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Development of a novel nutrient-rich solution for preserving small bowel during cold storage

by

Payam Salehi 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Experimental Surgery

Department of Surgery

Edmonton, Alberta
Fall 2005

Dedication

This thesis is dedicated to my wife, Sahar, whose love, support and understanding has allowed me to pursue my own dreams and aspirations. I thank her for all her encouragement over the past five years and I look forward to our future together.

Abstract

Despite the benefit of University of Wisconsin (UW) solution for preservation of other intra-abdominal organs, the maximum storage time for small bowel (SB) remains relatively brief. Optimal preservation of intestinal grafts is necessary to prevent early post-transplantation complications such as endotoxemia and bacterial translocation as well as stimulation of the immune system. This project addresses different aspects of a novel preservation technique using a nutrient-rich, amino acid-based (AA) solution which is tailored to the specific metabolic requirements of SB.

Our initial experiments revealed that the technique of hypothermic luminal perfusion with oxygenated standard UW solution improved graft storage quality, compared to clinical standard, vascular flush-only with UW solution. However, our data showed that there was a possible physical injury incurred as a direct result of continuous mechanical perfusion; mucosal integrity was markedly superior with only a brief 1h period of luminal perfusion. In a follow-up experiment, even a better result was observed when we used a similar strategy of brief perfusion with oxygenated AA solution. Since oxygen supply is essential to promote intestinal metabolism, in a separate set of experiments, we compared the effect of perfluorocarbon solution, a well-known oxygen carrier, with oxygenated AA solution. Surprisingly, we found similar improvements in energetics and mucosal function in both groups after a period of cold storage compared to clinical standard treatment. Apart from the beneficial effect of oxygen, there is a concomitant generation of oxygen free radicals during aerobic respiration which must be dealt with as a basal metabolic function. Hence, another study was performed to examine the possible improvement of SB preservation by combining known antioxidant agents with our AA solution.

In addition, we tested the effect of this novel preservation solution upon reperfusion. Interestingly, this treatment caused a lower neutrophil infiltration and superior mucosal integrity and function following a clinically relevant reperfusion period compared to the standard control treatment. Lastly, as a final test-bed of experimental proof for the beneficial effects of AA solution, a marked improvement in graft viability was observed when we examined the effectiveness of this preservation solution in a small animal model of orthotopic whole small bowel transplantation after 6 hour cold storage.

This novel preservation strategy using luminally administered amino acid-based solution, as a simple addition to the current standard procurement technique, may have implications for the successful preservation and transplantation of small bowel in the clinic.

Acknowledgement

I would like to express my sincere thanks to my supervisor, Dr. Thomas Churchill. His overly enthusiasm and scientific view on research and his mission for providing high-quality work, has made a deep impression on me. I owe him lots of gratitude for having me shown the way of research. Besides of being an excellent supervisor, Tom was as close as a relative and a good friend to me.

I wish to express gratitude and appreciation to all of my committee members, Dr. Richard Fedorak, Dr. Karen Madsen and Dr. Chris Cheeseman, for their detailed and constructive comments and immense help in planning and executing the work.

I warmly thank Dr. Jonathan Lakey for his valuable advice, friendly help and encouragement given throughout the graduate study.

I would also like to acknowledge with much appreciation the crucial role of Dr. Lin-Fu Zhu, Mr. John Walker and Dr. Jose Avila over the entire research project.

Many thanks to Ms. Kimberli Sawarin and Ms. Geneva Demeyer for excellent technical assistance.

Finally, over the past three years, I was financially supported by CIHR Clinical Research Initiative Fellowship Award, Alberta Heritage Foundation Studentship Award, Ivy Thomson and William A Thomson Graduate Scholarship and 75th Anniversary Award, Faculty of Medicine and Dentistry, University of Alberta.

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ATP: Adenosine Triphosphate

ADP: Adenosine Diphosphate

AMP: Adenosine 5'-Monophosphate

ANOVA: Analysis of Variance

ALP: Alkaline Phosphatase

CIT: Cold Ischemia Time

cAMP: Cyclic Adenosine Monophosphate

DMSO: Dimethyl Sulfoxide

EC: Energy Charge

EGF: Epidermal Growth Factor

EDTA: Ethylediamine Tetraacetic Acid

FI: Freshly Isolated

G-CSF: Granulocyte Colony-Stimulating Factor

HX: Hypoxanthine

HPN: Home Parental Nutrition

Isc: Intestinal Short-Circuit Current

IRI: Ischemia-Reperfusion Injury

LRSBTx: Living Related Small Bowel Transplant

MPO: Myeloperoxidase

MAP: Mean Arterial Pressure

MDA: Malondialdehyde

NOS: Nitric Oxide Syntheses

NADH: Nicotinamide Adenine Dinucleotide

NO: Nitric Oxide

OFR: Oxygen Free Radical

PARP: Poly(ADP-ribose) polymerase

PD: Potential Difference

PBS: Phosphate-Buffered-Sucrose

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

RBC: Red Blood Cell

RhGH: Recombinant Human Growth Hormone

RL: Ringer's Lactate

SBT: Small Bowel Transplantation

SE: Standard Error

SMA: Superior Mesenteric Artery

SOD: Superoxide Dismutase

SMV: Superior Mesenteric Vein

TPN: Total Parenteral Nutrition

TA: Total Adenylates

UW: University of Wisconsin

XO: Xanthine Oxidase

XD: Xanthine Dehydrogenase

Chapter 1

Introduction

Part 1) Small Bowel Transplantation

History of Small Bowel Transplantation

Nearly one century has passed since the first experimental intestinal transplantation by Alexis Carrel who described the surgical technique of transplanting intestinal segments into the neck of dogs in 1902 (1). The first experimental transplantations with long-term success were carried out by Lillehei and colleagues in 1959 who autotransplanted small bowel grafts in dogs after four hours of cold preservation (2). This inspired the first human intestinal transplants, which were performed by Ralph Deterling in Boston in 1964 (unpublished). The first reported human intestinal transplant was performed by Lillehei in 1967, and included the entire small bowel and right colon, with the superior mesenteric vessels being anastomosed to the left common iliac vessels (3). Unfortunately, the first eight reported attempts at clinical small bowel transplantation carried out between 1964 and 1970 were largely unsuccessful, with patients dying from technical complications, sepsis and rejection (4,5). Only one patient survived for more than one month (76 days) after an HLA-matched living related transplantation (6). After the introduction of total parenteral nutrition (TPN) in 1968, interest in clinical intestinal transplantation waned. However, with the realization that TPN could be associated with serious life-threatening complications, and with the development of safer and more potent immunosuppression in the 1980's, small bowel transplantation has returned to the clinical arena, and in the past decade has evolved into a reasonable alternative to TPN in patients with intestinal failure (7). Although the first intestinal transplant using cyclosporine, performed in 1985

by Zane Cohen (8) in Toronto was unsuccessful, in 1988 Deltz in Kiel, Germany performed what is considered to be the first successful intestinal transplant (9). Soon after, other successful outcomes were reported by the groups headed by Goulet in Paris (10), and Grant in London, Canada who had established the first intestinal transplant programs. There are now over 50 centers worldwide which have performed intestinal transplants, with more than 1000 transplants performed to date.

Current results of clinical small bowel transplantation

As of 2003, there have been a total of 989 small intestinal transplants carried out worldwide, including 433 isolated intestinal transplants, 386 combined liver/intestine, and 170 multivisceral (11). The majority of transplants have been carried out in children and infants. Overall patient and graft survival remains relatively low (less than 50 % five-year survival up to 2003), although of survivors, intestinal function has been completely restored in almost 80 per cent. Rejection is the major reason for graft failure, and sepsis remains the main cause of death. Small bowel transplantation has a high failure rate because of refractory graft rejection and sepsis. Several centers have reported improved outcomes with tacrolimus. To determine the status of small bowel transplantation, Grant et al reviewed the world experience since 2003 by using registry data (12). According to this report, as of May 2003, there were 484 recipients (52%) worldwide surviving intestinal transplantation. Sepsis has been the most common cause of death (49%), followed by rejection (11%), technical complications (9%), lymphoma (6%), and respiratory failure (6%). Survival has steadily improved since 1990 and is reported to be influenced by several factors. The era in which the patient received an intestinal transplant is important because graft survival has improved significantly over time. The size of the transplant center significantly contributes to survival, with improved patient

and graft survival reported at centers performing more than 10 intestinal transplantations, reflecting the importance of the learning curve for this complex procedure.

For patients receiving transplants after February 1998, The 1-year graft/patient survival rates were 65%/77% for intestinal grafts, 59%/60% for small-bowel and liver grafts, and 61%/66% for multivisceral grafts. Survival rates were significantly higher in patients who were called in from home for their transplant. One-year graft survival rates in home versus hospitalized patients were 70%/51% for intestine grafts ($P < 0.001$), 64%/50% for intestine and liver grafts ($P = 0.018$), and 71%/48% ($P = 0.010$) for multivisceral grafts. One-year patient survival rates in home versus hospitalized patients were 78%/72% for intestine grafts ($P = 0.03$), 67%/51% for intestine and liver grafts ($P = 0.008$), and 76%/54% ($P = 0.011$) for multivisceral grafts. The registry included almost all the intestinal transplantations between 1985 and 2003 (Figure 1-1).

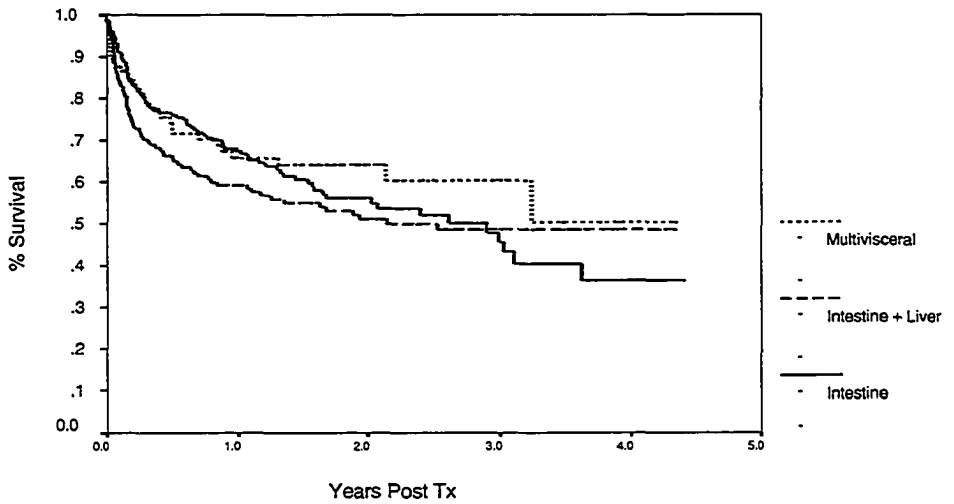


Figure 1-1. Actuarial Kaplan-Meier plot of graft survival rates after intestinal transplantation (data from Intestinal Transplant Registry website)(11).

Patients' survival rates were better after isolated intestinal transplantation than after combined small bowel/liver or multivisceral grafting (Figure 1-2).

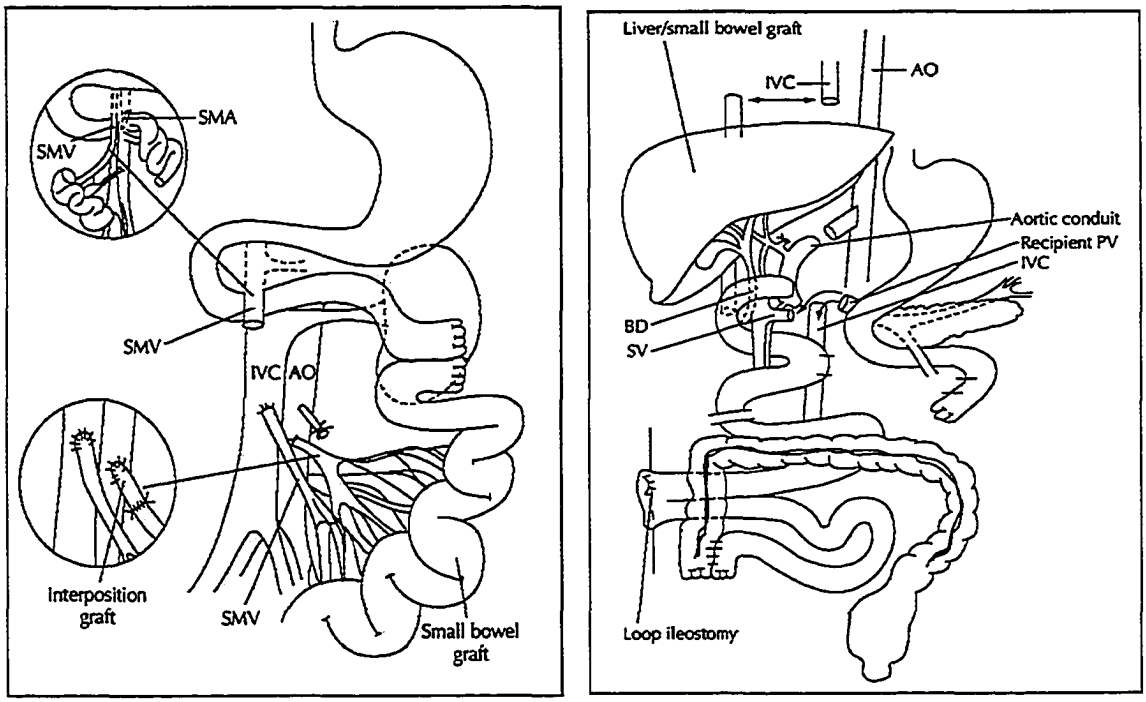


Figure 1-2. A) Isolated small bowel transplantation Graft, superior mesenteric artery (SMA) is anastomosed to the recipient aorta, with an interposition graft (*insert)* in case of a replaced hepatic artery from the SMA in the donor. The venous drainage can be systemic in the inferior vena cava (IVC) or to the portal circulation (*insert*). AO, aorta; IVC, inferior vena cava; SMA, superior mesenteric artery; SMV, superior mesenteric vein. B) Transplantation of the combined liver–small bowel graft, AO, aorta; BD, bile duct; IVC, inferior vena cava; SV, splenic vein. *(As published in Current Opinion in Organ Transplantation 1999; 4: 335-9)* (13)

This finding may be due to the difference in patients' status at the time of surgery, differences in technical complexity of these operations, and/or the greater ease of removing an isolated intestinal graft. Most of the deaths after intestinal transplantation were related to use of intense immunosuppression to prevent graft rejection. The high

death rate after isolated intestinal transplantation was a worry because, in most cases it should be possible to remove a failed small bowel graft and place the patient back on TPN. To become the standard treatment for intestinal failure, transplantation must offer greater safety, lower costs, and a better quality of life than TPN.

When should small bowel transplant be considered?

Small bowel transplantation (SBT) is currently limited to patients with irreversible intestinal failure who have developed life-threatening complications or have otherwise failed with TPN. Unfortunately, this limits the recipients to only the sickest of patients with intestinal failure, where transplantation offers the only hope for survival, and indeed many patients do not survive while waiting for a suitable donor (14). The most common indications in adults for intestinal transplantations are mesenteric ischemia, Crohn's disease, desmoid tumors, and trauma (15). Approximately two-thirds of potential candidates are children, many of which undergo combined liver/small bowel transplant, due to the increased incidence of hepatobiliary and metabolic complications of TPN, particularly in neonates. In children, gastroschisis, volvulus and necrotizing enterocolitis are the most common indications.

Preoperative assessment

A careful, multisystem, multidisciplinary assessment – including the gastrointestinal and hepatobiliary systems, nutritional status, anesthetic assessment, and psychological or developmental assessments – is necessary when considering a patient for small bowel transplantation.

The gastroenterological assessment must address whether the intestinal failure is indeed irreversible, and whether sufficient time for adaptation has been allowed. The anatomy of the gastrointestinal tract may be extensively altered due to numerous

previous surgeries and reconstruction. Upper and lower gastrointestinal radiology and endoscopy is usually required, both to elucidate the anatomy and to investigate the presence of residual gastrointestinal disease. An assessment of the motility of the remaining gastrointestinal tract should be carried out, including esophageal motility and gastric emptying studies, and motility studies of the remaining small and large bowel. Finally, the capacity of the peritoneal cavity, which may be diminished and contracted following massive intestinal resection, must be assessed.

The hepatobiliary assessment must include a thorough evaluation of hepatic structure and function to determine whether coexisting liver disease exists and whether it is deemed irreversible and will require a combined liver/small bowel transplant. If the liver disorder is reversible by medical management, such as treatment of sepsis or adjusting the parenteral nutrition, this should be instituted as soon as possible. Liver assessment should include test of liver function, a liver biopsy, evaluation of the portal vein to rule out thrombosis, and evaluation of the biliary tree by ultrasound or endoscopic retrograde cholangiopancreatography to rule out cholelithiasis or choledocholithiasis. Assessment of the nutritional status is important to identify and treat malnutrition and micronutrient deficiencies. This should include blood tests for protein, vitamins, essential fatty acids and trace elements. Tolerance of enteral nutrition and vascular access for parenteral nutrition is also assessed.

Contraindications

In general, intestinal transplants should not be performed individuals who have significant co-existent medical conditions that have no potential for improvement following transplantation. If the patient has active infection, malignancy, or HIV, transplantation is contraindicated. If there is evidence to indicate that a potential

recipient or the primary caregivers are not willing or able to reliably accept the responsibilities of the post-transplant management, transplantation is contraindicated.

Recipient surgery

Intestinal grafts are usually procured from ABO-identical, HLA-mismatched cadaveric donors as part of a multivisceral organ retrieval (16). The small intestine is very sensitive to ischemia so the maximum cold ischemia time is shorter than other solid organs, with revascularization ideally carried out within 6 hours. Typically, donors should weigh 20 to 30 % less than recipients, to match the smaller abdominal cavity of patients with short bowel syndrome who have had many previous surgeries. Preparation of donors includes administration of oral nystatin, neomycin, and trimethoprim-sulfamethoxazole, as well as intravenous broad-spectrum antibiotics, to decontaminate the bowel. Mechanical bowel preps are now avoided, as they tended to cause fluid-filled bowel distension making surgical manipulation more difficult. The procurement procedure involves separating the superior mesenteric vein and portal vein from the pancreas, which is divided at its neck. Tissue containing the lymphatic channels at the base of the mesentery is carefully clipped to avoid chylous ascities in the recipient. The small intestine is divided proximally at the proximal jejunum and distally at the terminal ileum.

After the bowel is stapled and divided, the SMA is flushed via the aorta with cold University of Wisconsin (UW) fluid, then the SMA is divided at its root with a cuff of aorta. In current clinical practice there is no luminal flush and the bowel is stored in cold UW solution while still filled intraluminally with enteric contents including potentially damaging gastric, pancreatic and biliary secretions and bacteria (17). In the recipient, the donor SMA is either anastamosed end-to-end to the recipient SMA, or end-to-side to the

infrarenal or supraceliac aorta with or without an interposition graft of donor artery for added length. The donor SMV (superior mesenteric vein) is ideally anastamosed with the correct anatomical and physiological drainage, i.e. either to the recipient SMV or portal vein, or alternately mesocaval drainage via an end-to-side anastamosis to the inferior vena cava. The theoretical metabolic consequences of systemic venous drainage is interruption of the enterohepatic circulation of bile salts, cholesterol and phospholipids, an elevated plasma ammonium level with potential neurologic sequelae, loss of direct flow of hepatotrophic factors, loss of filtering by the hepatic Kuppfer cells which are able to remove translocated bacteria from the portal blood stream, and finally interruption of the presentation of graft antigens to the liver which may promote tolerance of the recipient to the graft. Despite all these theoretical reasons to favour portal drainage, there is no reported difference in human survival between recipients of portal versus systemic drainage (18). Finally, intestinal continuity is established proximally, and distally either an end ileostomy or a diverting loop ileostomy with distal bowel anastamosis is created.

Post-transplant management (Immunosuppression)

Advances in immunosuppression over the last two decades is largely responsible for making intestinal transplantation clinically feasible. Current immunosuppressive regimens are continually evolving and improving. As of 1999, prednisone and tacrolimus was used in the majority of protocols, with some usage of mycophenolate mofetil, azathioprine, and cyclosporine (19).

Acute rejection is still the most prominent risk factor following small bowel transplantation. Unlike other organ transplantations, refractory acute rejection requiring graft removal was seen in approximately 20% of these patients. New

immunosuppressive agents including daclizumab, campath-1H, and rapamycin may reduce the incidence of acute and chronic rejection. However, with the increase in immunosuppression, there will be also an increased risk for bacterial, fungal, and viral infections.

Reperfusion injury has gained less attention, but may significantly activate the specific immune response and may promote acute allograft rejection. Early enteral nutrition has been shown to decrease postoperative complications including infections and technical complications. Furthermore enteral nutrition, especially immunonutrition and the application of lactobacilli, will restore the natural gut flora, improve mucosal barrier function, and decrease the release of cytokines and other mediators (20). Acute rejection may be successfully prevented by potent immunosuppression based on combined tacrolimus and rapamycin maintenance therapy. Early postoperative infections can be efficiently prevented by restoration of the natural gut flora and preservation of mucosal barrier function using immunonutrition and lactobacilli (21).

Procurement, Preservation and Reperfusion Injury

Clinical organ preservation necessitates a period of ischemia during which the damaging effects of hypoxia are initiated. The standard organ flush solutions have proven suboptimal in clinically relevant durations of cold storage of intestinal grafts, indicating that a customized approach to preservation is necessary in the small bowel (22). The biochemical and morphological alterations sustained during preservation are precursors to the more extensive structural and functional damage generated upon reperfusion, a phenomenon known as ischemia-reperfusion injury, which will be discussed in depth. It is apparent though that small bowel preservation and reperfusion injury lead to postoperative complications including graft dysfunction, bacterial

translocation through damaged mucosal barrier, sepsis, and also an increased risk for acute and chronic rejection (23). Thus, a major challenge of clinical and experimental transplantation continues to develop a procurement protocol tailored to SBT which minimizes ischemia-reperfusion injury, and ultimately affects improved clinical outcome by providing a definitive cure for intestinal failure.

Bacterial Translocation

The main sequel of both ischemia-reperfusion injury and sepsis is breakdown of the protective mucosal barrier, thus allowing translocation of bacteria, resulting in bacteremia and sepsis. Immunosuppression places patients at even higher risk of overwhelming systemic infections. The majority of SBT recipients have at least one infectious episode during their first three months post-transplant. In order to reduce the risk, prophylactic perioperative antibiotics are administered to donor and recipient, and cautious use of immunosuppression is employed. However, the most important factor in reducing the occurrence of sepsis and its complications is prevention, by improved preservation and immune suppression.

Future Prospects

Small bowel transplantation has the potential to be very successful, and perhaps may one day be more readily available for patients with intestinal failure on TPN. However, continued research in this field is essential to improve patient and graft survival. While the experience with living related donor intestinal transplantation has been very limited to date, some of the longest surviving grafts from the pre cyclosporine era were achieved when living related donors were utilized. The potential advantages of using living donors are: opportunity for better HLA matching and better control over ischemia times. The

potential disadvantages are that: putting the donor on the risk and also the allograft will consist of a shorter segment of bowel with smaller blood vessels.

There are many important challenges that must be addressed in the research arena; however ischemia-reperfusion injury and its close relation to ultimate graft function is an area with potentially significant impact applicable to clinical intestinal transplantation.

Part 2) Ischemia-Reperfusion Injury

Transplantation of any organ necessitates a period of interruption of blood flow. The consequent lack of oxygen and nutrient delivery results in some degree of cellular damage. A prerequisite for recovery of the ischemic organ is reestablishment of blood and energy supply to the tissue. Paradoxically, reperfusion of ischemic tissue induces further tissue injury and systemic metabolic consequences. This phenomenon, called ischemia-reperfusion injury, involves a complex interaction among oxygen free radicals, neutrophils, cytokines, and other mediators, with resultant direct injury at the cellular level. Clinically, ischemia-reperfusion injury (IRI) is a major cause of graft dysfunction or failure, and other complications including sepsis and death. Attenuation of IRI via interventions during the preservation and reperfusion stages is an integral aspect of intestinal transplant research, with the goal of more successful clinical small bowel transplantation.

Ischemia and Organ Preservation

Hypothermia decreases metabolic rate. Therefore, biochemical reactions are reduced and the rate of degradation of essential cellular components necessary for organ viability is reduced. Most enzyme systems show a 1.5- to 2.0-fold decrease in activity for every 10 °C decrease in temperature (24). However, although hypothermia is

essential during organ storage, a number of events can still occur leading to activation of inflammatory mediators that are ultimately deleterious to the preserved organ at the time of reperfusion.

Pathophysiology of ischemia

When tissue blood supply is interrupted, a series of biochemical events occur which lead to cellular and membrane dysfunction, intracellular edema and cell death (25). Oxygen is the basic cellular fuel essential for most cell functions. Aerobic metabolism occurs in the presence of oxygen, which allows for efficient generation of high energy phosphates, primarily adenosine 5'-triphosphate (ATP), which is necessary for normal cellular function. Ischemia rapidly results in tissue and cellular hypoxia. Under these conditions, anaerobic metabolism occurs which is a less efficient mechanism of generating energy substrate, yielding much less ATP per mole of substrate than aerobic metabolism (26). Cellular functions continue initially, and ATP is rapidly depleted as demand for ATP outweighs its supply during hypoxia. Depletion of cellular energy stores leads to inability to maintain transmembrane ionic gradients due to decreased function of plasma membrane ion pumps including the Na^+/K^+ATPase (27). This results in efflux of potassium ion and influx of sodium ion which causes membrane depolarization, causing voltage-dependent Ca^{2+} channels to open and an increased influx of calcium ion (28). Higher intracellular concentrations of Ca^{2+} activate membrane phospholipases which catalyze hydrolysis of cell-membrane and organelle phospholipids. Activation of mitochondrial phospholipases disrupt oxidative phosphorylation, further reducing ATP production. Unable to maintain homeostasis, damage to the cell membrane and increased sodium influx leads to fluid movement into the cell, cell swelling, lysis, and death.

Sodium Pump Inactivation

The sodium (Na^+/K^+-ATPase) pump is important to preserve proper intracellular electrolyte concentration (high K^+, low Na^+) and to maintain adequate clearance of alveolar fluid. Hypothermic storage results in the loss of function of the sodium pump, which returns to normal activity with rewarming to 37°C if the epithelial cells are not damaged. The loss of function of the sodium pump results in accumulation of sodium in the cell resulting in cell swelling. This is associated with an influx of chloride inside the cell and an efflux of K^+ out of the cell. Preservation solutions contain electrolytes and colloid to create an osmotic pressure gradient in an attempt to prevent hypothermia-induced cell swelling. The sodium pump activity has been shown to resume better functional activity at the time of rewarming if the lungs are preserved with extracellular-type preservation solutions that contain low K^+ and high Na^+ concentrations (29).

Morphologic and functional consequences of tissue ischemia

Tissue hypoxia plays a key role in formation of mucosal lesions when blood flow is interrupted or decreased (30,31). In an *in-vivo* model, Chiu *et al* demonstrated an evolving sequence of morphologic changes with longer periods of normothermic intestinal ischemia in dogs (32). Early changes occurred after 0.5 to 1 hour of partial superior mesenteric artery (SMA) occlusion, and consisted of the development of fluid-filled sub-epithelial Gruenhagen spaces, between the epithelial cells and their basement membrane, at the apices of intestinal villi. The epithelial cells themselves remained morphologically and biochemically normal when lifted off the basement membrane. With longer duration of ischemia, the subepithelial space extends down the sides of the villi and epithelial cells are further lifted, but still intact. Severe damage was seen after one hour of total SMA occlusion, or greater than 4 hours of partial occlusion, consisting of

denuded villi and disintegration and hemorrhage in the lamina propria. Park et al (33) similarly demonstrated in rats that normothermic ischemic periods of 20 minutes were not associated with any morphologic changes, but increasing degrees of injury paralleled longer duration of ischemia up to 90 minutes of total arterial occlusion. The sequence of histologic damage observed, beginning with epithelial lifting at villus tips, extending along the villus sides, followed by denudation of villi, loss of tissue in villus core, crypt layer infarction, and finally transmucosal and transmural necrosis, is reiterated in Park's classification of grading intestinal tissue injury (Figure 1-3).

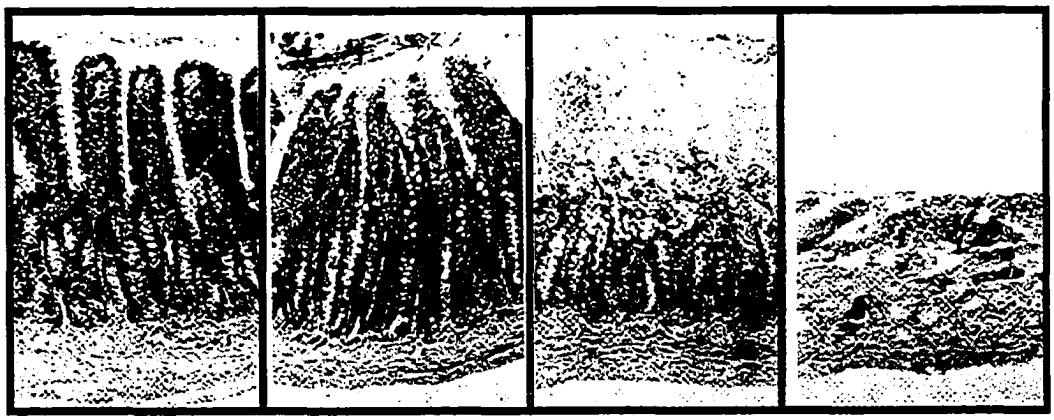


Figure 1-3. Small intestinal mucosal injury according to Park's classification (from left to right): injury limited to the subepithelial space at the tip of the villi (grade 1), denuded villi (grade 4), villus destruction with involvement of the crypt layer (grade 6), and mucosal necrosis with intact muscularis propria (grade 7)*(As published in Surgery 1990; 107: 574-80)* (33).

Chan et al (34) compared effects of normothermic, near-total ischemia on segments of pig jejunum and ileum, and demonstrated mild ischemic damage after 1.5

hours and maximal damage at 5 hours in the jejunum, whereas no evidence of histologic damage was seen up to 4.5 hours in the ileum, then ischemic injury progressed rapidly showing maximal damage after 6 hours of ischemia. This demonstrated that the ileum is more resistant to normothermic ischemia than the jejunum.

During hypothermia, cellular metabolism is slowed down by significantly, thus morphologic and functional results of ischemia develop much slower. Cold preservation of intestine for under nine hours resulted in only mild histologic damage, comparable to 20-30 minutes of normothermic ischemia (35-38). Histologic evaluation after ten to 24 hours of cold storage showed moderate to severe damage, comparable to 60 to 90 minutes of warm ischemia (39). Maintenance of important functional indices of the small intestine parallels degree of histologic damage, and also correlates with length of ischemia. Chiu et al (32) studied mucosal protective function and permeability in partial normothermic ischemia in dogs, by measuring luminal absorption of d-tubocurarine, a toxin not normally absorbed in the gastrointestinal tract which causes acute respiratory paralysis. They showed that increased permeability and impaired function only occurred after denudation of intestinal villi and exposure of the underlying lamina propria. Fujimoto et al (40) demonstrated increased permeability and conductance as measured by Ussing chambers after 10 hours of cold storage in rat small intestine, which correlated with severe histologic injury. Mueller et al demonstrated normal glutaminase and maltase (41) activity after 1, 6 and 12 hours of hypothermic ischemia in rats.

Reperfusion Injury

Intestinal ischemia is merely the first step in the cascade of cellular injury and tissue damage caused by ischemia-reperfusion injury (IRI). Several studies have demonstrated the phenomenon that mild intestinal damage sustained during ischemia is

greatly exacerbated upon reperfusion with oxygenated blood. In 1986, Parks and Granger (42) demonstrated that three hours of ischemia in cat small intestine followed by one hour of reperfusion resulted in significantly worse mucosal damage than four hours of ischemia alone. Mangino et al (43) likewise demonstrated that three hours of intestinal hypoperfusion in dogs produced only mild to moderate histologic injury, whereas reperfusion for one hour resulted in moderate to severe damage including crypt cell necrosis and a reduction in villus height and mucosal thickness. This evidence suggests that some reaction occurs upon return of oxygenated blood to ischemic tissue causing reperfusion injury.

Role of free radicals in ischemia and reperfusion

It has been currently recognized that reperfusion injury is associated with over-generation of reactive oxygen species (and also reactive nitrogen species, RNS) and free radical-mediated damage. Mitochondria are a main cellular site of free radical generation under normal conditions (44). There is an estimate that between 0.2 and 4% of the oxygen consumed by mitochondria is converted into reactive oxygen species. This is caused by "electron leak" at the respiratory chain, primarily leading to O_2^- generation (45). The greater the reducing state of the respiratory chain (which increases the availability of electrons leak) the greater the production of O_2^- radicals. This process is also dependent on the oxygen concentration and tension. Thus, during ischemic events the respiratory chain is kept in a reduced state since there is little oxygen (and some times no oxygen) to be converted to water by cytochrome oxidase. During reperfusion, the quick influx of oxygen to ischemic tissues causes over-generation of reactive oxygen species and overall damage to cell constituents. Mitochondria from several organs/tissues (including brain and heart) seem to be the main site of reactive oxygen

species generation at the time of reperfusion (46,47). The mitochondrial burst of oxyradical production can overwhelm existing cellular antioxidant defenses – either enzymatic or non-enzymatic - and cause damage to macromolecules including DNA, proteins and membrane lipids. Moreover, post-ischemic peroxidation of endoplasmic reticulum causes a further increase in cytoplasmic calcium concentration (partial loss of calcium homeostasis takes place during ischemia itself due to disruption of aerobic ATP supply) that can lead to uncontrolled activation of phospholipases and proteases (48). Calcium activation of nitric oxide synthase (NOS) may also prompt increased formation of $^{\bullet}NO$ and consequently of $ONOO^{-}$ (49,50). In mammalian systems undergoing reoxygenation or reperfusion, these free radical-induced events can lead to severe cell damage, apoptosis and organ failure.

Another important sites of ROS and/or RNS formation in post-ischemic tissues are the enzyme xanthine oxidase (produces primarily $O_2^{\bullet-}$ radicals, which can be converted to other reactive species) and activated phagocytes through NADPH oxidase (51). Even though this enzyme may be responsible for ROS generation in the canine heart (as well as in mammalian gut and liver – other organs that can be subjected to ischemia and reperfusion), its activity is just too low in human heart to be a relevant player in post-ischemic oxidative stress. The role of xanthine oxidase in reperfusion injury was originally proposed for intestinal ischemia in the mid 1980's. During ischemia, the breakdown of ATP (due to the hypoxic condition) causes accumulation of hypoxanthine and xanthine, which are substrates of xanthine oxidase. When oxygen is reintroduced to the system, xanthine oxidase may produce higher levels of $O_2^{\bullet-}$ and, consequently, of $^{\bullet}OH$ radicals.

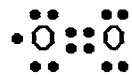
There is evidence that activated neutrophils do contribute to ROS/RNS generation and necrosis in post-ischemic myocardium (52). Moreover, the vascular endothelium can also be a relevant source of ROS/RNS in events of ischemia and reperfusion (53-55).

Mechanism of Reperfusion Injury: Oxygen Free Radicals

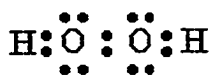
Oxygen free radicals, or reactive oxygen metabolites, are highly unstable, highly reactive molecules containing one or more unpaired electrons, produced by partial reduction of molecular oxygen (O_2) (56).

Electron configuration of oxygen free radicals and other related molecules

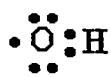
O_2 Molecular oxygen



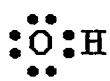
$\cdot O_2^-$ Superoxide radical



H_2O_2 Hydrogen peroxide

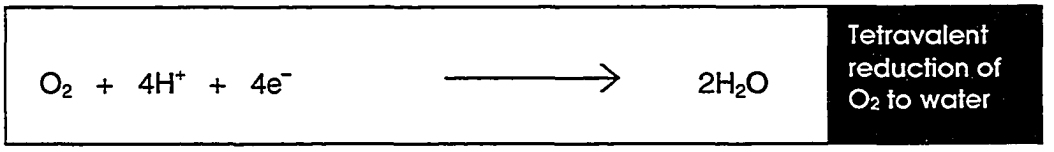


·OH Hydroxyl radical

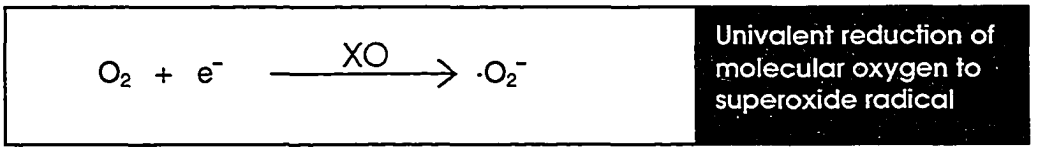


OH^- Hydroxide anion (not a free radical)

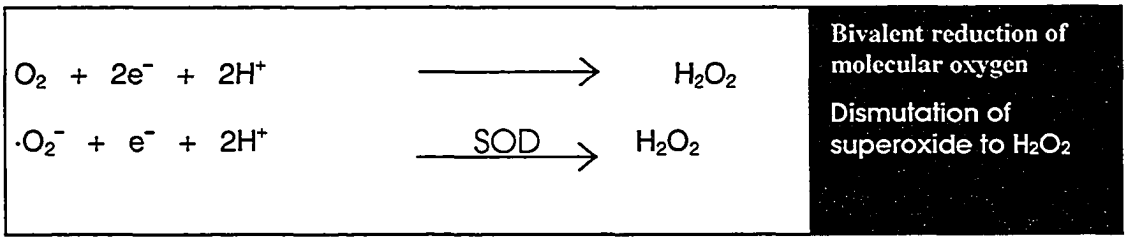
Under normal conditions, O_2 is reduced tetravalently to water (four electrons are donated to O_2 molecule in a single process) by the cytochrome system in mitochondria as a normal part of oxidative metabolism.



