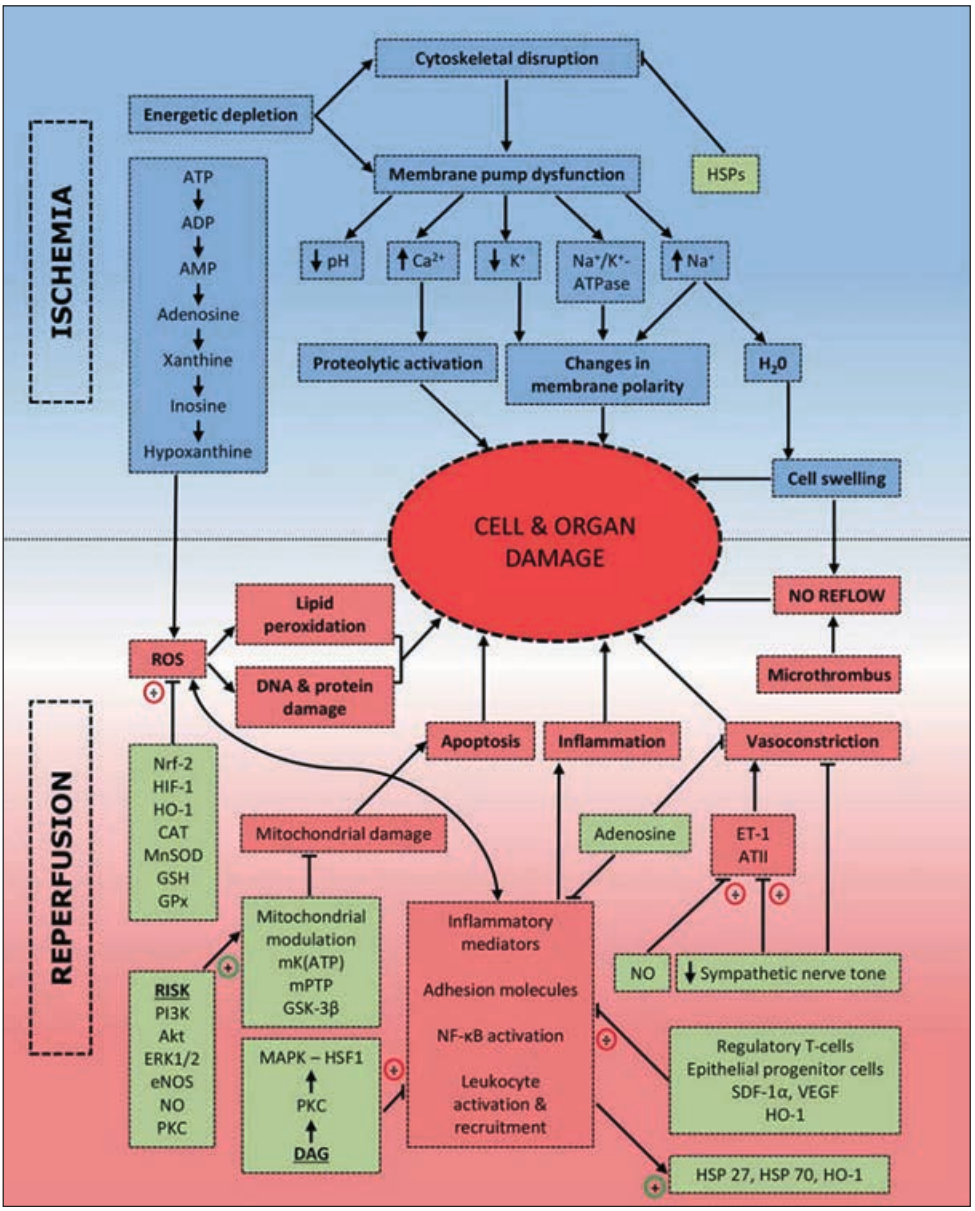

Figure 5. The main pathophysiological events during ischemia and reperfusion. Both stimulatory and inhibitory effects are depicted (denoted by the symbols + / green circle or ÷ / red circle, respectively). From *Kierulf-Lassen et al.*[66]

Ischemia-Reperfusion Injury (IRI)

The imbalance in metabolic supply and demand in the ischemic graft during the cold storage period results in tissue hypoxia and microvascular dysfunction. The

subsequent reperfusion augments the activation of the innate and adaptive immune responses.[67]

Reactive oxygen species (ROS)

After reperfusion, the earliest response is the generation of ROS. Reintroduction of oxygen paradoxically enhances cell injury due to alterations that take place during the hypoxic period. The most important of these changes takes place in the mitochondrial respiratory chain, where leakage of electrons causes the univalent reduction of oxygen and the formation of the superoxide anion radical (O^-_2). Superoxide dismutase catalyzes the conversion of two O^-_2 into molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). H_2O_2 is membrane permeable and can be degraded by catalase or glutathione peroxidase. When it instead reacts with iron, it forms the highly reactive hydroxyl radical, which can damage almost any cellular molecule in its immediate vicinity.[58] Enhanced ROS production has been detected within 20 seconds of reperfusion and can inflict damage to cellular components such as nucleic acids, proteins, mitochondria, and cellular membranes. ROS can cause damage directly to cellular components by causing oxidative stress, or they can act as mediators propagating and amplifying the inflammatory response. The stress caused by ROS may lead to necrosis, apoptosis, or trigger changes in cellular phenotypes in response to the injury (activation).

Upregulation of inflammatory mediators

Hypoxia and oxidative stress cause several transcription factors to be activated. Of particular importance for the innate immune response is nuclear factor-κB (NF-κB).[68] NF-κB has a central role in inflammation and innate immunity by regulating the expression of genes leading to the production of numerous important inflammatory mediators such as adhesion molecules, metalloproteinases, interleukins, tumor necrosis factor and colony-stimulating factors.[67]

Barrier injury allows the luminal contents to interact with the immune system

Cold ischemic damage alone, or in combination with IRI, may disrupt the epithelial barrier. Barrier disruption allows contact between luminal contents and submucosal structures, triggering a more severe inflammatory response (Figure 6). Microorganisms in the lumen express microbe-associated molecular patterns (MAMPs). When the integrity of an enterocyte is disrupted, damage-associated molecular patterns (DAMPs) like nucleic acids, heat-shock proteins (HSPs), and high-mobility group box chromosomal protein 1 (HMGB1) are released. DAMPs and MAMPs are recognized by pattern-recognition receptors (PRRs) on epithelial and immune cells, eliciting an inflammatory response.[67] This leads to increased expression of intracellular adhesion molecule-1 (ICAM-1) on the vascular endothelium. Neutrophils bind to this receptor and migrate to the villi and accumulate below the damaged epithelial lining. Their

function is to sterilize the wound. This is done by participating in phagocytosis and by releasing enzymes such as myeloperoxidase, which leads to the production of ROS.[6]

In the intestinal GCs, MAMPs elicit a secretory response in order to clear microbes from the immediate vicinity of the epithelial border.[69]

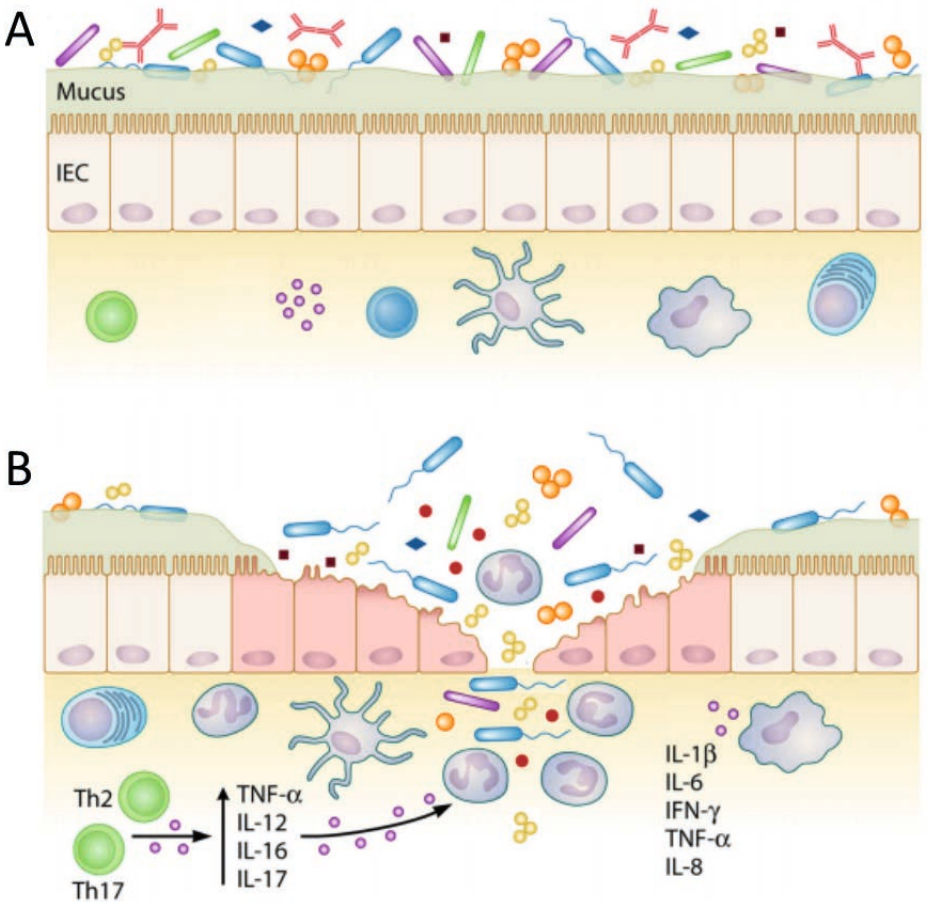

Figure 6. The intestinal mucosa before and after an ischemic barrier disruption with luminal content consisting of nutrients, microbiota, pancreatic enzymes, and bile salts. **(A)** The healthy mucosa is characterized by an intact barrier with healthy epithelial cells and a layer of mucus containing antimicrobial peptides; few immune cells; and a cytokine milieu dominated by anti-inflammatory cytokines. **(B)** Disruption of the barrier leads to interaction between luminal contents and the submucosal tissue of the intestine, causing a severe inflammatory host immune response. Th2 cells cause a humoral response. Th17 cells stimulate IL-17 production. Abbreviations: IEC - intestinal epithelial cell; IFN - interferon; IL - interleukin; Th -2/17, T-helper cells; TNF - tumor necrosis *factor. Modified from Hudson et al.*[55]

Lipopolysaccharide (LPS) or endotoxin, a component of Gram-negative bacteria membranes, binds to a PRR called Toll-like receptor 4 (TLR-4). This interaction causes the release of cytokines from monocytes and macrophages. LPS and TNF stimulation also increase apoptosis. LPS is capable of activating adaptive alloimmunity against graft antigens and may partly account for the high rejection rate and the difficulty to induce graft acceptance after ITx.[70]

Crosstalk between the innate and adaptive immune system

The primary response to IRI is by the innate immune system, but multiple pathways exacerbate the alloimmune response toward the graft resulting in a high rate of rejections. Complement factors cause T-cell priming and also contribute to tissue damage by chemokine and cytokine release leading to the recruitment of neutrophils, macrophages, and platelets. Translocating MAMP endotoxin (LPS) sensitizes the immune system towards graft rejection. Plasma proteins of the innate immune system bind to ischemic cells and allow recognition by antibodies resulting in complement activation and inflammation.

Increased activity in antigen-presenting cells increases sensitization to donor antigens. In these ways, the severity of the initial damage following IRI primes the adaptive immune system, increasing the risk of rejection.[6]

Damage to Paneth cells and stem cells

Ischemia causes endoplasmic reticulum stress in Paneth cells due to their high secretory activity. Paneth cells are essential in maintaining epithelial barrier defenses by secreting antimicrobial proteins into the luminal mucus layer, thereby playing a role in preventing bacterial translocation. IRI injury causes Paneth cell apoptosis and consequently impairs host defenses. While stem cells are generally quite ischemia resistant, the loss of Paneth cells may also impair the repair process since they produce factors vital for the survival of stem cells in the crypts.[45]

Mitogen-activated protein kinases (MAPK)

The MAPK superfamily consists of three main protein kinase families. Extracellular signal-regulated protein kinases (ERKs), the c-Jun NH2-terminal kinases (JNK), and the p38 family of kinases. Their name is a bit of a misnomer since most MAPKs are actually involved in the cellular response to potentially harmful stress stimuli such as hyperosmosis, oxidative and temperature stress, DNA damage, low osmolarity, and infection.[71] Upon activation, MAPKs translocate into the nucleus and activate various transcription factors regulating apoptosis, cell survival, or inflammation. JNK is considered to be part of the signaling cascade during apoptosis and necrosis[72],

whereas p38-MAPK has been linked to the pro-inflammatory response[73]. Both p38-MAPK and JNK are activated by phosphorylation during ischemia.[74]

Activation of cell death programs

Cell death programs are activated during cold storage and are further stimulated by IRI. How cell death is initiated is of consequence for the magnitude of the inflammatory response it generates.

Necrosis is an unregulated, passive, and energy-independent form of cell death. The cells break down in an uncontrolled manner and release their contents into the surrounding tissues, leading to a severe inflammatory response. Necrosis typically happens in response to severe ischemia or external noxious stimuli.

Apoptosis is a regulated, active, and energy-dependent form of cell death, which is crucial in order to remove damaged and old cells. It is particularly important in the intestine where the stem cells in the crypts are continually churning out new cells that migrate towards the apex of the villus. Cell death is an exceptionally tightly controlled process under normal circumstances.[43] Signals may trigger apoptosis from within the cell or by extracellular signals. These intrinsic and extrinsic pathways are different in their initial stages, but their main drivers, namely a family of cysteine proteases, converge when caspase-3 is activated.[63]

No-reflow phenomenon

Vascular endothelial cells are damaged by cold preservation and have been implicated in microcirculatory disturbances posttransplantation.[75] After IPI, endothelial cells alter their surface phenotype to one that is prothrombotic. Cell damage leads to the release of ATP into the extracellular environment. Extracellular nucleotides like ATP are early stimulators of inflammatory responses by endothelial cells, leading to platelet aggregation and microthrombi formation, thereby causing microvascular occlusion (i.e., no-reflow phenomenon).[76] The resultant lack of blood flow exacerbates local ischemic tissue injury. These responses are modulated by ectoenzymes that hydrolyze extracellular nucleotides to their respective nucleosides. The dominant ectonucleotidase expressed by the endothelium is CD39. Importantly, the biochemical activity of CD39 is lost at sites of acute vascular injury, such as that which follows IRI.[62]

MORPHOLOGY OF GRAFT INJURY

Histological features during intestinal ischemia

Ischemic injury to the intestine initially presents as progressive subepithelial edema, which first is apparent at the villus apex (Chiu-Park score Grade 1) and then extends towards the villus base, cleaving the epithelium from the lamina propria (Grade 2-3). These spaces are due to the detachment of the IECs from the basal membrane. When reperfusion is delayed, this will ultimately lead to the breakdown of the mucosal barrier and loss of villus tissue (Grade 4-5). Extended ischemia leads to changes and structural alterations in the deeper mucosal layers. Injury to the crypts will negatively affect the stem cell niche consisting of ISCs, Paneth cells, and mesenchymal cells, leaving no chance for regeneration and recovery (Grade 6-7). Finally, the damage becomes transmural, in which case the bowel will perforate after reperfusion (Grade 8). This sequence of events has been demonstrated during both normothermic and hypothermic ischemia and forms the basis of the Chiu-Park score for grading of the ischemic intestinal injury.[77-79]

Ultrastructural features during intestinal ischemia

The TJs become disrupted early on during the ischemic process. This leads to a paracellular influx of ions and water. At the base of the IEC, a zone of cytoplasm, virtually free of organelles, develops and enlarges[80] due to an influx of fluid in the infranuclear portion of the cells[65]. Intercellular fluid accumulates exerting a wedging action between cells and causing lifting of the epithelium from the basement membrane. This is visualized histologically as subepithelial edema. Ultimately, the intercellular membranes are fractured, leading to the release of the cells from the lamina propria, and their extrusion into the lumen of the bowel.[65]

During ischemia, the mitochondria swell, the endoplasmic reticulum dilates, microvilli shorten and degenerate, and the cytoplasm becomes vacuolated and lucent with decreased cytoplasmic granules (Figure 7).[81]

Grading intestinal morphological injury

Morphological injury to the intestine is assessed on formalin-fixed, hematoxylin and eosin (H&E) stained sections. The ideal grading system for ischemic injury should parallel the morphologic appearance of injury, with increasing severity of the insult. It should also be quick to learn, easy to use, and reliable, producing consistent results within and between observers.[79] While several scoring systems exist[82-84], the combined system of Chiu and Park[77] is demonstrated to be the most suitable grading system for ischemic intestinal damage (Table 2).[79]

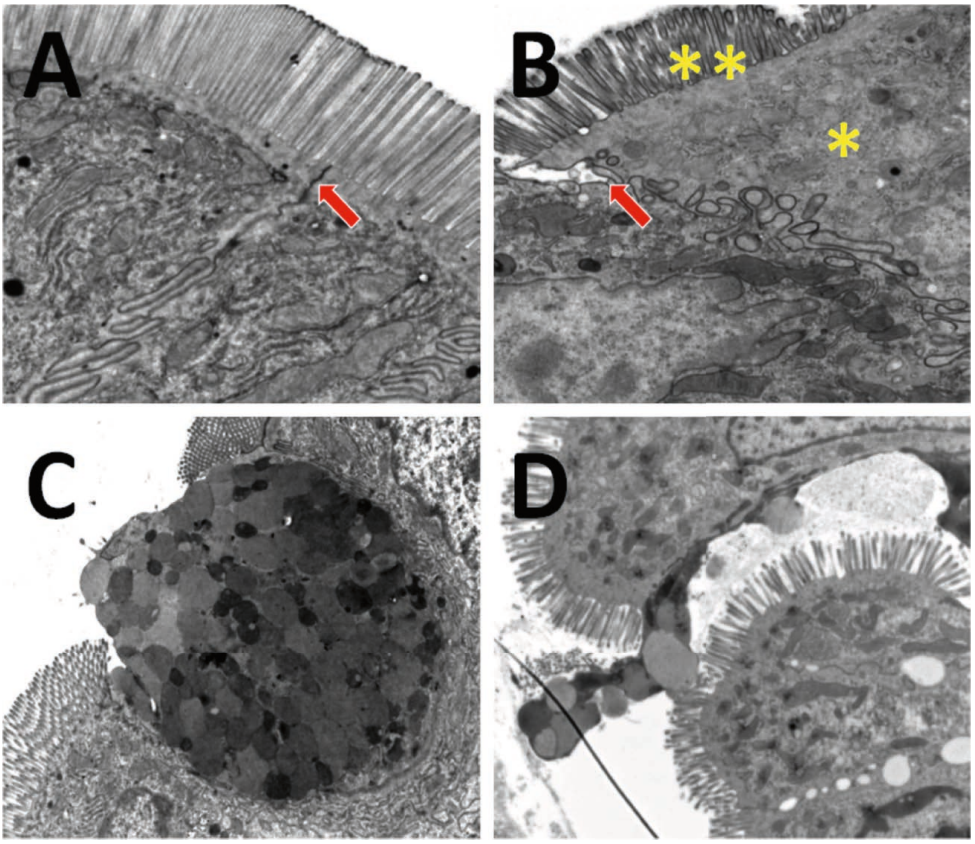

Figure 7. Electron micrographs of rat intestinal cells. **(A)** The apical side of two IECs prior to ischemia. The red arrow marks the intact TJ. **(B)** The apical side of two IECs after 14 hours of cold ischemia. Signs of ischemic damage include: Disruption of TJ (red arrow), lucent cytoplasm with decreased cytoplasmic granules (*), shortening and degeneration of microvilli (**) and degeneration of cell organelles. **(C)** GC with intact mucin granules. **(D)** GC discharging granules in response to ischemic stress. Pictures provided courtesy of Anna Casselbrant.