However even under physiologic conditions, a small proportion of O_2 (1 to 5 %) is reduced by a sequential univalent pathway, leading to the formation of OFR's containing unpaired electrons (57,58). Univalent reduction of O_2 forms the superoxide radical ($\cdot O_2^-$), and is catalyzed by xanthine oxidase (XO).



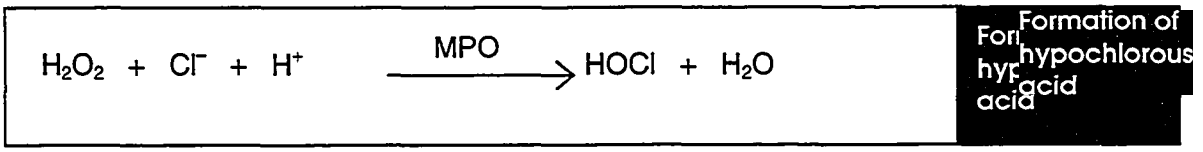
Superoxide on its own is not very cytotoxic, however the main consequence of initiating the univalent reduction of O_2 is the initiation of a series of reactions which yield more potent radical species. The superoxide molecule undergoes dismutation to hydrogen peroxide (H_2O_2), catalyzed by the endogenous free radical scavenger enzyme superoxide dismutase (SOD). Alternately, O_2 can undergo divalent reduction directly to H_2O_2.



The production of superoxide *in-vivo* is always accompanied by the production of hydrogen peroxide. Molecular oxygen cannot be directly reduced by three electrons, but $\cdot O_2^-$ and H_2O_2 react in the iron-catalyzed Haber-Weiss reaction to yield the extremely reactive and cytotoxic hydroxyl radical ($\cdot OH$)(59). Iron is abundant in the intestinal

mucosa, normally stored in enterocytes in the form of ferritin micelles. Superoxide can react with the Fe^{3+} form of iron in ferritin to liberate Fe^{2+}, which then reacts with H_2O_2 to form ·OH (60).

The hydroxyl radical wields its cytotoxicity by oxidization of sulfhydryl compounds which inactivates essential enzymes and membrane transport proteins, and by reacting with and damaging DNA and cytochromes and other proteins. Lipid peroxidation occurs when ·OH attacks polyunsaturated fatty acids in the cell membrane, forming membrane lipid peroxides (LOOH) which destabilize the lipid membrane and can lead to cell lysis. Other important OFR's include hypochlorous acid (HOCl), a cytotoxic oxidant formed by the reaction of H_2O_2 with chloride anion, catalyzed by the enzyme myeloperoxidase (MPO).



Hypochlorous acid is a powerful oxidizing and chlorinating agent which reacts with primary amines to yield *N*-chloro-derivatives, which exert their cytotoxicity by chlorination and damage of purine bases in DNA, and degredation of amino acids and proteins (61). To avoid tissue damage, cells have evolved endogenous control mechanisms to deal with physiologic formation of OFR's. Superoxide dismutase and catalase are endogenous OFR scavengers which catalyze the dismutation of $\cdot O_2^-$ to H_2O_2 and the reduction of H_2O_2 to water, respectively, thereby preventing the formation of the more cytotoxic hydroxide radical.

Endogenous scavengers of OFR's adequately protect tissue from damage under normal conditions, however situations with increased OFR formation will overwhelm

these intrinsic protective systems and result in cellular and tissue injury. Reperfusion of ischemic tissue is one such situation, as it is accompanied by a burst of OFR formation, which can be quantified in a reaction with luminol, which reacts with H_2O_2, $\cdot OH$ and HOCl, to produce measurable chemiluminescence (62).

Cytotoxicity of these oxygen metabolites [eg. superoxide radical, hydroxide radical, hydrogen peroxide] manifests itself by inactivating enzymes, membrane transport proteins, damaging DNA and lysis of cell membranes due to lipid peroxidation. The result is compromise of cellular compartments and membranes which potentiates cellular dysfunction and eventually, death. The intestine is extremely susceptible to OFR-mediated IRI due to its unique mucosal properties as well as biochemical changes initiated during ischemia which predispose the tissue to further injury upon subsequent reperfusion. The main source of OFR's in intestinal ischemia-reperfusion is the xanthine oxidase pathway (Figure 1-4)(63,64). Intestinal mucosal villi contain the highest concentration of xanthine oxidase (XO) of any tissue (65). In normal, non-ischemic cells XO exists predominantly as xanthine dehydrogenase (XD)(66), whose physiologic function is nucleic acid degredation, whereby XD reduces NAD^+ in the oxidation of purines. During ischemia however, XD is converted to XO (D-to-O conversion)(67). Parks et al (68) demonstrated a baseline level of XO in non-ischemic intestinal mucosa of 19 %. Following 1, 2 and 3 hours of ischemia, the proportion of XO increased to 34, 46 and 61 %, respectively, indicating that XO gradually increases with increasing duration of ischemia. In its XO form, the enzyme uses O_2 as an electron acceptor instead of NAD^+, and generates $\cdot O_2^-$ and H_2O_2 by the oxidation of hypoxanthine, which is abundant in ischemic intestinal mucosa.

Hypoxanthine + $2O_2$ + H_2O $\xrightarrow{XO}$ xanthine + $\cdot O_2^-$ + H_2O_2	Formation of OFR's by the xanthine oxidase pathway

The catabolism of ATP to hypoxanthine, the substrate for oxidative formation of free radicals by the XO pathway, is another biochemical change occurring during ischemia which predisposes the intestinal mucosa to a tremendous OFR-forming capacity. During ischemia, ATP is rapidly utilized as a cellular energy source, and is broken down to adenosine 5'-monophosphate (AMP). AMP is further catabolized to adenosine and then inosine which then forms hypoxanthine (HX). In an intestinal hypoperfusion model in cats, it's demonstrated a drastic rise in the concentration of HX in mucosal tissue during 120 minutes of partial ischemia.

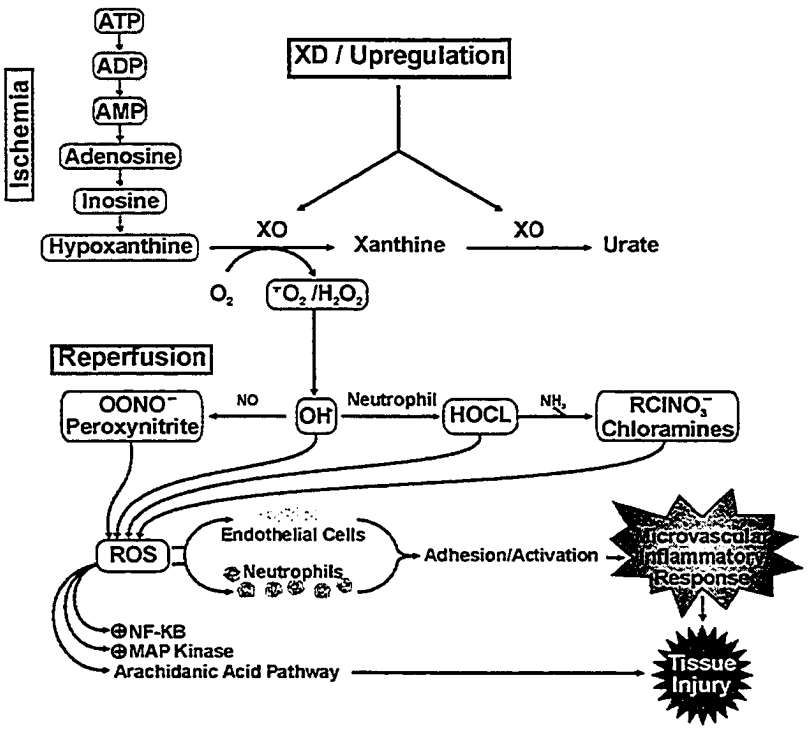


Figure 1-4. Mechanisms of oxygen free radical production during ischemia/reperfusion.

Upon reperfusion, the levels abruptly dropped towards baseline values. In a model of ischemia produced by endotoxemia, it's demonstrated that intestinal ischemia causes a 3-fold increase in hypoxanthine levels in arterial blood, and a 4.5-fold increase in portal venous blood, indicating generation of HX and subsequent leakage from damaged epithelial cells. Further evidence supporting the central role of xanthine oxidase and hypoxanthine in the pathogenesis of IRI is provided by Grogaard et al (69) who demonstrated in dogs that intraluminal infusion of XO and HX in the absence of ischemia resulted in the same morphologic and functional damage as seen in IRI. This injury was inhibited by treatment with allopurinol, an inhibitor of XO. Many studies have demonstrated that treatment with inhibitors of XO attenuates IRI. Parks et al (70) demonstrated that pre-treatment with allopurinol significantly attenuated the villus and crypt necrosis induced by 3 hours of partial ischemia. Parks and Granger (71) also showed that pre-treatment with intraluminal allopurinol or folate, both inhibtors of XO, significantly attenuate the increased intestinal permeability seen with IRI. It's been demonstrated a greater than 50 % reduction of OFR generation as measured by luminol chemiluminescence when allopurinol was added to intestinal mucosal tissue suspensions.

The intestinal mucosa is primed for OFR-generation and IRI by the accumulation of xanthine oxidase and one of its substrates, hypoxanthine, during ischemia. Upon subsequent reperfusion, there is an influx of the second substrate necessary for the XO pathway, namely molecular oxygen. Thus hypoxanthine is converted to xanthine and uric acid, thereby reducing O_2 to $\cdot O_2^-$. Korthus et al (72) demonstrated that reperfusion of ischemic skeletal muscle with hypoxic blood (arterial pO_2 3 to 5 mmHg) significantly attenuated reperfusion injury, and Parks and Granger obtained a similar result upon

reperfusion of intestine with normal saline. These experiments confirmed that oxygen is indeed an essential substrate in reperfusion injury.

Over evolutionary time, cells have developed endogenous control mechanisms to alleviate the damaging effects of OFR's. Superoxide dismutase and catalase are catalysts for the conversion of toxic superoxide to harmless water, thereby preventing oxidative damage (73). These endogenous scavengers (in addition to glutathione) adequately protect tissue from damage under normal conditions; however non-physiologic stressors such as organ storage and reperfusion overwhelm these protective systems and result in further exacerbation of injury. Pre-treatment of animals with SOD, the highly specific enzyme and OFR scavenger which converts O_2 to H_2O_2, has been shown to protect the intestinal mucosa from ischemia/reperfusion-induced histologic damage after 3 hours of partial ischemia followed by 1 hour of reperfusion. Treatment with catalase, the enzyme which detoxifies H_2O_2 to H_2O, and SOD attenuate the increased microvascular permeability induced by ischemia-reperfusion, and also inhibit the inflammatory cell inflammation which accompanies IRI (74). Catalase treatment has also been shown to decrease OFR production as measured by chemiluminescence in mucosal tissue suspensions after 1, 2 and 4 hours of cold ischemia and 30 to 120 minutes of reperfusion, as compared to non-treated tissue exposed to ischemia and reperfusion. Deferoxamine, an iron chelator, and apo-transferrin, an iron-binding protein, have been shown to attenuate the increased intestinal permeability when administered to cats before one hour of ischemia and 30 minutes of reperfusion (75). This confirms the important role of iron in the generation of OFR-mediated IRI by the iron-catalyzed Haber-Weiss reaction.

The role of glutathione as an OFR scavenger involves glutathione peroxidase, thereby enzymatically removing OFRs (61). The tripeptide thiol glutathione (GSH) has facile electron-donating capacity, linked to its sulfhydryl (-SH) group. Glutathione is an important water-phase antioxidant and essential cofactor for antioxidant enzymes; it provides protection also for the mitochondria against endogenous oxygen radicals. Its high electron-donating capacity combined with its high intracellular concentration endows GSH with great reducing power, which is used to regulate a complex thiol-exchange system. GSH depletion may be the ultimate factor determining vulnerability to oxidant attack.

Augmenting glutathione production is important since this endogenous antioxidant decreases during ischemia. Glutamine, a precursor of glutathione is able to enter the cell (unlike glutathione) and studies have demonstrated an effective (less OFR injury) increase in glutathione during IR (66). This leads to the question of the role of an amino acid-rich luminal solution that contains all three precursor amino acids (glutamate, cysteine, glycine). This important cellular protective mechanism may provide a direct link between nutrient supply and reduced IR injury. Glutathione exists in two forms: The antioxidant "reduced glutathione" tripeptide is conventionally called glutathione and abbreviated GSH; the oxidized form is a sulfur-sulfur linked compound, known as glutathione disulfide or GSSG. The GSSG/GSH ratio may be a sensitive indicator of oxidative stress. The reduced glutathione molecule consists of three amino acids - glutamic acid, cysteine, and glycine - covalently joined end-to-end. The sulfhydryl (-SH) group, which gives the molecule its electron-donating character, comes from the cysteine residue. Glutathione is present inside cells mainly in its reduced (electron-rich, antioxidant) GSH form. In the healthy cell GSSG, the oxidized (electron-poor) form,

rarely exceeds 10 percent of total cell glutathione. Intracellular GSH status appears to be a sensitive indicator of the cell's overall health, and of its ability to resist toxic challenge. Experimental GSH depletion can trigger suicide of the cell by a process known as apoptosis (Figure 1-5)(76).

Through its significant reducing power, GSH also makes major contributions to the recycling of other antioxidants that have become oxidized. This could be the basis by which GSH helps to conserve lipid-phase anti-oxidants such as alpha-tocopherol (vitamin E). Antioxidants are the body's premier resource for protection against the diverse free radical and other oxidative stressors to which it invariably becomes exposed (77). The antioxidant defense system is sophisticated and adaptive, and GSH is a central constituent of this system. Nowhere is its presence more important than in the mitochondria.

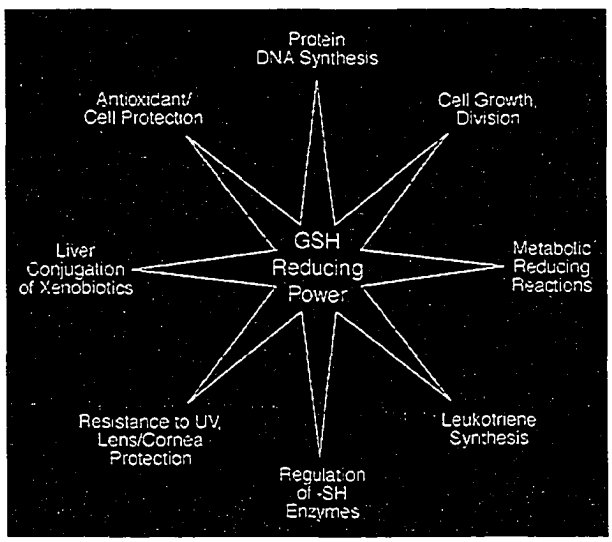


Figure 1-5. Cellular functions linked to reducing power of glutathione *(Kidd et al, Alternative Med Rev 1997; 2: 159)*(78).

Oxygen free radicals mediate IRI by a number of mechanisms, including direct cytotoxicity, as discussed above. However the most important effect of the early OFR's

is their role in the activation and infiltration of neutrophils, which are the most responsible mediators of tissue damage and dysfunction in IRI (Figure 1-6).

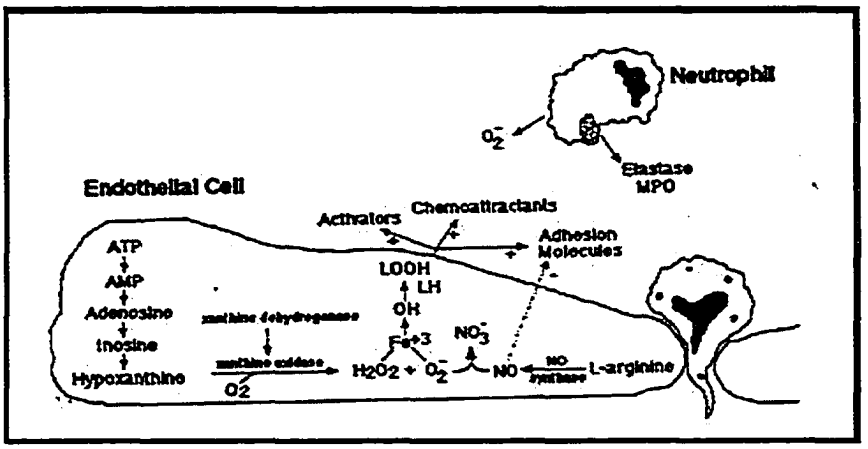


Figure 1-6. Oxidative stress events during ischemia/reperfusion.

The Role of Neutrophils in Ischemia-Reperfusion Injury

The inflammatory response during reperfusion inevitably leads to recruitment of inflammatory cells (79). The leukocyte appears to be the most important cell-type as it has been shown to adhere in great numbers to post-capillary venules of the small bowel graft upon reperfusion.

Ischemia triggers the activation of passenger macrophages, which release pro-inflammatory cytokines and mediate reperfusion injury during the early phase of reperfusion. IL-8, IL-12, IL-18, TNF-α, and IFN-γ will then activate recipient neutrophils and T-lymphocytes, which will trigger the delayed phase of reperfusion injury and perpetuate tissue damage. T-lymphocytes infiltrate tissue more rapidly than neutrophils and may also participate in the activation of recipient neutrophils after reperfusion (Figure 1-7).

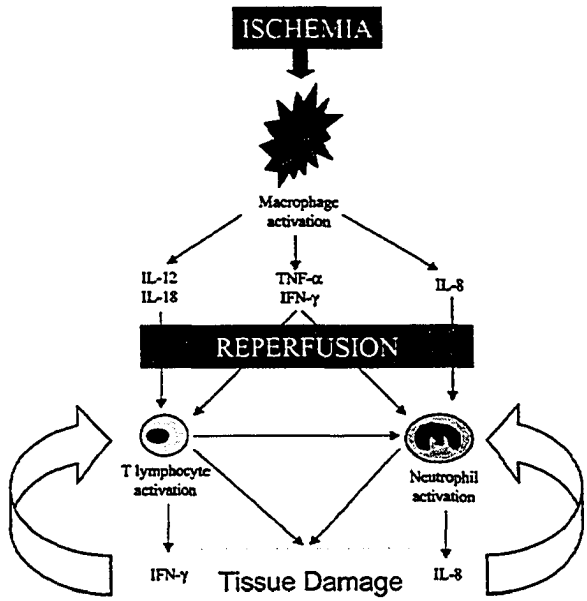


Figure 1-7. The potential mechanism of interaction between leukocyte activation and cytokine release during ischemia and reperfusion (*As published in de Perrot et al, Am J Respir Crit Care Med 2003; 167: 490-511*)(80).

Directly after reperfusion profound changes also in the shape of the endothelial cell because of edema and a drop in intracellular pH occur, what in turn promotes contraction of the cytoskeleton in the endothelial cells, significantly narrowing the lumen of the vessels (81). Hence, activated neutrophils adhere together with activated platelets to the damaged vessel wall, and due to their rigidity as well as diminished capillary diameter, they clog the microvasculature and considerably reduce tissue perfusion, creating a pathophysiological basis for no-reflow phenomenon (82). Platelet activation together with endothelial damage contributes to leukocyte recruitment and migration both by secretion of chemoattractants like PAF, PF4 or IL-8 and increasing adherence.

Neutrophils elicit their damaging effects predominantly by local infiltration of ischemic tissue upon reperfusion. Neutrophil infiltration has been demonstrated by

measuring myeloperoxidase (MPO), a biological marker for activated neutrophils. MPO is an enzyme found almost exclusively in neutrophils responsible for the conversion of H_2O_2 to HOCl. Grisham et al (83) demonstrated an 18-fold increase in neutrophil infiltration as measured by tissue MPO activity after reperfusion of ischemic tissue. This influx of neutrophils was attenuated by the pretreatment with the xanthine oxidase-inhibitor allopurinol or the OFR-scavenger SOD. These results suggest that XO-generated OFR's are responsible, either directly or indirectly, for the mucosal inflammatory infiltration of neutrophils during ischemia-reperfusion. No change has been shown in mucosal MPO activity following 48 hours of cold preservation in canine small intestine; however after 1 hour of reperfusion there was a significant increase in MPO activity, which was accompanied by the presence of severe histologic injury. Neutrophil infiltration was examined by direct MPO staining of intestinal tissue. They demonstrated a significant increase in number of activated neutrophils after 60 and 120 minutes of reperfusion in small intestine preserved for 2 hours in cold Ringer's lactate solution.

OFR's clearly play a role in the recruitment of neutrophils to post-ischemic tissue, and it is hypothesized that XO-derived OFR's initiate the production and release of pro-inflammatory agents, with subsequent attraction and activation of neutrophils. Neutrophil chemoattraction, migration, and infiltration at sites of inflammation is a complex process (Figure 1-8). In brief, neutrophil chemoattractants, including leukotriene B_4 (LTB_4), platelet activating factor (PAF) and interleukin-8 (IL-8), are important in directing the migration of neutrophils and activating local adhesion molecules on endothelial cell surfaces. Chemotaxis occurs when a soluble chemoattractant molecule diffuses away from the site of inflammation and induces neutrophil movement in the direction of increasing concentrations of the chemoattractant. Neutrophils express integrins, which

are ligands of endothelial adhesion molecules, which interact with selectins and intercellular adhesion molecules on the surface of endothelial cells at the site of inflammation. Selectins (E-selectin and P-selectin) are adhesion molecules responsible for the initial attachment of leukocytes to endothelial cells, and mediate the phenomenon of leukocyte rolling along the vessel wall. Firm attachment of the neutrophil to the endothelial wall occurs by the interaction of a specific neutrophil integrin (CD11/CD18) with an intercellular adhesion molecule (ICAM-1) on the endothelial surface. Transendothelial migration from the vessel lumen to the extracellular space is also mediated by chemoattractants, including IL-8 released from endothelial cells at inflammatory sites (84).

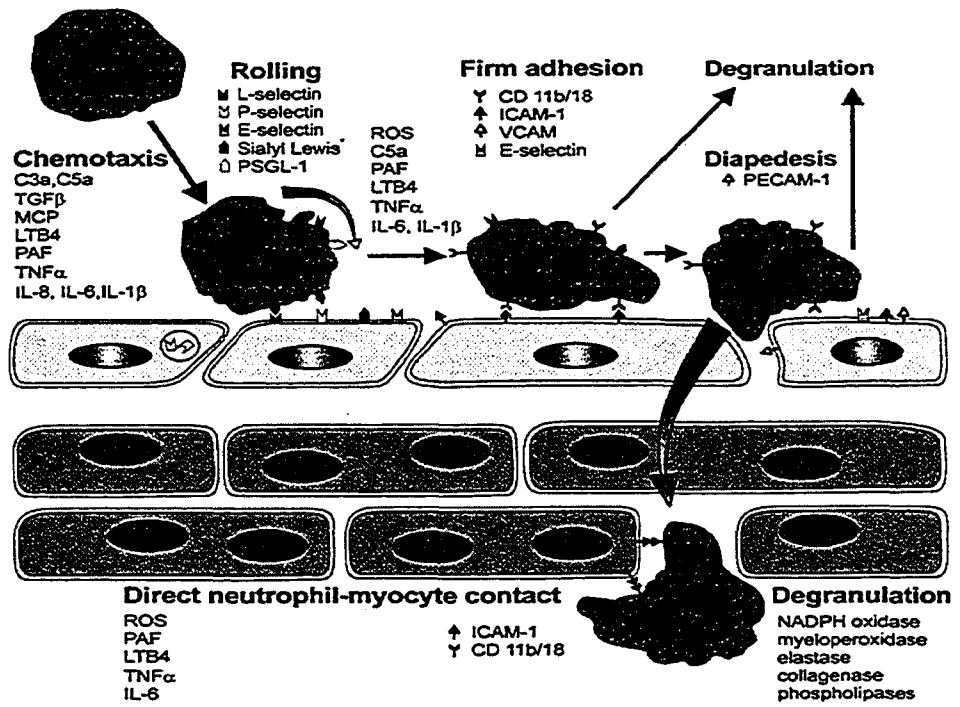


Figure 1-8. Stages of neutrophil activation. *(Kaminski et al, Int J Cardiol 2002; 86: 41-59)* (85).

Evidence of direct neutrophil interaction with endothelium in the setting of ischemia-reperfusion has been demonstrated using intravital microscopy. Massberg et al examined leukocyte-endothelial interactions in intestinal mucosa in a model of rat syngeneic small intestinal transplantation with 6 to 24 hours of cold preservation and 90 minutes of reperfusion (36). They observed the number of rolling leukocytes and adherent leukocytes dramatically increased in submucosal post-capillary venules and intramural collecting venules, as compared to sham-operated animals, with up to 600 adherent leukocytes per mm^2 in transplanted rats compared to less than 100 per mm^2 in sham controls.

In summary, activated neutrophils mediate tissue damage by three main mechanisms during IRI: (1) release of proteolytic enzymes which directly degrade the endothelial basement membrane and extracellular matrix, including elastase, collagenases, and proteases, (2) generation and release of OFR's, and (3) impeding blood flow in mucosal capillaries and post-capillary venules, causing the no reflow phenomenon, which will be discussed later. Neutrophils themselves are a source of OFR's, producing $\cdot O_2^-$ by the NADPH oxidase-catalyzed reduction of O_2. There are approximately 10 million neutrophils per gram of intestinal tissue, thus the intestinal mucosa can generate a huge cytotoxic $\cdot O_2^-$ flux (86). As previously mentioned, production of $\cdot O_2^-$ is acompanied by H_2O_2 due to the dismutation of $\cdot O_2^-$ by SOD. Hypochlorous acid is then formed from H_2O_2, catalyzed by MPO released from neutrophils. These OFR's trigger further OFR-formation including $\cdot OH$ and *N*-chloroamines, which go on to cause tissue damage.

The Role of Endothelial Cells in Reperfusion Injury

The vascular endothelium is an active site in the mediation of IRI, both as a source and a target of destructive mediators. Vascular endothelial cells produce and release hormones and endothelial factors which regulate vascular tone, platelet aggregation, and neutrophil infiltration (87).

Metabolites of arachidonic acid serve as a crucial link between OFR production and neutrophil infiltration. OFR's themselves are not direct neutrophil chemoattracts, however they initiate the production of LTB_4 and PAF, both of which are powerful chemoattractants (88,89). OFR's interact with mucosal endothelial cell membranes to activate phospholipase A_2, which leads to the formation of arachidonic acid in the lipid membrane. Arachidonic acid is metabolized by one of two pathways: the lipoxygenase pathway which leads to the formation of leukotrienes including LTB_4, and the cyclooxygenase pathway which forms thromboxane A_2 and B_2 and prostaglandins. Platelet activating factor is also formed from a membrane phospholipid by the action of phospholipase A_2.

LTB_4 is a powerful neutrophil chemoattractant which binds to specific receptors on neutrophils and elicits various responses including expression and activation of the adhesion molecule CD18 and generation of OFR's and proteases (90). PAF is produced in endothelial cell plasma membrane and stimulates the aggregation and activation of platelets, and the adhesion of neutrophils. Kubes et al (91) demonstrated a five-fold increase in mucosal PAF levels during reperfusion of ischemic tissue, and pre-treatment with PAF antagonists attenuated the IR-associated adhesion and infiltration of neutrophils. Thromboxane A_2 is produced and released by platelets from endogenous arachidonic acid metabolism, and is a potent vasoconstrictor and stimulator of platelet

aggregation. A fourfold increase has been demonstrated in mucosal production of thromboxane following 3 hours of ischemia and one hour of reperfusion of dog ileum.

Nitric Oxide (NO)

An important effector mechanism of macrophages depends on the induction of nitric oxide synthase (iNOS), generating nitric oxide (from L-arginine)(92). NO is rapidly converted to other reactive intermediates, reacts with superoxide to form highly reactive peroxynitrite (93). Many lines of evidence indicate that NO contributes to cytotoxicity required for elimination of tumors and invading micro-organisms (94,95). Many investigators have taken an interest in NO to suppress lymphocyte/T-cell proliferation, antibody production, graft-versus-host disease, mucosal damage, hemorrhage and vasodilatation (96-98). IFNγ is a potent inducer of NOS in macrophages and induction is enhanced by TNFα, among other agents (99). Besides the role of NO in immune defense, NO is involved with inflammation and autoimmune tissue destruction. Inhibition of hypoxia-induced vasoconstriction and platelet adhesion/aggregation are both key processes of particular relevance to ischemia/reperfusion injury following organ transplantation. Due to the cytotoxic nature of NO in host defense, it has been postulated that an inappropriate or nonspecific host cell attack may occur during non-physiologic stresses which may lead to inflammatory and autoimmune responses. Inhibition of NOS has been shown to protect isolated cells following activation of NO production. Cell death may be a combination of inhibition of mitochondrial function and/or DNA damage; both a direct result of NO production.

DNA repair mechanisms

A key mechanism by which IR affects cellular ATP and increases intestinal permeability involves OFR production and the subsequent formation of peroxynitrite.

Peroxynitrite is a potent trigger of DNA single strand breakage (100); DNA damage in turn activates the nuclear enzyme poly(ADP-ribose)polymerase (PARP)(101). PARP binds to damaged DNA and consumes NAD^+ to synthesize poly(ADP-ribose) chains on a variety of nuclear target proteins in a futile cycle which depletes cellular NAD^+ (102). Depletion of NAD^+ suppresses mitochondrial respiration and exhausts both NAD^+ and ATP pools. In a heavily damaged cell with massive activation of PARP, depletion of NAD^+ can have death-inducing consequences. Hence, PARP activation will influence energy-dependent processes, such as those encountered following SB transplantation; a period that relies heavily upon energy-consuming processes involved in maintenance of barrier function and mucosal regeneration.

Chemical inhibition of PARP with 3-aminobenzamide (3-AB) confers protection against ischemia/reperfusion injury in numerous models (103,104). Data from our lab links PARP utilization of adenylates and increased intestinal permeability in a mouse model of chronic inflammation (105). IL-10 gene-deficient mice develop a chronic colitis with increased intestinal permeability coupled with evidence of oxidative stress, and increased levels of PARP. IL-10 gene-deficient mice injected with 3-AB exhibited significantly greater ATP and total adenylate levels compared to their non-treated counterparts, and perhaps more importantly exhibited a return to normal intestinal morphology.

Some studies have suggested a link between PARP activation and the signalling pathways involved in pro-inflammatory gene expression and, furthermore, inhibition of PARP with 3-AB down-regulates the induction of iNOS, and TNFα secretion (106,107). Hence, PARP inhibition may have multiple beneficial effects of direct relevance to the preservation of small bowel grafts for transplantation; these include the down-regulation

of pro-inflammatory cytokines, reduced neutrophil infiltration, conservation of ATP & NAD^+; and normalization of intestinal permeability.

No "Reflow" Phenomenon

Many experiments have demonstrated a decrease in total intestinal blood flow during reperfusion. Papparella et al (108) showed intestinal blood flow decreased by 59 %, 50 % and 40 % respectively with no recovery during the entire reperfusion period of 120 minutes, or a continued decline in the intestinal blood flow throughout the 60 to 180 minute period of reperfusion. Additionally it has been shown that systemic mean arterial pressure (MAP) does not change after reperfusion, or there is only a brief dip in MAP for 15 minutes followed by a recovery to baseline values, thus indicating that there is another factor accounting for the diminished intestinal blood flow.

The "no reflow" phenomenon has been implicated as the explanation of this observed decrease in intestinal blood flow, and furthermore no reflow has been found to significantly contribute to much of the mucosal damage following intestinal ischemia and reperfusion. Menger et al (109) used intravital microscopy following 4 hours of ischemia and two hours of reperfusion in hamster striated muscle, and demonstrated a decreased functional capillary density, (a measure of the number of perfusing capillaries as seen with a fluorescent marker in a given area), to 30 % of baseline, indicating no reflow in 70 % of capillaries following ischemia-reperfusion. In addition, they demonstrated a decrease in red blood cell (RBC) velocity in capillaries and post-capillary venules during the two hours of reperfusion, with partial recovery at 24 hours post ischemia. This diminished microcirculatory blood flow was correlated with increased leukocyte interaction (rolling and adhesion) with vessel walls, and with more severe histologic damage. In a rat small bowel transplant model, Massberg *et al* examined the

microcirculation using *in vivo* fluorescence microscopy following 6 to 24 hours of cold preservation. They showed a decreasing functional capillary density and capillary RBC velocity in the mucosa and muscular microcirculation with increasing lengths of cold ischemia time. This was correlated with number of rolling and adherent leukocytes in submucosal postcapillary venules. They also demonstrated a decrease in number of villi perfused proportional to cold ischemia time, with 100 % perfusion in sham operated animals, 99.8 % in the 6-hour preservation group, 89.7 % in the 12-hour group, 87.9 % in the 18-hour group, and then a drop to 40 % villi perfused in the 24-hour preservation group.

The hypothesis generated from these experiments points to the central role of neutrophils in mediating no reflow. Neutrophils, as discussed, are chemoattracted to areas of IRI indirectly by OFR's, and then neutrophils interact and adhere to endothelial cells by direct ligand-receptor interaction. This accumulation of neutrophils in the microcirculation can cause mechanical impedance to blood flow as demonstrated by RBC velocity, and furthermore promote platelet aggregation which further plugs the microcirculation. A second mechanism is that neutrophils damage the endothelial lining and basement membrane by release of proteases, collagenases and elastases, which causes an increase in capillary permeability and a leak of fluid from the plasma to the interstitium. This result in increased viscocity in the microcirculation with a consequent decreased velocity and ultimately complete cessation of blood cell flow through the microcirculation, hence, no reflow.

The end result of ischemia-reperfusion-induced OFR generation and neutrophil infiltration is structural damage to the mucosal barrier, accompanied by functional changes as well. As many studies have illustrated, the degree of mucosal damage

parallels the extent of OFR and other mediator production and neutrophil infiltration, and is consistently considerably more severe upon ischemia *and* reperfusion than ischemia alone.

Protective mucosal barrier function

One of the most critical functions of the intestine is to provide a protective barrier from translocation of microorganisms. In a 10-year prospective study, Cheadle et al (110) surveyed patients on the surgical service and found that transplant patients are at greatest risk of sepsis and septic complications. Sigurdsson et al (111) carried out a retrospective review of pediatric intestinal transplants over nine years, and found an average rate of bacteremia of 2.1 episodes per patient. Enteric organisms were the most common pathogen isolated, with 76 gram negative rods and 36 enterococci, out of 133 positive blood cultures. Another report (112) demonstrated that bacterial isolates in blood are the same as stool isolates in 81 % of multivisceral transplant patients with sepsis, indicating bacterial translocation is the most common mechanism of developing bacteremia. In an experimental model of pig SBT, Fryer et al (113) demonstrated a 60 to 100 % rate of bacteremia on the third day post-transplant, with no significant difference in animals with systemic venous drainage versus portal drainage, and no difference in immunosuppressed animals. In addition, viable enteric bacteria were recovered from virtually all portal lymph nodes, mesenteric nodes and liver samples on post-operative day three, thus indicating bacterial translocation is prevalent following SBT.

Energetics

The epithelial permeability barrier is maintained largely by the tight junction region close to the apical surface of intestinal epithelial cells. The tight junction consists of a number of proteins which are dynamic, energy-requiring structures. These proteins

are modulated by alterations in pH (114), calcium concentration (115), cAMP levels (116), temperature (117), and osmolarity (118). In addition, agents that deplete cellular ATP also cause a dilation of the tight junction at the ultrastructural level, a perturbation of the actin cytoskeleton, an increase in epithelial permeability, and an increase in transepithelial flux of macromolecules (118). This disruption of the permeability barrier seen in cells subjected to ATP depletion has been shown to be caused by a disassembly of the protein components of the tight junction, with a subsequent association of these proteins into large, non-functional complexes within the cytoplasm. Importantly, this disassembly is reversible; repletion of cells with ATP leads to a re-assembly of the tight junctions and restored epithelial barrier function. Thus, the increase in intestinal permeability seen in ischemia/reperfusion could be due to a reduction in cellular ATP and an inability of epithelial cells to maintain tight junctions. This in turn results in increased bacterial translocation, dramatic increases in inflammation and the potential for life-threatening infection.