Table 2: The Chiu-Park grading system used for the evaluation of the preservation injury. From *Park et al.*[77]

Chiu-Park grading system			
Score	Description	Score	Description
0	Normal mucosa	5	Loss of villus tissue
1	Subepithelial space at villus tip	6	Crypt layer infarction
2	More extended subepithelial space	7	Transmucosal infarction
3	Epithelial lifting along villus sides	8	Transmural infarction
4	Denuded villi		

Morphological features during reperfusion

After suffering an ischemic insult, the subsequent reperfusion can paradoxically increase tissue injury and contribute to severe inflammation. After reperfusion, the damage incurred during cold ischemia increases by 1-2 grades on the Chiu-Park scale.[51,68,85,86] The damage is seen first at the villus tips and then, with increasing injury severity, progresses towards the crypts. Up to a certain level of damage, physiological repair mechanisms can rapidly (within 1 hour) return the mucosa to a morphologically intact state and quickly replenish the protective mucus layer.[85] The detachment of epithelial cells at the villus tip does not necessarily cause a breach in the lining. The lamina propria retracts and contractile (non-muscle myosin) fibers pull the remaining sheet of epithelial cells closed, sealing the wound like a purse-string, and the distal portion is shed (Figure 8).[51]

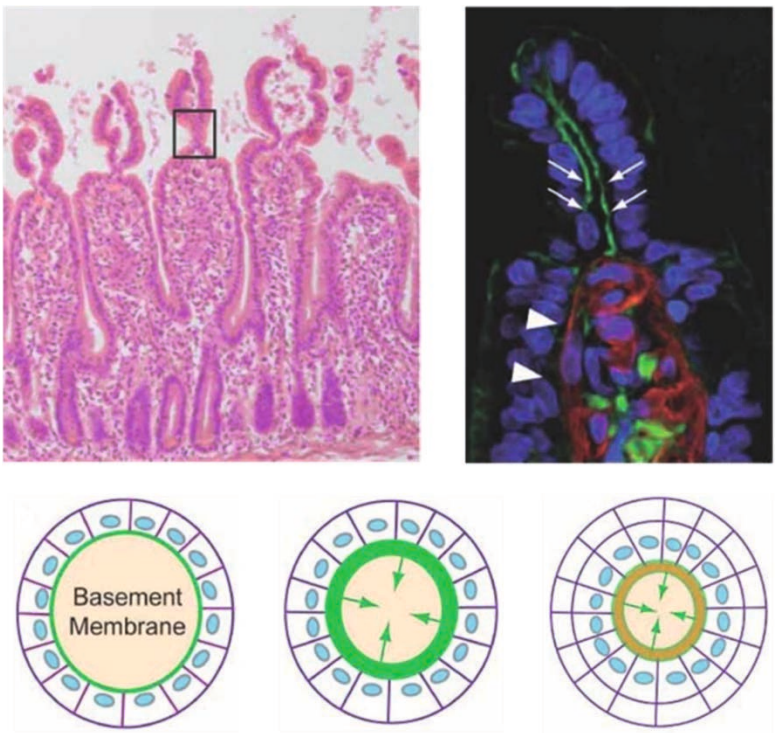

Figure 8. Lamina propria retraction and purse-string closure of the epithelial defect. **(A)** H&E section showing constriction of the epithelial sheet, preventing denudation of the basement membrane. **(B)** Green staining indicates phosphorylated myosin light chains (pMLC) (white arrows), which denotes active contraction. No such contraction can be seen basally to the detached epithelium (white arrowheads) **(C)** Schematic representation of a transverse section, demonstrating how pMLC-mediated contraction reduces the virtual wound surface, limiting the exposure of the lamina propria to potentially harmful luminal content. Adapted *from Grootjans et al.*[87]

This orderly process is a physiological adaptation that may be overwhelmed in the transplant setting for three reasons. Firstly, the oxygen tension gradient in the villi selectively protects the crypt level from ischemia.[47] This allows the epithelium on the distal part of the villi to be discarded and replenished by the ISCs in the crypt. In contrast, during cold storage of a graft, all layers are ischemic, and the damage is more equally distributed. Secondly, the insult from the cold storage period may be severe enough to completely disrupt the epithelial covering of the lamina propria bringing it into contact with luminal contents. Lastly, purse-string closure of epithelial defects is mediated by contractile fibers, which are energy-dependent. During cold storage, there is not enough energy to close even small defects. Mucosal defects allow contact between luminal contents and the lamina propria, which in turn sets off a more severe immune response following reperfusion, further propagating tissue damage.

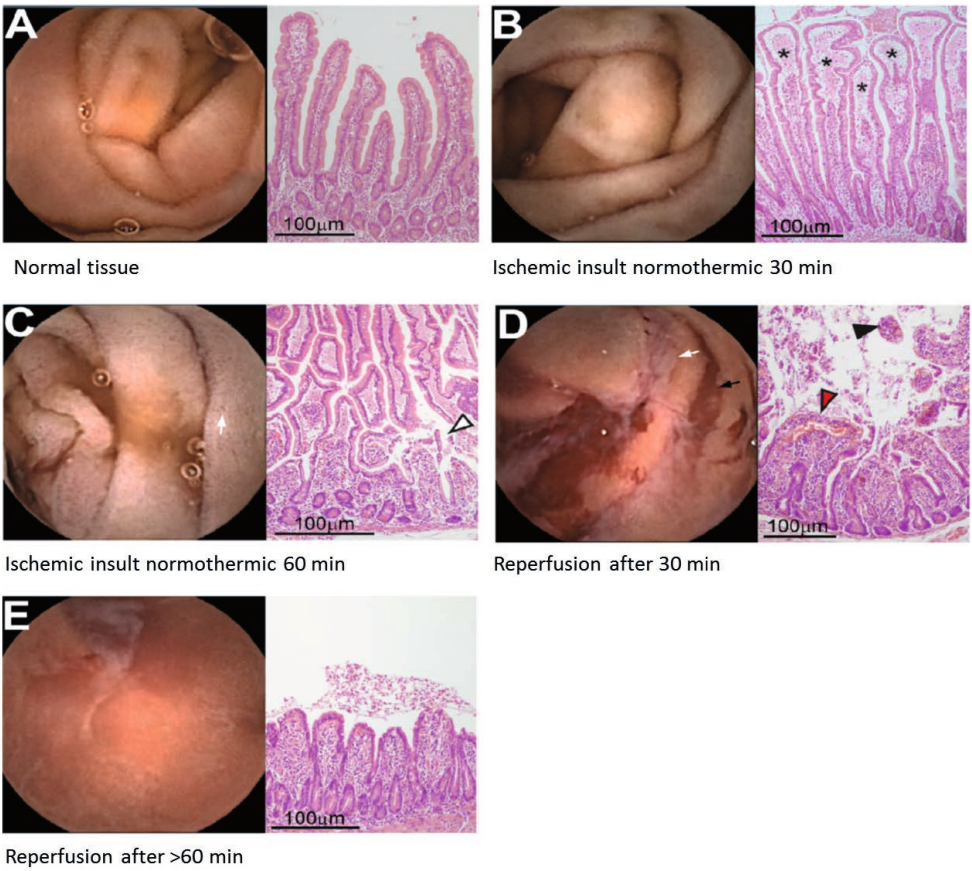

Figure 9. Concordance between the endoscopic and histological appearance of IRI. **(A)** Normal endoscopic view and histological appearance. **(B)** After 30 minutes of normothermic ischemia, subepithelial spaces appear (*), indicating retraction of the basement membrane. This relates to the pale discoloration of mucosa in the endoscopic view. **(C)** After 60 minutes of normothermic ischemia,

breaches in the epithelial lining can be observed (arrowhead), which relates to the appearance of punctate lesions in the endoscopic view (white arrow). (D) After 30 minutes of reperfusion, hemorrhage and shedding of IR-damaged epithelial cells becomes evident in both histological view (right panel: red arrowhead and black arrowhead, respectively), and endoscopic view (left panel: black arrow and white arrow, respectively). (E) After 60 minutes of reperfusion, villus atrophy is observed in conjunction with gaps in the epithelial lining and luminal debris. This relates to the loss of villous structure in the endoscopic view and the 'white clouds' of intraluminal debris. Adapted *from Grootjans et al.*[85]

As shown in Figure 9, after reperfusion, there is an initial worsening of the injury.[68,85,86,88] Following this, the repair process should ideally lead to the shedding of the distal epithelium followed by purse-string closure, resulting in shortening and flattening of the villi. However, if crypt damage occurs, repair becomes more difficult. If ischemic damage is severe (affecting the crypt layer), complete restoration is unlikely, and chronic changes are likely to occur.[86,89]

PROTECTIVE STRATEGIES

Organ preservation

A transplantable organ is exposed to multiple insults on its way from the donor to the recipient, which may negatively influence its function. In the donor, the organ will be influenced by the events surrounding the donor's death.[56,57] This may be a period of circulatory instability or cardiac arrest and resuscitation. The procurement operation involves a period of relatively warm ischemia prior to storage in cold preservation solution. The cold storage period imparts damage proportional to its length.[32,90] The transplantation itself involves a period of gradual rewarming and increasing metabolic demands and accelerating ischemic damage. The accumulated damage incurred up to the point of reperfusion sets the stage for the severity of the reperfusion injury.[58] The purpose of organ preservation strategies is to limit the damage incurred by an organ prior to reperfusion in the recipient. Limiting preservation injury is, currently, only moderately successful.

Preservation modalities

Organ preservation may be static or dynamic, hypothermic or normothermic. Simple static cold storage (SCS) is the primary method for the preservation of most organs. Hypothermic machine perfusion has acquired a robust evidence base in renal transplantation[91,92] and is currently being implemented clinically in liver transplantation, albeit with modest evidence of benefit[93]. Normothermic perfusion techniques have been around for decades but have not been generally adopted clinically yet. There has recently been a resurgence of interest in these techniques, with many interesting experimental findings being published. They have yet to acquire a sufficiently robust evidence base to warrant the increased cost and complexity of routine clinical implementation. With modern pump systems, increased compactness, and advanced sensors and software control, they do, however, show considerable promise for the future.

Oxygenation of intestinal grafts during SCS with a perfluorocarbon as an oxygen carrier has shown some promise in experimental studies.[94,95]

Regardless of preservation modality, the procurement of organs during the donor operation from deceased donors is currently preceded by an infusion of chilled preservation solution through the arterial circulation in a volume sufficient to clear blood and cool the organs substantially (10-15°C) before final removal from the body. The technique currently most common for the preservation of organs is still SCS.

While not an organ preservation technique per se, normothermic regional perfusion (NRP) is increasingly being utilized in donation after circulatory death (DCD) programs and provides normothermic circulation locally in the donor's abdominal aorta. NRP is followed by the introduction of standard cold preservation solutions. It is a promising alternative to rapid recovery techniques with excellent results for kidneys and livers.[96,97] At present, no intestines procured and transplanted after NRP have been reported.

The effects of hypothermia

In clinical organ transplantation, cooling is the first line of defense against hypoxic injury. Cooling is necessary to reduce cellular metabolism and oxygen-requirements in order to prevent tissue injury. While metabolic rate is substantially reduced, some activity is retained.[98] Most enzyme systems show a 1.5-2.0 fold decrease in activity for every 10°C decrease in temperature. A decrease from 37°C to 2°C should, therefore, reduce metabolism significantly but does not stop it. Ischemia progresses, causing cellular damage, albeit at a slower rate. Moreover, structural changes to the cytoskeleton progress during cold ischemia. Despite this, hypothermic organ preservation remains the current mainstay of organ preservation.

Alleviating IRI in the intestinal graft

As with other transplantable organs, intravascular flushing via arterial cannulation using an acellular preservation solution has been the main approach for intestinal preservation. The IEC layer is sensitive to ischemic injury, and its disruption is a source of severe postoperative morbidity.

Hypothermia slows ATP depletion and reduces anaerobic glycolysis, thus reducing the rate of ischemic damage. The damage does continue, albeit at a slower rate. Therefore, reducing CIT is one of the most important measures to reduce subsequent IRI[32] and improving long term graft survival[90].

Perfusion solutions are designed to counteract the effects of hypothermic ischemia, particularly, the electrolyte shifts secondary to membrane pump dysfunction (mainly the Na^+/K^+ ATPase).

A vascular preservation solution should have the following properties:[98]

1. Prevent osmotic water shifts and reduce hypothermia-induced cell swelling.
2. Prevent acidosis
3. Prevent interstitial edema

4. Attenuate oxidative stress
5. Provide energy and maintain ATP levels

While commonly used preservation solutions generally follow these principles, they differ in composition (Table 3).

Table 3. The contents of the vascular preservation solutions UW, HTK, and IGL-1® and the laxative Movicol® PEG 3350.

Contents of solutions					
	UW	HTK	IGL-1	Movicol	
Sodium (mmol/L)	30	15	125	65	cation
Potassium (mmol/L)	125	10	25	5,4	cation
Magnesium (mmol/L)	5	4	5	-	cation
Calcium (mmol/L)	-	0,015	-	-	cation
Chloride (mmol/L)	20	30	-	53	anion
Sulphate (mmol/L)	5	-	5	-	anion
Lactobionate (mmol/L)	100	-	100	-	anion, impermeant
Phosphate (mmol/L)	25	-	25	-	anion, impermeant
Raffinose (mmol/L)	30	-	30	-	Impermeant
Ketoglutarate (mmol/L)	-	1	-	-	Energetics, impermeant
Adenosine (mmol/L)	5	-	5	-	Energetics
Tryptophan (mmol/L)	-	2	-	-	Energetics, Antioxidant
Glutathione (mmol/L)	3	-	3	-	Antioxidant
Allopurinol (mmol/L)	1	-	1	-	Antioxidant
Histidine (mmol/L)	-	198	-	-	Buffer
Bicarbonate (mmol/L)	-	-	-	17	Buffer
Mannitol (mmol/L)	-	30	-	-	Osmotic agent
Hydroxyetyl starch g/L)	50	-	-	-	Osmotic agent
PEG-35000 (g/L)	-	-	1	-	Osmotic agent
PEG-3350 (g/L)	-	-	-	105	Osmotic agent
pH	7,4	7,0-7,2	7,4	8,1	
Osmolality (mOsm/kg)	320	310	290	240	

Solutions currently in use for intestinal transplantation

Several preservation solutions are used for intestinal graft preservation, apparently with similar results despite their widely differing contents.[99]

University of Wisconsin (UW) cold storage solution revolutionized abdominal organ preservation in the late 1980s and still represents the gold standard. Its composition resembles the intracellular milieu and contains a high potassium concentration (125 mmol/l) in addition to high-molecular-weight impermeants such as lactobionic acid and raffinose which prevents intracellular edema secondary to ischemia. It provides oncotic support with hydroxyethyl starch (HES) and possibly mitigates oxidative stress with the redox agents: glutathione and allopurinol. Phosphate acts as a hydrogen ion buffer. The main drawback of UW, besides being surprisingly viscous, is its high potassium content, which requires a flush before reperfusion to avoid recipient hyperkalemia.

Histidine-tryptophan-ketoglutarate solution (HTK) uses histidine as a buffer and ketoglutarate as an impermeant. Its electrolyte composition resembles the extracellular milieu with high sodium and low potassium and therefore requires no flush. HTK solution provides similar results to the UW solution. It has low viscosity and clears blood more easily from small vessels than UW.[99]

IGL-1® is a newly designed preservation solution for abdominal organs. It has almost identical ingredients as the UW solution, with the exception of the electrolyte composition, which combines low potassium and high sodium content avoiding the requirement for a pre-reperfusion flush. It also uses a high molecular weight PEG instead of HES as an oncotic agent. In liver transplantation, IGL-1® has demonstrated comparable results to UW, but no data is available on the results of intestinal preservation using this type of solution.

Luminal preservation

The luminal route is an attractive opportunity for additional organ protection. Luminal interventions could dilute and evacuate the intestinal content, introduce molecules that protect the intestinal barrier, and delay osmotically driven fluid shifts between the lumen and the villi. Delivering substances that maintain cell metabolism could diminish certain aspects of the ischemic injury.

Multiple experimental studies have explored each of these rationales.[100] The introduction of any solution for luminal preservation dilutes and, depending on the protocol, may remove intestinal contents. Teasing out the effect of dilution and the removal of contents would require a control group with a solution that does not affect cold ischemic tissues. The composition of such a solution is not immediately apparent. In the absence of metabolically active processes in the IEC layer, solutions may have other effects than expected. Attempts with UW[101,102], a high-sodium laxative[103], and a low-sodium electrolyte solution[104] were unsuccessful.

Based on these studies, it is apparent that net fluid shifts can originate from the lumen despite adequate osmotic support being provided in the vascular compartment. This is likely due to alterations in mucosal permeability characteristics, caused by cold storage.

Ischemia causes a dilation of TJs, which may allow the hypertonic milieu in the villi to pull water in, as evidenced by histological and ultrastructural findings.[65,77,78]

Attempts to protect the IEC layer with low-sodium PEG-based solutions have been successful in experimental models.[103-106] Besides luminal oncotic support, possible explanations include the clearing and diluting of intestinal contents, protecting TJs, stabilizing the mucus layer, preventing the activation of membrane epitopes, and forming a protective hydrogel and stabilizing the IEC membranes.

Intraluminal delivery of nutrient-rich preservation solutions has also repeatedly resulted in superior morphology after extended preservation times and in some cases, improved bioenergetics.[102,105,107,108] This suggests that addressing the luminal compartment using a nutritive approach may prove beneficial in terms of both maintaining acceptable graft morphology and allowing the safe prolongation of the cold ischemia time. Since the cellular interactions maintaining the mucosal barrier, including the functioning of tight junctions, are energy-dependent processes, nutritive approaches may represent a promising strategy.[100] To take the logical step and combine the methods to both protect the IEC layer from external insult while simultaneously supporting metabolism is theoretically appealing.

Polyethylene glycol (PEG)

PEGs are synthetic, non-branched, uncharged, and hydrophilic polymers. They have been in medical use for several decades and are considered safe and physiologically inert. PEGs have a variety of everyday uses in medicine, from drug delivery systems to laxatives. There has been considerable interest and success in using PEGs in the context of organ preservation, but their mechanism of action is debated. The physical and chemical properties of PEGs depend on their molecular mass and linear conformation.

PEGs are not absorbed in the gastrointestinal tract, are nontoxic, resist digestive enzymes, have no effect on bacterial enzymes, and are accurately measurable. Surprisingly small amounts of PEG are required to replace the osmotic activity of other solutes (e.g., mannitol) – i.e., less than can be explained by molecular weight and number of molecules.[109] In the typical physiological setting, PEG substantially reduces sodium and water absorption in the lumen despite the large osmotic gradient between the lumen and the villi.[110] Water absorption is prevented by PEG completely if luminal sodium concentrations are low.[111] One of the most used medical products

is Golytely®, a high-sodium PEG 3350 formulation used for bowel preparation before a colonoscopy. This formulation was used by the Pittsburgh group for lavage of the intestinal graft prior to intestinal transplantation in the early 90s but later abandoned due to edema formation.[30,112] Later work has elucidated the importance of the sodium concentration in PEG solutions in the setting of IPI.[103]

It is believed that the capacity of PEGs to form compact structures and their hydrophobic character increase with the molecular mass.[59] These properties would recommend high molecular mass PEG solutions as more suitable for forming protective hydrogel films, yet beneficial effects have also been reported with PEG solutions with molecular masses ranging broadly between 400 and 20,000 Da.[113]

PEG solutions may bind to epithelial cells, changing fundamental barrier function properties such as surface hydrophobicity and epithelial permeability.[114] PEGs have been found to protect the intestinal epithelium against Pseudomonas aeruginosa sepsis[115], radiation damage[114], bile-salt mediated barrier disruption[116], and decrease microbial interactions with intestinal epithelium[117].

Immunocamouflage is the concept by which non-immunogenic molecules, such as PEG, create a barrier between allogenic epitopes on the cell membrane and the circulating cells and antibodies of the recipient[59]. It may, however, be necessary for PEG to covalently bind to the cell membrane to realize this particular effect[118,119].

AGING OF THE INTESTINE

The small intestine develops age-related morphological changes. Older intestines seem to develop a progressive intestinal barrier dysfunction, which may be associated with mucosal atrophy, damage to TJ structure, and altered TJ protein composition. Histological changes associated with aging include shortened and scattered villi, decreased mucosal thickness, decreased villus height and width, and a reduced villus density in aging rats[120], but the literature is inconsistent[121]. Altered TJ integrity is one of the main mechanisms behind the progression of IPI.[103] Intestinal epithelial barrier function, and its resistance to IPI may decrease with advancing donor age.

Intestinal homeostasis and regeneration are maintained by IESCs located in the crypts. In the aging intestine, stem cell activity and regeneration are impaired. Some of the mechanisms suggested are DNA damage in the IESCs themselves and reduced stimulatory signaling activity from Paneth cells and mesenchymal cells in the stem-cell niche.[45] Also, stem cells are influenced negatively by systemic circulating factors, which increase with senescence.[122]

AIMS OF THE THESIS

The specific aims of this thesis were:

- To compare how preservation injury develops in rat, porcine, and human tissues.
- To study, in a rodent model, if the size of PEG molecules affects luminal preservation.
- To investigate, in a rodent model, if the age of an intestinal donor affects the preservation injury incurred during cold ischemia and to evaluate the effect of luminal preservation in various age groups.
- To assess whether luminal preservation of the human small intestine is protective like that observed in animal studies.

MATERIALS AND METHODS

The main methods used in this thesis are briefly described and discussed below. More detailed descriptions of protocols are found in the "Materials and methods" sections of papers I-IV.

Animals

Rats (Paper I, II and III)

Sprague-Dawley (SD) rats of around three months of age were used for all rat studies. Additionally, for Paper III, nine-months-old rats were purchased and kept in the University Animal Quarters until they reached the desired age (14 and 20 months). Animals had unrestricted access to water and rat chow and were not fasted before surgery in any of the studies or groups. The studies were reviewed and approved by the local committee of the Swedish Animal Welfare Agency (#135/07 and #1040/2017).

Pigs (Paper II)

Landrace pigs of either sex (n = 7), weighing around 30 kg, were housed and operated on at the Pius Branzeu Center in Timisoara, Romania. All experiments were reviewed and approved by the Ethics and Deontology Committee for Research on Animals of the University of Medicine and Pharmacy, Timisoara, Romania (13008/9 May 2013).