Mucosal ATP is an important assessment of intestinal graft viability, as it reflects the energy-synthesizing capacity of the cell, and ultimately the enterocyte's ability to recover its normal cellular homeostatic functions following ischemia. Irreversible energy failure is demonstrated by a severe decline in ATP levels during ischemia, with no recovery upon reperfusion, as shown in a study by Vejchapipat et al (119) following as little as 90 minutes of warm ischemia. They found ATP levels are a useful indicator of tissue viability after reperfusion. ATP was found to be a sensitive indicator of IRI in a study where they found mucosal ATP levels abruptly decrease during 30 minutes of warm ischemia and partially recover during reperfusion over 150 minutes, correlating well with histologic damage, correlating well with histologic damage. Yamada et al (120)

showed a decline in ATP levels proportional to the length of cold preservation (from 6 hours to 48 hours) of rat small intestine. Recovery of ATP after 30 minutes of reperfusion also correlated very well with length of cold ischemia time (CIT), demonstrating a recovery to 60 % of baseline ATP levels in the 6-hour preservation group, compared to virtually no recovery in the 48-hour group.

Regeneration

Regeneration is recovery of the intestinal mucosa, both structurally and functionally, following ischemia-reperfusion injury, and is absolutely necessary for ultimate recovery following small bowel transplantation. Many studies have demonstrated the enormous regenerative capacity of the intestinal mucosa. In 1972, Robinson et al (121) demonstrated considerable histologic damage in the intestinal mucosa following one hour of arterial and venous occlusion, causing deepithelialization of villus tips, mucosal edema, vascular stasis, and hemorrhagic infiltration in the lamina propria. Following 24 hours however, there was complete structural recovery and restored vascularization to the villus core. Regeneration following intestinal transplantation has been demonstrated in rats, whereby a moderate grade of histologic damage was seen in the 120 minutes of reperfusion following 1, 2 or 4 hours of hypothermic preservation, and complete histologic recovery was observed 24 hours later in the 1 and 2-hour cold preservation group, and near complete recovery in the 4-hour group. Severe histologic damage including complete denudation of villi and some crypt cell injury following small bowel transplantation has been demonstrated in dogs with 24 hours of cold ischemia. In spite of this the jejunal mucosal structure completely recovered in 28 days, and the ileal mucosa had also greatly improved and showed marked regeneration. They also showed an increase in mucosal levels of ornithine

decarboxylase, the rate-limiting enzyme in polyamine synthesis, which is a marker for cellular proliferation, as polyamines stimulate cellular DNA and RNA synthesis. These studies demonstrate that no matter the degree of villus injury, if the pluripotent cells at the crypt base remain intact, cell proliferation and regeneration is still possible, however structural regeneration takes longer with more severe initial ischemia-reperfusion damage. Miner et al interestingly demonstrated a proliferation of goblet cells in intestinal villi 24 hours following a 30-minute SMA occlusion. It is suggested that this may be due to the protective function of mucous glycoproteins which have been shown to scavenge OFR's (122).

Reperfusion injury and antioxidants

Several studies have also shown that addition of antioxidants to perfusion solutions can have beneficial effects against reperfusion injury in heart, kidney and brain. This is a relevant strategy because endogenous antioxidant capacity can be overwhelmed by oxyradical overgeneration during reoxygenation. Moreover, in some cases, the activity of certain endogenous antioxidant enzymes is actually decreased during ischemia of mammalian organs.

Addition of antioxidants after the onset of reperfusion is, however, without much effect (this is because over-production of free radicals has already occurred). Several antioxidants have been capable to reduce post-ischemic oxidative stress in heart, brain and kidney; examples are SOD, polyethylene glycol SOD (PEG-SOD has higher plasma half-live than SOD), SC-52608 (a low-molecular weight SOD-mimetic), U74006F (a chain break antioxidant), the oxyradical scavengers dimethylurea and *N*-2-mercaptopropionyl glycine, and the iron chelator deferoxamine. This last one demonstrates the involvement of transition metal ions in free radical production during

reperfusion, possibly by catalyzing Fenton reactions. Delocalization of iron during ischemia in several organs (the causes are still unsolved), such as kidney, seems to be of relevance for iron-mediated oxidative stress and cell damage during oxygen-reperfusion.

Many studies with the use of exogenous antioxidants have given numerous disappointing results in experiments dealing with myocardial infarction. In the case of brain ischemia/reperfusion, not all studies with antioxidants have demonstrated neuroprotection. That was possibly caused by the ability (or not) of the antioxidants to gain access to the sites of free radical production during reperfusion. On the other hand, the use of exogenous antioxidants and allopurinol has been beneficial in improving the efficiency of experimental organ transplants, in special kidney, after cold (0-5°C), hypothermic (5-30°C) or warm ischemic storage (37°C). Cold or hypothermic storage are known to prolong kidney metabolic viability by delaying ATP depletion and the loss of calcium homeostasis.

Many experiments have studied preservation conditions and their effect on intestinal transplantation. Zhu *et al* (123) demonstrated improved outcome with luminal preservation, showing improved survival after transplantation in grafts preserved with vascular and luminal Ringer's lactate (RL) (80 % 5-day survival) compared to vascular preservation alone (67 % 5-day survival). Others have compared different vascular preservation solutions. Schweizer et a*l* (124) demonstrated histologic damage in rat small bowel autotransplantation after preservation with UW is less severe than preservation with Eurocollins solution. Gundlach et al (125) also demonstrated that UW vascular preservation is superior to RL or Eurocollins, as measured by improved recovery of ATP levels upon reperfusion, and decreased severity of mucosal structural

damage. Sasaki et al (126) showed that the addition of 2 % or 4 % glutamine to the vascular preservation solution (UW or normal saline) improved histologic grade of injury following 18 hours of cold ischemia and transplantation in rats. Glucose transport was also improved (127). In 1970 Chiu et al (32) demonstrated the benefit of luminal administration of glucose in protection from ischemia, whereby 60 minute SMA occlusion with the immediate luminal administration of 10 % glucose in water improved the degree of histologic injury and mucosal ATP content. In 1996, Kokudo et al (128) demonstrated a similar benefit of glucose in the luminal preservation solution during small bowel transplantation. They demonstrated improved electrophysiologic function of mucosa as measured by Ussing chambers when 2.5% glucose was added to RL as the luminal preservation solution, with vascular RL for 12 hours of hypothermic storage.

Many interventions to improve IRI and SBT have been targeted at OFR's. As already discussed, many studies have demonstrated the beneficial effects on all aspects of IRI by pretreatment with superoxide dismutase (SOD), catalase, deferoxamine, and allopurinol, all of which interfere with the xanthine-oxidase pathway of OFR generation.

Regeneration of small intestinal mucosa after ischemia-reperfusion injury

Transcriptional of immediate early genes, such as c-fos and c-jun, have been observed after ischemia-reperfusion (I/R) injury of liver and heart, and it is thought that proteins coded by those genes induce apoptosis of proliferation of the post-ischemic cells. Taguchi et al (129) have shown that c-Fos and c-Jun increase after small intestinal transplantation. The aim of their study was to prove the relationship between transcriptional activation of immediate early genes and cellular responses, such as apoptosis or proliferation, in the rat small intestinal epithelium after I/R stress.

They isolated whole small intestines of male Lewis rats and clamped for 30 minutes, and then reperfused. Tissue specimens were taken at 0, 15, 30, 60, 90, 120, 180, 240, and 360 minutes after reperfusion. Total RNA was extracted from mucosa, and expression of c-fos and c-jun were quantified by semi-quantitative RT-PCR method. Proliferation of the epithelial cell was visualized by immunohistochemisty for proliferating cell nuclear antigen (PCNA), and apoptosis was detected by in situ terminal deoxynucleotidyl-transferase-mediated dUTP biotin nick end labeling (TUNEL) method.

The c-fos expression was activated transiently, peaked at 30 minutes after reperfusion, and immediately declined. Activation of c-jun was slower, peaking at 90 minutes after reperfusion, with an expression prolonged more than c-fos. The number of PCNA-positive cells increased at 60 to 360 minutes after reperfusion, and TUNEL-positive cells increased later than PCNA-positive cells, at 120 to 360 minutes after reperfusion (Figure 1-9).

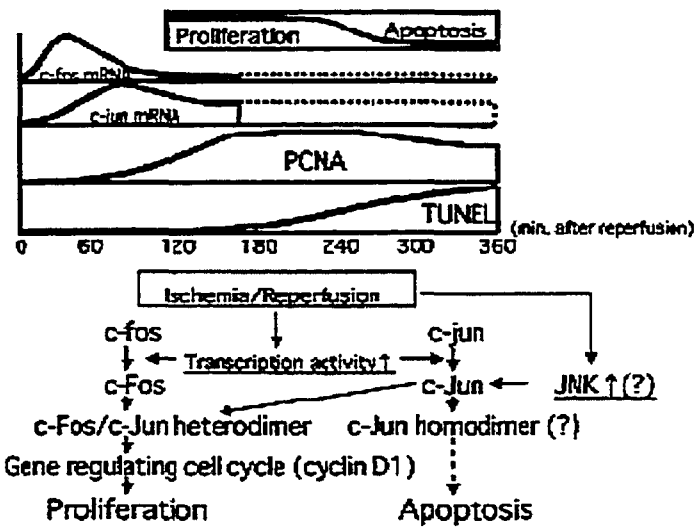


Figure 1-9. Expression of c-fos and c-jun in relation to proliferation and apoptosis of enterocyte after ischemia and reperfusion.

Patterns of mRNA expression of c-fos and c-jun in rat intestine after ischemia-reperfusion were similar to those in mouse liver (130). After I/R stress, transcriptional activation of c-fos and c-jun was observed in the small intestinal epithelium. Sequential expression patterns of those genes were believed to be related to the cellular responses such as proliferation and apoptosis. Increase of c-jun expression correlated with increase of TUNEL-positive cells, therefore c-jun was suggested to be one of the factors that induce apoptosis.

Conclusion

Ischemic injury occurring during hypothermic preservation, and reperfusion injury generated upon revascularization of the graft, are clearly important factors in ultimate graft and patient (or laboratory animal) survival. Although extensive research has been carried out in the fields of organ preservation and reperfusion injury, clearly there is still much room for improvement, as evidenced by the inferior long term patient and graft survival following small bowel transplant compared to other solid organ transplants.

The small intestine is unique in solid organ transplants, in that there are two accessible routes of administration of preservation solutions, namely vascular and luminal. Surprisingly, there has been very little research in the area of luminal preservation, in spite of the fact that enterocytes, especially at villus tips, derive much of their nutrient support from the lumen. With current preservation techniques, both experimentally and clinically, there is invariably villus tip injury, if not complete epithelial denudation and lamina propria damage. Preliminary studies in our lab have been enlightening, demonstrating significant benefits of preservation with a luminal solution tailored to the metabolic requirements of the enterocyte, compared to vascular preservation alone. Olsen et al demonstrated that the addition of impermeant (both osmotic and oncotic) to

simple crystalloid solutions could provide better SB graft quality when compared to the current clinical standard, vascular flush with UW solution (131). Olsen et al also examined the effect of vascular flush with simultaneous addition of N,N-bis[2-hydroxyethyl]-2-aminoethane sulfonic acid (BES), a known buffering agent, and gln supplementation by addressing the fundamental metabolic principle of pH homeostasis (132). The study strongly supported that the beneficial effects of gln-enriched UW solution could be amplified when combined with an effective buffering agent such as BES. As small bowel was typically obtained at the time of multi-visceral organ procurement which relied on a common vascular flush, use of such a solution was not clinically applicable.

So, they hypothesized that addition of a luminally-delivered solution, formulated on amino acid requirements for energy- and non-energy-related reactions would provide site-specific preservation of mucosal energetics, barrier function and morphology throughout an extended period of cold storage. A series of experiments documented that a luminal-delivered solution, formulated on physiologic SB requirements, could provide targeted preservation of the SB mucosa (40). Following demonstrating a beneficial role for the luminal administration of preservation solutions in a small animal model, direct clinical applicability using human SB was examined (133). That study supported luminal administration of preservation solutions for improvement of human SB graft quality during clinically relevant periods of cold storage.

The next logical step following these results will be to determine if these beneficial effects seen following preservation alone are translated into attenuation of injury during reperfusion, and subsequently improved regeneration and restoration of normal intestinal function. Many studies have demonstrated that the degree of injury

sustained during hypothermic ischemia, related to the duration of cold storage, correlate well with degree of reperfusion injury, both morphologically and functionally. Thus we expect that the addition of luminal preservation will indeed ameliorate reperfusion injury. However there is undoubtedly still more room for improvement. Thus, we further propose to administer agents which have been shown to attenuate ischemia-reperfusion injury. We expect, based on previous research, that these agents will protect intestinal grafts from ischemia-reperfusion injury. We expect that grafts, if protected from IRI, are more successful at regeneration and restoration of normal intestinal functions. Our ultimate goal is to develop a protocol which can be translated to the clinical realm, encompassing organ preservation with combined vascular and luminal routes, and pretreatment with modifiers of IRI, and potentially post-transplant administration of agents that will promote regeneration.

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Chapter 2

Methodology

General Experimental Procedures

Rodents were housed in the animal facility located at the S.M.R.I. and cared humanly in accordance with the Canada Council on Animal Care published guidelines. The studies were approved by the University of Alberta Health Sciences Animal Welfare Committee. Experiments involve 200-300g male Sprague-Dawley (n = 4-6 per group). Rats were fasted overnight in cages with raised floors to minimize coprophagy. Water was provided *ad libitum.*

Surgical Procedures

Under anesthesia (1-2% halothane or 1-3 % isoflurane), the small bowel in Sprague-Dawley rats was exposed via midline laparotomy.

Storage-type experiments

After clamping the supraceliac aorta, the superior mesenteric artery (SMA) was flushed with 10 ml cold University of Wisconsin solution. The suprahepatic vena cava was transected to facilitate the outflow of both blood and perfusate. The entire jejunum and ileum was subsequently harvested. Luminal solutions (20 ml; equivalent to ~1.0 ml/g tissue) were then placed into the lumen of the bowel allowing the effluent to exit uninhibited. Each end was immediately ligated with 3-0 silk, leaving the bowel filled without turgor with the same solution. The bowel was then stored in 30 ml of solution and stored on ice in a 4°C incubator.

Ischemia time-points: Tissue samples (1-2 g) were taken at 4, 8, 12 and 24 hours post vascular flush.

Reperfusion-type experiments

The superior mesenteric artery (SMA) was clamped for a period of 60 min ischemia for reperfusion studies. Time points (for tissue sampling) encompassed a short-term period of reperfusion (0 to 90 min) in order to observe immediate changes in energetics, OFR and related events. The advantage of this simple surgical technique for reperfusion was that we could easily carry this out in the lab and SMA occlusion resulted in >95% reduction in intestinal blood flow (blood desaturation occurs within seconds). Furthermore, interaction between the events of IR injury leading to inflammation (neutrophil infiltration) was observed in this whole animal model. Repeated sampling of SB was limited by removal of tissue thereby compromising recovery of remaining segment in the animal.

The time points chosen for tissue sampling were based on specific time-related events of metabolic, structural and functional recovery; scheduled time points and relevant events.

Short-term reperfusion time-points:

0 min – metabolic, structural, functional injury following 60 min ischemia;

15 min – initial 'burst' OFR production and metabolic recovery;

60 min– short term metabolic (adenylate) recovery;

90 min – initial recovery from oxidative damage.

SB Transplantation - Experiments in the small animal SB transplant model focus on alleviating IR injury and maximizing regeneration; this include the luminal administration of an AA solution prior to and during reperfusion. Our department's animal microsurgery lab (Dr. Lin-Fu Zhu, Microsurgeon) is well established as part of Dr. Norman Kneteman's (Director of Transplantation) research program. Dr. Zhu has 12 years experience in

animal microsurgery and has performed an estimated career total of 2500 organ transplants (with ~250 SB transplants) and all required facilities exist at the S.M.R.I. Orthotopic SB transplants according to established microsurgical techniques were performed with syngeneic rats (Lewis-Lewis).

Donor operation: Under anesthesia, SB was exposed and associated arteries/veins ligated. The SB was isolated and attached only to its vascular pedicle including the portal vein and infrarenal aorta. The aorta was ligated proximal to SMA and the graft flushed with 5-10 ml vascular preservation solution (UW); the portal vein provided an outlet. If a luminal preservation solution was to be administered, then 20 ml solution was permitted to flow through the SB lumen. After luminal flush, graft ends were tied to 'capture' remaining solution. The SMA with a cuff of aorta was resected and the graft removed and stored in 0-4 °C solution for 6 h.

Recipient Operation: The recipient's intestine was exposed and IVC/aorta cross-clamped. Donor bowel was brought in for engraftment. Portal vein was attached to the IVC (end-to-side fashion). Following aortic anastomosis, blood flow was re-established in the transplanted graft. The recipient's small intestine was removed after ligation of all blood vessels. The wound was closed. Hydration and body temperature were maintained throughout recovery. Tissues were sampled at the following times:

0 min – metabolic, structural, function injury after 6 h cold storage;

35 min– initial period of oxidative injury;

1 and 3 days – initial repair and metabolic/oxidative recovery;

7 d – extensive regeneration and repair of mucosa. The animals were sacrificed by day 14^{th}.

Composition of amino acid solution

Table 2-1: Progression of our Nutrient-rich, amino acid-based (AA) Solution.

	Version of AA Solution	
	Chapters 4-6	**Chapters 7-9**
Lactobionate	20	20
Adenosine	5	5
Bes	15	15
Glutamine	35	35
Glucose	20	20
Glutamate	20	20
Aspartate	20	20
Arginine	10	10
Glycine	10	10
Valine	10	10
Asparagine	10	10
Threonine	10	10
Lysine	10	10
Serine	10	10
Methionine	5	5
Ornithine	5	5
Leucine	5	5
Isoleucine	5	5
Histidine	5	5
Cysteine	5	-
Cystine	-	5
Proline	5	5
Hydroxybutyrate	3	3
Tyrosine	1	1
Tryptophan	1	1
Dextran (67.3 kdal)	5%	5%
Allopurinol	1	1
Trolox	-	1
3-Aminobenzamide	-	1

Numbers shown are in mM amounts unless otherwise stated. The pH of solutions was adjusted to 7.40 ± 0.01 using NaOH.

Reason for Modification

Supplementation with highly effective chemical antioxidant, Trolox; reduces oxygen radical-mediated injury. Addition of PARP inhibitor, 3-aminobenzamide (3AB); conserves cellular ATP. Cystine was substituted for cysteine to avoid potential toxic effects.

Experimental Endpoints - At specified times following reperfusion, tissue samples were snap-frozen in liquid nitrogen for analysis of parameters (A-H) below, leaving the remainder ('fresh' tissue) for structural and functional assessment (H, I). For transplanted animals, tissue was analyzed in a similar manner depending on experimental design and focus.

a) **Energetics:** Adenylates – ATP, ADP, AMP, Total Adenylates, ATP/ADP ratio;

b) **Metabolic status:** End products – of amino acid metabolism: ammonia, alanine; of carbohydrate metabolism: lactate;

c) **Oxidative Damage:** Lipid peroxidation – malondialdehyde (MDA);

d) **OFR Sources:** Myeloperoxidase (neutrophil);

e) **OFR Scavenger:** Glutathione– endogenous scavenger influenced by amino acids

h) **Structure:** Light Microscopy – gross histology graded by Park's classification;

i) **Barrier Function:** In vitro permeability (Ussing chambers) and mucosal electrophysiology;

Tissue Preparation for metabolite assays

The samples of small bowel tissue (~ 100 mg) were weighed out in polypropylene test tubes and were then stored on dry ice until being homogenized in 6% perchloric acid (containing 1mM EDTA which chelates heavy metals) using an Ultra-Turrax hemogenizer. A 50 µl aliquot of the homogenate was removed and placed in 950 µl 0.15 M NaOH solution for protein determination. The samples were then immediately centrifuged at 4°C for 15 min at 20000g to remove precipitated proteins. The supernatant was then transferred to a 1.5 ml microcentrifuge tube, neutralized with the addition of 3 M KOH/ 0.4 M Tris/ 0.3 M KCl and then centrifuged. The supernatants were placed on ice and immediately assayed for ATP, ADP, and AMP. These assays were

performed enzymatically based on the absorbance of NADH at 340 nm using a Dynatech MRX plate reader.

Metabolite assay

All metabolite assays used are based on oxidation or reduction of the purine nucleotide NAD(H) monitored spectrophotometrically at 340 nm. An aliquot of the sample extract of known volume was placed in 200 µl of the appropriate reaction mixture in well of a 96-well plate. The initial absorbance of this mixture was recorded, and the appropriate enzyme was then added to initiate the reaction. The progress of the reaction was monitored using standard amounts of the measured metabolite, and upon completion of the reaction, a final absorbance reading was recorded. Blank wells containing only reaction mixture and enzyme with no tissue extract were also recorded to be factored into the final calculation.

A calibration curve for the absorbance of NAD(H) for the plate reader was produced using standard amounts of NAD(H). The amount of NAD(H) converted in the well was then measured using the plate reader by calculating the absolute difference between the final and initial readings.

Oxidation of substrate:

$$A + B \rightarrow C + D$$

$$C + NAD^+ \rightarrow E + NADH + H^+$$

Reduction of substrate:

$$A + B \rightarrow C + D$$

$$C + NADH \rightarrow E + NAD^+$$

The molar quantity of NAD(H) converted, as measured by plate reader, was therefore equal to the molar quantity of the measured substrate, with the exception of AMP which was one half the molar quantity converted.

Measuring ATP:

Solutions:

A. Tris buffer (100 mM): Dissolve 12.11 g Trizma ® base (minimum 99.9% titration, CAS 77-86-1, Sigma Chemicals) in 800 mL of distilled water and adjust the pH to 8.0 using a 2 M NaOH solution or diluted HCl solution (final volume up to 1 L).

B. Magnesium Sulfate solution (100 mM): Dissolve 12.32 g of Magnesium Sulfate in 500 mL of distilled water.

C. Glucose solution (100 mM): Dissolve 0.18 g Glucose in 10 mL of distilled water.

D. ATP Standard (0.6 mM, Adenosine 5-Triphosphate disodium salt, Sigma Chemicals): Prepare 1.5 mL of a 60 mM standard in Tris buffer and dilute to 0.6 mM. The pH of the standard should be 8.0.

Assay Procedure:

Plate all the samples (100 µL for rat small bowel extractions) and standards (10, 20, 30, 40, 50, 80, 100 µL) and then prepare the reaction mixture by combining 1.0 mM β-Nicotinamide Adenine Dinucleotide, 5 mM Magnesium Sulfate, 2 mM Glucose, and Glucose-6-Phospate Dehydrogenase (Leuconostoc, 0.5 units per assay) and volume up with Tris buffer. Pipette 200 µL of the reaction mixture in each well. Record the initial absorbance at 340 nm using a Dynex MRX TC plate reader. Add 20 µL of Hexokinase (0.5 units/assay) to each well. Read the absorbance every 30 minutes until completion (approximately 2-3 hours). The change in optical density due to ATP utilization was calculated by using Microplate Analysis Program, version 1.75 (1).

ATP + glucose → ADP + glucose-6-phosphate (hexokinase)

glucose-6-phosphate + NAD^+ → 6-P-gluconolactone + NADH + H^+ (G6PDH)

Measuring ADP and AMP:

Solutions:

A. Imidazole buffer (100 mM): Dissolve 6.808 g Imidazole (1,3-Diaza-2,4-cyclopentadiene, ACS 288-32-4) in 800 mL of distilled water and adjust the pH to 7.0 using a 2 M NaOH solution or diluted HCl solution (final volume up to 1L).

B. Magnesium Sulfate solution (100 mM): Dissolve 12.32 g of Magnesium Sulfate in 500 mL of distilled water.

C. Glucose solution (100 mM): Dissolve 0.18 g Glucose in 10 mL of distilled water.

D. AMP Standard (0.6 mM, Adenosine 5'-Monophosphate Sodium, minimum 99%, Sigma Chemicals): Prepare 3.0 mL of a 60 mM standard in Imidazole buffer and dilute to 0.6 mM.

E. ADP standard (0.6 mM, Adenosine 5'-Diphosphate Sodium, Sigma Chemicals): Prepare 3.0 mL of a 60mM standard in Imidazole buffer and dilute to 0.6mM.

Assay Procedure:

Plate the samples (75 µL for rat small bowel extractions) and standards. Prepare the reaction mixture by dissolving NADH (β-Nicotinamide Adenine Dinucleotide (reduced form, disodium salt), 0.4 mM), PEP (Phospho (enol) pyruvate, 0.5 mM), ATP (Adenosine 5'-Triphosphate, 0.4mM), MgSO4 (5 mM, Solution B), KCl (50 mM), and LDH (L-Lactic Dehydrogenase (type II rabbit muscle), 0.5 units/assay) with Imidazole buffer (solution A). Add 200 µL of the reaction mixture to each well and read the initial absorbance at 340 nm on a Dynex MRX TC plate reader. Add 0.5units/assay Pyruvate Kinase. The plate was read every 15 minutes until reaction completion in approximately 1-2 hours. The optical density change due to ADP utilization was calculated giving a value for the molar quantity of ADP in each well. This reading was used as both the final ADP

absorbance and the initial AMP absorbance. 0.5 units/assay myokinase was added and the change in optical density due to AMP utilization was recorded.

AMP + ATP → 2 AMP (myokinase)

ADP + phosphoenolpyrovate → ATP + pyruvate (pyruvate kinase)

Pyruvate + NADH + H^+ → lactate NAD^+ + H_2O (lactate dehydrogenase)

Lactate assay procedure

Prepare Hydrazine buffer reaction mixture (pH = 9.6) by dissolving Hydrazine (150 mM) and NAD+ (1.0 Mm) in distilled water. NaOH was used to bring the pH to 9.6. Plate samples/standards in a 96-well plate. Add 200 µL of hydrazine buffer to each well. The initial absorbance is read at 340 nm on a Dynex MRX TC plate reader. Add LDH (L-Lactic Dehydrogenase from bovine heart, 1 unit per assay). Read the absorbance every hour for approximately 4 hours or until reaction was complete. The change in optical density due to AMP utilization was recorded.

Lactate + NAD^+ → pyruvate + NADH + H^+ (lactate dehydrogenase)

Pyruvate + hydrazine → pyruvate-hydrazone

Alanine assay

Solutions:

A. Tris buffer (100 mM): Dissolve 12.11 g Trizma ® base (minimum 99.9% titration, CAS 77-86-1, Sigma Chemicals) in 800 mL of distilled water and adjust the pH to 8.0 (final volume 1 L).

B. Alanine Standard (L-Alanine, minimum 98%, Sigma Chemicals, 0.6 mM): Prepare 1 mL of a 600 mM standard in Tris buffer. Use serial dilutions to create a final standard concentration of 0.6 mM.

Assay Procedure:

Plate the samples (100 µL) and standards. Prepare the reaction mixture by dissolving NADH (β-Nicotinamide Adenine Dinucleotide (reduced form, disodium salt), 0.4mM), α-Ketoglutaric acid, (Sigma Chemicals, 1.0 mM), and LDH (L-Lactic Dehydrogenase (type II rabbit muscle), 2 units/assay) with Tris buffer. Add 200 µl of the Reaction mixture to each well. Wait 20 minutes, as there may be pyruvate present resulting in an inaccurate increase in the optical density reading, before reading the initial absorbance at 340 nm. Add 1 unit/assay Alanine Transaminase (glutamate pyruvate transaminase, Sigma chemicals). Read absorbance after ½ hour, then check every 15 minutes thereafter, to ensure reaction goes to completion (up to 3 hours).

Alanine + α-ketoglutarate → pyruvate + glutamate (alanine transaminase)

Pyruvate + NADH + H^+ → lactate + NAD^+

Measuring Malondialdehyde (MDA) (2,3)**:**

Reagents:

1) 7 % SDS (sodium dodecyl sulfate, 7 g in 100 ml) [3.5 g SDS in 50 ml H_2o]
2) 0.1 M HCl [10 ml of 1M HCl made up to 100 ml H_2o]
3) 10 % Phosphotungstic acid
4) 0.67 % TBA (2-Thiobarbituric acid) [0.67 g in 100 ml H_2o + 1 ml of 1M NaOH and heat to 50-60°C to dissolve, > 20 min, light yellow]
5) PBS (K_2HPO_4): 50 mM

Combination of the reaction mixture:

- 0.2 ml of 7% SDS, 2.0 ml of 0.1M HCl, 0.3 ml 10% Phosphotungstic acid, 1.0 ml 0.67% TBA.

Assay procedure:

Weigh tissue (e.g. 0.1 g, keep the tissues in liquid nitrogen). Hemogenize with 1 ml PBS buffer and keep them on ice (to measure protein take 50 µl of supernatant in an eppendorf containing 950 µl NaOH). Spin for 15 min at 15000 g at 0°C. Take 500 µl of the supernatant and transfer into polypropylene tubes. Then, add 875 µl of the reaction mixture to each tube and vortex. Boil for 1 hr at 95°C, add 750 µl N-Butanol and vortex. Spin again at 1000 g for 1 min. Take 100 µl and read it in duplicate using a fluorometer (Excitation: 530/25, Emission: 575/15).

Preparing standards:

1) Mix 100 µl of concentrated MDA (1,1,3,3-tetraethoxy propane) with 4.9 ml of 0.1 M HCl.
2) Incubate at 37°C (water bath) for 15 min.
3) Dilute this 1:1000 (10 µl made up to 10 ml PBS) to give 122 µM MDA.
4) Make all the standards (16, 8, 4, 2, 0 µM), treat them exactly like the samples.

Measuring reduced glutathione (4)**:**

Reagents:

- Monochlorobimane stock solution: Dissolve 25 mg of mcbn in the vial in 2.2 ml of absolute ethanol (final concentration of 50 mM).
- GST stock solution (G-6511): dissolve contents in 4.0 ml to yield a 100 U/ml stock.
- Imidazole buffer: 100 mM, PH=7.0.

Assay Procedure:

Weigh tissue (e.g. 0.1 g, keep the tissues in dry ice). Hemogenize with 1 ml 6% PCA (containing 1mM EDTA, chelates some of the heavy metals that inhibits reactions). To measure protein take 50 µl of supernatant in Eppendorf (labeled) containing 950 µl 15 mM NaOH. Spin down at 0°C / 15000 g for 15 min. Then, take 400 µl of supernatant to Eppendorfs. Neutralize with 3 M KOH, 0.3 M Imidazole (about 240 µl). Centrifuge and store the extract. Plate100 µl of each sample for reading. Add 150 µl of buffer containing mcbn (100 µl of mcbn in 15 ml imidazole buffer). Then, add 1 µl GSH S-trasferase (GST) to each well. Incubate in a dark place for 30 min (at room temperature) and read after 30, 60, 90 min using a fluorometer; Wave length: Excitation: 380/20 – Emission: 485/20 nm.

Standards:

- Prepare 0, 10, 25, 50, 100, 200 µM of GSH standard in Imidazole buffer (100 mM). Treat standards exactly like the samples.

Measuring myeloperoxidase activity (5)**:**

Reagents:

- 50 mM KH2PO4, pH=6.0, using 2 M NaOH or HCl solution (Room Temperature).
- 1 % Hexadecytrimethylammonia Bromide (HTAB) in potassium phosphate buffer.
- 1% H_2O_2 (Make fresh for each assay, store in dark on ice).
- Take 33.3 µL of hydrogen peroxide stock (30%) and dilute to 1ml using distilled H2O.
- PBS (K_2HPO_4): 50 mM (FW 174.2 in 1 liter=1 M, 8.71 g in 1 L in this case).

(Note: just before the test add some crystals of protease inhibitor, PMSF, to the buffer)

Reaction Mixture: To make 10 ml:

1 mM O-Dianisidine (3.2 mg)

0.5 mM Hydrogen Peroxide (12.5 µl of 1% solution, final concentration of 0.0005%)

Phosphate Buffer (10 ml)

Assay procedure:

Weigh out 100 mg of tissue in 15 ml tube and grind it with 1 ml PBS. Take 700 µl of the homogenate into 15 ml polypropylene capped tubes. Take 50 µl of homogenate into 950 µl NaOH (in an eppendorf) for protein assay.

Keep sample (leftover, about 250 µl) cold on ice prior to adding 300 µl HTAB solution and homogenizing. Homogenize tissue for up to 30 seconds (two times). HTAB is a detergent that releases MPO from the primary granules of the neutrophil. Spin the samples 5 min at 20,000 g at 2°C. Transfer supernatant to eppendorf tube and place on ice. Pipette 10 µl samples in a plate (1:20 dilution for rat small bowel). Add 250 µl of reaction mixture to each well.

Read the plate using the MPO kinetic assay (read every 15 sec x 120) and measure the change in absorbance at 450 nm (room temperature). One unit of MPO activity is defined as that degrading 1 µmol of peroxide per minute at 25 °C. Analyze data curves using mpa (microplate analysis program).

No standard is required.

Measuring tissue protein level (6)**:**

All the biochemical values reported in these series of experimets were standardized according to the tissue protein levels.

Reagents:

- LOWRY "A": Dissolve 0.5 g of cupric sulfate, pentahydrate and 1.0 g of Sodium citrate with distilled water. Then, dilute up to full volume (50 ml) using distilled water.

- LOWRY "B": Dissolve 10.0 g of sodium carbonate and 2.0 g of sodium hydroxide and dissolve in distilled water. Top up to full volume (250 ml) using distilled water.
- Reaction mixture: Mix 1 mL Lowry A with 49 mL Lowry B (Make fresh for each assay).

Standards:

Weigh 200 mg of Albumin from bovine serum, minimum 96% electrophoresis (CAS 9048-46-8, Sigma Chemicals) directly into a 5 ml volumetric flask. Add a very small amount of sterile saline and carefully swirl to dissolve. When BSA is dissolved, add enough sterile saline to bring solution up to full volume. Invert slowly many times to ensure proper mixing of solution.

Dilute 4% BSA to make a 0.04% solution of BSA by taking 10 µl of the 4% solution, and dilute it with 900 µl of sterile saline. The solution can be stored in the –20 °C freezer for up to 3 months.

Assay Procedure:

- Grind tissue sample (100 mg) in 1 mL 6% Perchloric acid and immediately remove 50µl of sample from homogenate.
- Place into 950 µl of a 0.15 M NaOH solution and store in 4 °C for minimum 48 hours.
- Mix well and be certain diluted sample containing homogenate is clear enough for test and centrifuge before use.
- Pipette 10-30 µl of the diluted homogenate mixture (for rat small bowel), into a microwell plate. Pipette the 0.04% BSA standard (in triplicate).
- Add 200 µl of Reaction mixture to each well. Be certain to mix each well using a 200 µl multipipettor. Leave plates for 10 minutes.

- Add 20 µl of Folin-Ciocalteu's phenol reagent, 2 N (Sigma Chemicals) reagent to each well, and mix well (very important) –use 200 µl multipipettor again.
- Leave plates for an additional 30 minutes.
- Read plates using plate reader, with 750 nm filter using a Dynex MRX TC plate reader.

***In-Vitro* studies of small bowel function:**

The advantage of *in vitro* techniques for studies of the intestine are many: the mucosa can be studied isolated, eliminating many of uncertain factors with *in vivo* permeability studies; exactly defined segments of the bowel are studied; experiments can provide insight into the mechanisms and routs of transepithelial transport and more importantly in case of ischemia/reperfusion, the bowel viability and function can be estimated without transplantation which is difficult experimental procedure.

The drawbacks are obvious: the mucosa is taken out of its natural environment and deprived of its circulation, lymph drainage and neuroendocrine control, all of which could have effects on permeability results. Metabolic changes occur rapidly after excision of tissue and also proper handling of the tissue is essential for valid and reproducible results.

Ussing Chamber:

Ussing chamber was introduced in 1951 by the Danish physiologists Ussing and Zerhan (7). This has been important for the understanding of ion transport in the intestine. The principle is shown in Figure 2-1.

A flat sheet of mucosa between two half-chambers filled with continuously oxygenated buffer. Temperature is kept at 37 °C. The use of two pairs electrodes makes it possible to measure the electrophysiological properties of the epithelium during the experiment.

The marker molecule (^{3}H-Mannitol) is added to mucosal buffer and at certain time intervals, samples are removed from the serosal (submucosal) buffer for assessment of permeation. The U-shaped tube secures an equal hydrostatic pressure on both sides of the chamber and thus, avoids damage caused by bending of the tissue.