Human organ donors

In Paper II and IV, human intestinal segments were obtained from deceased brain dead (DBD) multiorgan donors who donated organs for clinical transplantation and received the standard management for organ donors. The donors (or next of kin) previously consented for tissue use for medical research. The use of human tissue in this study was reviewed and approved by the regional ethical review committee (Dnr 204-17).

Surgical Procedures

Intestinal procurement and luminal preservation in rats (Paper I, II, III)

Rat intestines were obtained after in situ perfusion with 8-10 mL cold preservation solution. The aorta was isolated and ligated above the bifurcation and above the emergence of the superior mesenteric artery. Retrograde, in situ perfusion of the intestine through the infrarenal aorta was initiated within 10 seconds of the aortic ligation. Luminal preservation was performed as previously described by the Edmonton group.[102,123] In brief, cold PEG solution was gently flushed through the lumen, and the

effluent was allowed to freely exit the distal end until clear (approximately 10 mL). The end of the intestinal segment was then ligated with 3-0 silk, and a further 2-3 mL of the solution was infused, leaving the bowel filled without turgor and allowing an even distribution of the solution throughout the entire length of the harvested graft. The intestinal content was left in place in the control groups. Graft ends were tightly ligated with 3-0 silk ligature, and the intestines were stored in ice-chilled perfusion solution.

Intestinal procurement in pigs (Paper II)

Following colectomy, the portal vein and the SMA were isolated. The entire jejunoileum was then perfused with 1.5 L of cold HTK solution (Custodiol®) through the infrarenal aorta. The venous effluent was vented by opening the diaphragm and cutting the right atrium.

Intestinal procurement and luminal preservation in humans (Paper II, IV)

Organ procurement was done in a standard fashion with retrograde aortic perfusion using HTK solution (Paper II) or IGL-1® (Paper IV). Venous effluent was vented via a suction tube placed in the inferior vena cava. The last meter of the small intestine was resected immediately after in situ perfusion. Bowel ends were stapled off, and a segment of the procured intestine was directly placed in an organ isolation bag with cold preservation solution without any luminal intervention (Paper II and control group in Paper IV).

Paper IV had an experimental group with luminal preservation. Through a small incision near one of the stapled ends, a low sodium PEG 3350-containing solution (Movicol®, Norgine, UK) was introduced in the lumen of the intestinal segment and the intestine was gently filled, then flushed free of solution and contents. The procedure was repeated once while taking great care to handle the intestine only by its mesentery. After that, the length of the bowel was measured, and a solution volume corresponding to 1 mL/cm was introduced in the intestine. Finally, the opened end was stapled off, and the bowel was placed in an organ isolation bag with cold IGL-1®.

Preservation solutions

The small intestine was perfused with and stored in University of Wisconsin solution (Paper I), HTK solution (Paper II and III) and IGL-1® (Paper IV). The contents of the three solutions are detailed in Table 3. Luminal preservation was performed using a commercially available solution (Movicol®, Norgine, UK) in Papers I, III, and IV. Customized solutions where PEG-3350 was replaced by either PEG-10000 or PEG 20000 while maintaining an identical electrolyte content with Movicol® were used in Paper II.

Histology, histochemistry, and immunohistochemistry

Formalin-fixed, paraffin-embedded tissue was used throughout the study for standard histology, histochemistry, or immunohistochemistry. The ischemic injury was assessed on sections stained with hematoxylin and eosin (H&E) using the Chiu/Park score (Table 2).[77] Intestinal mucosal GCs were stained using Alcian Blue (Paper I,II,III), and the positive cells from the villus-crypt junction to the villus tip were counted on multiple fields and sections. Assessment of age-related changes in organs other than the intestine (Paper III) was performed using H&E, elastin-Van Gieson, and von Kossa stains (for the aorta), or Periodic acid Schiff (PAS) staining (for the kidney). Neutrophil staining (Paper II) was performed using Naphtol AS-D chloroacetate esterase. Immunohistochemistry was used to study the TJ proteins zonula occludens-1 (ZO-1) (Paper I,II, and IV) and claudin-3 (Paper II and IV) as well as active caspase-3 as a measure of apoptosis (Paper I and II)).

Western blot

Western blot protein analysis was used for the semiquantitative assessment of several types of proteins. In Paper I, Western Blot analysis has been used for studying the activation (phosphorylation) of two mitogen-activated protein kinases (MAPK): p38 and JNK2. In Paper II, the method has been used for the study of several TJ proteins (claudin-3, claudin-4, tricellulin, ZO-1, occludin) and that of cytokeratin-8. In Paper III, the technique was employed for the semiquantitative assessment of claudin-3, claudin-4, tricellulin, and occludin.

Ussing chamber

An Ussing chamber system allows the measuring of the barrier and transport functions in epithelial tissues. In this setting, enterocytes function similarly to resistors in an electrical circuit coupled in parallel. Damage to the integrity of an enterocyte, analogous to the loss of a resistor, leads to a decrease in overall resistance and, therefore, a decrease in potential difference (PD). In Paper I and III, whole-thickness rat small intestine was mounted in conventional Ussing chambers (Warner Instruments, Hamden, CT, USA), and where the electrophysiologic parameters PD and epithelial electrical resistance (Rep) were measured over a period of 20 minutes. The epithelial ion current (Iep) was calculated via Ohms law (Iep=PD/Rep). The Ussing chambers have also allowed the study of transepithelial permeability for fluorescein sodium salt (FSS; Mw-376kDa) (Sigma-Aldrich, Stockholm, Sweden) or fluorescein isothiocyanate-dextran (FD4; Mw-4000kDa) (Sigma-Aldrich).

Lyophilization and assessment of edema

In Paper IV, full-thickness intestinal samples with a weight of around 250mg were freeze-dried for 24 hours using the Lyovac GT 2 (Leybold-Heraeus, GmbH, Germany).

They were weighed before and immediately after the freeze-drying process. The results were expressed as:

$$\%\ H_2O \text{ in the tissues} = \left\{\left(1 - \frac{\text{Dry weight}}{\text{Wet weight}}\right) \times 100\right\}$$

Statistical methods

Nonparametric methods were used for statistical comparisons. Statistical differences between independent groups were calculated using the Kruskal–Wallis test corrected for multiple comparisons using the Tukey test, followed by the Mann–Whitney U test (GraphPad Prism6; GraphPad Software, La Jolla, CA). Changes within groups were tested with Friedman test for repeated measures, followed by the Wilcoxon test. Differences between groups, in the frequency of areas with severe histological damage, were evaluated using the Chi-square test (WinSTAT® add-in for Excel, Robert Fitch Software, Cambridge, USA). Data were presented as median (range) unless otherwise stated. Results were considered statistically significant at $p < 0.05$.

RESULTS

Paper I - Luminal polyethylene glycol alleviates intestinal preservation injury irrespective of molecular size

Microscopic examination revealed significant villus injury and subepithelial edema already after 4 hours in intestines that were treated with vascular perfusion alone (the control group - group 1) while all three groups (group 2-4) of intestines receiving luminal treatment showed very discrete changes (Figure 10). After eight and 14 hours, changes in the LP groups were still discrete, whereas the injury progressed in the control group. The size of the PEG molecule used did not yield a significant difference in injury score at any time point.

The same trend held true when assessing GCs. A significant decrease over time was found in the control group but not in the three LP groups. No difference was found between LP groups.

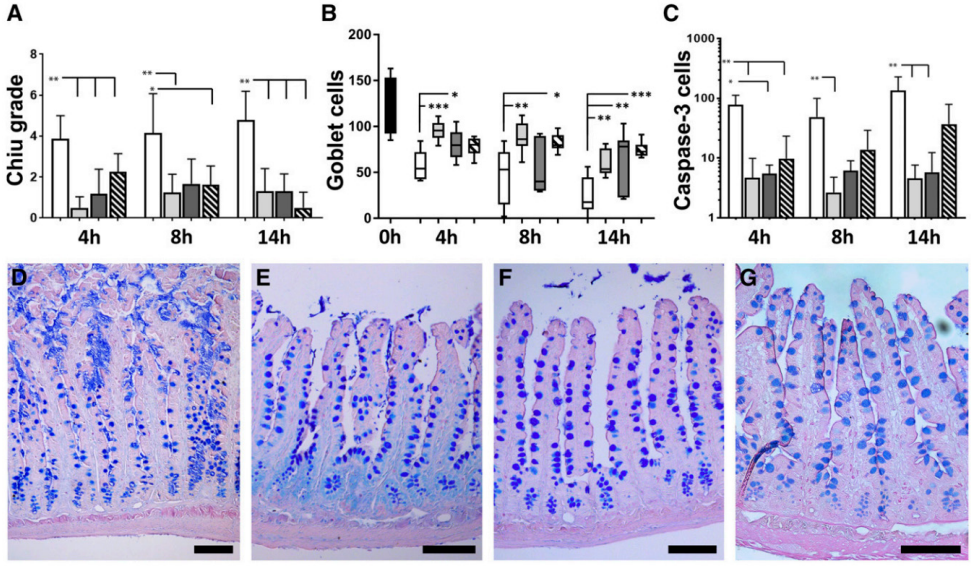

Fig. 10. Microscopic evaluation of the intestinal grafts. **(A)** Preservation injury evaluated using the Chiu/Park grading scale. **(B)** Summary of the goblet cell count using Alcian Blue staining. **(C)** Enterocyte apoptosis quantified by caspase-3–positive cells in group 1 (no luminal preservation, white bar), group 2 (PEG 3350 Da, light gray bar), group 3 (PEG 10,000 Da, dark gray bar), and group 4 (PEG 20,000 Da, hatched bar). **(D)** Representative microphotographs of Alcian blue staining showing severely damaged villus architecture, goblet cell depletion, and luminal mucus discharge in group 1. **(E–G)** Maintained villi and mucus-repleted goblet cells in **(E)** group 2, **(F)** group 3, and **(G)** group 4 after 14 hours of cold preservation. Original magnification 100×; scale bar 100 μm. *$p < 0.05$; **$p < 0.01$ ***$p < 0.001$.

Cells positive for active caspase-3, indicating apoptosis, were infrequent in normal tissue. In the control group, the number of positive cells increased rapidly with increasing CIT. The groups receiving LP had markedly less apoptotic activity at all time points.

Normal intestines revealed a thin, continuous reticular fluorescent signal outlining the mucosal contour at the most apical part of the basolateral membrane representing ZO-1. The signal was detected from the crypt bottom to the tip of the villus. In the control group, this signal was absent in the distal third of the villus after 4 hours but well preserved in the LP groups. Progressive changes were found at the later time-points in all groups, but they developed more slowly in the LP groups compared to control.

Phosphorylated p38-MAPK was found throughout cold storage in the control group and the two LP groups with the smaller PEG sizes (groups 1-3). The maximal activation (highest ratio between phosphorylated and total p38) was found after 4 hours of preservation irrespective of the luminal treatment. At this point, intestines in group 3 had significantly lower p38-MAPK activation compared with group 1. At later time points, groups 1–3 revealed minimal p38-MAPK activation at the same levels found in normal, non-ischemic tissue.

Phosphorylation of JNK occurred during cold storage with a maximal activation after 4 hours of preservation and, similarly to p38-MAPK, decreased at later time points.

In the LP group with the largest PEG molecule, both p38-MAPK and JNK could also be detected, but the immunobands were fuzzy and poorly defined. Furthermore, phosphorylation of these proteins could not be detected at all.

Electrical parameters decreased from the baseline value to the end of the experiment, indicating an increasing epithelial injury. After 4 hours of preservation time, both PD and Iep were significantly lower in group 1 and group 2 compared with groups 3 and 4 as well as with fresh tissue. After 8 and 14 hours of cold storage, PD and Iep decreased further in group 1 (>50%), undergoing vascular perfusion alone, compared with groups 3 and 4. The Rep was lower in all groups compared with fresh tissue (p-value <0.05) and did not differ between groups, except for the 8h time point when Rep was higher in groups 3 and 4 compared with groups 1 (controls, no luminal treatment) and 2 (PEG-3350). In summary, the electrophysiological characteristics were improved by the LP, although there was no clear superiority for any of the PEG solutions used.

The permeability of the FSS probe increased with the preservation time without showing any consistent differences between the various groups. The permeability of FD4 remained low and comparable with the fresh intestinal tissue, irrespective of the preservation time and solution.

Paper 2 – Intestinal preservation injury: A comparison between rat, porcine and human intestines

After cold-storage of intestinal segments, subepithelial lifting and edema were found in all species. However, significant differences were noted in the extent and timeline of these alterations. Rat intestines developed subepithelial edema and epithelial shedding already after eight hours of CIT, whereas the porcine intestines showed the lowest degree of injury with mild edema or even normal histology. At all time-points, human intestines exhibited a lesser injury than in rats, but higher than in pigs (Figure 11).

GC counts also indicated different patterns between the three species. Control rat intestine had a higher number of GCs than pig intestine. In rat and human intestines, GCs decreased over time, whereas the amount of GCs in porcine intestines did not differ significantly from the baseline throughout the entire experiment. Normal pig intestines had significantly higher enterocyte density and significantly fewer polymorphonuclear neutrophils (PMN) in the villi compared to rat and human intestines.

In all three species, ZO-1 was detected as an intense, thin fluorescent signal at the most apical part of the basolateral membrane, from the crypts to the villus tips. Claudin-3 was visualized as a thin, reticular signal along the entire basolateral membrane colocalizing with ZO-1 at the most apical part of the basolateral membrane. Rat intestines lost ZO-1 staining at the apex after 8 hours, while human and pig intestines did not. At this time point, Claudin-3 became hazier in rats but retained its sharp appearance in humans and pigs. These changes progressed at 14 hours in rats only. After 24 hours, all rat intestines completely lacked villus staining for ZO-1, while both porcine and human intestines revealed immunofluorescent staining frequently reaching villus tips. In both pig and human intestines, claudin-3 revealed more diffuse, discontinuous staining along the basolateral membrane with an evident subjunctional staining gradient.

All proteins analyzed by Western blot were detected in rat, pig, and human samples; generally, all proteins studied were found to have the lowest expression in the rat small intestine. After eight hours of cold storage, the expression of claudin-3, claudin-4, tricellulin, and ZO-1 was significantly higher in human samples compared to rat samples. This difference persisted after fourteen and 24 h for claudin-4 but subsided

for claudin-3, tricellulin, and ZO-1. In four out of six TJ-proteins studied (occludin, tricellulin, claudin-3, ZO-1), no differences between species were found after 14 h and 24 h of cold storage.

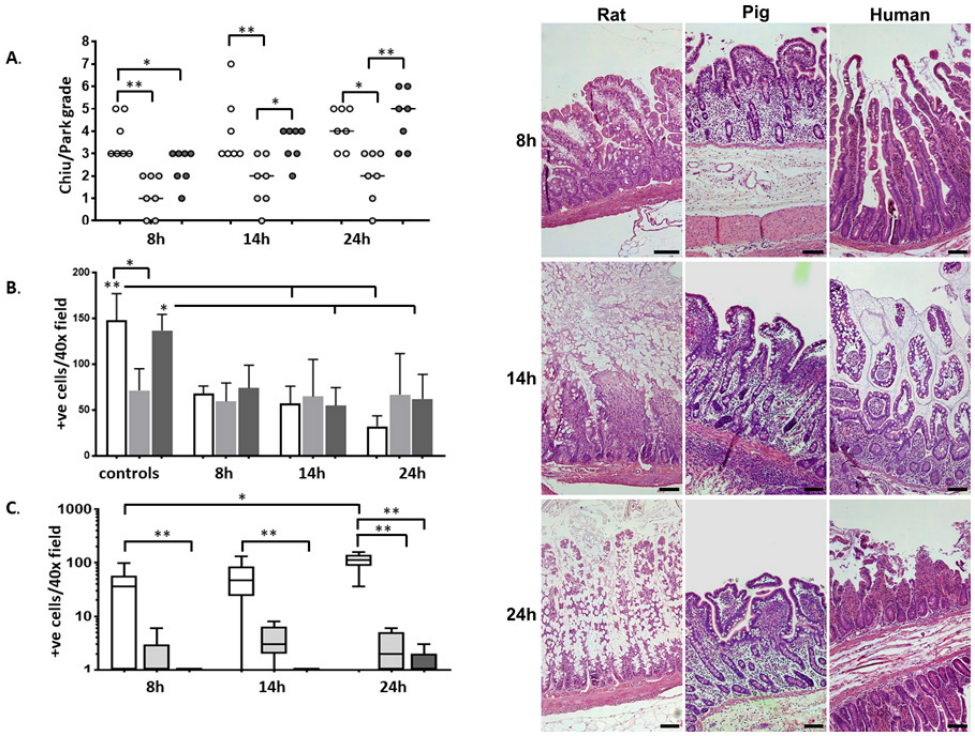

Figure 11. Light microscopy of rat (white), pig (light grey), and human (dark grey) intestines after different periods of cold storage (CS). **(A)** Summary of the tissue injury (Chiu/Park score) induced by the CS with each dot representing one individual (n=7) and the bar showing the median value. **(B)** Goblet cell count. **(C)** Enterocyte apoptosis quantified by caspase- 3 positive cells (box plot showing the median, 5-95 percentile, and lowest and highest values at each time point). * $p < 0.05$, ** $p<0.01$. A large number of apoptotic enterocytes (positive for active caspase-3) were found in rat intestines after 8h of CS. Rat intestines had more apoptotic enterocytes than human intestines at all time points (Fig.1C). Right: representative microphotographs from each species at each of the three time-points (H&E stain, original magnification x100, scale bar 100 µm).

Paper 3 – The impact of age and luminal preservation on the development of intestinal preservation injury in rats.

The evolution and severity of the injury score were similar in the grafts from young, adult, or old rats when only vascular perfusion was applied. However, the addition of LP significantly lowered the injury score after 4h of cold storage for old rats. After 8h and 14h of cold storage, the addition of LP significantly reduced the injury score in

all three age groups. No difference in GC count was found between age groups at any time point, but LP delayed mucosal GC depletion in all age groups (Figure 12).

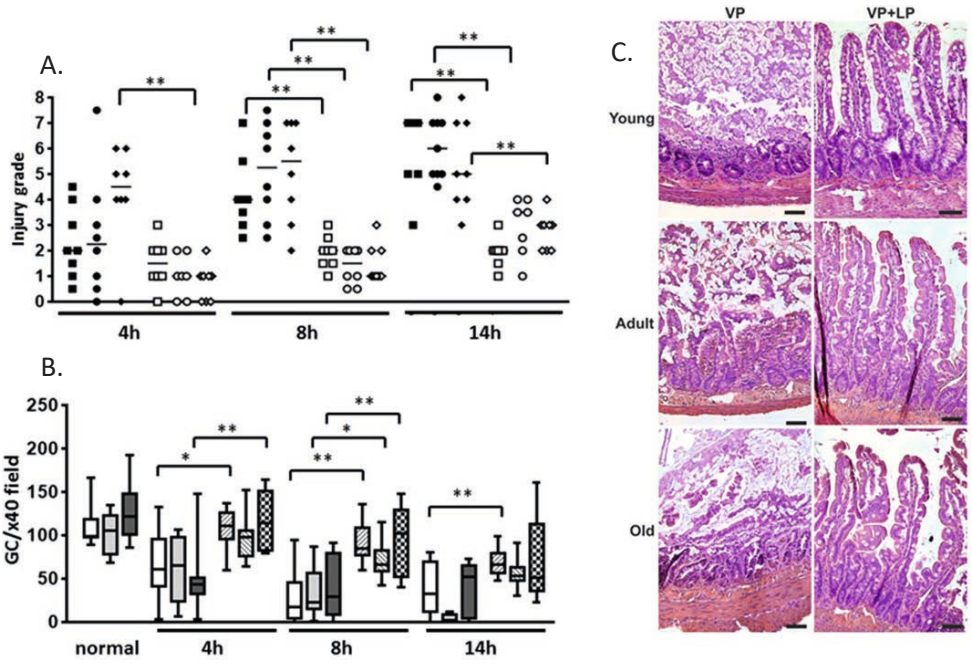

Fig 12. Light microscopy examination of the intestinal grafts after cold storage. **(A)** The tissue injury induced by the intestinal cold storage in young (square), adult (circle), and old (diamond) donors undergoing vascular perfusion (VP, closed symbols) only or combined vascular and luminal preservation (LP, open symbols).; **(B)** summary of the GC count using Alcian Blue staining; **(C)** representative microphotographs from the last time point (14 hours), original magnification x200, scale bar 50 μm, * $p<0.05$, ** $p<0.01$

Villus density decreased significantly with increasing age; no differences in villus length, villus width, or mucosal thickness was found between the three age groups. Aortas from old animals (20 months) displayed a reduced thickness of the vascular medial layer compared to the aortas of young animals (3 months). The number of medial elastic fibers, as well as the number of vascular smooth muscle cells, was also reduced in the aortas of old rats. They also showed microcalcifications in the media layer, resembling Mönckeberg's arteriosclerosis. The kidneys of old rats also revealed histological signs of both glomerulosclerosis and tubulosclerosis.