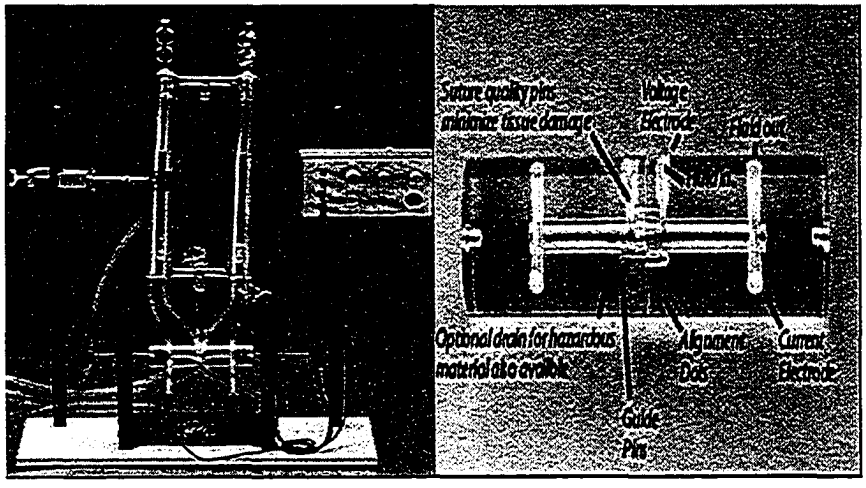


Figure 2-1. Continuously circulating Ussing chamber.

Tissue preparation

Tissues designated "unstripped" or "stripped" depending on whether the mucosa is dissected free from the muscle layers or not. The stripped preparations, thus consists of the epithelium, the underlying lamina propria and the muscularis mucosa. For studies of permeability, stripped tissue is preferred because of shorter diffusion distance. Although in our studies, after 12 h cold preservation, it was too difficult to strip the bowel without damaging the epithelium. So, we used unstripped segments.

After mounting in the chambers, tissues are allowed to equilibrate for 30-60 minutes to re-establish ion transport processes before experiments are started.

Electrophysiology

Epithelial tissues transport ions and thus generate a transepithelial voltage as a result of the asymmetric distribution of ion channels on the apical and basolateral membranes of epithelial cells. The ability to maintain a transepithelial potential difference (PD) is dependent on the activity of all the electrogenic ion pumps in the epithelial cell membranes, mainly Na^+/K^+ ATPase, and on the epithelial barrier function.

Transmural intestinal short-circuit current (Isc) is measured over a surface area of 0.9 cm^2. The spontaneous trans-epithelial potential difference (PD) is determined, and the tissue is clamped at zero voltage by continuously introducing an appropriate Isc with an automatic voltage clamp (DVC 1000 World Precision Instruments, New Haven, CT, USA), except for 5-10 s every 5 min when PD is measured by removing the voltage clamp. Tissue ion conductance (G) is calculated from PD and Isc according to Ohm's Law (8). A stimulated increase in Isc is induced by addition of the adenylate cyclase-activating agent, forskolin (10^{-5} mol/L), to the serosal surface. Epithelial responsiveness is defined as the maximal increase in Isc to occur within 5 min of exposure to the secretagogue. PD is expressed as millivolts (mV), G as millisiemens/cm^2 (mS/cm^2), and Isc as microamperes per square centimeter ($\mu A/cm^2$).

All electrophysiology and permeability data depend on surface area (i.e. villus height). So, animals with the same age, weight and diet were used for each experiment. In addition, histologic evaluation was used to compare mucosal surface area between different samples within a particular experiment.

Histology (9)**:**

Solutions:

A. Scott's Solution

a. Magnesium Sulfate: 10 g

b. Sodium Bicarbonate: 2 g

c. Dilute up to 1000 mL using tap water.

B. Block Softening Solution

a. 95-100% Ethanol (630 mL)

b. Distilled water (270 mL)

c. Glycerol (100 mL, ACS 56-81-5, Sigma Chemicals)

C. Chromium Alum Solution (For Ribbon Sections)

a. Gelatin powder (1 g)

b. Chromium Potassium Sulfate powder (0.1 g)

c. 100 mL of Distilled Water

d. Add a few thymol crystals to thwart bacterial growth (note they will not dissolve).

D. Eosin Staining Solution

a. Eosin Yellowish Solution, 1%W/V , Fisher Scientific (200 mL)

b. 95% Ethanol (600 mL)

c. Glacial Acetic Acid (4 mL, ACS 003-78, BDH)

E. Acid Alcohol Dip

a. Make 70% ethanol by diluting 100% ethanol with distilled water.

b. Add 0.25 % HCl acid to the 70% ethanol

Tissue preparation: Place small amounts of tissue (approximately 2-5 mm of tissue) into OmniSette Tissue cassette (Fisher HealthCare). Cassettes are then stored in 10% buffered Formalin acetate (Fisher Scientific) until processing begins. Tissues are processed using a Leica TP 1020 Automatic Tissue Processor.

Embedding fixed tissue sample in paraffin

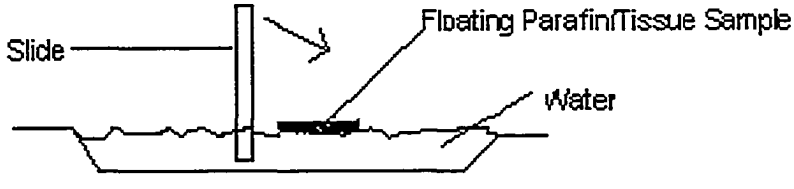
1. Remove cassettes containing fixed tissue samples from processing unit.
2. Place cassettes into paraffin bath (temp. set at between 58 and 63 °C).
3. Warm a metal molding tray, and then quickly open cassette and position tissue sample into the base of the tray, using heated forceps. Tissue should be positioned with the opening to the lumen facing upward and downward (doughnut appearance when looking downward onto tissue sample).
4. Add liquid paraffin (58 to 63 °C) on top of tissue sample. Let paraffin solidify very slightly at this stage, add cassette base, quickly add sufficient liquid paraffin to cover the holes on top of the base, and allow paraffin to solidify on a cooling metal tray (approximately 12-20 °C).
5. Once wax has completely solidified, pull cassette from metal molding tray.
6. Use a sharp knife to remove excess wax from edges of cassette.
7. Store blocks until ready for cutting.

Microtome

8. Cut into blocks until surface of the tissue has been reached. This is called roughing the block.
9. Place roughed blocks into an alcohol/glycerol, block softening bath (on ice) for at least 15 minutes.
10. Place cassette into the Microtome. Begin cranking the wheel of the microtome. The sample block will advance automatically on its own, at a pre-set thickness level. Ensure that blades are secure and nick-free.
11. Once a couple slices are cut, and the depth of the slice is satisfactory, gently grasp the end of the paraffin ribbon with forceps, and gently support the ribbon

while continuing to slice out about a 10 cm long ribbon of connecting tissue/paraffin slices.

12. Once a ribbon is produced, take either the rounded end of forceps, or a smooth wooden stick, to help support the ribbon as it is moved to a water bath containing 1-1.5 L of water, and 80-100 ml of diluted Chrom Alum solution (solution C), preheated to 45 °C. The temperature of the water bath is crucial, too warm and sample will melt away, too cool and paraffin will take on an opaque, crumbly appearance.
13. Use a wooden stick to gently split apart the floating paraffin slices.
14. Float a pre-labeled slide (pencil or stick on labels are best) angled with top end towards you, in order to attract slices of paraffin/tissue. Once the end of the paraffin/tissue slice sticks to the slide, gently turn bottom end of slide upward. Paraffin/tissue sample should then stick to the slide.



15. Quickly view the slide under microscope, but do not let slide/sample dry out.
16. Repeat steps 11 through 15, until sample is satisfactory.
17. Periodically, rub a Kim wipe over the surface of the water bath in order to skim off any excess wax or samples.
18. Place slide at an upright angle, in order to let excess water run off the slide.
19. Leave slides to air dry for 48 hours minimum.

Dewaxing/Staining:

1. Place slides containing dried on samples into a slide rack, and then place into a xylene dip for 1-2 minutes. Be certain to swish rack gently.
2. Dip slides into fresh xylene for an additional 1-2 minutes.
3. Dip slides into fresh xylene dip for a final 1-2 minutes.
4. Dip slides into absolute alcohol dip (15 seconds)
5. Dip slides into a second absolute alcohol dip for 15 seconds Dip slides into a 95% alcohol dip for 15 seconds.
6. Dip slides into second 95% alcohol dip (15 seconds).
7. Dip slides into water rinse bucket and allow fresh water to run into bucket until all alcohol runs clear off slides.
8. Place slides into Hematoxylin Stain (Gill's Formulation #2, Fisher Scientific) for 45 seconds.
9. Rinse with water.
10. Quickly dip slides into 0.25% acid/alcohol dip.
11. Then immediately rinse (gently) with water, to remove the acid/alcohol dip.
12. Dip slides into Scott's solution (Solution A). The specimens on the slide should turn blue within a second or two.
13. Rinse with water.
14. Place slides into Eosin stain (15 seconds).
15. Dip slides into 95% alcohol dip (15 seconds).
16. Dip slides into 95% alcohol (10 seconds).
17. Dip slides into fresh absolute alcohol (10 seconds).
18. Dip slides into fresh absolute alcohol (10 seconds).

19. Dip slides into a final, fresh absolute alcohol dip (10 seconds).
20. Dip slides into xylene dip to remove alcohol (15 seconds).
21. Dip slides into fresh xylene dip (10 seconds).
22. Dip slides into fresh xylene dip (10 seconds). Store slides in final xylene dip until slip covers are ready to be placed onto slide.

Cover slips:

23. Remove single slide from xylene dip.
24. Take a small amount of Permount® (Fisher Scientific) onto a wooden swab stick, and place onto slide near specimen.
25. Immediately tip slide to one end, in order to allow mounting media to gently run overtop the specimen on the slide.
26. Quickly position glass cover slip over the center of the slide, being certain to cover the specimen.
27. Place the completed slide on a flat surface to dry for 48 hours minimum.

Statistics

Metabolite data are reported as 'µmol/g protein' and presented as means ± SE. Statistical differences between groups are determined using analysis of variance, followed by Tukey's honest significant difference (HSD) *post hoc* comparison test; $p < 0.05$ is reported. Histology scores are compared using the Kruskal-Wallis test; $p < 0.05$ is reported. Survival is calculated by the method of Kaplan-Meier. The impact of different treatments on animal survival is evaluated using the log-rank test.

Analysis of variance: One (unsatisfactory) procedure for comparing a quantitative variable among multiple groups is to perform multiple t-tests. The problem is each t-test has a type I error rate of α. However, over the series of multiple t-tests type I error grows

with each new comparison. With three comparisons α grows from 0.05 to 0.14. The analysis of variance (ANOVA) is a procedure for comparing multiple populations that avoids inflation of type I error. Because there is only one comparison, the type I error rate remains exactly at α.

The test statistic is F, which equals to mean square treatment divided by mean square error. When we accept the null hypothesis (no difference is present), F value is about 1.0. A significant F-value tells you only that the means are not all equal (i.e., reject the null hypothesis). We still do not know exactly which means are significantly different from which other ones. Hence, we need *post hoc* tests. The most widely used post hoc test in medical sciences is Tukey's Honestly Significant Difference or HSD test. There are many types of post hoc tests all based on different assumptions and for different purposes. Tukey's HSD is a versatile, easily calculated technique that allows us to answer just about any follow up question from the ANOVA.

Assumptions for ANOVA: Firstly, all the groups must have the same variance (hemogenity of variances). We can test the validity of this assumption using Leven's test. Secondly, the samples should be independent of one another. Also, each group should be assigned randomly to a certain treatment.

Kruskal-Wallis test: Park's classification is used to compare histology samples among different groups. Since this is an ordinal scale and the variances are not equal, we have to use a non-parametric test called Kruskal-Wallis test.

Assumptions: Groups are randomly assigned to treatments. Also, the measurement scale employed is at least ordinal.

The test statistic H is calculated by:

$$H = \frac{12}{n(n+1)} \sum_{i=1}^{k} \frac{R_i^2}{n_i} - 3(n+1)$$

n_i (i = 1, 2, ..., k) represents the sample size for each of the k groups (i.e., samples) in the data and R_i is the sum of the ranks for group i.

Survival data analysis

This module describes methods of summarizing and graphing the survival experience in one or more groups of patients/animals using time to death (or to the occurrence of the event) and for comparing this experience between two or more groups.

Assumptions: There are clear, well defined starting and end points (death or closing date of the study) for each case. The cases are from a common population (homogeneous).

Numerous procedures have been reported for the comparison of two or more independent survival curves. One procedure is called Peto Peto log-rank test, Matel's log-rank test, or simply log-rank test. This test is a direct product of the Mantel-Haenszel procedure.

Assumptions: The samples are independent randomly assigned to different treatments. The censoring patterns for the observations are the same for different groups.

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Chapter 3

A novel technique of hypothermic luminal perfusion for small bowel preservation*

Introduction

The success rate of small bowel transplantation (SBT) is nowhere near that of other commonly transplanted organs (1). Currently, it is the only option for patients who can no longer be maintained on intravenous nutritional support (Total Parenteral Nutrition) as a result of line infections and problems of venous access (2,3). The impracticality of transplanting SB limits the procedure to patients with irreversible intestinal failure who have developed life-threatening SB failure. Despite the obvious demands for improved preservation solutions and/or techniques for SB, the current overall graft survival remains low: under 40% five-year survival (4). Thus, it is still an unreliable process that offers patients little hope for long term survival. The main obstacles to developing a reliable SBT model are those of bacterial translocation across the deteriorating mucosal barrier and control of allograft rejection; two-thirds of patient mortality is due to sepsis or immune rejection (4,5).

The most significant recent advancement in solid organ storage has been the development of the University of Wisconsin (UW) solution (6). It is used to vascularly flush and store the donor organ in the organ retrieval procedure. Despite its effectiveness in other intra-abdominal organs, its value in SB preservation has been limited. The maximum 'safe' storage time for SB is brief (6-8 hours) and moreover, graft

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* A version of this chapter has been published in *Transplantation* 2003; 76 (1): 71-6 (Zhu JZ, Castillo EG, Salehi P, Avila J, Lakey JR, Churchill TA). I gratefully acknowledge our summer students, Zhu J and Castillo E, for their major role in this study.

quality is often compromised even with short periods of ischemia. Without an effective solution for SB, the UW solution is by default the current clinical standard.

Oxygenation techniques such as luminal insufflation with hyperbaric oxygenation (delivery of oxygen without solution) and continuous vascular perfusion have been attempted as alternatives to static cold storage (7-10). However, the use of insufflation and vascular perfusion techniques remains controversial due to issues of complexity and risk of injury during continuous delivery of gaseous oxygen to the lumen and continuous mechanical insult to the vascular bed. Never the less, the requirement for oxygen delivery to the tissue is perhaps one of the most important factors in maintaining tissue viability. We hypothesized that oxygenated luminal perfusion with UW solution would facilitate energy production and therefore preserve an intact mucosal barrier at prolonged times of storage.

Materials and Methods

Male Sprague-Dawley rats (200-300 g) were obtained from the University of Alberta and used as bowel donors. All experiments were conducted in accordance with the regulations and policies of the Canadian Council on Animal Care. Chemical agents were AR-grade and were purchased from Sigma Chemical (Oakville, Canada).

Surgical procedure and procurement of the small intestine: Rats were fasted overnight in cages with raised floors to minimize coprophagy. Water was provided *ad libitum*. Prior to laparotomy, rats received an intraperitoneal dose of 65mg/250g pentobarbital (Somnotol; MTC Pharmaceuticals, Cambridge, Ontario, Canada), followed by inhalational halothane (1-2%) to maintain anesthesia. A midline laparotomy was performed and the aorta exposed infrarenally and at the level of the celiac trunk. The supraceliac aorta was then clamped and 10 ml of modified University of Wisconsin (UW)

solution (equivalent to ~0.5 ml/g tissue) was administered via the infrarenal aorta. The suprahepatic vena cava was transected to facilitate the outflow of both blood and perfusate. The entire jejunum and ileum was subsequently harvested. Luminal solutions (20 ml; equivalent to ~1.0 ml/g tissue) were then placed into the lumen of the bowel allowing the effluent to exit uninhibited. Each end was immediately ligated with 3-0 silk, leaving the bowel filled without turgor with the same solution. The bowel was then stored in 30 ml of solution and stored on ice in a 4°C incubator. Bowel that was treated with perfusion was luminally flushed as above and then transferred to the recirculating perfusion apparatus and perfused with an additional 80 ml of solution (+ 5 µg/ml Ciprofloxacin) at ~1 ml/g/min (solution was bubbled with 100% oxygen) at 4 °C. Tissue samples (1-2 g) were taken at 4, 8, 12 and 24 hours post vascular flush. To arrest metabolic activity, samples were snap frozen in liquid nitrogen, and subsequently stored at –65°C until processed. Freshly isolated (FI) tissue was obtained from a separate group of animals to provide an approximation of *in vivo* metabolites and 'normal' histology.

Experimental groups: All experimental groups (n=4) were flushed vascularly with UW solution except for freshly isolated tissue and then treated luminally as below:

Group 1 – no luminal treatment [clinical control, UWV];

Group 2 – UW solution [UWL];

Group 3 – 1 h perfusion then static storage with UW [UWP1];

Group 4 – 24 h perfusion with UW [UWP24];

Sample Preparation and Metabolite Assay: Frozen SB samples were weighed and then extracted 1:5 w/v in perchloric acid containing 1mM EDTA. The precipitated protein was removed by centrifugation (20 min at 20,000xg). Acid extracts were neutralized by the

addition of 3 M potassium hydroxide/ 0.4 M Tris/ 0.3M potassium Chloride and then recentrifuged (20min at 14,000xg). Aliquots of neutralized extracts were immediately processed via standard enzyme-linked metabolite assays (11). Spectrophotometric analysis was then performed to measure the absorbance of NADH at 340 nm, providing quantification of ATP, ADP, AMP, lactate, and ammonia. Values are reported as µmol per gram wet weight.

Malondialdehyde: Frozen tissue (100 mg) was homogenized 1:10 in phosphate-buffered saline. The homogenate was then processed and fluorescence was compared to standard amounts of MDA according to Yagi (12,13).

Histology: Full-thickness samples were fixed in a 10% buffered formalin solution, embedded in paraffin, cut 3- to 5-µm thick, and stained with hematoxylin and eosin. Histologic damage was assessed using Park's histologic classification of intestinal tissue injury (14).

Statistical analysis: Metabolite data were reported as means ± SE for each group. Statistical differences between groups were determined using analysis of variance, followed by Tukey's Honest Significant Difference *post hoc* comparison test; $p<0.05$ was reported. Histology scores were compared using the Kruskal-Wallis test; $p<0.05$ was reported.

Results

Energetics

ATP (Figure 3-1): Within 4 h, ATP values dropped by 40-50% in groups 1 & 2 and by 25% in Group 3, whereas continuous perfusion in Group 4 resulted in elevated ATP values unchanged from FI levels throughout 12 h. After 12 h, ATP in Group 4 was significantly higher than the corresponding values in all other groups (1.56 vs 0.46 to

0.75 µmol/g, $p<0.05$). After 24 h, levels in Group 4 were at least 2 fold greater than other groups (ie. 0.85 versus 0.22-0.35 µmol/g).

Total Adenylates (Figure 3-1): In groups 3 & 4, Total Adenylate levels (TA = ATP+ADP+AMP) remained unchanged from freshly isolated values (3.4 µmol/g) after 4 h; these values were significantly higher than corresponding values in Groups 1 & 2 ($p<0.05$). Interestingly, the same trend was observed after 8-12 h cold storage. After 24 h, Groups 2-4 retained higher levels compared to control (Group 1); 2.2 for all three groups vs 1.2 µmol/g ($p<0.05$).

Energy charge (Figure 3-2): The ratio of Energy Charge (EC) reflects the proportion of total adenylates that exist in a form that is immediately available for cellular work; [EC=(ATP+ADP/2)/TA] [15]. Within 4 h, EC dropped from freshly isolated values of 0.69 in all groups except Group 4. After 12 h, EC levels were higher in Groups 2 & 3 compared to Group 1 (0.51 for both vs 0.39, $p<0.05$). However, after 24 h, no significant difference was observed in these groups compared to control; values were 0.43-0.47. Continuous perfusion (Group 4) resulted in maintenance of EC at FI levels throughout the entire 24 h time course.

ATP/ADP (Figure 3-2): By 4 h, all groups except for Group 4 were markedly lower than FI values of 1.3 ranging from 0.51 to 0.58. Continuous perfusion (Group 4) resulted in ATP/ADP ratios not significantly different from FI levels at all time points throughout 24 h.

Lactate (Figure 3-3): Anaerobic metabolism was measured quantitatively via lactate accumulation during the 24 h experimental time course. By 4 h, the value of 1.8 µmol/g for FI tissues was significantly lower than all experimental groups. After 12 h lactate levels were significantly higher in Groups 2 & 3 compared to control (9.4 & 11.0 vs 6.9

μmol/g, $p<0.05$). The same trend was observed after 24 h for groups 2 & 3. Correspondingly, continuous perfusion (Group 4) resulted in the lowest levels throughout the 24 h cold storage compared to all other groups ($p<0.05$); values remained lower than 3.3 μmol/g at all time points.

Ammonia (Figure 3-3): Within 4 h, all experimental groups demonstrated significantly higher values of ammonia than the FI level of 3.7 μmol/g; values ranged from 4.8 to 6.2 μmol/g in Groups 1 (control) & 4, respectively. After 8-12 h, although all groups exhibited higher ammonia levels than control (Group 1), values of 6.5-7.5 in Group 3 were the highest levels of all groups at these time points ($p<0.05$). After 24 h, continuous perfusion (Group 4) was found to have the lowest level of tissue ammonia (5.4 μmol/g).

Peroxidative Damage

Malondialdehyde (Figure 3-4): Levels of this peroxidation product in Group 1 increased more than two-fold over the first 4 h and remained elevated throughout the entire 24 h period of storage; values rose from FI values of 66 to 145 nmol/g. Group 2 tissues, however, remained low and were at no time greater than FI values throughout 24 h. With 1 h perfusion (Group 3), levels increased by 50% after 4 h storage and subsequently returned to FI levels. With continuous perfusion (Group 4), values after 4-12 h were similarly elevated as in Group 1 tissues and returned to FI values after 24 h storage. In general, as the time of oxygenated luminal perfusion increased, MDA levels also increased at 4, 12 & 24 h storage.

Histologic Injury

Difference in the degree of mucosal injury was evident within 12 h cold storage in all groups (Table 3-1; Figure 3-5). Compared to Groups 1 & 2, injury was significantly lower in Groups 3 & 4; median Park's histologic grades were grade 6 in Groups 1 & 2 and

grade 4 in Groups 3 & 4, $p<0.05$. After 24 h, mucosal integrity was markedly superior in Group 3 tissues subjected to brief 1 hour perfusion followed by static storage (grade 4.5); including the denudation and loss of villus tissue. This was in contrast to Groups 2 & 4 which exhibited extensive villus destruction with crypt layer infarction (grade 6). Group 1 exhibited severe mucosal necrosis and infarction of the mucosal layer (grade 7), however 2/4 histology samples showed evidence of complete infarction of the muscularis propria (grade 8).

Discussion

Clinically, the period between harvesting a donor organ and transplantation into a recipient involves cold static storage (16). During this period, the organ is devoid of blood supply and oxygen. This phase of cold ischemia is, at present, unavoidable and results in the gradual deterioration of absorptive properties and barrier function, eventually progressing into irreversible damage. Pools of ATP are rapidly depleted during ischemia since many energy-utilizing processes are still in operation even at hypothermia (17). Cellular energetics, such as ATP and total adenylates, have been found to reflect the quality of small bowel more than any other solid organ during storage. The maintenance of high-energy phosphorylated compounds is inversely correlated with structural and functional damage (18). The epithelial layer is maintained mainly by the tight junction region close to the apical surface of intestinal epithelial cells. These tight junctions consist of numerous proteins that are dynamic, energy-requiring structures. Agents that result in ATP hydrolysis also lead to dilation of the tight junctions, an increase in epithelial permeability, and an increase in transepithelial flux of macromolecules (19). Normally, toxic contents within the lumen are confined to the lumen by the mucosal barrier. However, depleted cellular ATP reserves results in an

inability of epithelial cells to maintain tight junctions. The consequences of a poorly functioning graft are those of bacterial translocation leading to sepsis in the recipient.

Despite poor graft preservation quality, the current clinical practice involves a common arterial vascular flush with UW as part of a multi-organ procurement procedure followed by static storage. One major problem with this method is the accumulation of metabolic end products such as lactate and ammonia. In previous studies, we have demonstrated that by retaining a significant volume of preservation solution inside the lumen, the metabolic and structural integrity of the graft is considerably improved, since end products are unable to accumulate to toxic levels (20). Compared to a vascular flush alone, luminal flushing with even a simple crystalloid solution has shown improved mucosal function over varying storage periods (21). This has been postulated to occur by dilution of resident enteric contents, which contain feces and other cytotoxic agents, including bacterial endotoxins as well as biliary and pancreatic secretions (22,23).

In the present communication, ATP levels were consistently higher in luminally-treated groups compared to the clinical control [UWV]; this effect was more pronounced in tissues subjected to continuous perfusion. Even after 24 h, total adenylate levels and energy charge ratios in luminally-treated groups were maintained above that for the control group. Upon examination of lactate levels, there were significantly higher concentrations of lactate in the luminally-flushed groups except for 24 h continuous perfusion group. Because lactate production is used as a reflection of glycolytic activity, increasing quantities of lactate in these groups signaled continued anaerobic catabolism for energy production. Lower levels of lactate in tissues subjected to continuous perfusion was likely a result of continuous removal of waste products and is not necessarily due to the absence of substrate; although our lab has determined that there

is ample glycogen in fresh bowel (50 μmol/g), potential alterations in the associated enzyme machinery (ie. glycogen phosphorylase) remain to be clarified. The maintenance of low concentrations of end products has several beneficial implications. Regulatory enzymes in the glycolytic pathway has been shown to be inhibited both by pH decline and by lactate (24,25). Thus, removal of end products such as lactate, H^+ and NH_3 may contribute to increased energy production via conservation of the phosphorylated state of key regulatory enzymes, such as phosphofructokinase (26), among others that are pH-sensitive.

In terms of energetics, the beneficial effect of continuous perfusion far exceeds that of static storage. Levels of ATP and total adenylates were both consistently higher in the perfusion groups. With respect to the ATP and TA data, it is clear that the main factor in improved energetics is luminal delivery of oxygen and/or the simultaneous removal of end products. The combination of available luminal solution and ample amounts of oxygen allow for higher graft quality. ATP/ADP ratios and energy charge values reflect the proportion of adenylates that are readily available for cellular work in addition to the status of mitochondrial oxidative-phosphorylation; both parameters were consistently higher in tissues subjected to continuous perfusion in the current study. Oxygen and substrate delivery to the metabolically active epithelium is fundamental to the preservation of mucosal structure and function. The primary stressor involved in organ storage for transplantation is unequivocally a problem of limited oxygen. Our attempts in the current communication to supply adequate oxygen to the mucosal surface via continuous luminal perfusion at hypothermia were successful in terms of maintenance of metabolic homeostasis. However, our data suggests that the mucosal layer can only tolerate a limited period of luminal perfusion at hypothermia; mechanical

disruption of the mucosal surface is likely responsible for the poorer architecture of tissues subjected to 24 h perfusion. Continuous luminal perfusion at warmer temperatures may prove more advantages since alterations in membrane fluidity may not be a problem in terms of mechanical perturbation, although we suspect that issues of bacterial overgrowth may arise.

The generation of malondialdehyde results from the interaction of OFRs with lipid metabolism during normal oxidative metabolism. This product of lipid peroxidation is not only an indicator of peroxidative injury but is also a reactive agent that can subsequently react with cellular lipids and proteins. In this study, static storage of SB resulted in a significant increase in MDA after only 4 h. There are two ways that OFRs can be produced in our isolated experimental system. First, purine catabolism as a result of ATP hydrolysis leading to a net decline in total adenylate levels to lower energy purine catabolites (eventually producing hypoxanthine). The enzyme xanthine oxidase converts hypoxanthine to xanthine in the presence of molecular oxygen and in doing so, generates H_2O_2 (a precursor to radical formation) (27). Although oxygen levels may be rapidly consumed during entry into cold storage, one of the consuming processes is xanthine oxidase. Interestingly, the UW solution used in this experiment contained 1 mM allopurinol, a purported inhibitor of xanthine oxidase, however total adenylates continued to drop at all time points throughout the 24 h period of storage. Why allopurinol was ineffective in small bowel tissues subjected to vascular flush alone is most likely related to the delivery of insufficient allopurinol to the site of purine catabolism. Luminal flush with an additional volume of preservation solution (containing allopurinol) resulted in only minor decreases in TA values throughout the time courses. Interestingly, there was a positive correlation of MDA level with perfusion period; 24 h

perfusion resulted in the highest MDA levels of the luminally-treated specimens. This was not entirely unexpected, since the basal production of OFRs is directly related to electron transport system activity. As much as 4% of oxygen metabolized captures single electrons that leak out of the mitochondria during complex III activity and result in the generation of superoxide radicals (27). Consequently, as the amount of oxygen delivered to the tissue increases, so does the amount of OFR's and hence, resultant MDA levels.

Intestinal injury following ischemia is a common clinical complication that is most often assessed by histologic evaluation of standard hematoxylin and eosin stained tissue sections. Typically, ischemia leads to progressive damage starting at the villus tips, extending to the regenerative crypt cells after prolonged periods (28). The Crypts of Lieberkuhn play an important role in re-epithelialization once issues of oxygen and substrate delivery have been resolved upon reperfusion. According to our data, although 24 h continuous perfusion provides improved graft energetics, the brief 1 h perfusion group exhibited markedly superior histology picture throughout 24 h storage at 4°C. With a limited 1 h period of oxygenated perfusion, only the upper 2/3 of the villi were adversely affected, leaving the metabolically active crypts intact. Continuous oxygenated luminal perfusion, however, resulted in extensive villus destruction accompanied by crypt infarction; histologic injury in both perfusion groups is in contrast to the severe mucosal necrosis in control tissues; complete transmural infarction occurred in 2/4 specimens. The integrity of the small bowel epithelium is an important barrier to foreign antigens and infections. It is now recognized that prevention of mucosal atrophy improves outcome after surgery, and loss of the mucosal barrier can result in systemic complications following after small bowel transplantation. The incidence of post-operative sepsis

leading to mortality is currently 55% when using a vascular flush of UW solution; the current clinical standard involves no luminal treatment or flush (4,5).

In summary, improvements in graft quality are clearly demonstrated with a technique of hypothermic luminal perfusion with the gold standard UW solution. However, our data suggests that there is a delicate balance between the luminal delivery of oxygen and the physical injury incurred as a direct result of mechanical perfusion. The detrimental effect of continuous perfusion on mucosal architecture may be related to the disruption of an already sensitive tissue layer; this injury may be further exacerbated at hypothermia. In this study, mucosal integrity was markedly superior with a brief 1h period of luminal perfusion. A similar strategy of brief luminal perfusion may improve graft quality in the clinical arena.

Table 3-1. Histological grades of intestinal injury.

	Grade	Median
FI	0,0,0,0	0
12 h		
Group 1	4,5,7,8	6
Group 2	3,6,6,7	6
Group 3	3,4,4,5	4*
Group 4	3,4,4,5	4*
24 h		
Group 1	5,6,8,8	7
Group 2	4,6,6,7	6
Group 3	2,4,5,5	4.5*
Group 4	5,6,6,7	6

FI denotes freshly isolated tissue. Group 1, no luminal treatment; Group 2, UW luminal treatment; Group 3, 1 h perfusion then static storage with UW; Group 4, 24 h perfusion with UW. All values after 12 or 24 h were significantly greater than FI ($p<0.05$). * $p<0.05$, compared to Group 1.

Park's classification: 0 = normal mucosa; 1 = subepithelial space; 2 = extended subepithelial space; 3 = epithelial lifting; 4 = denuded villi; 5 = loss of villus tissue; 6 = crypt infarction; 7= transmucosal infarction; 8= transmural infarction.

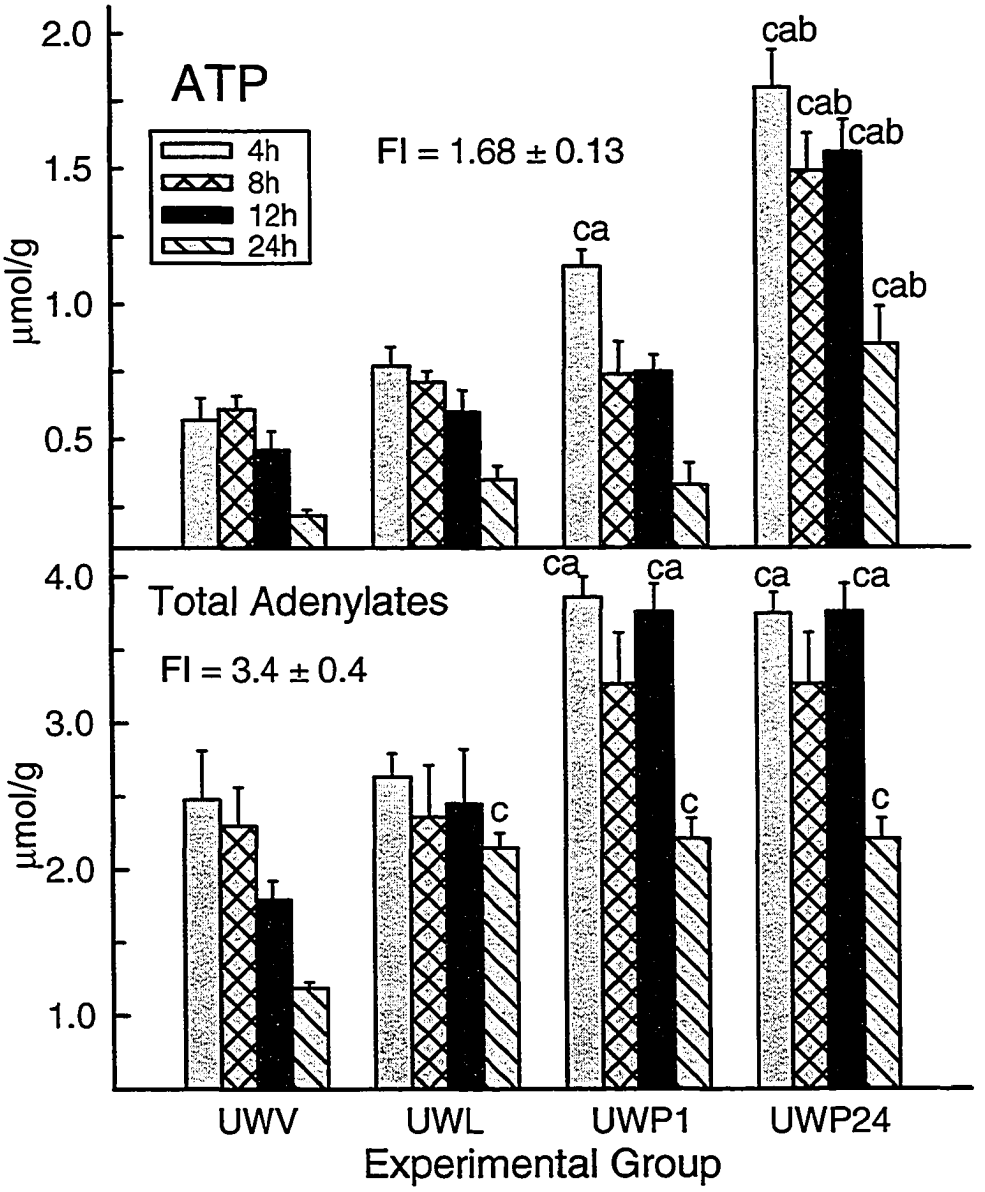

Figure 3-1. Effect of luminally-administered UW solution on levels of ATP and Total Adenylates. c - Significantly different from control group (UWV); $P<0.05$. a, b – significantly different compared to UWL and UWP1, respectively; $P<0.05$.

UWV, no luminal treatment; UWL, UW luminal treatment; UWP1, 1 h perfusion then static storage with UW; UWP24, continuous perfusion with UW.