In the Ussing chamber experiments, the PD decreased with increasing preservation time in all groups. The PD was significantly higher in all the groups receiving LP at 4h and 8h. After 14h, this trend was weaker. Epithelial resistance (Rep) mirrored PD findings and decreased with longer preservation time. Intestinal permeability increased with time in all groups regardless of age. The permeability for the FSS

probe increased with longer preservation time. FSS-probe permeation tended to be highest in untreated old intestines when compared to old intestines with LP. The FD4-probe permeability increased with longer preservation time in both young and old intestines.

In normal intestines, Western blot protein analysis found several age-related differences in TJ protein expression: adult intestines had significantly higher tricellulin expression than young- and old intestines; old intestines revealed significantly lower occludin expression compared to young intestines. The expression of all four TJ proteins studied decreased over the fourteen hours of preservation, irrespective of age, or luminal treatment. LP did not confer any meaningful difference.

Paper 4 – Luminal preservation of the human small bowel using a polyethylene-glycol based solution

Please refer to the manuscript in the last section for complete results. Results of unpublished works may not be included in this part of the thesis due to copyright issues in advance of journal submission.

In short, the results of a preliminary analysis indicated an improvement of the histological score and a reduction in caspase- 3 and 9 activity when using LP compared to controls. The morphologic improvement was significant only after 4 and 24 hours of cold storage ($p<0.05$). There was possibly a trend towards lesser injury in the grafts receiving LP after 8 and 14 hours ($p = 0.05$ and $p = 0.06$, respectively).

GENERAL DISCUSSIONS

Cold storage of intestinal grafts causes a time-dependent breakdown of the mucosal barrier. This sets the stage for subsequent IRI, whose severity is principally dictated by the extent of the cold storage injury. A significant mucosal injury develops within several hours, limiting the time the small bowel can be stored outside the body before transplantation. This short, poorly tolerated CIT creates a temporal and logistical challenge. Any unforeseen difficulties in performing the recipient evisceration or the backtable graft preparation can cause delays that prolong the CIT of the intestinal graft.

Does PEG size influence the quality of preservation in luminal solutions?

Previous experimental work by our group has found that intraluminal use of a low-sodium PEG solution can ameliorate cold storage injury.[103,104,124] This effect was found to be due to the addition of PEG to an electrolyte solution since an identical solution without PEG was significantly less efficacious.[104] The size of the PEG molecule influences its biochemical properties[59], with larger PEGs theoretically being able to confer additional benefit. We evaluated this hypothesis in Paper I by using three different molecular weights of PEG in an otherwise identical solution, in the setting of LP. Similarly to our previous papers, the current results showed that luminal solutions with PEG significantly ameliorated the magnitude of tissue damage associated with IPI. The molecular mass of the PEG molecule did not influence the results in a meaningful manner in most parameters assessed.

One putative protective mechanism could be the formation of a protective hydrogel at the luminal interface limiting prolonged mucosal contact with intestinal contents, as well as the interaction of the PEG molecules with both the mucus layer and the enterocytes.[59] The interaction of PEGs with biological surfaces is a well-known phenomenon.[59,109,115,125,126]

Another possible mechanism of action ascribed to PEG could be the pegylation of the cell surface proteins (i.e., camouflage). Surface modification of cells with a PEG-containing solution has been demonstrated to impair the signals initiating T-cell activation, cytokine production, and T-cell proliferation.[119] Similarly, vascular perfusion and storage with a PEG-based solution appeared to protect kidneys from monocytes/macrophages and $CD4^+$ T-cell infiltrations after reperfusion, further suggesting that PEG creates a barrier that prevents the recognition of antigenic sites in the recipient.[127] In one of the very few studies combining ITx and PEGs, perfusion and storage of porcine small intestine using IGL-1® has resulted in improved survival

at day 8 after transplantation (50% vs. 16%), less caspase-3 activity 2 hours after reperfusion as well as reduced overall incidence of acute rejection (38% vs. 84%).[128] The real significance of these results is unclear, as the preservation time was very short (below 5 hours), and many of the animals expired already by day 4 supposedly due to acute rejection.

Given the change in physicochemical properties with increasing molecular weight, several studies have assessed whether the protective properties of the various PEGs depend on the size of the PEG molecule. A study comparing the effect of two PEGs with high molecular weight (30kDa and 50kDa) indicated that PEG30kDa provides better protection following extended cold preservation in a porcine kidney autotransplantation model.[129] The same group later showed that smaller size PEG (20kDa) also provided similar protection.[127] It is important to note that the two PEG-based organ preservation solutions currently in use are both based on high molecular weight PEGs (20kDa and 35 kDa). The concentration of PEG in the solutions varies greatly (15g/L for PEG 20kDa in SCOT® vs. 1 g/L for PEG 35 kDa in IGL-1®).[130]

A final explanation could be that the PEGs we used, despite their relatively small molecular weight, were able to act as colloids in the lumen. Being able to maintain their osmotic/oncotic properties in the lumen would delay fluid shifts from the lumen to the villi, despite impairment of TJs due to energy depletion. If this is the case, then PEGs have unique properties, not necessarily related to their molecular weights (3.35-20 kDa) or hydrodynamic radii (1.8-4.8 nm), since dextrans of far higher molecular weight (73-1185 kDa) and hydrodynamic radii (6.5-20 nm) are unable to impart this protective effect.[131,132]

In our study, the groups receiving PEGs revealed a different pattern of several molecular events compared to the control group. Improvements in all LP groups were noted in TJ structures as well as morphological and electrophysiological parameters. Apoptosis was less frequent in groups receiving luminal PEG solutions, a finding somehow resembling the findings of Badet et al. reporting less apoptotic cells in kidneys preserved using IGL-1®, a PEG 35kDa-based solution.[133]

Determining whether the protective mechanisms of PEG in the intestine are due to dilution and evacuation of luminal contents or other mechanisms intrinsic to the PEG molecule is difficult. Previous LP experiments using a solution with a similar electrolyte composition to the solution used herein, but lacking PEG, have led to worse results than both the standard method (vascular perfusion and preservation) and LP using a PEG-containing solution.[104] Interestingly, whereas a large PEG seems to have decreased the activation of p38-MAPK activation in a porcine proximal

tubular epithelial cell line[134], our study did not reveal any impact of PEG 3.35kDa on p38-MAPK activation.

How do animal models for intestinal preservation compare to human tissues?

When carrying out experimental work on animal models, it is crucial to know how well the findings can be extrapolated to the clinical setting, as there are essential differences between the common experimental models and humans. Until the early 1990s, the canine model was the gold standard for preclinical experiments in many areas, including ITx.[135] Ethical considerations have led to its gradual replacement with pigs and small animals, leaving an additional gap in knowledge regarding many translational biological and pharmacological phenomena. Previous work has inferred a higher resilience of porcine intestines towards intestinal ischemia as compared to rats.[104,136,137] The results of Paper II indicate that mucosal morphological changes develop more rapidly in human and rat intestines when compared to porcine tissues, with the rat intestines demonstrating the highest degrees of morphological damage. Both human and porcine intestines developed significant submucosal edema, while this histological feature was not observed in rodents.

Our findings indicate that using pig intestines for preservation studies would require more extended cold storage periods than in rats to attain a comparable tissue injury. A direct consequence is a need for a critical reassessment of previous studies using segmental in situ ischemia-reperfusion or small bowel transplantation using short CIT.[138-140]

Since juvenile pigs appear to tolerate ischemia better than humans, one should take this into consideration when interpreting the results of studies utilizing this model.

Caspase-3 activation and increased apoptosis, following cold storage of various rodent organs, have been reported earlier.[106,141,142] We found abundant cells positive for active caspase-3 in rat intestines but not in human or porcine samples. This is an intriguing finding. While the caspase-3 dependent apoptotic pathway is essential in humans, there may be other caspase-independent cell death pathways at work during hypothermic ischemia.[43]

Another explanation is that the activation of cell death pathways is delayed in human and porcine tissues during cold storage, and activated caspases are only present at lower levels during this time. Apoptosis is a feature of reperfusion injury in the human kidney[143], but necrosis may be a more prominent type of cell death than apoptosis in ischemic human intestines[144]. For Paper IV, an assay based method for caspase detection was used due to these findings. There are undeniably unequal

susceptibilities to ischemic injury in human, porcine tissues, and rat tissues. The different magnitude of caspase-cascade activation during cold storage could suggest that injury mechanisms are directed through different pathways in different species. There may also be significant interspecies differences concerning the timing of pathway activation. Another explanation could be that human and porcine tissues are less prone to caspase-3 and MAPK activation during hypothermia.[74]

In the current study, we used the distal small intestine in all three species. This approach gives consistency to our findings since regional differences in ischemic susceptibility (jejunum vs. ileum) have previously been reported.[26,145]

A potential drawback of Paper II was the use of human intestines from brain dead organ donors. This setting was both necessary and relevant as it mirrored the clinical situation of intestinal procurement and preservation for transplantation. Nonetheless, this may have induced additional changes and differences compared to young and healthy rats and pigs. Brain death induces both local and systemic inflammatory processes that disadvantage the human specimens in this study.[56,57] Inducing brain death in animal models, especially in pigs, is associated with frequent methodological problems[146], and therefore, we opted to use a standard experimental model in order to keep our experiments more broadly relevant. The same rationale was applied to other factors affecting the typical human donor, such as a period of hemodynamic instability, cardiac arrest, trauma, or medical interventions (vasopressors, fluid resuscitation) that may potentially have affected the intestine. Discordant age between study subjects (young animals vs. middle-aged organ donors) could also be regarded as another potential confounding factor. However, mirroring all these various attributes in an experimental model would be both futile and inconsistent. For the sake of reproducibility, we opted for standard experimental models. Another important consideration is that the antibodies used in the study may have different affinities to their respective antigens in different species. This implies that a higher protein expression detected on the Western blot does not necessarily reflect the absolute level of tissue expression of a protein, but may also be influenced by the antibody-antigen affinity, which may differ between species.

Do intestinal grafts from older donors develop more severe IPI?

Age is assumed to be an essential donor attribute, but there is no hard evidence to suggest that intestines from older donors are unequivocally unsuitable.[26,27] Since the volume of intestinal transplantation is low compared to other organs, there is not a lack of available donor grafts per se. However, the recent focus on the detrimental effects of preexisting and de-novo donor-specific antibodies (DSA) for long-term graft function, may lead to requirements for better HLA matching.[147,148] The potential consequence of such matching requirements is the need for a larger donor pool. In

order to achieve this, one could expand the geographical donor organ catchment area at the cost of increased CIT, or reassess previously accepted criteria for intestinal donation such as age.