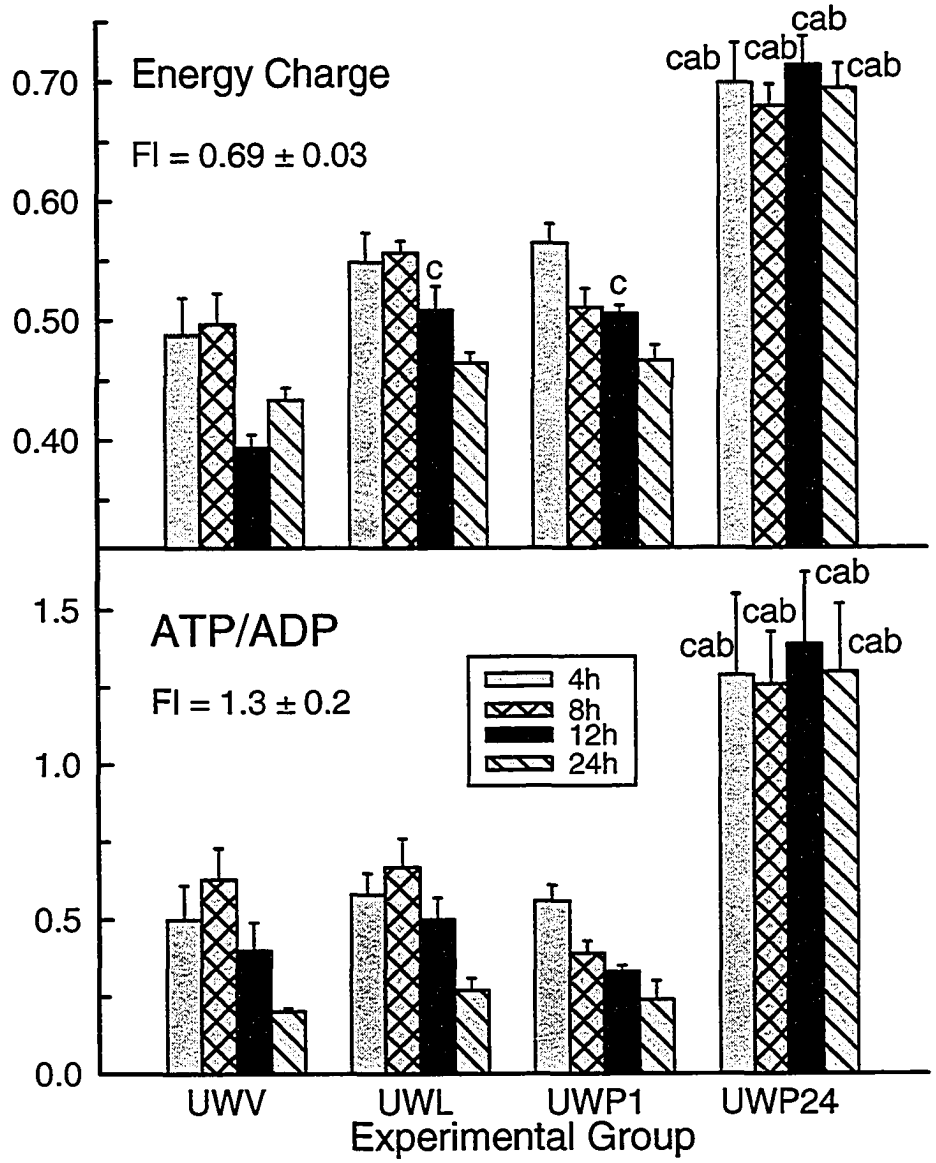

Figure 3-2. Effect of luminally-administered UW solution on Energy Charge and ATP/ADP ratios. **c** - Significantly different from control group (UWV); $P<0.05$. Statistical comparisons are as detailed for Figure 3-1. UWV, no luminal treatment; UWL, UW luminal treatment; UWP1, 1 h perfusion then static storage with UW; UWP24, continuous perfusion with UW.

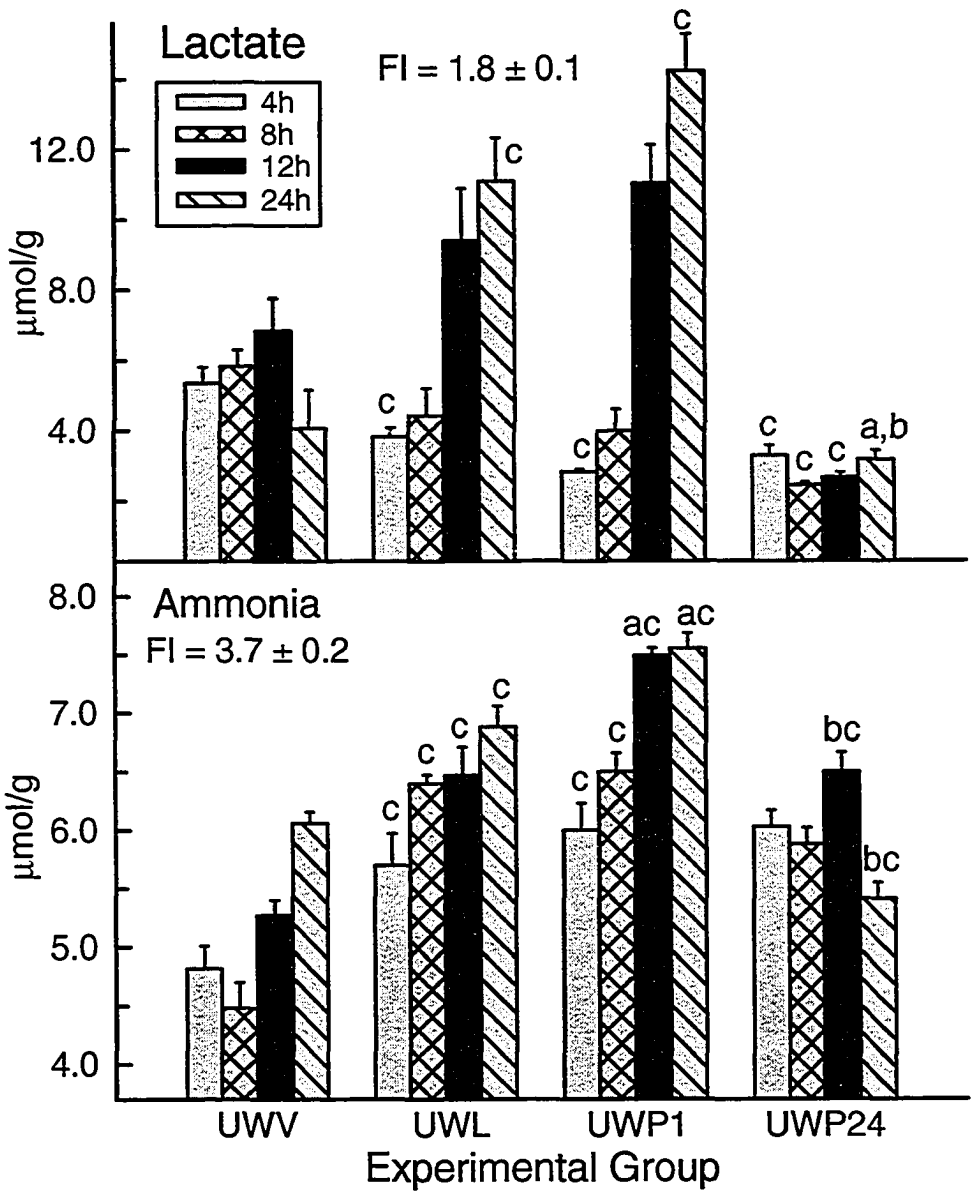

Figure 3-3. Effect of luminally-administered UW solution on levels of lactate and ammonia. ***c*** - Significantly different from control group (UWV); $P<0.05$. Statistical comparisons are as detailed for Figure 3-1.

UWV, no luminal treatment; UWL, UW luminal treatment; UWP1, 1 h perfusion then static storage with UW; UWP24, continuous perfusion with UW.

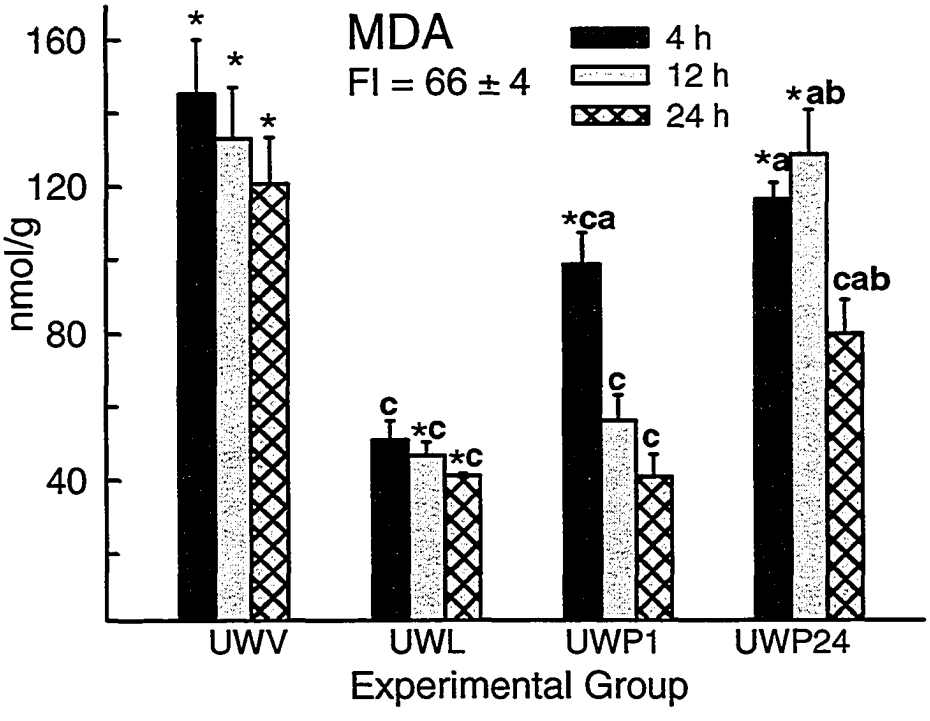

Figure 3-4. Effect of luminally-administered UW solution on levels of malondialdehyde. * - Significantly different from freshly isolated values; $P<0.05$. **c, a, b** – Significantly different compared to control (UWV), UWL and UWP1, respectively; $P<0.05$. UWV, no luminal treatment; UWL, UW luminal treatment; UWP1, 1 h perfusion then static storage with UW; UWP24, continuous perfusion with UW.

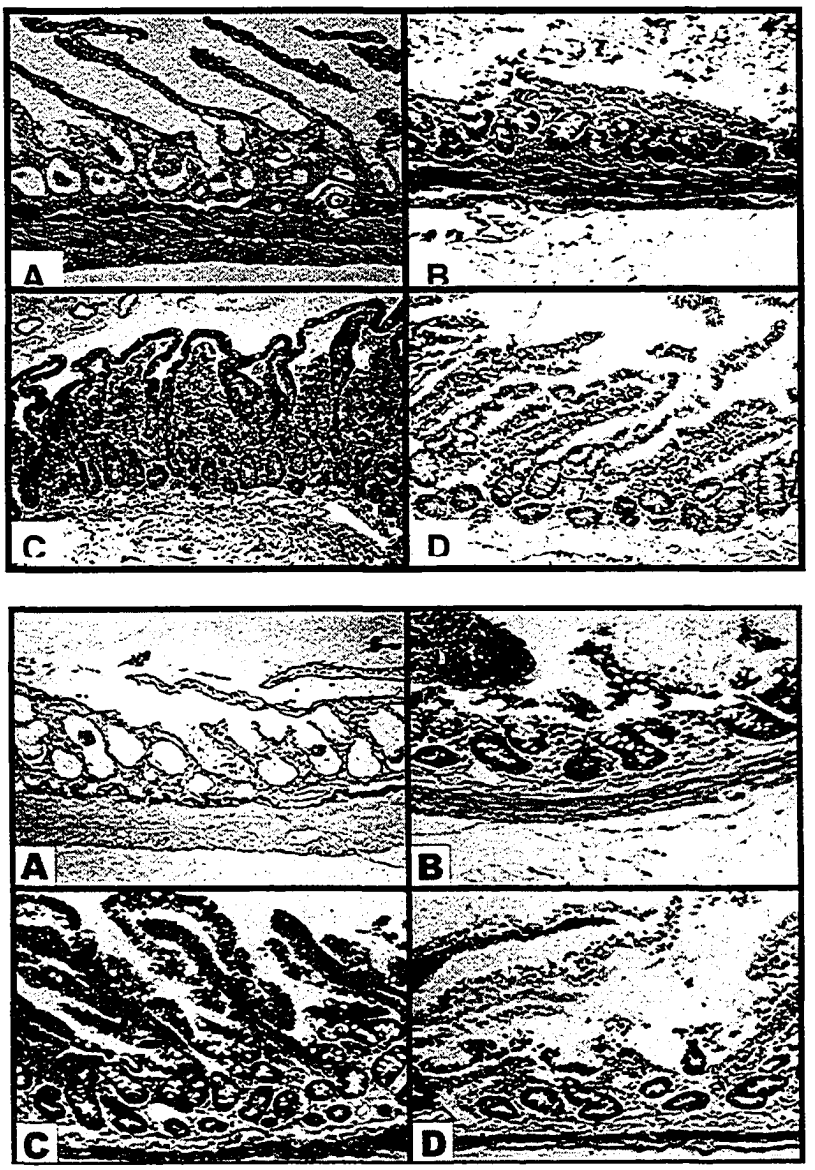

Figure 3-5. Histology of Small Bowel after 12 h (upper panel) and 24 h (lower panel) cold storage.

A. Group 1, no luminal treatment; **B.** Group 2, UW luminal treatment; **C.** Group 3, 1 h perfusion then static storage with UW; **D.** Group 4, continuous perfusion with UW.

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Chapter 4

Preservation of the small bowel mucosal barrier via luminal perfusion with a proven nutrient-rich solution*

Introduction

Small bowel transplantation (SBT) is the only treatment option available to patients with irreversible intestinal failure who have developed life-threatening complications (i.e. IV-line infection leading to sepsis) or have otherwise failed with total parenteral nutrition (1-3). Not only is the health of these patients compromised already, but success of SBT is currently nowhere near the success rates of the other commonly transplanted organs. Overall graft survival remains low (less than 40 % five year survival) (4).

Major obstacles to the development of reliable and safe SBT are primarily those of bacterial infection and allograft rejection; 67% of patient mortality is due to sepsis (55%) or rejection (12%) (3). Following transplantation, sepsis is the direct consequence of bacterial translocation across an injured small bowel (SB) graft (5,6). Unfortunately, the mucosal epithelium is extremely susceptible to even brief periods of ischemia. Hence, graft viability related to preservation is one of the most important factors in successful transplantation. Despite the benefit of the University of Wisconsin (UW) solution for preservation of other intra-abdominal organs, the maximum storage time for SB remains relatively brief (6-8 hours); furthermore graft quality is often compromised (7). No single preservation solution has proven truly effective for SB and equivalent results can be achieved with simple crystalloid solutions (Normal Saline) or with complex

solutions such as UW solution (8,9). Thus, it is by default that the current clinical standard for SB consists of a vascular flush with UW solution as part of multi-visceral organ procurement.

Development of a preservation solution that prevents mucosal injury during static cold storage and facilitates the regeneration of the epithelial barrier upon reperfusion will provide a critical step towards reliable SBT in the clinical arena. Techniques involving the removal of luminal contents prior to cold storage via luminal flush aid in the elimination of bile acids, digestive enzymes and bacterial endotoxins (10). Supplementation with essential nutrients (primarily amino acids) can further increase the benefit of luminal flushing prior to standard storage techniques. Recently, our lab has developed an effective nutrient-rich preservation solution that is specifically tailored to the physiological amino acid requirements of the SB (11,12).

Despite success with our novel amino acid-based preservation solution for use in the static storage of SB, substrate limitations including oxygen delivery remain a significant problem in maintaining mucosal barrier function. Historically, luminal insufflation with hyperbaric oxygenation (delivery of oxygen without solution) and continuous vascular perfusion have been attempted as alternatives to static cold storage (13-15). However, the use of insufflation and vascular perfusion techniques remains controversial due to issues of complexity and risk of injury during continuous delivery of gaseous oxygen to the lumen and continuous mechanical insult to the vascular bed.

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* A version of this chapter has been published in *Transplantation* 2003; 76 (6): 911-7 (Salehi P, Zhu JZ, Castillo EG, Avila J, Lakey J, Churchill TA).

In the present communication, we tested the hypothesis that the mucosal barrier can be successfully preserved by combining a proven nutrient-rich luminal preservation solution with the benefit of continuous perfusion.

Materials and Methods

Male Sprague-Dawley rats (200-300 g) were obtained from the University of Alberta and used as bowel donors. All experiments were conducted in accordance with the regulations and policies of the Canadian Council on Animal Care. Chemical agents were AR-grade and were purchased from Sigma Chemical (Oakville, Canada).

Surgical procedure and procurement of the small intestine: Rats were fasted 10-12 h overnight in cages with raised floors to minimize coprophagy. Water was provided *ad libitum*. Prior to laparotomy, rats received an intraperitoneal dose of pentobarbital (65mg/250g; Somnotol; MTC Pharmaceuticals, Cambridge, Ontario, Canada), followed by inhalational halothane (0.5-2%) to maintain anesthesia. A midline laparotomy was performed and the aorta exposed infrarenally and at the level of the celiac trunk. The supraceliac aorta was then clamped and 10 ml of modified University of Wisconsin (UW) solution (equivalent to ~0.5 ml/g tissue; Table 1) was administered via the infrarenal aorta. The suprahepatic vena cava was transected to facilitate the outflow of both blood and perfusate. The entire jejunum and ileum was subsequently harvested. Luminal amino acid (AA) solution (20 ml; equivalent to ~1.0 ml/g tissue, Table 4-1) was then placed into the lumen of the bowel allowing the effluent to exit uninhibited. Each end was immediately ligated with 3-0 silk, leaving the bowel filled without turgor with the same solution. The bowel was then stored in 30 ml of solution and stored on ice in a 4°C incubator. Bowel that was treated with perfusion (1 h and 24 h) was luminally flushed as above and then transferred to the recirculating perfusion apparatus (Figure 4-1) and

perfused with an additional 80 ml of solution (+ 5 µg/ml Ciprofloxacin) at ~1 ml/g/min (solution was bubbled with 100% oxygen). For bowel that was perfused for only 1 h, the ends of the segment were tied off at the conclusion of 1h and then statically stored in 30 ml preservation solution on ice. Tissue samples (1-2 g) were taken at 4, 8, 12 and 24 hours post vascular flush. To arrest metabolic activity, samples were snap frozen in liquid nitrogen, and subsequently stored at –65°C until processed. Freshly isolated (FI) tissue was obtained from a separate group of animals to provide an approximation of *in vivo* metabolites and 'normal' histology.

Experimental groups: All experimental groups (n=4) were flushed vascularly with UW solution (except for freshly isolated tissue) and then treated luminally as below:

Group 1 – no luminal treatment [clinical control];

Group 2 – AA solution;

Group 3 – 1 h perfusion then static storage with AA;

Group 4 – 24 h perfusion with AA;

Sample Preparation and Metabolite Assay: Frozen SB samples were weighed and then extracted 1:5 w/v in perchloric acid containing 1mM EDTA. The precipitated protein was removed by centrifugation (20 min at 20,000xg). Acid extracts were neutralized by the addition of 3 M KOH/ 0.4 M Tris/ 0.3M KCl and then recentrifuged (20min at 14,000xg). Aliquots of neutralized extracts were immediately processed via standard enzyme-linked metabolite assays (16). Spectrophotometric analysis was then performed to measure the absorbance of NADH at 340 nm, providing quantification of ATP, ADP, AMP, lactate, and ammonia. Values are reported as µmol per gram wet weight.

Malondialdehyde (MDA): Frozen tissue (100 mg) was homogenized 1:10 in phosphate-buffered saline. The homogenate was then processed and fluorescence was compared to standard amounts of MDA according to Yagi (17,18).

Histology: Full-thickness samples were fixed in a 10% buffered formalin solution, embedded in paraffin, cut 4 to 5-μm thick, and stained with hematoxylin and eosin. Histologic damage was assessed using Park's histologic classification of intestinal tissue injury (19).

Statistical analysis: Metabolite data were reported as means ± SE for each group. Statistical differences between groups were determined using analysis of variance, followed by Tukey's Honest Significant Difference (HSD) *post hoc* comparison test; $p<0.05$ was reported. Histology scores were compared using the Kruskal-Wallis test; $p<0.05$ was reported.

Results

Energetics

ATP (Figure 4-2): After 4 h, ATP values ranged from 1.0 to 2.0 μmol/g, with Groups 2, 3 and 4 being significantly different from control Group (0.57 μmol/g, $p<0.05$). Within 4 h, ATP levels dropped by 40-65% in Groups 1 and 2 and by 25-30% in Group 3; values in Group 4 remained unchanged from freshly isolated tissue throughout 12 h time period. After 8 h, Groups 3 and 4 exhibited higher ATP values compared to control Group (1.0 and 2.2 vs 0.6 μmol/g, respectively, $p<0.05$). After 12 h ATP values in Groups 1-3 ranged from 0.5 to 0.9; this was significantly lower than the corresponding value of 1.5 μmol/g in Group 4 ($p<0.05$). Even after 24 h, Group 4 ATP levels were more than 2 fold greater than Group 1-3 ($p<0.05$).

Total Adenylates (Figure 4-2): After 4 h, total adenylate values (TA=ATP+ADP+AMP) dropped from a freshly isolated (FI) value of 3.4 µmol/g in Group 1; Groups 2-4 exhibited increases compared to FI. Within 8 h, elevated TA values were observed in Groups 3 and 4 compared to Groups 1 and 2 ($p<0.05$). However, by 12 h, Groups 3 and 4 were significantly higher than only Group 1 (3.2 and 2.9 vs 1.7 µmol/g, $p<0.05$). Following 24 h, there was no significant difference between any of the groups.

Energy charge (Figure 4-3): Energy charge [EC=(ATP+ADP/2)/Total Adenylates] value for FI specimens was 0.69 ± 0.03. FI value was notably different from Groups 1-3 ($p<0.05$) after 4 h; values ranged from 0.49 to 0.58. Throughout 24 h, Group 4 showed significantly higher EC values compared to Groups 1-3 at all times ($p<0.05$), remaining unchanged from freshly isolated levels.

ATP/ADP Ratio (Figure 4-3): FI tissue had an ATP/ADP ratio of 1.3 ± 0.2, significantly different from all Groups following 4 h ($p<0.05$). Group 4 exhibited a higher ATP/ADP values compare to Groups 1-3 throughout the entire 24 h; values were 1.5-2.0 in Group 4 compared to <0.75 in Groups 1-3.

Lactate Accumulation (Figure 4-4): Within 4 h, levels of this anaerobic endproduct were elevated compared to FI. After 8 h and 12 h, Groups 2 and 3 exhibited higher lactate levels compared to control (Group 1). Interestingly, lactate levels for Group 4 were notably lower than for Groups 1-3 during the entire 24 h ($P<0.05$).

Ammonia (Figure 4-4): Within 4 h, all experimental Groups showed a greater accumulation of ammonia than FI tissue ($p<0.05$). Throughout the 24 h time course, Groups 2-4 demonstrated 50-70% higher ammonia levels than Group 1 ($p<0.05$).

Lipid peroxidation

Malondialdehyde (MDA; Figure 4-5): The level of MDA in freshly isolated tissues was 572 ± 48 nmol/g. After a 2.2 fold increase by 4 h storage, control group values fell to levels significantly lower than in Groups 2-4 ($P<0.05$); final 12-24 h values were not significantly different than FI in this group. Group 3 did not exhibit an increase in MDA until 12 h; thereupon rising to 2 fold FI values ($P<0.05$). In Group 4, MDA levels were consistently elevated; statistical significance was apparent between Group 4 and at least one other group throughout the entire 24 h period.

Histology

The histologic changes produced by ischemia in all Groups are summarized in Table 4-2. After 12 h, histologic integrity was markedly superior in Groups 2, 3 and 4 (median park's grades 3, 2 and 3.5, respectively; Figure 4-6). Within this time-period, crypt layer infarction was obvious in Group 1 (grade 6). After 24 h, the best Park's grade was observed in Group 3 ($p<0.05$, compared to other Groups; Figure 4-7). Tissues subjected to 1 h perfusion prior to static storage exhibited relatively normal intestinal architecture with extension of subepithelial space and minor-moderate lifting, with a median histopathologic grade of 2. Massive lifting down sides of villi with some denudation in tips were observed in Group 2 (grade 3.5). Continuously perfused tissues subjected to 24 h ischemia exhibited an obvious disintegration of lamina propria with infarction of the crypt layer or mucosal layer in 2/4 samples. The worst mucosal damage (i.e. transmural infarction, grade 8) was clearly observed in Group 1 (control).

Discussion

Intestinal injury due to ischemia is a common clinical problem, observed in volvulus, gastroschisis, mesenteric occlusion, necrotizing enterocolitis and during

various surgical procedures such as aortic aneurysm repair, cardiopulmonary bypass and of particular relevance to this study, small bowel transplantation. With as much as 55% of post transplant mortality caused by septic complications, maintenance of the mucosal barrier is critical in preventing the movement of bacteria from 'outside' the body into the blood-stream (4,20). A preservation solution or technique that substantially reduces or even eliminates mucosal injury incurred during cold storage will provide an integral step towards the development of a reliable SBT in the clinic. Despite poor graft preservation quality, the current clinical practice involves a common arterial vascular flush with UW as part of a multi-organ procurement procedure followed by static storage (7). Clinically, the period between harvesting a donor organ and transplantation into a recipient involves static cold storage; a period during which nutrient and oxygen supplies are halted. Energy levels within the tissue are soon depleted during ischemic storage since many energy-utilizing processes are still in operation even at hypothermia (21). This phase of cold ischemia is, at present, unavoidable and results in the gradual deterioration of absorptive properties that are essential for normal bowel function, eventually progressing into irreversible damage.

The mucosal barrier is maintained largely by the tight junction region close to the apical surface of intestinal epithelial cells. The tight junction consists of a number of proteins, which are dynamic and energy-requiring structures. Depleting cellular energy reserves (ATP and Total Adenylates) results in an inability of epithelial cells to maintain this barrier to the toxic bacteria captive within the lumen (22). Cellular energetics, such as ATP and total adenylates, have been found to reflect quality of small bowel more than any other solid organ during prolonged storage. Dilation of tight junctions is the causal link to an increase in trans-epithelial flux of macromolecules and enteric bacteria (22).

With prolonged the periods of hypoxia experienced during clinical small bowel storage, functional and structural integrity rapidly declines with time, eventually leading to the loss of the entire barrier. This results in direct contact with luminal microorganisms, leading to bacterial translocation and an antigenic response. The end result is bacterial translocation, an elevated inflammatory response and the potential for life-threatening infections (23-25).

It has been long recognized that continuous hypothermic perfusion of organs such as kidney and liver, under optimal conditions of oxygenation, perfusate flow and substrate supply can yield excellent preservation (26). In the present study, we had hypothesized that although luminal flushing provides improved mucosal integrity (versus vascular flush), its preservation capacity would be dramatically enhanced when the luminal solution is saturated with oxygen and continuously perfused. The metabolism of amino acids proceeds through the TCA cycle, which is heavily dependent on the availability of oxygen. In luminal flushing followed by static storage, there is a minute amount of oxygen initially dissolved in the solution. However, since the solution is confined to the lumen, oxygen reserves are depleted by oxidative phosphorylation and are not replenished. Eventually, this decline forces the tissue to utilize a much less efficient anaerobic metabolism. Without oxygen, there is a progressive impairment of mitochondrial function by an increase in uncoupled respiration. Hypoxic stress due to the static nature of organ storage is responsible for the uncoupling of respiration by the collapse of mitochondrial membrane potential and the ensuing increase in membrane permeability (27). This can be avoided if the mitochondria are maintained in an active oxidative state by continuous oxygenated perfusion, even at hypothermic temperatures. Since the luminal solution employed in the present study has an abundance of nutrients,

the limiting factor in maintaining graft quality is the availability of oxygen. Under optimal conditions of oxygenation, perfusate flow, and substrate supply, the nutrient-abundant solution should be theoretically capable of producing excellent preservation of intestinal mucosa.

Our attempts in the current communication to supply adequate oxygen and nutrients to the mucosal surface via continuous luminal perfusion at hypothermia were successful in terms of maintenance of metabolic homeostasis. In terms of energetics, continuous perfusion far exceeds that of static storage. ATP levels were consistently higher in the perfusion groups. As well, total adenylates were sustained at a higher concentration. The maintenance of purines in a higher energy phosphorylated form (ATP, ADP, or AMP) is necessary for the efficient regeneration of ATP once blood flow is re-established and reperfusion is initiated. ATP/ADP ratios are typically used to reflect the quality of mitochondrial oxidative phosphorylation and energy charge values reflect the proportion of adenylates that are available for cellular work. In our data, ATP/ADP and EC values were higher in perfusion groups. This indicates a greater reserve of high energy molecules and thus, better preserved tissue. It is not a novel concept that oxygen and substrate delivery to the metabolically active epithelium is fundamental to the preservation of mucosal structure and function. Indeed, the primary stressor involved in organ storage for transplantation is unequivocally a problem of limited oxygen. However, there was an obvious limit to the benefit of oxygen delivery when encompassed in the technique of hypothermic luminal perfusion.

One important factor limiting the benefit of continuous oxygenated perfusion is the production of oxygen free radicals. Malondialdehyde (MDA) is not only an index of lipid peroxidation but is also a reactive agent that can subsequently react with cellular

proteins (and lipids), thereby damaging the cell at multiple levels (28). In this study, static storage of SB resulted in a significant increase in MDA after 4 h; levels returned to FI values after 12 h. Presumably, this decline is a result of further interaction with cellular components. Interestingly, tissues subjected to varying periods of perfusion exhibited elevated MDA levels. There was a positive correlation for MDA levels with degree of perfusion; 24 h perfusion resulted in the highest MDA levels of the luminally-treated specimens. This was not entirely unexpected, since the basal production of OFRs is directly related to electron transport system activity. As much as 4% of oxygen metabolized captures single electrons that leak out of the mitochondria during complex III activity and result in the generation of superoxide radicals (29). Consequently, as the amount of oxygen delivered to the tissue increases, so does the amount of electron-capturing oxygen and hence, resultant OFR levels. Despite elevated MDA levels in tissues perfused for 1 h prior to static storage, this group exhibited only minor histologic injury. Clearly there is a balance between delivering oxygen for energy production and the inadvertent production of detrimental by-products.

The intestinal mucosa is very sensitive to even brief periods of ischemia. Ultrastructural investigations have revealed alterations in the microvilli of apical epithelial within 5 minutes after the onset of ischemia (30,31). After 30 minutes warm ischemia, the upper two-third of villi are completely denuded of epithelial cells, whereas the crypts remain relatively intact (32). However, prolonged ischemic insult progressively leads to crypt infarction and eventually to infarction of the submucosa and muscularis layers. The crypt cells play a vital role in the re-epithelialization of denuded villi once issues of limited oxygen and substrate delivery have been resolved upon reperfusion. Moreover, some investigators demonstrated that the oxygen dissolved in the fluid introduced into the

lumen was an important factor in the protection of the mucosa against ischemic damage (33). According to our data, although 24 h continuous perfusion provides improved tissue energetics, the brief 1 h perfusion group exhibited histology that was markedly superior throughout 24 h storage at 4°C. With the limited 1 h period of perfusion, only the upper portion of villi was adversely affected, leaving the metabolically active crypts of Lieberkuhn intact. Continuous luminal perfusion, however, resulted in extensive disintegration of lamina propria accompanied by crypt infarction. The data presented in this study suggests that despite superior metabolic status facilitated by the delivery of oxygen and nutrients to the mucosal layer, the physical action of continuous luminal perfusion [in conjunction with an elevated level of lipid peroxidation] was the causal agent in the observed mucosal injury. Our data suggests that the mucosal layer can only tolerate a limited period of luminal perfusion at hypothermia; disruption of the mucosal surface due to mechanical or oxidative stress is apparent after 24 h perfusion.

Luminal flushing even with a simple crystalloid solution has been shown to improve the metabolic and structural integrity of the mucosal layer over varying periods of static storage compared to vascular flushing alone. This has been postulated to occur by dilution of resident enteric contents, which contain feces and other cytotoxic agents, including bacterial endotoxins as well as biliary and pancreatic secretions (10). Supplementation of the luminal solution with essential nutrients, such as amino acids, further increases the benefit of luminal flushing. Numerous studies involving animal models have demonstrated that the lumen, rather than the vasculature, is the route by which the majority of nutrient absorption occurs in the body; amino acids absorption through the lumen varies from 35% to 99% (34). The nutrient-rich preservation solution employed in this study has been specifically tailored to the physiological amino acid

requirements of the SB. The composition of amino acids in the solution cater to both metabolic (energy production) and synthetic (synthesis of critical molecules) aspects of intestinal metabolism (32,35). Specifically, numerous amino acids have been noted to play key roles in a variety of cellular housekeeping processes. For example, arg, glu, and pro are all precursors of ornithine, which is required for epithelial cell proliferation, differentiation, and repair (36). Furthermore, glu, gly, and cys are precursors to glutathione, which aids in the defense of intestinal mucosa against toxic and peroxidative damage (37). Arg, a precursor of nitric oxide, plays an important role in the regulation of intestinal blood flow and epithelial cell repair (38). Other amino acids, including thr, lys, met, tryp, ser and gly have been implicated in major protein synthetic roles (32). When combined in a solution, these amino acids play an important adjunctive role in maintaining overall mucosal viability during conditions of hypothermic hypoxia.

In conclusion, an ideal preservation solution should preserve the structure of intestinal epithelium during the ischemic period, prevent of tissue injury due to oxygen free radicals and be able to regenerate high-energy phosphate compounds. This study demonstrates that perfusion clearly improves tissue energetics, however mucosal integrity is markedly superior with only a brief 1 h period of perfusion; this limits injury due to mechanical and oxidative stress. A similar strategy of brief perfusion with oxygenated amino acid solution may improve small bowel graft structure from cold preservation injury in the clinical setting.

Table 4-1. Composition of Preservation Solutions.

	UW	AA
Lactobionate	100	20
Raffinose	30	-
Adenosine	5	5
$MgSO_4$	5	-
KH_2PO_4	25	-
Allopurinol	1	1
Dextran (67.3 kdal)	5 %	5 %
BES	-	15
Glutamine	-	35
Glucose	-	20
Glutamate	-	20
Aspartate	-	20
Arginine	-	10
Glycine	-	10
Valine	-	10
Asparagine	-	10
Threonine	-	10
Lysine	-	10
Serine	-	10
Methionine	-	5
Ornithine	-	5
Leucine	-	5
Isoleucine	-	5
Histidine	-	5
Cysteine	-	5
Proline	-	5
Hydroxybutyrate	-	3
Tyrosine	-	1
Tryptophan	-	1

Values are 'mM' amounts unless otherwise stated.

Solution pH was adjusted to 7.40 ± 0.01 using NaOH.

BES = N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid.

Table 4-2. Histological grades of intestinal injury.

	Group	Grade	Median
	FI	0,0,0,0	0
12 h	**1**	4,5,7,8	6
	2	2,3,3,4	3 c
	3	1,2,2,2	2 c
	4	3,3,4,4	3.5 c
24 h	**1**	5,6,8,8	7
	2	3,3,4,5	3.5 c
	3	2,2,2,3	2 c
	4	4,5,6,7	5.5 c,b

FI denotes freshly isolated tissue. All values after 12 or 24 h were significantly greater than FI ($p<0.05$). **c,b** - significantly different compared to Group 1 and Group 3, respectively; $p<0.05$.

Park's classification: 0 = normal mucosa; 1 = subepithelial space; 2 = extended subepithelial space; 3 = complete epithelial lifting; 4 = denuded villi; 5 = loss of villus tissue; 6 = crypt infarction; 7= transmucosal infarction; 8= transmural infarction.

Group 1, no luminal treatment; Group 2, AA luminal treatment; Group 3, 1 h perfusion then static storage with AA; Group 4, 24 h perfusion with AA.

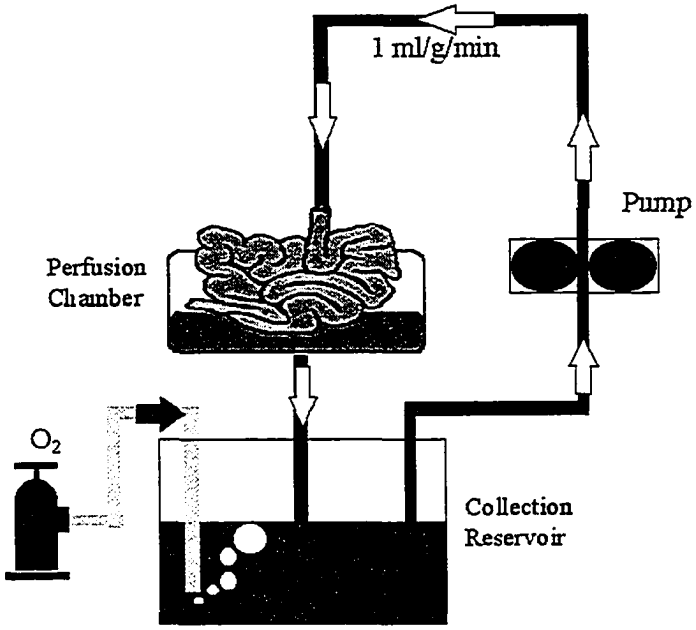

Figure 4-1. Diagram of Perfusion Apparatus. Temperature was maintained at 2-4 °C and flow rate was 1 ml/g/min.

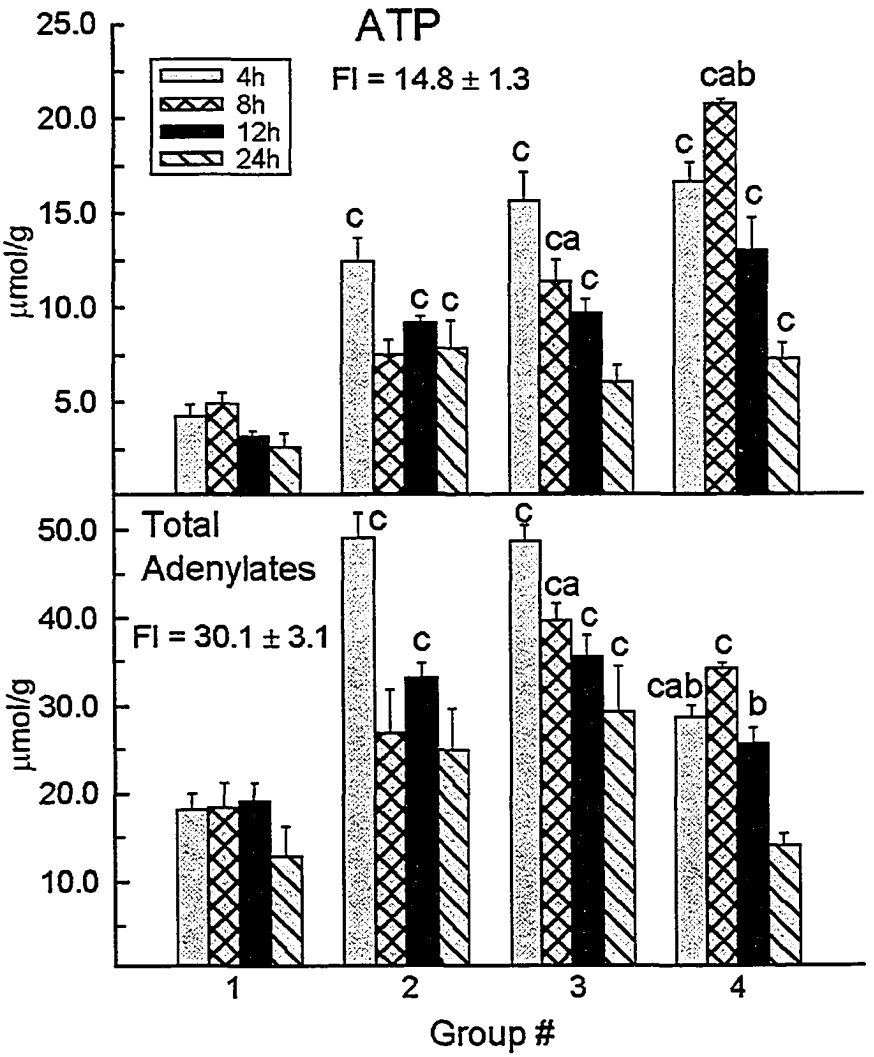

Figure 4-2. Effect of luminal AA solution on ATP and Total Adenylates.

c, a, b – significantly different compared to Group 1 (control), Group 2 and Group 3, respectively; $P<0.05$.

Group 1, no luminal treatment; Group 2, AA luminal treatment; Group 3, 1 h perfusion then static storage with AA; Group 4, 24 h perfusion with AA.

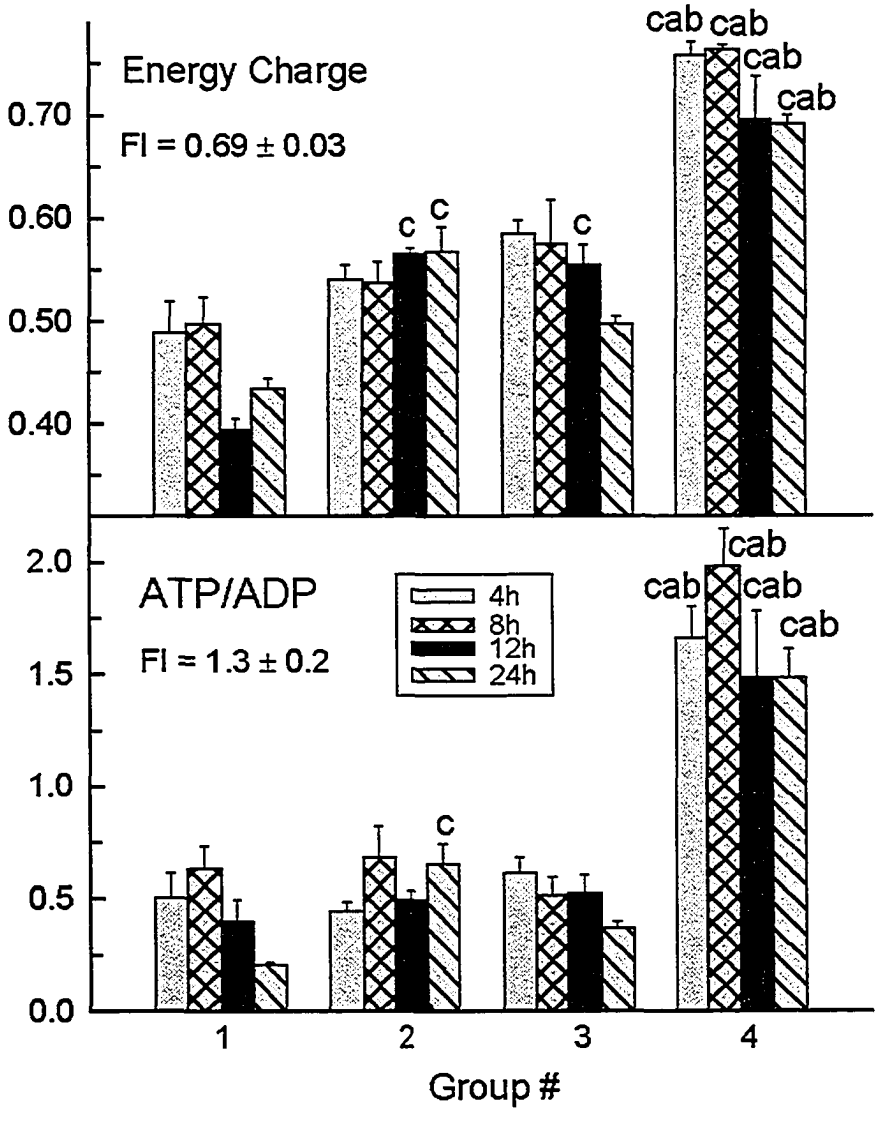

Figure 4-3. Effect of luminal AA solution on Energy Charge and ATP/ADP ratios.

c, a, b – significantly different compared to Group 1 (control), Group 2 and Group 3, respectively; $P<0.05$.

Group 1, no luminal treatment; Group 2, AA luminal treatment; Group 3, 1 h perfusion then static storage with AA; Group 4, 24 h perfusion with AA.

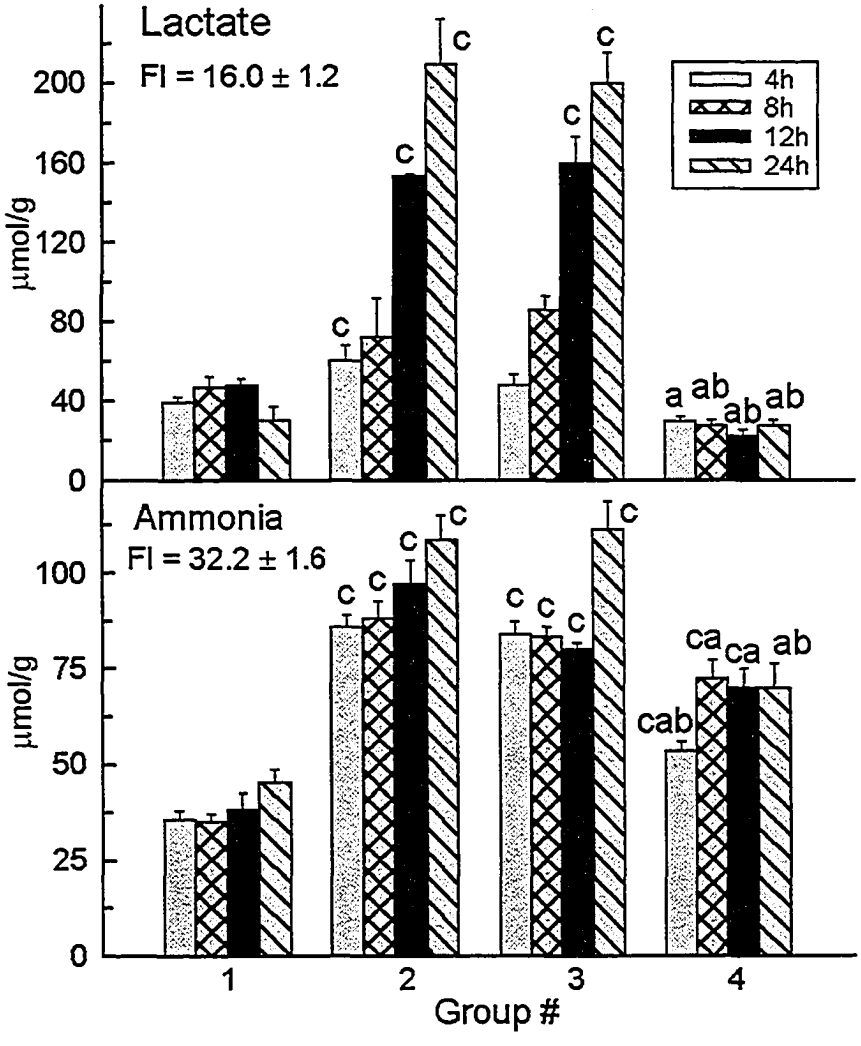

Figure 4-4. Effect of luminal AA solution on end products, lactate and ammonia.

c, a, b – significantly different compared to Group 1 (control), Group 2 and Group 3, respectively; $P<0.05$.

Group 1, no luminal treatment; Group 2, AA luminal treatment; Group 3, 1 h perfusion then static storage with AA; Group 4, 24 h perfusion with AA.

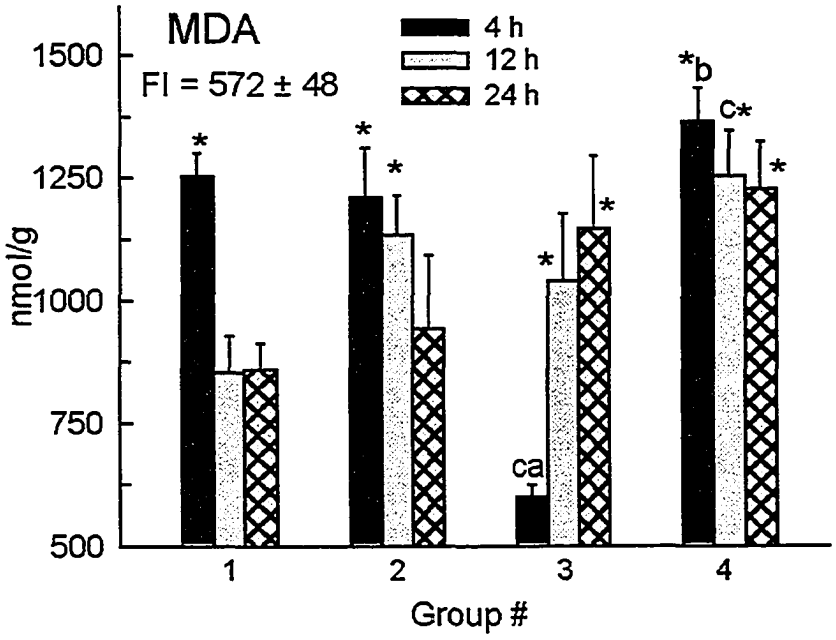

Figure 4-5. Effect of luminal AA solution on the peroxidation product, malondialdehyde (MDA). * - Significantly different from freshly isolated values; $P<0.05$. **c, a, b** – significantly different compared to Group 1 (control), Group 2 and Group 3, respectively; $P<0.05$.

Group 1, no luminal treatment; Group 2, AA luminal treatment; Group 3, 1 h perfusion then static storage with AA; Group 4, 24 h perfusion with AA.

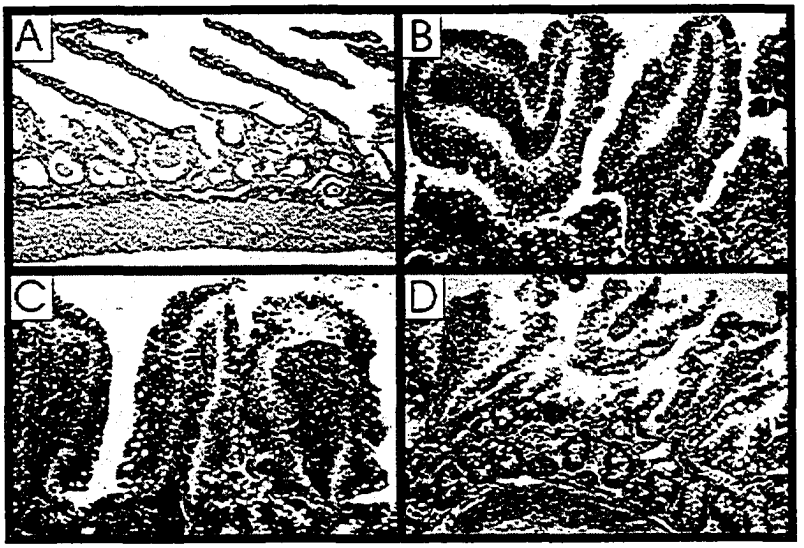

Figure 4-6. Histology of Small Bowel after 12 h Storage.

A. Group 1, grade 7 – transmucosal infarction; **B.** Group 2, grade 3 – complete epithelial clefting; C. Group 3, grade 2 – extended epithelial clefting; D. Group 4, grade 6 – loss of villi and crypt infarction.

Group 1, no luminal treatment; Group 2, AA luminal treatment; Group 3, 1 h perfusion then static storage with AA; Group 4, continuous perfusion with AA.

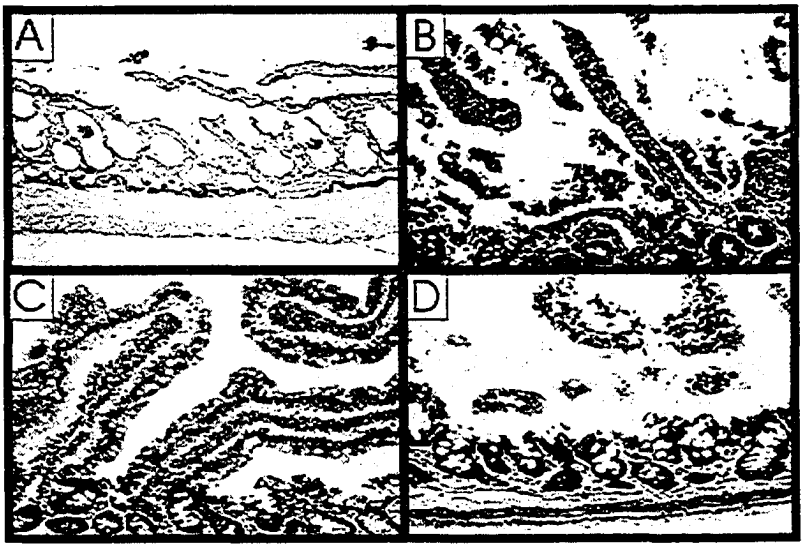

Figure 4-7. Histology of Small Bowel after 24 h Storage.

A. Group 1, grade 8 – transmucosal and transmural infarction; **B.** Group 2, grade 4 – denuded villi; **C.** Group 3, grade 2 – extended epithelial clefting; **D.** Group 4, grade 6 – loss of villi and crypt infarction.

Group 1, no luminal treatment; Group 2, AA luminal treatment; Group 3, 1 h perfusion then static storage with AA; Group 4, 24 h perfusion with AA.

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Chapter 5

Nutrient-related prophylaxis for intestinal ischemia-reperfusion injury*

Introduction

Intestinal injury as a result of ischemia and subsequent reperfusion (I/R) plays a key role in various surgical procedures and clinical conditions: aortic aneurysm repair, shock, small bowel transplantation (SBT), and necrotizing enterocolitis in neonates (1,2). An inadequate oxygen supply induces tissue injury eventually leading to irreversible cellular dysfunction. Although reperfusion is essential to prevent anoxic cell death, it is often associated with additional and severe cellular damage. Ischemia/reperfusion injury has been divided into an initial phase due to the synthesis of oxygen free radicals (OFRs), followed by a later phase involving neutrophils and the release of inflammatory mediators (3). During ischemia, catabolism of ATP eventually results in increased hypoxanthine. This leads to a xanthine oxidase-mediated burst of superoxide molecules, causing epithelial cell damage by peroxidation of cell membrane as well as the accumulation and activation of neutrophils (4). Activated neutrophils adhere to the endothelial cells, clog the capillaries, and release more OFRs and various enzymes including myeloperoxidase and proteases as part of a defense mechanism to degrade injured tissue (5). Upon exposure to a non-physiological stress such as intestinal ischemia precipitated as a complication during surgery, endogenous defense mechanisms may become overwhelmed, resulting in decreased mucosal function, increased barrier permeability and all-encompassing decay of mucosal structure and function.

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* A version of this chapter has been published in *Am J Transplant* 2004; 4 (5): 728-37 (Salehi P, Madsen K, Zhu J, Castillo E, Avila J, Lakey JR, Churchill TA).

Development of a luminal preservation solution that prevents mucosal injury during ischemia and facilitates the regeneration of the epithelial barrier upon reperfusion will provide a critical step towards remedying clinical problems that involve intestinal ischemia. Techniques involving the removal of luminal contents prior to ischemia via luminal flush aid in the elimination of bile acids, digestive enzymes and bacterial endotoxins (6). Supplementation with essential nutrients (primarily amino acids) can further increase the benefit of luminal flushing even during periods of limited oxygenation. Recently, our lab has developed an effective nutrient-rich preservation solution that is specifically tailored to the metabolic requirements of the SB (7,8). Although this strategy clearly protects the intestinal mucosa during transient periods of ischemia, evidence is lacking as to its effectiveness following reperfusion. The purpose of this study was to determine the role of this novel solution in ameliorating I/R injury in an in-vivo model of intestinal ischemia.

Materials and Methods

Male Sprague-Dawley rats (200-300 g) were obtained from the University of Alberta and used as bowel donors. All experiments were conducted in accordance with the regulations and policies of the Canadian Council on Animal Care. Chemical agents were AR-grade and were purchased from Sigma Chemical (Oakville, Canada).

Surgical procedure and procurement of the small intestine: Rats were fasted 10-12 h overnight in cages with raised floors to minimize coprophagy. Water was provided *ad libitum*. Rats were anesthetized with inhalational halothane (0.5-2%) and oxygen gas mixture. Rectal temperature was monitored and maintained at 35-37°C using a heated mat. Through a small midline incision the entire bowel was eviscerated. An incision made on the first part of duodenum to insert a cannula. Luminal amino acid (AA) (20 ml;

equivalent to ~1.0 ml/g tissue) or Ringer's solutions were then placed into the lumen of the bowel. Solution was allowed to flush out enteric contents via transection of the terminal ileum. A tie was then placed around the distal ileum to contain the remaining solution throughout the duration of the 60 min ischemia. Total ischemia was induced by clamping the superior mesenteric artery (SMA) for 60 min, after which time the occlusive clip was removed and reperfusion was then monitored over 60 min. Tissue samples (1-2 g) were taken at post ischemic time (0) and 15, 30, and 60 min reperfusion period. To arrest metabolic activity, samples were snap frozen in liquid nitrogen, and subsequently stored at –65°C until processed. Freshly isolated (FI) tissue was obtained from a separate group of animals to provide an approximation of *in vivo* metabolites and 'normal' histology.

Experimental groups: All experimental groups (n=4) were pretreated luminally as below:

Group 1 – none [clinical control];

Group 2 – Ringer's solution;

Group 3 – an amino acid (AA) solution;

Composition of Solutions: Ringer's solution contained: sodium chloride (103 mM), sodium lactate (28 mM), potassium chloride (4 mM), calcium chloride (1.8 mM). AA solution contained: lactobionate (20 mM), adenosine (5 mM), allopurinol (1 mM), 5% dextran (67.3 kdal), BES [N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] (15 mM), glutamine (35 mM), glucose/glutamate/aspartate (20 mM each), arginine/glycine/valine/asparagine/threonine/lysine/serine (10 mM each), methionine/ornithine/leucine/isoleucine/histidine/cysteine/proline (5 mM each), hydroxybutyrate (3 mM), tyrosine/tryptophan (1 mM each); pH, 7.40.

Sample Preparation and Metabolite Assay: Frozen samples were weighed and then extracted 1:5 w/v in perchloric acid containing 1 mM EDTA. The precipitated protein was removed by centrifugation (20 min, 20,000xg). Acid extracts were neutralized by the addition of 3 M KOH/ 0.4 M Tris/ 0.3M KCl and then recentrifuged (20min, 14,000xg). Aliquots of neutralized extracts were immediately processed via standard enzyme-linked metabolite assays (9). Spectrophotometric analysis was then performed to measure the absorbance of NADH at 340 nm, providing quantification of ATP, ADP, AMP, alanine, and ammonia.

Malondialdehyde (MDA): Frozen tissue (100 mg) was homogenized 1:10 in phosphate-buffered saline. The homogenate was then processed and fluorescence was compared to standard amounts of MDA according to Yagi (10).

Measurement of myeloperoxidase (MPO) activity: Frozen tissue (100-200 mg) was homogenized in hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM phosphate buffer, pH 6.0). The homogenate was processed and MPO activity was measured spectrophotometrically according to Krawisz et al (11).

Glutathione assay: The level of glutathione in the tissue was determined as a measurement of protection against oxidative stress. Reduced glutathione (GSH) was fluorometrically quantitated in bowel tissue homogenates using the monochlorobimane assay (12). Briefly, monochlorobimane (100 µM final concentration) and glutathione S transferase (0.2 units) were added to 500 µl tissue homogenate and incubated for 30 min at 37°C in order to form a fluorescent adduct with GSH. Samples were read on a fluorescence plate reader [excitation 380 nm; emission 485 nm].

Histology: Full-thickness samples were fixed in a 10% buffered formalin solution, embedded in paraffin, cut 4 to 5-µm thick, and stained with hematoxylin and eosin.

Histologic damage was assessed using Park's histologic classification of intestinal tissue injury (13).

Functional assessment – in Vitro electrical measurements: Ileal segments were taken after 60 min reperfusion, stripped of their serosa and muscular layers. The mucosa was mounted in Ussing chambers exposing mucosal and submucosal surfaces to 10 mL of oxygenated Krebs buffer with an ionic composition of: Na^+, 143 mM; K^+, 5mM; Mg^{2+}, 1.1 mM; Ca^{2+}, 1.25 mM; Hco_3^-, 25 mM; Cl^-, 123.7 mM; Hpo_4^-, 1.95 mM; and fructose 20 mM with 95% o_2 and 5% co_2 and pH=7.35. Transmural intestinal short-circuit current (Isc) was then measured over a surface area of 0.9 cm^2. The spontaneous transepithelial potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate Isc with an automatic voltage clamp (DVC 1000 World Precision Instruments, New Haven, CT), except for 5-10 seconds every 5 minutes when PD was measured by removing the voltage clamp. Tissue ion conductance (G) was calculated from PD and Isc according to Ohm's Law (14). Increase in Isc was induced by addition of the adenylate cyclase-activating agent, forskolin (10^{-5} mol/L), to the serosal surface. Epithelial responsiveness was defined as the maximal increase in Isc to occur within 5 minutes of exposure to the secretagogue. PD is expressed as millivolts (mV), G as millisiemens/cm2 (mS/cm^2), and Isc as microamperes per square centimeter ($\mu A/cm^2$).

Manitol permeability: An Ussing chamber was used to measure the permeability of mannitol in each ileal specimen. A quantity of 10 µCi of [3H] mannitol (Dupont, NET101) was added to the mucosal side of the reservoir. After an equilibration period (20 min), samples were taken from the mucosal and serosal sides. Ten and 20 min after equilibration, samples were again taken from both mucosal and serosal sides, and

assessed for tritiated mannitol radioactivity in a scintillation counter to determine mannitol flux across the tissue (15).

Statistical analysis: Metabolite data were reported as 'μmol/g protein' and presented as means ± SE. Statistical differences between groups were determined using analysis of variance, followed by Tukey's Honest Significant Difference (HSD) *post hoc* comparison test; $p<0.05$ was reported. Histology scores were compared using the Kruskal-Wallis test; $p<0.05$ was reported.

Results

Energetics

ATP (Figure 5-1A): After 15 min reperfusion, ATP values ranged from 5.3 to 10.0 μmol/g protein, with groups 1 to 3 being significantly different from freshly isolated values (14.9±1.5 μmol/g, $p<0.05$). ATP levels were preserved over 60 min warm ischemia (WI) in Group 3 and recovered to 100% freshly isolated values after 60 min reperfusion. After 30 min reperfusion, Group 3 exhibited higher ATP values compared to Groups 1 and 2 (13.1 vs 7.9 and 7.8 μmol/g, $p<0.05$). Even after 60 min reperfusion period, Group 3 ATP was approximately 2-fold greater than Groups 1 and 2 ($p<0.05$).

Total Adenylates (Figure 5-1B): Total adenylate level (TA = ATP + ADP + AMP) was 30.1±3.6 μmol/g protein in freshly isolated tissues. TA values were preserved over 60 min WI in Group 3 with no subsequent decreases in values throughout the entire 60 min reperfusion period. Values in Group 3 were consistently greater than control tissues at all times. TA values in Group 1 dropped to 70% of FI values following 60 min WI (21±1.1 μmol/g protein) and continued to drop during reperfusion; final values were 55% of FI levels after 60 min reperfusion (16.3±1.7 μmol/g protein).

Endproducts

Ammonia (Table 5-1): Ammonia, product of amino acid deamination, reflected a greater involvement of amino acid metabolism in Group 3; ammonia levels were 40% higher than control in Group 3 after 60 min warm ischemia ($p<0.05$). Ammonia cleared within 15 min reperfusion in this Group and remained low and not significantly different from Groups 1 and 2 throughout the remainder of reperfusion period.

Alanine (Table 5-1): Alanine, a product of amino acid metabolism in the bowel, was elevated in Group 3 tissues after WI and throughout reperfusion. Even after 60 min reperfusion alanine levels were higher in Group 3 compared to corresponding values in Groups 1 and 2 ($p<0.05$).

Enzyme Activities Relating to Glutamine Metabolism

The activities of several enzymes associated with glutamine metabolism were assessed (Table 5-2). In untreated tissues, levels of alanine aminotransferase (ALT), glutamate dehydrogenase (GDH), and glutaminase, either remained constant or exhibited a decline upon reperfusion. In luminal treated groups, either with Ringer's or AA solutions, there were significant increases with at least 2 of the three enzymes. The only group that demonstrated significant elevation of the primary enzyme associated with glutamine metabolism in the intestine, glutaminase, was Group 3, nutrient-treated tissues. In this group, glutaminase activity rose by as much as 42 % above control tissues between 30 and 60 min reperfusion ($P<0.05$).

Endogenous Antioxidant and Lipid Peroxidation

Glutathione (Figure 5-2A): GSH level was 6.4 ± 0.6 µmol/g protein in freshly isolated tissues. After 60 min warm ischemia, Group 3 exhibited higher levels of GSH compared to Groups 1 & 2 (8.7 vs 4.2 and 5.1 µmol/g protein, respectively, $p<0.05$). GSH values

tended to increase in both Groups 2 & 3 during reperfusion. GSH levels were significantly higher in Group 3 tissues compared to Groups 1 & 2 throughout the entire reperfusion time-course.

Malondialdehyde (Figure 5-2B): The extent of lipid peroxidation was determined by measuring malondialdehyde (MDA). The level of MDA in freshly isolated tissues was 286 ± 24 nmol/g protein. Only Group 3 tissues exhibited elevated MDA levels after 60 min WI; values were 40% greater than Groups 1 and 2 (436 vs 311 and 310 nmol/g protein, respectively, $p<0.05$). After 15 min reperfusion, levels of this product of lipid peroxidation resumed values that were not significantly different from Groups 1 and 2.

Polymorphonuclear Leukocyte (PMN) Infiltration

Myeloperoxidase activity (Figure 5-3): Neutrophil recruitment, assessed by myeloperoxidase (MPO) activity was 29 ± 2 U/g protein in freshly isolated tissues. During reperfusion time MPO activity tended to increase in all groups. However, MPO activity was markedly reduced over the first 30 min reperfusion in Group 3; MPO values were 56% of control after 30 min. No significant differences were detected after 60 min reperfusion.

Histology

The histologic changes produced by ischemia and subsequent reperfusion in all Groups are summarized in Table 5-3. Occlusion of the mesenteric artery followed by reperfusion produced significant structural changes in Groups 1 and 2. After 60 min warm ischemia, histologic integrity was markedly superior in Group 3 compared to groups 1 and 2 (median park's grades 2.5 vs 6 and 5, respectively, $p<0.05$). Histology indicated superior preservation of mucosal architecture in Group 3 tissues throughout the entire reperfusion period, particularly at 30 min reperfusion time point; median grade 4 in Group 3 vs grade

6 in Groups 1 & 2. After 60 min reperfusion, Groups 1 and 2 exhibited an obvious disintegration of lamina propria with infarction of the crypt layer (grade 6) whereas, lifting down sides of villi with some denudation in tips were observed in Group 3 (grade 5). A significant finding was the high incidence of massive hemorrhagic regions in both control and Ringer's treated tissues; these regions were absent in nutrient-treated tissues (Group 3). Representative regions of hemorrhage and mucosal architecture after 60 min reperfusion are depicted in Figure 5-4.

Mucosal Barrier and Electrophysiology

Mannitol flux. (Figure 5-5A) Group 3 exhibited significantly lower permeability compared with both Ringer's solution (Group 2) and control group (11.6±1.3 vs 31.0±3.2 and 61.5±9.0 nmol/cm^2/h, $p<0.05$). These data suggest that barrier function of AA-treated tissues was maintained at fresh tissue values (22.2±1.6 nmol/cm^2/h).

Conductance. (Figure 5-5B) Conductance levels in untreated control tissues rose dramatically compared to other experimental groups after 60 min reperfusion. Although luminal flushing had a marked effect independent of solution composition when compared to control group, AA treated tissues (Group 3) exhibited the lowest conductance values of all groups ($p<0.05$).

Potential difference. (Figure 5-5C) The ability to maintain a PD is a characteristic shared by all transporting epithelia and is dependent on the electrogenic ion pumps in the epithelial cell membrane and on the epithelial barrier function. Following reperfusion, Groups 1 and 2 tissues had PD values <1 mV, indicative of severely injured tissue. In sharp contrast, AA-treated samples were markedly superior at 2.1 ± 0.6 mV (similar to fresh tissue values), indicating a maintenance of ion transport processes across the mucosal membrane.

Response to forskolin. (Figure 5-5D) Short-circuit current is the measure of the net effect of all ion activities across the mucosal layer. The delta Isc response to forskolin in tissues treated with AA solution (Group 3) was markedly greater than that of tissues treated with Ringer's solution (Group 2) and control group (19.4 vs 4.1 and 1.0 $\Delta\mu A/cm^2$, $p<0.05$).

Discussion

The mucosal barrier is maintained largely by the tight junction region close to the apical surface of intestinal epithelial cells. The tight junction consists of a number of proteins, which are dynamic and energy-requiring structures. Depleting cellular energy reserves results in an inability of epithelial cells to maintain this barrier to the toxic bacteria captive within the lumen (22). Cellular energetics, such as ATP and total adenylates, have been found to reflect structural/functional quality of small bowel more than any other solid organ during ischemic episodes. Dilation of tight junctions is the causal link to an increase in trans-epithelial flux of macromolecules and enteric bacteria (16). With prolonged the periods of hypoxia experienced during clinical ischemia of the intestine, functional and structural integrity rapidly decline with time, eventually leading to the loss of the entire barrier. As a direct result of this intimate relationship between tissue energetics and maintenance of structural integrity, parameters of energy metabolism correlate exceptionally well with grade of histologic injury and with mucosal permeability.

In the current study, luminal treatment with the amino acid-based solution resulted in considerable protection of the intestinal mucosa throughout ischemia and upon reperfusion. ATP levels were elevated at the conclusion of the 60 min period of ischemia compared to untreated and resumed quantities that were 110% that of fresh tissue values. Furthermore, total adenylate quantities were maintained at fresh tissue

values and as time of reperfusion progressed, levels were always greater than control. The significance of this is a potential reduction in xanthine oxidase derived oxygen free radicals resulting from increased purine catabolism. As well, increased phosphorylated purine levels also permits superior recovery of ATP upon reperfusion, as was observed in this study. Presumably tissues subjected to no treatment or luminal Ringer's must rely on either the 'salvage pathway' or de novo synthesis for replenishment of ATP levels during reperfusion; both pathways being reliant on energy/substrate input and/or multiple enzyme reactions to facilitate regeneration of ATP. Presumably, maintenance of energetics involved an up-regulation of enzymes relating to glutamine metabolism, as was observed in this study. Increased production of the primary by-product of intestinal amino acid metabolism, alanine, supported the idea that luminal delivery of nutrient amino acids promotes metabolism even during ischemia. The presence of alanine is an excellent marker for intestinal amino acid metabolism since, the processing of many amino acids results in the net production of alanine; which then is transported via the blood to the liver for gluconeogenesis.

The barrier function of the normal intestinal mucosa is of utmost importance to prevent permeation of potentially noxious substances, including endotoxins and bacteria (17,18). The Ussing chamber technique is widely used to characterize gastrointestinal ion transport and consequently to examine intestinal permeability and function in animal models. Because the Ussing chamber is an isolated system, it provides a controlled environment in which to analyze intestinal segments without the limitations that are inherent of an in vivo approach. Using this system we determined that the nutrient-treated group was significantly less permeable to radiolabelled mannitol after 60 min reperfusion. Small water-soluble molecules such as mannitol permeate the intestinal

mucosa by passive diffusion between epithelial cells. Hence, the permeation of these molecules mainly depends on the integrity of tight junctions. To further evaluate the effects of the experimental solutions on the intestinal barrier, the Ussing chamber permits the measurement of various bioelectrical parameters; including potential difference (PD), an indicator of tissue viability, and G, a measure of tissue ion conductance. The amino acid treated group exhibited markedly lower ion conductance and higher tissue viability. The ability to maintain a spontaneous transepithelial potential difference is a characteristic shared by all transporting epithelia and is dependent on the electrogenic ion pumps in the epithelial cell membranes, mainly the Na^+/K^+ pump, and on the epithelial barrier function. Moreover, well-maintained epithelial function of amino acid treated tissues was confirmed by greater increases in short circuit current (Isc) after forskolin induction. Stimulation of chloride secretion via this potent adenylate cyclase activator is typically utilized during Ussing chamber studies to assess quality of the intestinal segment following in vitro manipulations; as tissue integrity becomes compromised, so does the ability to stimulate chloride secretion via forskolin. Hence, along with the array of electrophysiological parameters assessed, an elevated Isc forskolin response indicated a superior maintenance of functional integrity. Histologic examination of the specimens correlated with the electrophysiology measurements throughout reperfusion. In the current study, nutrient-treated tissue exhibited histologic architecture that was markedly superior throughout the entire reperfusion period; lifting downsides of villi, leaving the metabolically active crypts of Lieberkuhn intact.