The intestinal mucosal barrier provides an essential line of defense against the enteric environment. Barrier function may become impaired with increasing age.[120] While the literature is not consistent in describing what these changes may be, some histologic changes reminiscent of mucosal atrophy may be evident.[121,149] In our study, we did not find any significant changes in villus height, width, or mucosal thickness. However, we did find a significant reduction in villus density with increasing age. On the molecular level, aging is associated with remodeling of intestinal epithelial TJs.[120] Several alterations have previously been reported, but we were only able to show decreasing expression of occludin with increasing age. While Paper III did not investigate stem cell function or regenerative capacity, it is well described that aging causes a reduction in IESC capacity for regeneration.[122] This is due to a combination of DNA damage to the stem cells themselves as well as age-induced changes to the cells that indirectly control the IESCs like Paneth cells and mesenchymal cells in the IESC niche.[45] Humoral factors may influence stem cell capacity, leading to the alluring possibility that older stem cells can be somewhat rejuvenated by a younger host.[122]

The mucus layer prevents bacteria and luminal enzymes from gaining direct access to the epithelium. The highly glycosylated mucins that comprise its backbone are continuously secreted by GCs in the epithelial layer. The GCs release mucins continuously, and in response to stressors, in order to protect the mucosa. However, they can become overwhelmed if subjected to repeated or severe stress.[52] Similarly to a previous report, Paper III showed that luminal PEG solutions improved the overall mucosal morphology, and preserved the mucin stores in the mucosal GCs.[106] The effects of LP on TJ protein levels were subtle, and the only significant difference between intestines receiving LP and the control intestines was noted on tricellulin, a protein located at the luminal, outermost part of the junctional complex.

While LP consistently improved the electrophysiological parameters and reduced GC depletion of the intestinal mucosa regardless of the age of the donor animal, it did not consistently affect TJ protein levels. This leads us to speculate whether the protective effects of LP are only partly dependent on shielding the TJ proteins and possibly more due to oncotic effects or interactions with mucus. This speculation is supported by the fact that superior morphology matched improvements in the electrophysiological parameters. Mucus in the small intestine is loosely attached and easily displaced, and we speculate that PEG may be able to stabilize it. However, assessing the mucus layer in detail is unfortunately not possible with standard, formalin-based tissue fixation techniques. Looking at mucus alterations during preservation and investigating the

ability of PEG to interact with and stabilize the mucus layer using additional techniques is an exciting area for future work.

The use of LP with PEG may have delayed fluid shifts from the luminal compartment to the subepithelial space and lamina propria. If, in fact, the PEG molecules stay exclusively in the luminal compartment, this is a plausible explanation. The current study, however, tells us nothing about the location of the PEG.

Our aging study indicates that older intestines are not predisposed to more severe injury on the tissue- or molecular level when compared to younger intestines. There was a slight tendency towards a higher grade of early injury and increased permeability in the intestines from old rats. While this was somehow expected due to indirect evidence from other studies, it did not become prominent and clear cut under the strictly controlled conditions of the experiment. The use of LP completely abrogated this difference, suggesting that LP may be especially useful in grafts from older donors. While promising, it is important to interpret these results with caution since they only shed light on the cold ischemic period. The regenerative capacity dictated by IESCs can only be meaningfully assessed in reperfusion and survival studies. Such experiments would introduce other valuable endpoints such as reperfusion injury, regenerative capacity, and animal survival.

A final consideration is the difficulty in matching human and rat age. Several age conversion formulae are available, but the aging relationship is not linear.[150,151] The advanced microscopic age-related changes found in the aorta and kidneys of old rats, confirm the validity of the aging model used in this study. However, it is crucial to keep in mind that different organ systems may have comparatively different aging velocities.

Does luminal preservation protect human intestinal grafts?

While several studies have shown a convincing effect of LP with a low sodium PEG-based solution in rats[103,104,124], this has not been tested on the human intestine. A study in porcine intestines showed more discrete protection, partly due to the slow progression of morphological damage. The same study noted that large LP filling volumes contributed to increased edema formation.[152] Interspecies physiological differences require preclinical confirmation of safety and efficacy in human tissues. The results in the current study imply a modest yet consistent level of protection induced by LP in human intestines. A particular safety concern in human tissues was edema formation; no significant difference was identified in this respect. The use of a PEG-based LP solution, which is commercially available globally, represents a pragmatic approach that combines efficacy, safety, and the avoidance of logistical, financial, and regulatory constraints to its implementation.

Using vascular perfusion solutions for LP is appealing for its simplicity and has been studied.[123,153,154] Luminal UW solution provided some protection for both the rat and human intestine, but its high potassium content could potentially favor hyperkalemia, requiring wash-out. Interestingly, the only study where rat intestines were transplanted, all rats receiving grafts with luminal UW died shortly after surgery, whereas 80% of the rats receiving grafts undergoing LP with an amino-acid based solution survived the 14-day study period.[101] Newer solutions like Custodiol® and IGL-1® have not been tested for LP. Although IGL-1® contains PEG, it also has a high sodium content, which is detrimental in the context of LP.[103]

Edema is a dominant feature of IPI. It is caused by fluid shifts between the intestinal lumen and the tissue due to barrier damage and ischemia-related dysfunction of channels and pumps.[67] A principal concern when using a LP solution is promoting tissue edema. Luminal lavage with Golytely®, a high sodium PEG-based solution, was discontinued in the 1990s due to graft edema.[30,112] The detrimental role of high sodium has also been shown in an experimental study.[103] The study on human intestines showed that LP with a low-sodium PEG formulation did not cause excess edema.

IPI is the result of multiple molecular alterations involving the enterocytes and the underlying lamina propria, all triggered by the energetic failure secondary to ischemia.[67] As in other tissues, IRI will further aggravate the damage. Studies have found the increase in damage to have a magnitude of 1-2 degrees on the Chiu/Park scale.[68,77,101] Thus, a moderate preservation injury after cold storage may progress to epithelial disruption after reperfusion.[101] In Paper IV, LP delayed the mucosal injury during cold storage, improving the chances for maintained integrity of the mucosal lining after reperfusion. We found areas of high-grade injury to be much more prevalent in intestinal segments without LP. Such areas of high-grade injury may cause chronic changes if the crypt layer is involved since stem cell niches function as autonomous units and do not take over for one another.[45,122]

The proximal small intestine may be more sensitive to ischemic injury than the distal small intestine, as previously shown in studies in rats[154], pigs[155], and humans[26]. In order to avoid this variation between the two intestinal regions as a confounding factor, all the studies herein exclusively used distal small intestine, which is the most ischemia resistant part. Thus, the injury grades may become higher if preserving more proximal segments, as is the case in clinical transplantation. In human samples, this may have contributed to the modest difference in average injury scores between bowel segments with or without LP. Notably, there was a difference in the frequency of advanced focal injury. Intestinal segments without LP had areas of advanced injury (epithelial breakdown, ulcerations) four times more often than segments with LP. Barrier disruption increases the risk of bacterial translocation, which is a significant cause of complications after ITx.[37] We believe that even minor improvements may

translate into clinical advantages downstream due to the potential impact on the remote organ injury[156] and even the occurrence and severity of rejection[70].

Strengths of the human LP study include using segments from the same anatomical area and simultaneous use of intestine from the same donor in both the intervention and the control group.

A limitation of the study is that a significant proportion of intestinal donors included were outside the accepted OPTN criteria for intestinal transplantation.[26] However, since intestines from the same donor were studied both with and without LP, the donors served as their own controls. Another more significant limitation is the absence of reperfusion, which would be necessary to study the impact of LP on IRI.

CONCLUSIONS

With the support of the studies presented in this thesis, I conclude that:

1. The rat is a sensitive model for ischemia research, but it has some important differences compared to humans and pigs.
2. When comparing the effect of low, medium, and high molecular weight PEGs during luminal preservation, an improvement was found regardless of molecular weight.
3. Advanced age does not appear to be a negative factor for the development of intestinal preservation injury.
4. Luminal preservation with a commercially available, PEG-3350 based solution reduces the intestinal preservation injury in human intestines without promoting edema.

FUTURE PERSPECTIVES

The effects of intestinal preservation on mucus

Mucus is an essential part of the intestinal defense system that has not received much attention in the context of intestinal preservation for transplantation. We plan to describe the effects of preservation and transplantation on this essential part of the intestinal barrier. The interactions between PEG and mucus may explain some of its protective roles. Studying these interactions may give a mechanistic explanation for the protective role seen after the luminal installation of PEG.

The mucosal injury and recovery after intestinal transplantation

Our previous studies have repeatedly revealed a milder intestinal preservation injury following LP, but no groups have explored how this advantage will evolve after the transplantation of these grafts. We plan to transplant rat intestines with and without LP and follow the graft and the recipients up to one week after transplantation. We will assess the initial reperfusion injury as well as the mucosal recovery with respect to several histological and molecular parameters.

Subgroups benefiting from luminal preservation

LP may be more useful in some donors than others. Pre-donation factors that predispose to injury like cardiovascular instability may be more amenable to protection with luminal PEG. A preliminary subgroup analysis in our current material indicates that young patients who have undergone cardiac arrest may benefit the most. In order to elucidate this, we will continue our collection of donor specimens in order to do an adequately powered analysis with a suitable control group.

Colon preservation

The colon is increasingly being transplanted along with intestinal grafts, but little is known about the effect of preservation on this part of the gastrointestinal tract. Using rat and human tissues, we will systematically describe the mucosal alterations from a histological and molecular standpoint.

REFLECTIVE STATEMENTS

- While animal studies may provide an excellent way to get an initial understanding of the processes involved in preservation injury, there is no substitute for data on human tissues. As we have shown there are substantial differences between conventional animal models and humans. When examining the more complex molecular pathways involved in IPI and IRI, it becomes essential to use human tissues to avoid the pitfalls of significant physiological interspecies differences.
- The lumen is an easy and obvious route to get direct access to the most sensitive part of the intestinal graft, i.e., the mucosa. There is considerable evidence in animal models that LP with PEG delays and/or reduces the amount of damage incurred during the cold storage period. Our preliminary results appear to confirm these findings in human tissues, as well. Following publication, these results will have to be confirmed by other groups. One ongoing study is the Belgian/Dutch multicenter trial LUMINTRAL, where results may be published in the near future. This study is also limited by only assessing the IPI phase. If more information is to be gleaned concerning the effect of LP, an experimental transplantation model should be used.
- The mechanism by which PEG solutions work is unknown. Whether it is due to oncotic effects, the clearance of luminal contents, interactions with mucus, or a membrane-stabilizing effect inherent to the PEG molecule is unclear. Future mechanistic studies will have to elucidate this. Regardless of the underlying mechanism clearing the intestinal contents must be done safely, and this is not as straightforward as it may seem. Important considerations are both the electrolyte composition and the requirement for a low electrolyte but at the same time, high-osmolality solution in the cold ischemic phase.

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