In recent years in our lab, we have focused our research on the effects of substrate required for homeostatic maintenance in mucosal epithelial cells during periods of low oxygen. Luminal flushing even with a simple crystalloid solution has been

shown to improve the metabolic and structural integrity of the mucosal layer over varying periods of ischemia. This has been postulated to occur by dilution of resident enteric contents, which contain feces and other cytotoxic agents, including bacterial endotoxins as well as biliary and pancreatic secretions (10). Supplementation of the luminal solution with essential nutrients, such as amino acids, further increases the benefit of luminal flushing. Numerous studies involving animal models have demonstrated that the lumen, rather than the vasculature, is the route by which the majority of nutrient absorption occurs in the body; amino acids absorption through the lumen varies from 35% to 99% (20). The nutrient-rich preservation solution employed in this study has been specifically tailored to the physiological amino acid requirements of the SB. The composition of amino acids in the solution cater to both metabolic (energy production) and synthetic (synthesis of critical molecules) aspects of intestinal metabolism (21,22). Specifically, numerous amino acids have been noted to play key roles in a variety of cellular housekeeping processes. For example, arg, glu, and pro are all precursors of ornithine (a precursor to polyamines), which is required for epithelial cell proliferation, differentiation, and repair (23). Endogenous growth factors (polyamines) are up-regulated in response to luminal nutrient administration (via enteroglucagon in terminal ileum). Polyamines stimulate cellular RNA and DNA synthesis and promote cellular proliferation, thus facilitating regeneration and adaptation. Furthermore, glu, gly, and cys are precursors to glutathione, which aids in the defense of intestinal mucosa against toxic and peroxidative damage (24). Arg, a precursor of nitric oxide, plays an important role in the regulation of intestinal blood flow and epithelial cell repair (27). Other amino acids, including thr, lys, met, tryp, ser and gly have been implicated in major protein synthetic roles (26). When combined in a solution, these amino acids play an important

adjunctive role in maintaining overall mucosal viability during conditions of ischemia. Reperfusion following ischemia is the time when issues of nutrient supply are of utmost concern to a regenerating mucosa.

The importance of glutathione largely revolves around augmenting production of this intrinsic OFR scavenger. Augmenting production is important in that glutathione decreases in ischemic environments. Glutamine, a precursor of glutathione is able to enter the cell (unlike glutathione) and studies have demonstrated an effective increase in glutathione during IR. This leads to the question of the role of an amino acid-rich luminal solution that contains all three precursor amino acids (glutamate, cysteine, glycine). Interestingly in this study, glutathione levels in tissues treated with AA solution increased ~2 fold over fresh tissue values, supplying evidence that luminal delivery of the nutrient-rich solution used in this experiment facilitates intracellular antioxidant augmentation. Interestingly, both luminal treatment groups (Ringer's and AA solutions) exhibited increased glutathione levels. This may suggest that the physical presence of a solution within the lumen may have an up-regulatory effect on amino acid metabolism, as was observed with measured enzymes involved in glutamine metabolism in Ringer's treated tissue; all three glutamine-related enzymes assayed exhibited significant increases at at least 1 time point throughout reperfusion in tissues treated with AA solution. Presumably the limiting factor in the group treated with Ringer's solution was endogenous amino acid levels. Never-the-less, treatment with luminal amino acids resulted in the greatest elevation of endogenous glutathione, presumably by replenishing depleted endogenous amino acid stores. This important cellular protective mechanism may provide a direct link between nutrient supply and reduced IR injury.

Despite the amazing capacity of the SB for nutrient-dependent regeneration, there are other potentially deleterious events related to IR injury that must be addressed in order to promote recovery from ischemia. OFR's are highly unstable, highly reactive molecules which form in ischemic tissue upon re-exposure to oxygen; this is due in part to the partial reduction of molecular oxygen typically catalyzed via two enzymes: NADPH peroxidase (myeloperoxidase in neutrophils) and xanthine oxidase (XO; in intestinal mucosa). Another major OFR generating system is directly related to endogenous electron transport system (ETS) activity in the epithelium. As much as 4% of oxygen metabolized captures single electrons that leak out of the mitochondria during complex III activity and result in the generation of superoxide radicals (25). Consequently, as the activity of the ETS increases due to the presence of amino acid nutrients, so does the amount of electron-capturing oxygen and hence, resultant OFR levels. Cytotoxicity of these oxygen metabolites [eg. superoxide radical, hydroxide radical, hydrogen peroxide] manifests itself by inactivating enzymes, membrane transport proteins, damaging DNA and lysis of cell membranes due to lipid peroxidation. The result is compromise of cellular compartments and membranes which potentiates cellular dysfunction and eventually, death. OFR induced injury has been well established as a fundamental mechanism in IRI in SB, kidney, lung, heart, and liver. The intestine is extremely susceptible to OFR-mediated IRI due to an abundance of xanthine oxidase (XO) in mucosal epithelial cells and an inherent sensitivity of intestinal mucosal cells to ischemic injury. In pathologic states such as ischemia, xanthine dehydrogenase is converted to XO leading to increased oxidation of hypoxanthine, especially during conditions of intestinal hypoxia when ATP is actively being depleted. Interestingly, levels of XO are proportional to duration of ischemia; thereby providing direct relevance for research

targeted at improving strategies targeted at improving recovery from SB ischemia. Supplementation or administration of chemical or enzymatic antioxidants has been the focus of much research over the past several decades and due to the nature of mucosal metabolism, OFR-directed cytotoxicity is a primary target for alleviation of IRI in SB. In our study, the amino-acid treated group had accumulated significantly higher levels of the lipid peroxidation product, malondialdehyde (MDA), than the other groups throughout ischemia. However, MDA levels were rapidly cleared and resumed values similar to groups 1 and 2. Although treatment with the nutrient-rich AA solution was the cause of increased oxidative damage during ischemia, upon reperfusion this treatment was apparently also a factor in the efficient reversal of oxidative injury incurred during ischemia and reperfusion.

The inflammatory response during reperfusion inevitably leads to recruitment of neutrophils. Neutrophils are believed to play an important role in intestinal ischemia-reperfusion injury, can further potentiate the cellular injury initiated by ischemia (26,27). Upon adherence, neutrophils are activated and release multiple degradative enzymes (myeloperoxidase, elastase, and collagenase) which then potentiate cell damage. Inflammation due to neutrophil infiltration is a precursor to cytokine-directed necrosis. Hence, strategies focusing on reducing neutrophil involvement during IR are primary factors affecting recovery and regeneration of ischemically-injured mucosa. In the present study, the extent of neutrophil infiltration was significantly reduced over 30 min reperfusion in amino acid-treated tissues, indicating the possibility of lower graft immunogenicity over this time frame. Presumably, reduced neutrophil localization implies reduced chemotactic factors and leukotrienes influencing the attraction of neutrophils to the site of injury. Although, diminished neutrophil recruitment supports the

link between IR injury and inflammatory response, additional adjunctive therapies with a suitable inhibitor of neutrophil recruitment or mast cell stabilizer may be required to fully protect the injured mucosa inflammatory attack. Although neutrophil infiltration is a natural response to injured tissue which is typically well-tolerated, a major insult where large segments of intestine are affected may result in an overwhelming neutrophil response. For such an organ as the intestine which has an amazing recuperative capacity, a period during which regeneration is permitted or even promoted in the presence of luminal nutrients may be the major determinant in facilitating mucosal recovery following IR.

Although the incidence of acute mesenteric ischemia is low (<2 %) of all acute laparotomies, when it does occur, mortality of the patient is very high. Following aorto-iliac reconstructive surgery (for aortic aneurysm repair) ischemic mucosal lesions can be detected in more than 10 % of cases; over 7 % of patients present with intestinal ischemia as a clinical complication. Due to difficult diagnosis and delayed treatment, by the time an explorative laparotomy can be performed, it is usually too late and large portions of the intestine have already become necrotic and/or gangrenous. The only surgical practice that results in intestinal ischemia as a primary problem in 100% of cases is the ex vivo storage of bowel for the purposes of transplantation. Reducing the deleterious effects of cold ischemia is a major problem affecting clinical outcomes following transplantation. Ischemia-reperfusion injury and the subsequent amplification of host immune attack is a fundamental obstacle to successful preservation of this sensitive organ. Therapies targeting reducing IR injury generally involve short surgical times and have not in the past resorted to direct treatment of the lumen prior to surgeries involving a predicted period of intestinal ischemia. The nutrient-rich solution presented in

the current communication may provide an effective strategy for the treatment of the intestine during these types of surgical procedures to alleviate the potentially catastrophic complications precipitated by coincidental intestinal ischemia.

Table 5-1. End-products of Intestinal Metabolism.

	Group 1	Group 2	Group 3
Ammonia			
FI	32.2 ± 1.8		
0 m	63 ± 3 *	48 ± 3 ***c**	88 ± 3 ***cI**
15 m	46 ± 5 *	45 ± 4 *	53 ± 4 *
30 m	43 ± 3 *	48 ± 2 *	54 ± 1 ***c**
60 m	50 ± 4 *	50 ± 2 *	57 ± 2 *
Alanine			
FI	8.5 ± 0.9		
0 m	26 ± 2 *	20 ± 2 *	34 ± 1 ***cI**
15 m	24 ± 3 *	23 ± 1 *	32 ± 2 ***cI**
30 m	19 ± 2 *	16 ± 1 *	31 ± 2 ***cI**
60 m	26 ± 2 *	17 ± 2 ***c**	35 ± 2 ***cI**

Values are 'µmol/g protein' and presented as means ± SEM. FI –denotes values from freshly isolated tissue. * -Significantly different from fresh tissue values; $P<0.05$. **c,I** – Significantly different from control group (group 1) and group 2, respectively; $P<0.05$. Group 1, no treatment; Group 2, treated with luminal Ringer's solution; Group 3, treated with luminal amino acid solution.

Table 5-2. Enzymes involved in glutamine/glutamate metabolism.

	Group 1	Group 2	Group 3
Alanine aminotransferase			
FI	167 ± 15		
0 m	234 ± 15 *	126 ± 9 **c**	178 ± 9 **cI**
15 m	101 ± 10 *	129 ± 12	183 ± 11 **cI**
30 m	128 ± 6 *	159 ± 16	230 ± 16 ***cI**
60 m	132 ± 7 *	218 ± 13 ***c**	251 ± 22 ***c**
Glutamate Dehydrogenase			
FI	3.5 ± 0.1		
0 m	3.3 ± 0.1	2.9 ± 0.1 *	3.1 ± 0.2
15 m	2.7 ± 0.1 *	3.3 ± 0.1 **c**	3.2 ± 0.1 **c**
30 m	2.7 ± 0.1 *	3.3 ± 0.1 **c**	3.0 ± 0.2
60 m	2.9 ± 0.1 *	2.6 ± 0.1 *	2.9 ± 0.2
Glutaminase			
FI	25 ± 2		
0 m	42 ± 5 *	42 ± 3 *	38 ± 5 *
15 m	36 ± 5	47 ± 4 *	48 ± 5 *
30 m	34 ± 2	39 ± 3 *	54 ± 3 ***cI**
60 m	34 ± 3	33 ± 5	49 ± 1 ***cI**

Values are 'IU/g protein' and presented as means ± SEM. FI –denotes values from freshly isolated tissue. * -Significantly different from FI; $P<0.05$. **c,I** –Significantly different from control group (group 1) and group 2, respectively; $P<0.05$.

Group 1, no treatment; Group 2, treated with luminal Ringer's solution; Group 3, treated with luminal amino acid solution.

Table 5-3. Histological grades of intestinal injury upon reperfusion.

	Group	Grade	Median
	FI	0,0,0,0	0
0 min	**1**	5,6,6,7	6
	2	4,6,6,4	5
	3	2,3,2,4	$2.5^{c,b}$
30 min	**1**	6,7,7,6	6
	2	7,6,6,6	6
	3	5,2,3,5	$4^{c,b}$
60 min	**1**	6,7,7,6	6
	2	6,6,5,6	6
	3	4,6,5,5	5

FI denotes freshly isolated tissue. **c,b** - Significantly different compared to Group 1 and Group 2, respectively; $p<0.05$. Park's classification: 0 = normal mucosa; 1 = subepithelial space; 2 = extended subepithelial space; 3 = complete epithelial lifting; 4 = denuded villi; 5 = loss of villus tissue; 6 = crypt infarction; 7= transmucosal infarction; 8= transmural infarction.

Group 1, no treatment; Group 2, treated with luminal Ringer's solution; Group 3, treated with luminal amino acid solution.

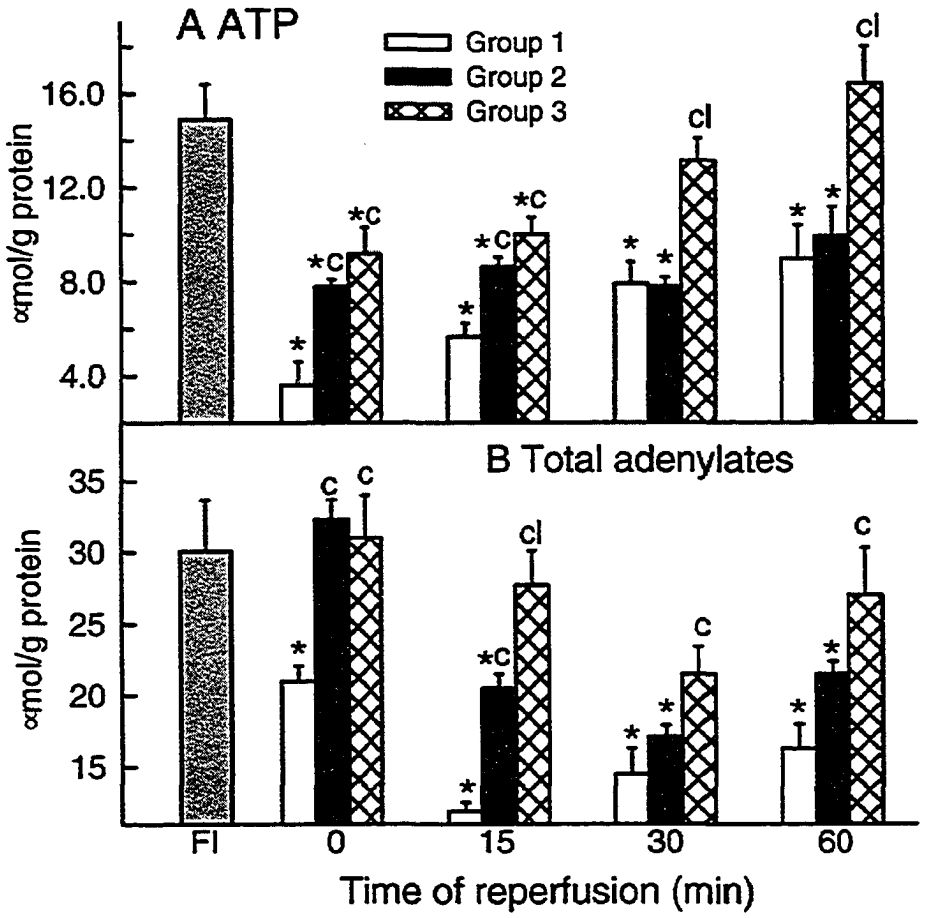

Figure 5-1: Effect of luminal treatment on levels of ATP and total adenylates during 60-min reperfusion. c,l: Significantly different compared with group 1 (control) and group 2, respectively; $p < 0.05$. *: Significantly different compared with freshly isolated (FI) tissue values; $p < 0.05$.

Group 1, no treatment; Group 2, treated with luminal Ringer's solution; Group 3, treated with luminal amino acid solution.

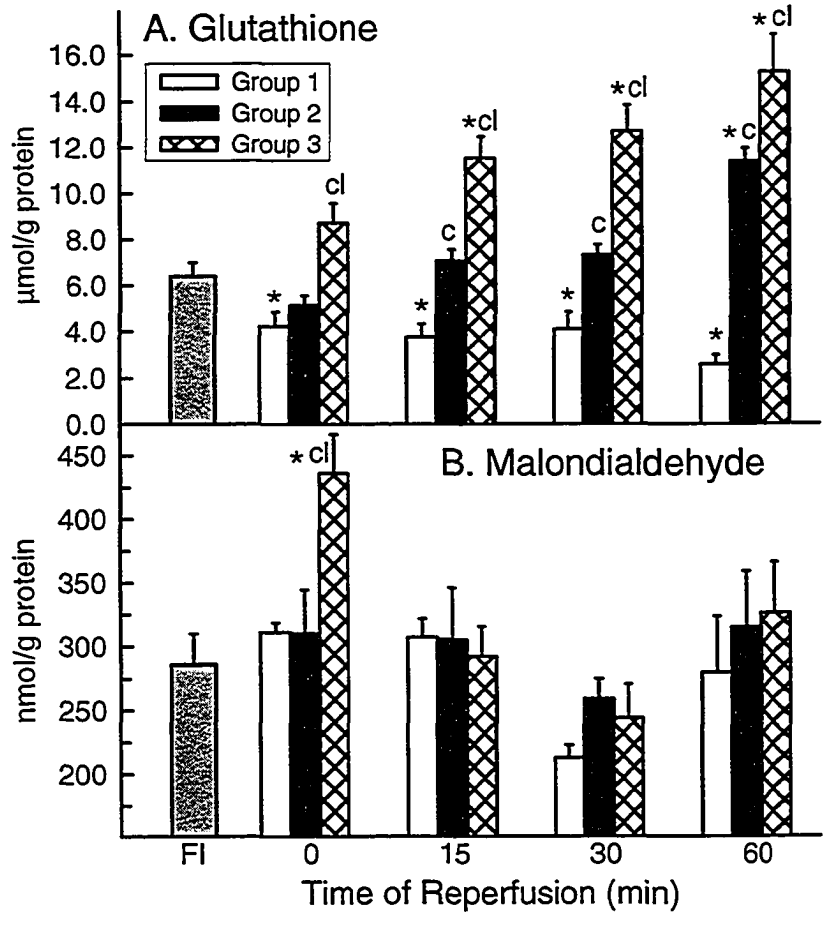

Figure 5-2: Effect of luminal treatment on levels of glutathione (GSH) and malondialdehyde (MDA) levels. c,l: Significantly different compared with group 1 (control) and group 2, respectively; $p < 0.05$. *: Significantly different compared with freshly isolated (FI) tissue values; $p < 0.05$.

Group 1, no treatment; Group 2, treated with luminal Ringer's solution; Group 3, treated with luminal amino acid solution.

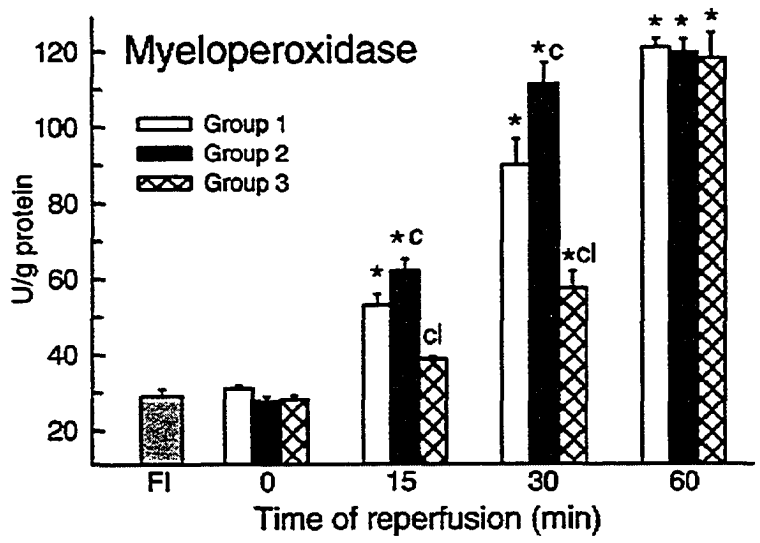

Figure 5-3: Effect of luminal treatment on myeloperoxidase (MPO) activity. c,I: Significantly different compared with group 1 (control) and group 2, respectively; $p < 0.05$. *: Significantly different compared with freshly isolated (FI) tissue values; $p < 0.05$.

Group 1, no treatment; Group 2, treated with luminal Ringer's solution; Group 3, treated with luminal amino acid solution.

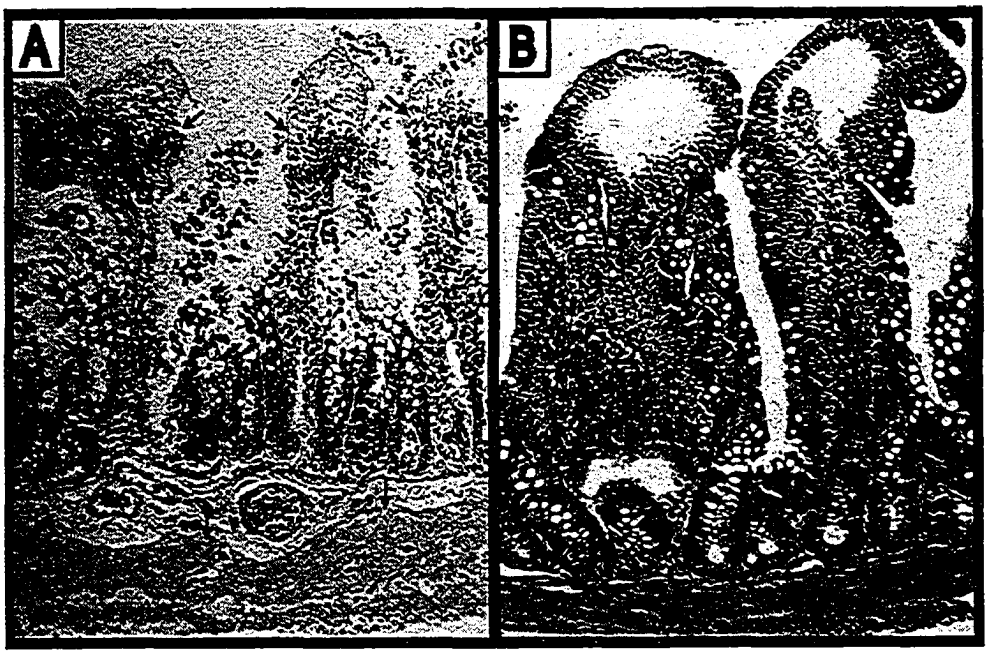

Figure 5-4: Histology after 60-min reperfusion. (A) Untreated control (Group 1); arrows denote regions of hemorrhage at villus tips and edema at the submucosal layer. (B) Amino acid (AA)-treated tissue (Group 3); moderate degree of clefting at the villus apex is evident, villi and mucosal architecture is otherwise representative of healthy tissue. 10×; hematoxylin & eosin staining.

Group 1, no treatment; Group 2, treated with luminal Ringer's solution; Group 3, treated with luminal amino acid solution.

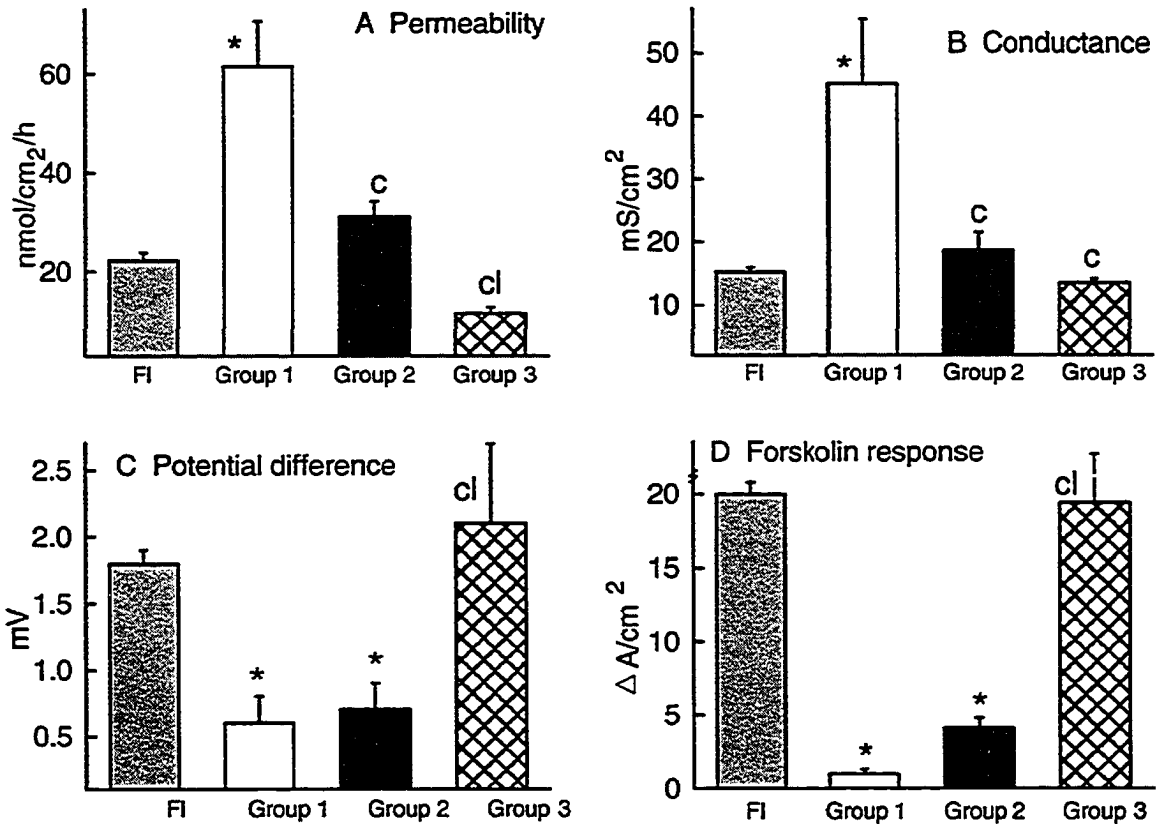

Figure 5-5: Effect of luminal treatment on (A) permeability (B) conductance (C) potential difference, and (D) short circuit current response to forskolin. c,l: Significantly different compared with group 1 (control) and group 2, respectively; $p < 0.05$. *: Significantly different compared with freshly isolated (FI) tissue values; $p < 0.05$.

Group 1, no treatment; Group 2, treated with luminal Ringer's solution; Group 3, treated with luminal amino acid solution.

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Chapter 6

Ameliorating small bowel injury using a cavitary two-layer preservation method with perfluorocarbon and a nutrient-rich solution*

Introduction

Small bowel transplantation (SBT) is the only treatment option for those patients with intestinal failure that can no longer be maintained on total parenteral nutrition (TPN) as a result of line infections and problems of venous access (1,2). Unfortunately, not only is the health of these patients compromised already, but success of SBT is currently nowhere near the success rates of the other commonly transplanted organs. Overall survival rates up to 1999 have remained low (<40% five-year survival) with complications of bacterial infection and allograft rejection accounting for 2/3 of patient mortality. Encouraging new data indicate that post-transplant survival is steadily improving. Intestinal Transplant Registry data (up to 2001-2002) indicate that 2.5 year graft survival is 52% & 66% for intestinal and multi-visceral transplants (3). Recently, even better graft survival has been reported at one center; 71% 3 year survival for isolated intestinal transplants (4). These improvements are due primarily to increased surgeon/center experience and better medical management (diagnosis of rejection & immunosuppression). Nevertheless, SB injury, whether due to preservation injury before transplantation or upon reperfusion after transplantation, may lead to a loss of intestinal barrier function and subsequent bacterial translocation with a dramatic increase in inflammation and the potential for life-threatening infection (5).

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* A version of this chapter has been published in *Am J Transplant* 2004; 4 (9): 1421-8 (Salehi P, Tsujimura, Walker J, Avila J, Madsen K, Lakey J, Kuroda Y, Churchill TA).

Kuroda et al. have demonstrated that the oxygenation of the heart and pancreas as well as small bowel by the cavitary two-layer method involving perfluorochemical and UW solution allows more ATP production and makes it possible to prolong the preservation time (6-8). PFC is an oxygen carrier designed to release oxygen into the surrounding tissue more effectively. The two-layer method supplies sufficient oxygen continuously to the graft and allows ATP production in the graft using both endogenous substrates and exogenous adenosine during the storage (9,10). The use of continuously oxygenated perfluorocarbons, known for their high solubility coefficients for oxygen, in a two-layer culture with standard University of Wisconsin preservation media, has extended the acceptable range of cold ischemia time.

Over the past few years, our lab has developed a novel amino acid-rich solution which is specifically tailored to the metabolic requirements of SB. We have clearly shown a superior preservation of mucosal integrity and energetics via the strategy of intraluminal administration of the nutrient-rich solution at the time of organ procurement/harvest (11-13). The purpose of this study was to investigate the possibility of improving the small bowel graft quality during storage by combining the strategy of both continuous oxygenation and nutrient supply through a cavitary two-layer method.

Methods

Male Sprague-Dawley rats (200-300 g) were obtained from the University of Alberta and used as bowel donors. All experiments were conducted in accordance with the regulations and policies of the Canadian Council on Animal Care. Chemical agents were AR-grade and were purchased from Sigma Chemical (Oakville, Canada). Perfluorodecalin was obtained from FluoroMed, LP (Texas).

Surgical procedure and procurement of the small intestine: Rats were fasted 10-12 h overnight in cages with raised floors to minimize coprophagy. Water was provided *ad libitum*. Prior to laparotomy, rats received an intraperitoneal dose of pentobarbital (65mg/250g; Somnotol; MTC Pharmaceuticals, Cambridge, Ontario, Canada), followed by inhalational halothane (0.5-2%) to maintain anesthesia. A midline laparotomy was performed and the aorta exposed infrarenally and at the level of the celiac trunk. The supraceliac aorta was then clamped and 10 ml of modified University of Wisconsin (UW) solution (equivalent to ~0.5 ml/g tissue) was administered via the infrarenal aorta. The suprahepatic vena cava was transected to facilitate the outflow of both blood and perfusate. The entire jejunum and ileum was subsequently harvested. Amino acid (AA) or UW solution (40 ml; equivalent to ~2.0 ml/g tissue) was then flushed through the lumen with effluent exiting the open end uninhibited. Once thoroughly flushed, each end was immediately ligated with 3-0 silk, leaving the bowel filled without turgor with each respective solution. The bowel was then submersed in 30-40 ml of perfluorodecalin, UW or amino acid solution and stored on ice in a 4°C incubator. Tissue samples (1-2 g) were taken at 0, 4, 12 and 24 hours post vascular flush. To arrest metabolic activity, samples were snap frozen in liquid nitrogen, and subsequently stored at –65°C until processed.

Experimental groups: All experimental groups (n=6) were flushed vascularly with UW solution and then treated luminally as below:

Group 1 – no luminal treatment [clinical control] then stored in UW solution;

Group 2 – UW solution then stored in PFC solution;

Group 3 – AA solution then stored in PFC solution;

Group 4 – AA solution then stored in AA solution.

Perfluorocarbon was pre-saturated with 100% oxygen prior to experimentation; in Group 4, AA solution in storage pot was pre-saturated with oxygen. For histology and Ussing chamber studies an additional group of freshly isolated (FI) bowel was sampled for comparative purposes.

Composition of Solutions: Modified UW solution contained: Lactobionic acid (100 mM), raffinose (39 mM), KOH (100mM), NaOH (15 mM), KH_2PO_4 (25 mM), $MgSO_4$ (5 mM), adenosine (5 mM), allopurinol (1mM), 5% dextran, pH, 7.4. AA solution contained: lactobionate (20 mM), adenosine (5 mM), allopurinol (1 mM), 5% dextran (67.3 kdal), BES [N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] (15 mM), glutamine (35 mM), glucose/glutamate/aspartate (20 mM each), arginine/glycine/valine/asparagine/lysine/ threonine/serine (10 mM each), methionine/ornithine/leucine/isoleucine/histidine/proline/ cysteine (5 mM each), hydroxybutyrate (3 mM), tyrosine/tryptophan (1 mM each), pH, 7.4.

Sample Preparation and Metabolite Assay: Frozen SB samples were weighed and then extracted 1:5 w/v in perchloric acid containing 1mM EDTA. The precipitated protein was removed by centrifugation (20 min at 20,000xg). Acid extracts were neutralized by the addition of 3 M KOH/ 0.4 M Tris/ 0.3M KCl and then recentrifuged (20min at 14,000xg). Aliquots of neutralized extracts were immediately processed via standard enzyme-linked metabolite assays (14). Spectrophotometric analysis was then performed to measure the absorbance of NADH at 340 nm, providing quantification of ATP, ADP, AMP, lactate, and ammonia. Values are reported as μmol per gram protein.

Malondialdehyde (MDA): Frozen tissue (100 mg) was homogenized 1:10 in phosphate-buffered saline. The homogenate was then processed and fluorescence was compared to standard amounts of MDA according to Ohkawa (15).

Glutathione assay: The level of glutathione in the tissue was determined as a measurement of protection against oxidative stress. Reduced glutathione (GSH) was fluorometrically quantitated in bowel tissue homogenates using the monochlorobimane assay (16). Briefly, monochlorobimane (100 µM final concentration) and glutathione S transferase (0.2 units) were added to 500 µl tissue homogenate and incubated for 30 min at 37°C in order to form a fluorescent adduct with GSH. Samples were read on a fluorescence plate reader [excitation 380 nm; emission 485 nm].

Histology: Full-thickness samples were fixed in a 10% buffered formalin solution, embedded in paraffin, cut 4 to 5-µm thick, and stained with hematoxylin and eosin. Histologic damage was assessed using Park's histologic classification of intestinal tissue injury (17).

Functional assessment – in Vitro electrical measurements: Ileal segments were taken after 12 h cold storage, stripped of their serosa and muscular layers. The mucosa was mounted in Ussing chambers exposing mucosal and submucosal surfaces to 10 mL of oxygenated Krebs buffer with an ionic composition of: Na^+, 143 mM; K^+, 5mM; Mg^{2+}, 1.1 mM; Ca^{2+}, 1.25 mM; HCO_3^-, 25 mM; Cl^-, 123.7 mM; HPO_4^-, 1.95 mM; and fructose 20 mM with 95% O_2 and 5% CO_2 and pH=7.35. Transmural intestinal short-circuit current (Isc) was then measured over a surface area of 0.9 cm^2. The spontaneous transepithelial potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate Isc with an automatic voltage clamp (DVC 1000 World Precision Instruments, New Haven, CT), except for 5-10

seconds every 5 minutes when PD was measured by removing the voltage clamp. Increase in Isc was induced by addition of the adenylate cyclase-activating agent, forskolin (10^{-5} mol/L), to the serosal surface. Epithelial responsiveness was defined as the maximal increase in Isc to occur within 5 minutes of exposure to the secretagogue. PD is expressed as millivolts (mV) and Isc as microamperes per square centimeter ($\mu A/cm^2$).

Mannitol permeability: An Ussing chamber was used to measure the permeability of mannitol in each ileal specimen. A quantity of 10 µCi of [^{3}H]mannitol (Dupont, NET101) was added to the mucosal side of the reservoir. After an equilibration period (20min), samples were taken from the mucosal and serosal sides. Ten and 20 min after equilibration, samples were again taken from both mucosal and serosal sides, and assessed for tritiated mannitol radioactivity in a scintillation counter to determine mannitol flux across the tissue (18).

Statistical analysis: Metabolite data were reported as 'µmol/g protein' and presented as means ± SE. Statistical differences between groups were determined using analysis of variance, followed by Student-Newman-Keuls' (SNK) *post hoc* comparison test; $p<0.05$ was reported. Histology scores were compared using the Kruskal-Wallis test; $p<0.05$ was reported.

Results

Energetics

Adenosine triphosphate (Figure 6-1). ATP levels in all experimental groups (Groups 2-4) were consistently greater than control (Group 1) throughout 24 h storage. Of particular clinical relevance, after 12 h, Groups 3 and 4 showed significantly higher ATP values compared to Group 2 (7.4 and 7.5 µmol/g in Groups 3 and 4, respectively, vs 3.7 µmol/g

in Group 2, $p<0.05$). After 24 h, final ATP levels in Groups 3 and 4 were 3-fold greater than Group 2 (4.2 and 4.8 vs 1.4 µmol/g, $p<0.05$).

ATP/ADP Ratio (Figure 6-1). Group 2 values were consistently greater than Group 1 over the first 12 h storage ($P<0.05$). However, after 12 h Groups 3 and 4 exhibited ratios that were considerably greater than Groups 1 & 2; values were 0.51-0.66 in Groups 3 and 4 compared to 0.03-0.20 in Groups 1 and 2 ($p<0.05$).

Energy Charge (Figure 6-1). Energy charge (EC = [ATP+ADP/2]/Total Adenylates) values in Groups 2-4 were markedly higher than control (Group 1) at all time points throughout 24 h. After 12 h, EC level was higher in Group 3 compared with Groups 2 and 4 (0.63 vs 0.51 and 0.53, $p<0.05$).

End products

Lactate (Figure 6-2). Anaerobic metabolism was assessed by lactate accumulation during the 24 h experimental time course. After 12 h and 24 h, levels were significantly higher in Groups 2-4 than in Group 1. Notably, levels were 35% greater in Group 4 than Group 3 after 12 h storage (85 vs 63 µmol/g, $p<0.05$).

Ammonia (Figure 6-2). After 12 h storage, only Group 4 exhibited a significant accumulation of this by-product of amino acid metabolism ($P<0.05$). After 24 h, all experimental groups showed relatively high ammonia values with no significant difference.

Antioxidant and lipid peroxidation levels

Glutathione (Figure 6-3). Glutathione (reduced form; GSH) levels in control group dropped significantly to 16-49 % within 4 h storage. During the first 12 h storage, Groups 2-4 exhibited significantly greater GSH than control. Only Group 4 levels remained

elevated throughout the entire 24 h period; levels were significantly greater than Groups 1-3 after 24 h (75 nmol/g in Group 4 vs 47-49 nmol/g in Groups 1-3; $P<0.05$).

Malondialdehyde (Figure 6-3). In Group 1, levels of this lipid peroxidation by-product increased significantly within 4 h and remained high throughout 24 h storage. Most notably, after 12 h, only the two groups treated with AA solution (Groups 3 and 4) exhibited significant increases over Group 1 ($P<0.05$). Interestingly, by 24 h, MDA levels in Group 4 had dropped to 63 % (all other groups ranged from 122 to 145 %).

Mucosal permeability and Electrophysiology

Mannitol flux (Figure 6-4A). Permeability of the mucosal barrier to mannitol in Group 1 (control) was more than 3 fold greater than in freshly isolated tissues (67 vs 22 nmol/cm^2/h, $p<0.05$). Barrier function of the mucosa in Groups 2-4 was equivalent to that of freshly isolated tissue even after 12 h cold storage.

Short circuit current (Figure 6-4B). Isc levels in all groups were significantly lower than freshly isolated values following 12 h storage. Groups 3 and 4 exhibited the highest values of Isc among the experimental groups; the values were > 3 fold greater than control group (14.1 and 18.3 in Groups 3 & 4 vs 3.8 μA/cm^2 in Group 1; $p<0.05$).

Potential difference (Figure 6-4C). Following 12 h cold storage, Groups 1 and 2 tissues exhibited PD values less than 0.5 mV, indicative of severely injured tissue. PD levels in Groups 3 & 4 were markedly higher than Groups 1 & 2 (not significantly different from freshly isolated tissue), indicating a maintenance of ion support processes across the mucosal membrane.

Forskolin response (Figure 6-4D). As mucosal barrier and ion pump function deteriorates, the ability of the membrane to generate a substantial current in response to forskolin also declines. After 12 h, Isc response to forskolin was lower in all experimental

groups compared to freshly isolated tissue. In sharp contrast, Isc changes in response to forskolin in Groups 3 & 4 were significantly greater than that of Groups 1 & 2 (11.8 & 13.9 vs 0.0 & 2.3 $\Delta\mu A/cm^2$, $p<0.05$).

Histology

Histologic changes throughout the cold ischemia period are summarized in Table 6-1. After 12 h, histologic integrity of mucosal layer was markedly superior in Groups 2, 3 and 4; median grades were 1.5, 1, 1.5 respectively. Specimens in Group 1 (control) exhibited various levels of injury including villus denudation, crypt layer infarction and even transmural infarction (median grade = 5). Following 24 h cold storage, both Groups 1 & 2 exhibited transmucosal and transmural infarction; median grade for both groups was 8. Despite a median grade of 5 for Group 3 after 24 h, 50% of specimens exhibited crypt and transmucosal infarction (grade 7) whereas the remaining 50 % had relatively minor epithelial clefting with intact cryptal regions. Group 4 showed superior maintenance of villus and mucosal morphology with 100% of the specimens incurring only minor degrees of epithelial clefting (median grade = 2).

Discussion

During clinical organ procurement, the blood supply and hence oxygen is cut off from the organ. A common arterial flush as part of multi-viscera procurement is used to clear the blood from the vasculature and cool the organ to near-zero temperatures (19). As residual oxygen levels decline, metabolism of the stored tissue shifts from the oxidative phosphorylation to a much less efficient anaerobic means of producing ATP (20). Since energy-utilizing processes are still in operation, although at significantly reduced enzymatic rates due to thermodynamic considerations, energy-utilizing processes remain in operation and as a result energy levels rapidly decline within the

tissue. At a metabolic level, energy is required to maintain intact and functional mitochondria, the site of oxidative phosphorylation. Consequently, the deleterious effects of cold storage encompass the impairment and degeneration of mitochondria; upon reperfusion this can translate to poor recovery of the tissue (21). In terms of maintaining mucosal integrity, barrier function is governed by energy-dependent tight junction regions at the apical surface of intestinal epithelial cells (22). Depleting cellular energy reserves results in an inability of epithelial cells to maintain this barrier. With prolonged the periods of hypoxia experienced during small bowel storage, functional and structural integrity rapidly decline with time, eventually leading to increases in trans-epithelial flux of potentially noxious macromolecules and enteric bacteria (22,23). Clinically, the failure to maintain an intact and functional mucosal barrier leads to an increased risk of septic complications in the recipient (24). A previous study, investigating the benefits of a two-layer method of perfluorocarbon preservation supports this positive correlation between cellular energetics and graft quality; bowel with superior energetic profiles resulted in superior transplant outcomes (25). As a direct result of the intimate relationship between energetics and mucosal integrity, improvements in energy metabolism directly translate to improvements in intestinal permeability and electrophysiology.

A fundamental problem with the storage of small bowel for transplantation is the preservation of mucosal structure and function during periods of low oxygen. Luminal flushing with a simple crystalloid solution has been shown to improve the quality of the mucosal layer over varying periods of ischemia (12,13,26). Most likely, this occurs as a result of dilution of toxic enteric contents, containing feces, bacterial endotoxins and biliary/pancreatic secretions. Supplementation of the luminal solution with essential nutrients, such as amino acids, further increases the benefit of luminal flushing (11,12).

The nutrient-rich preservation solution employed in this study has been specifically tailored to the physiological amino acid requirements of the SB (11-13). The composition of amino acids in the solution cater to both metabolic (energy production) and synthetic (synthesis of critical molecules) aspects of intestinal metabolism (27-31). In this study, there was a definite advantage of luminal UW solution in combination with PFC over control specimens (flushed vascularly with UW solution). All energetic parameters assessed, ATP, ATP/ADP and energy charge indicated a reduction in the degeneration of metabolic status. Previous studies have documented a significant positive effect on tissue viability following a cavitary two-layer method of storage (6-8). Hence, the maintenance of mucosal permeability after 12 h storage in this group, despite poorer electrophysiology parameters, was not unexpected; improved histology with no specimens exhibiting crypt or mucosal infarction following 12 h supported these findings. This beneficial effect was further amplified by incorporating an intraluminal solution rich in nutrients. The amino acid-rich (AA) solution clearly surpassed the UW solution in terms of promoting aerobic ATP generation; this effect was particularly pronounced after 12-24 h storage. Permeability and electrophysiology of the stored mucosal samples paralleled tissue energetic status. Even after 12 h storage, the maintenance of energy levels at near initial values translated to markedly superior preservation of mucosal electrophysiology and a well-maintained barrier. The ability to maintain a spontaneous transepithelial potential difference is a characteristic shared by all transporting epithelia and is dependent on electrogenic ion pumps in the epithelial cell membranes, mainly the Na^+/K^+ pump, and on the epithelial barrier (32). Moreover, well-maintained epithelial function of AA-treated tissues was confirmed by a sustained ability of the mucosa to respond to external stimuli; alterations in net ion flux (Isc) were substantially greater than

UW treated tissues. Interestingly, between the two AA-treated groups, positive effects on tissue energetics and mucosal permeability/electrophysiology were independent of the external oxygen-saturated solution in which the organs were immersed. Gross morphologic examination of AA-treated specimens revealed a superior preservation of the mucosa following 12 h; an intact epithelial layer was maintained with only minor disturbances in overall villus architecture. Interestingly, after 24 h storage, 100% of samples exhibited varying degrees of epithelial clefting leaving the underlying lamina propria and cryptal regions intact. Although, there is a substantial cost investment in using commercially available perfluorocarbons as oxygen carriers (~$1000 US/liter), the reduced metabolic demand facilitated by low temperature may permit the use of an aqueous solution for the delivery of adequate oxygen. Not only would this simplify the procedure, but any incumbent injury arising from physical manipulation may be avoided; damage to the tissue may result from forcible submersion in dense perfluorocarbon. Issues of 'scaling up' and diffusional limitations may become apparent in the storage of porcine/canine bowel that may highlight areas of concern prior to clinical application.

Although oxygen is a necessary substrate for the promotion of normal intestinal metabolism, there is a concomitant generation of oxygen free radicals (OFRs) during aerobic respiration that must be dealt with as a basal metabolic function (21). A primary source of OFR's during aerobic metabolism is related to the electron transport chain (ETC). As much as 2-4% of oxygen metabolized captures single electrons that leak out of the mitochondria during complex III activity and result in the generation of superoxide radicals (21). Consequently, as the activity of the ETS increases due to the presence of carbon substrate and oxygen, so does the amount of electron-capturing oxygen and hence, resultant OFR levels. Cytotoxicity of these oxygen metabolites [eg. superoxide

radical, hydroxide radical, hydrogen peroxide] manifests itself by inactivating enzymes, membrane transport proteins, damaging DNA and lysis of cell membranes due to lipid peroxidation (33,34). The result is compromise of cellular compartments and membranes which further potentiates cellular dysfunction and eventually, death. Under normal conditions (ie. fully oxygenated and normal temperatures), endogenous anti-oxidant mechanisms are able to neutralize the potential deleterious effects of OFRs. Malondialdehyde (MDA) is a by-product of lipid peroxidation and can further react with cellular proteins and lipids, thereby damaging the cell at multiple levels (35). Hence, MDA levels are often used as a quantitative index of OFR generation. Interestingly, after 12 h storage in tissues treated with AA solution, MDA levels were elevated despite apparently sufficient quantities of reduced glutathione, one of the single most important cellular antioxidant mechanisms essential for normal cell proliferation and function (31). It was only after more time had elapsed (after 24 h) that this enzyme-dependant mechanism was able to reduce levels of this peroxidation by-product. This observation is important since the induction of apoptosis has been found to be particularly responsive to elevated OFR levels (36). These results suggest that future formulations of a nutrient-rich preservation solution may benefit from administration of a chemical anti-oxidant in order to alleviate some of the potential injury incurred during the first 12 h storage; antioxidant mechanisms related to enzymatic processes (ie. superoxide dismutase and catalase) may prove ineffective during hypothermic storage.

The use of oxygen insufflation and continuous vascular/luminal perfusion as techniques to deliver oxygen to the tissue remains controversial because of issues of complexity and risk of mechanical injury to the lumen and vascular bed (11,26,37,38). Never the less, the requirement for oxygen delivery is one of the most important factors

in maintaining tissue viability. PFCs are unique in that they possess an inherent high gas solubility thus enabling the simultaneous delivery of oxygen and removal of carbon dioxide (39,40). Of particular interest to this study, perfluorodecalin (the PFC used in this experiment) dissolves 14.4 times as much oxygen as aqueous solutions (39,41). Oxygen solubility is 49 ml of oxygen per 100 ml of perfluorodecalin; aqueous solutions in 100% oxygen atmosphere contain no more than 3.4 ml O_2 per 100 ml (42). The advantage of perfluorochemicals is the ability of the solution to directly transfer oxygen by direct diffusion while not being influenced by changes in environmental temperatures and/or pH. The only potential drawback (other than cost) of PFCs is their relative immiscible nature and the high specific gravity (1.93 g/ml) which may present obstacles of diffusional barriers in large tissues (some regions of an organ may be exposed to PFC while others are not) and physical injury due to forcing a delicate tissue under the surface of a dense immiscible liquid (42). Despite marked differences in oxygen solubility of PFC and aqueous solutions, energy metabolism and its resultant effect on mucosal barrier and electrophysiology appear to be equivalent in our small animal model of organ preservation. However, in order to apply these findings in a meaningful way to the clinical arena, testing in a small animal transplant study is required to determine graft recovery and long-term viability following reperfusion. Furthermore, we recommend that future studies investigating the use of perfluorocarbons also examine the possibility that an oxygen-saturated aqueous solution may contain adequate oxygen for maintaining tissue/organ viability at hypothermic temperatures. The use of aqueous solutions for oxygen delivery would represent a significant reduction in overall cost and would avoid adding unnecessary complexity to the preservation technique employed.

Table 6-1. Histological grades of intestinal injury following 12 h and 24 h cold storage in all experimental groups. Group 1, no luminal treatment then stored in UW solution; Group 2, luminal UW solution then stored in PFC solution; Group 3 – luminal AA solution then stored in PFC solution; Group 4, luminal AA solution then stored in AA solution.

	Group	Grade	Median
	FI	0,0,0,0	0
12 h	**1**	1,6,8,4	5
	2	5,1,0,2	1.5
	3	0,0,2,2	1
	4	0,1,2,2	1.5
24 h	**1**	8,8,8,8	8
	2	8,8,8,8	8
	3	7,3,7,3	5
	4	3,0,3,1	2

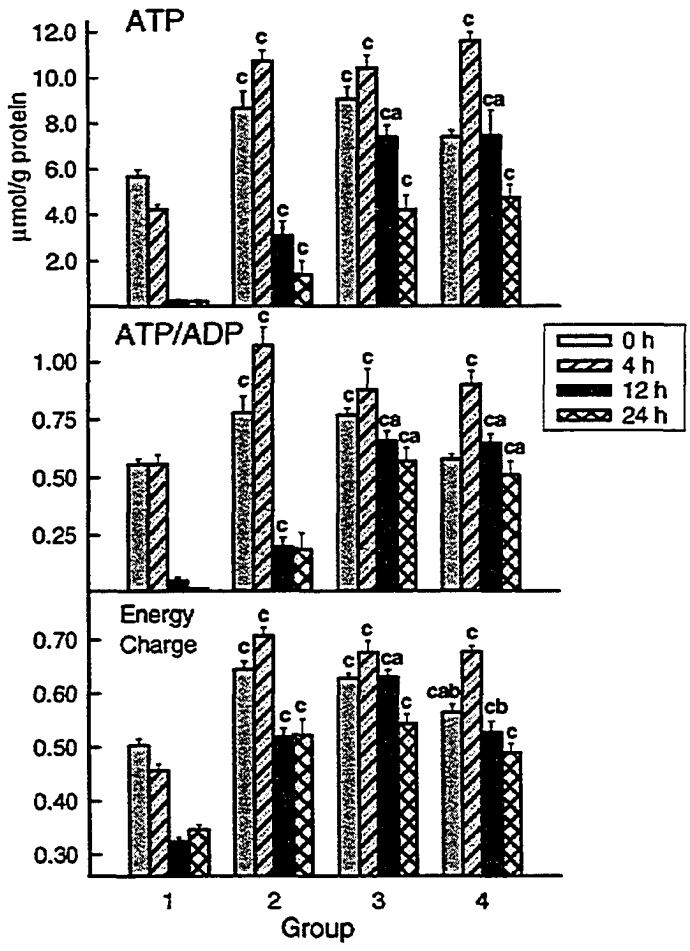

Figure 6-1. Effect of two-layer storage on levels of ATP, ATP/ADP and energy charge ratios. ***c,a,b*** -Significantly different compared to group 1 (control), group 2, group 3, respectively; $P<0.05$.

Group 1, no luminal treatment then stored in UW solution; Group 2, luminal UW solution then stored in PFC solution; Group 3 – luminal AA solution then stored in PFC solution; Group 4, luminal AA solution then stored in AA solution.

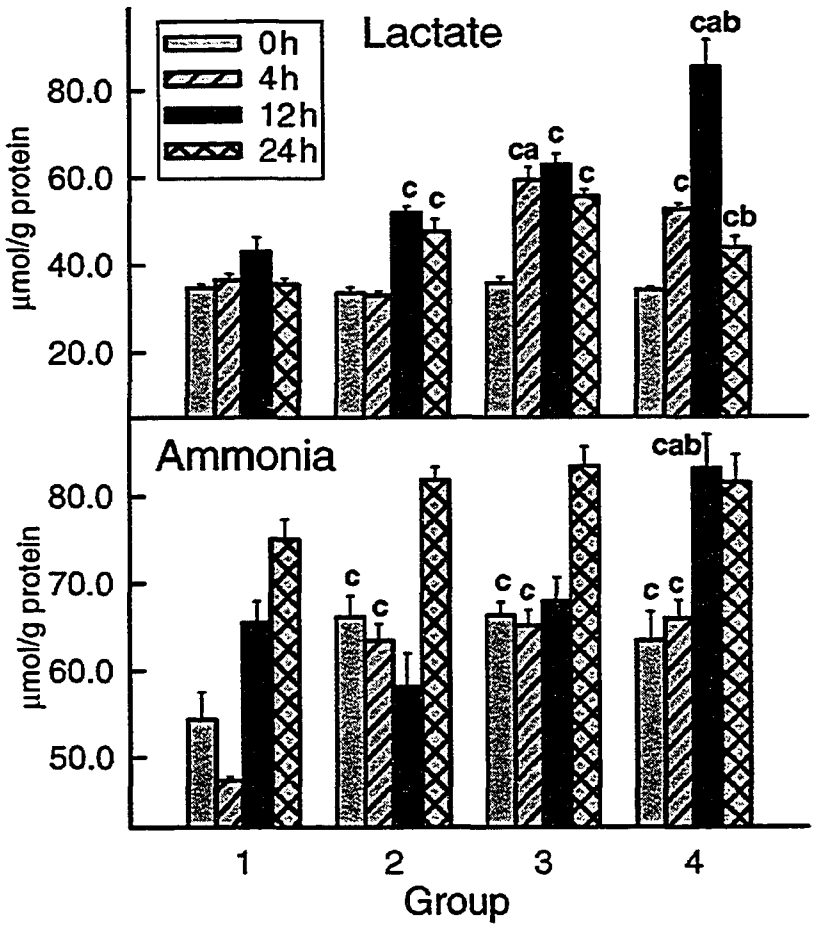

Figure 6-2. Effect of two-layer storage on levels of lactate and ammonia. ***c,a,b*** - Significantly different compared to group 1 (control), group 2, group 3, respectively; $P<0.05$.

Group 1, no luminal treatment then stored in UW solution; Group 2, luminal UW solution then stored in PFC solution; Group 3 – luminal AA solution then stored in PFC solution; Group 4, luminal AA solution then stored in AA solution.

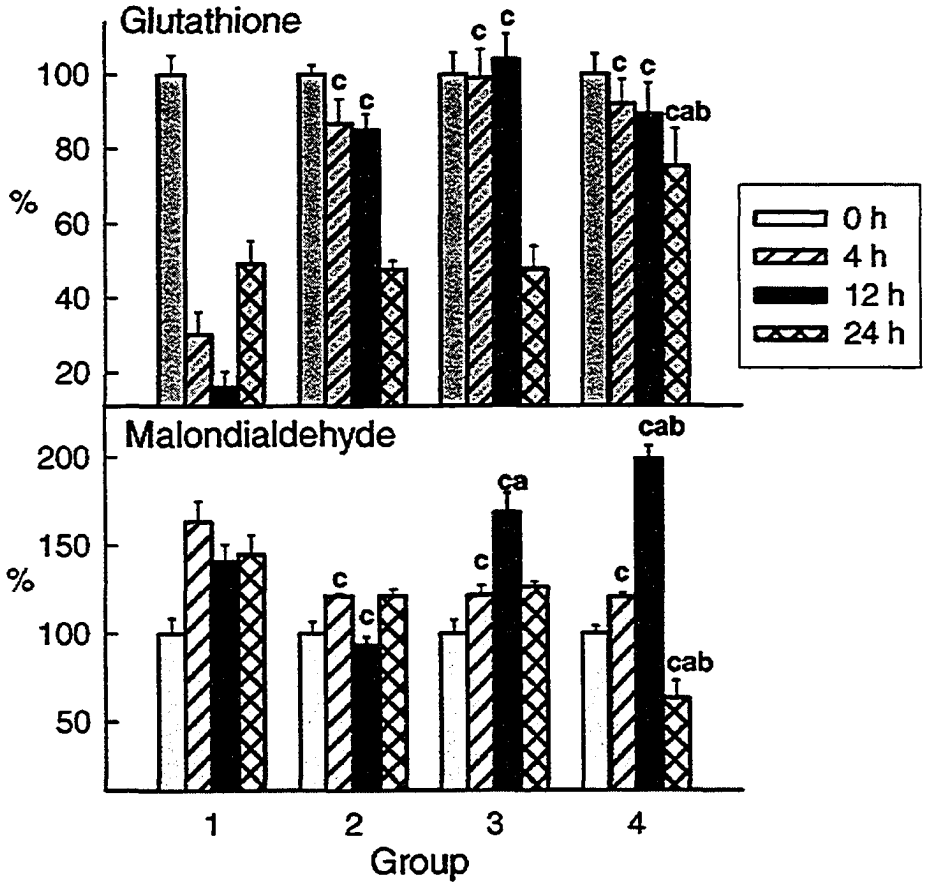

Figure 6-3. Effect of two-layer storage on levels of glutathione and malondialdehyde. ***c,a,b*** -Significantly different compared to group 1 (control), group 2, group 3, respectively; $P<0.05$.

Group 1, no luminal treatment then stored in UW solution; Group 2, luminal UW solution then stored in PFC solution; Group 3 – luminal AA solution then stored in PFC solution; Group 4, luminal AA solution then stored in AA solution.

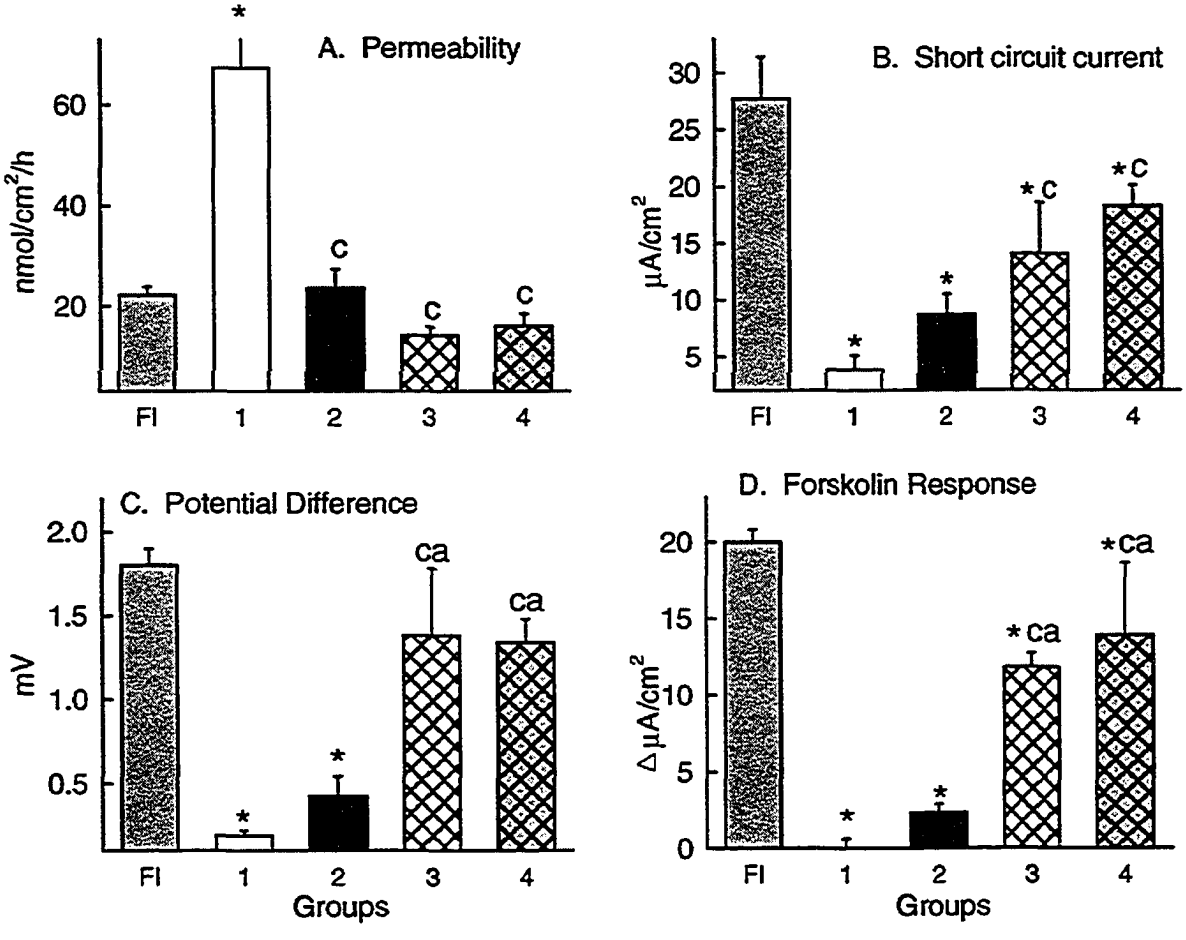

Figure 6-4. Effect of two-layer storage on (A) mannitol permeability, (B) short circuit current, (C) potential difference, and (D) short circuit current response to forskolin stimulation. ***c,a,b*** -Significantly different compared to group 1 (control), group 2, group 3, respectively; $P<0.05$.

Group 1, no luminal treatment then stored in UW solution; Group 2, luminal UW solution then stored in PFC solution; Group 3 – luminal AA solution then stored in PFC solution; Group 4, luminal AA solution then stored in AA solution.

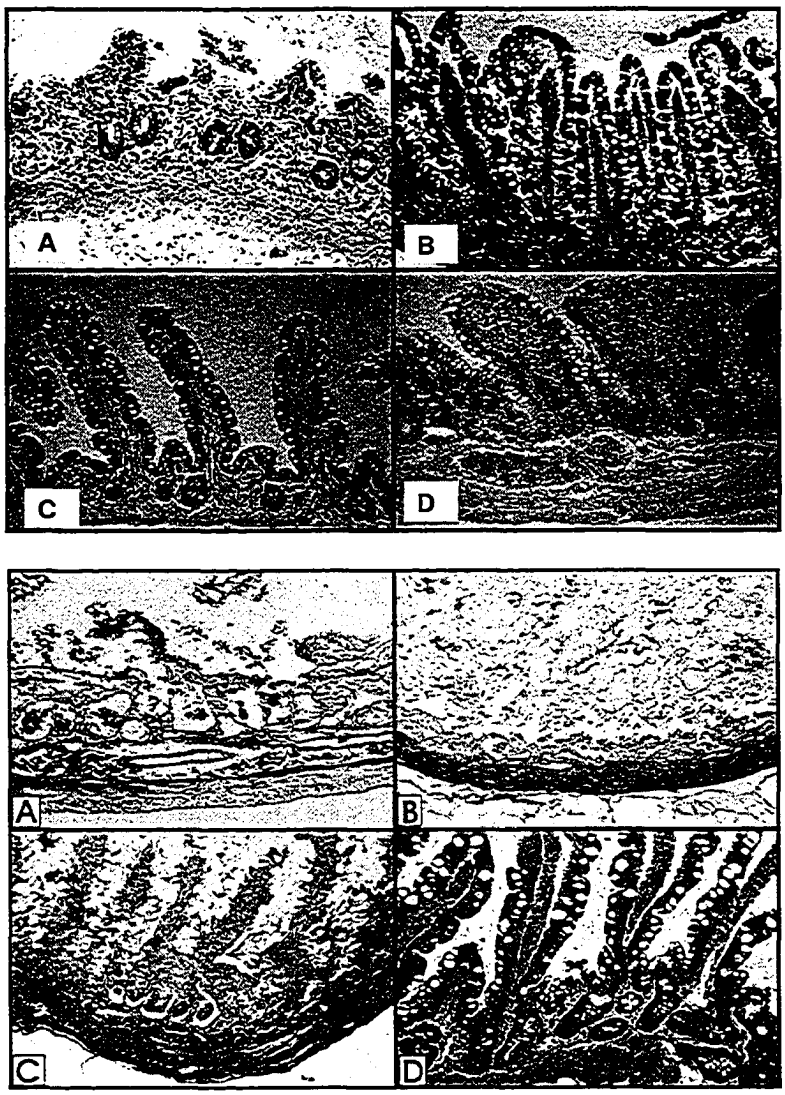

Figure 6-5. Most severe grade of histologic injury following 12 h (upper panel) and 24 h (lower panel) cold storage. A) untreated control (Group 1); B) Group 2, luminal UW solution then stored in PFC solution; C) Group 3, luminal AA solution then stored in PFC solution; D) Group 4, luminal AA solution then stored in AA solution.

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Chapter 7

Preserving the mucosal barrier via nutrient and antioxidant treatment during small bowel storage*

Introduction

Small bowel transplantation is a life-saving procedure for patients with intestinal failure who have developed severe complications or have otherwise failed with parenteral nutrition (1,2). It has been successfully carried out in various animal models, yet clinically, there are still many obstacles to improve patient and graft survival. Sepsis is one of the major causes of morbidity and mortality after intestinal transplantation and it may occur as a result of loss of intestinal barrier function and subsequent bacterial translocation due to poor organ preservation (3,4). While recent advances in solid organ preservation (i.e. development of University of Wisconsin (UW) solution) allows prolonged hypothermic ischemia in renal and liver transplantation, small bowel preservation, even for a short time, compromises graft function dramatically (5).

During ischemia, catabolism of ATP eventually results in increased hypoxanthine. This leads to a xanthine oxidase-mediated burst of superoxide molecules (6,7,). Increased production of superoxide radicals leads to the generation of hydrogen peroxide which can further produce cellular damage. These oxygen free radicals may also interact in the presence of transition metal catalysts to form the highly toxic hydroxyl radical and other oxidizing species and cause lipid peroxidation of cell membranes (8).

* A version of this chapter has been accepted for publication in *Surgery* 2005 (Salehi P, Walker W, Madsen K, Churchill TA).

This hypothesis has resulted in quite a number of studies suggesting a beneficial role for antioxidant therapy in reducing ischemia/reperfusion injury (9,10).

Over the past few years, our laboratory has developed a novel amino acid-rich preservation solution which is particularly tailored to the physiologic requirement of SB. We clearly have demonstrated the beneficial effects of intraluminal administration of nutrient-rich solution on mucosal integrity after intestinal cold ischemia (11-13). We hypothesized that one way to further reduce organ damage during cold storage was to prevent oxidative stress. In this study, we aimed at assessing the effects of nutrients (amino acids) and antioxidant supply (Trolox, a water-soluble analogue of vitamin E and superoxide sidmutase/catalase) during small bowel cold preservation.

Materials and Methods

Male Sprague-Dawley rats (200-300 g) were obtained from the University of Alberta and used as bowel donors. All experiments were conducted in accordance with the regulations and policies of the Canadian Council on Animal Care. Chemical agents were AR-grade and were purchased from Sigma Chemical (Oakville, Canada).

Surgical procedure and procurement of the small intestine: Rats were fasted 10-12 h overnight in cages with raised floors to minimize coprophagy. Water was provided *ad libitum*. Prior to laparotomy, rats received an intraperitoneal dose of pentobarbital (65mg/250g; Somnotol; MTC Pharmaceuticals, Cambridge, Ontario, Canada), followed by inhalational halothane (0.5-2%) to maintain anesthesia. A midline laparotomy was performed and the aorta exposed infrarenally and at the level of the celiac trunk. The supraceliac aorta was then clamped and 10 ml of modified University of Wisconsin (UW) solution (equivalent to ~0.5 ml/g tissue) was administered via the infrarenal aorta. The suprahepatic vena cava was transected to facilitate the outflow of both blood and

perfusate. The entire jejunum and ileum was subsequently harvested. In the groups treated luminally, UW or amino acid (AA) solution (20 ml; equivalent to ~1.0 ml/g tissue) was then placed into the lumen of the bowel allowing the effluent to exit uninhibited. Each end was immediately ligated with 3-0 silk, leaving the bowel filled without turgor with the same solution. The bowel was then stored in an additional 40 ml of each respective solution and stored on ice in a 4°C incubator. Tissue samples (1-2 g) were taken at 0, 4, 12 and 24 hours post vascular flush. To arrest metabolic activity, samples were snap frozen in liquid nitrogen, and subsequently stored at –65°C until processed.

Experimental groups: All experimental Groups (n = 6 in each) were flushed vascularly with UW solution and then treated luminally as follows:

Group 1: no luminal treatment (clinical control), then stored in UW solution;

Group 2: 1 h perfusion with AA solution, then static storage in AA solution;

Group 3: 1 h perfusion with AA solution + trolox, then static storage in AA solution containing trolox;

Group 4: 1 h perfusion with AA solution + SOD/catalase, then static storage in AA solution containing SOD/catalase.

In group 3 animals, trolox was also administered intraperitoneally (15 mg/kg, 1mM), just before induction of anesthesia. In group 4, SOD/catalase (30/300 KU/kg, 30/300 KU/L) was administered intravenously through penile vein.

Composition of Solutions: Modified UW solution contained: lactobionic acid (100 mM), raffinose (30 mM), KOH (100mM), NaOH (15 mM), KH_2PO_4 (25 mM), $MgSO_4$ (5 mM), adenosine (5 mM), allopurinol (1mM), dextran (67.3 kdal; 5%), pH 7.4. AA solution contained: lactobionate (20 mM), adenosine (5 mM), allopurinol (1 mM), dextran (67.3

kdal; 5%), BES [N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] (15 mM), glutamine (35 mM), glucose/glutamate/aspartate (20 mM each), arginine/glycine/valine/ asparagine/lysine/threonine/serine (10 mM each), methionine/ornithine/leucine/ isoleucine/histidine/proline/cysteine (5 mM each), tyrosine/ tryptophan (1 mM each), hydroxybutyrate (3 mM), Trolox (1 mM), 3-aminobenzamide (1 mM), pH 7.4.

Sample Preparation and Metabolite Assay: Frozen SB samples were weighed and then extracted 1:5 w/v in perchloric acid containing 1mM EDTA. The precipitated protein was removed by centrifugation (20 min at 20,000xg). Acid extracts were neutralized by the addition of 3 M KOH/ 0.4 M Tris/ 0.3M KCl and then recentrifuged (20min at 14,000xg). Aliquots of neutralized extracts were immediately processed via standard enzyme-linked metabolite assays (14). Spectrophotometric analysis was then performed to measure the absorbance of NADH at 340 nm, providing quantification of ATP, ADP, and AMP. Values are reported as µmol per gram protein. Protein was measured according to Lowry et al (15).

Malondialdehyde (MDA): Frozen tissue (100 mg) was homogenized 1:10 in phosphate-buffered saline. The homogenate was then processed and fluorescence was compared to standard amounts of MDA (1,1,3,3-tetra-ethoxypropane according to Ohkawa (16).

Glutathione (GSH): Frozen tissues (100 mg) were homogenized 1:5 w/v in 6% perchloric acid containing 1mM EDTA. Precipitated protein was removed by centrifugation (20 min, 14,000xg). Acid extracts were neutralized with 3 M KOH/0.3 M imidazole and recentrifuged (20 min, 14,000xg). Extracts were analyzed based on fluorescence and compared to standard amounts of reduced glutathione according to Fernandez-Checa and Kaplowitz (17).

Histology: Full-thickness biopsies were fixed in 10% buffered formalin solution, embedded in paraffin, cut to 5 μm, and stained with hematoxylin and eosin. Histologic damage was assessed using Park's histologic classification of intestinal tissue injury (18).

Functional assessment: in vitro electrical measurements: Ileal segments were taken after 12-h cold storage, stripped of their serosa and muscular layers. The mucosa was mounted in Ussing chambers exposing mucosal and submucosal surfaces to 10 mL of oxygenated Krebs' buffer with an ionic composition of: Na^+, 143 mM; K^+, 5 mM; Mg^{2+}, 1.1 mM; Ca^{2+}, 1.25 mM; HCO_3^-, 25 mM; Cl^-, 123.7 mM; HPO_4^-, 1.95 mM; and fructose 20 mM 95% O_2 and 5% CO_2 and pH = 7.35. Transmural intestinal short-circuit current (Isc) was then measured over a surface area of 0.9 cm^2. The spontaneous transepithelial potential difference (PD) was determined, and the tissue was clamped at zero voltage by continuously introducing an appropriate Isc with an automatic voltage clamp (DVC 1000 World Precision Instruments, New Haven, CT), except for 5-10 s every 5 min when PD was measured by removing the voltage clamp. Potential difference is expressed as millivolts (mV) and Isc as microamperes per square centimeter ($\mu A/cm^2$).

Mannitol permeability: An Ussing chamber was used to measure the permeability of mannitol in each ileal specimen. A quantity of 10 μCi of (^3H) mannitol (Dupont, NET101) was added to the mucosal side of the reservoir. After an equilibration period (20 min), samples were taken from the mucosal and serosal sides. Ten and 20 min after equilibration, samples were again taken from both the mucosal and serosal sides, and assessed for tritiated mannitol radioactivity in a scintillation counter to determine the mannitol flux across the tissue (19).

Statistical analysis: Metabolite data were reported as means ± SE for each group. Statistical differences between groups were determined using analysis of variance, followed by Tukey's Honest Significant Difference (HSD) *post hoc* comparison test; $p<0.05$ was reported. Histology scores were compared using the Kruskal-Wallis test; $p<0.05$ was reported.

Results

Energetics

ATP (figure 7-1a): After 4 h cold storage, ATP levels in amino acid treated tissues (groups 2-4) were approximately threefold greater than in control group ($p<0.01$). Within 12 h, ATP levels dropped by 40% in Group 2 and by 60% in Group 4; values in Group 3 remained unchanged from freshly isolated (FI) tissue throughout 12 h time period (12.8±1.3 in Group 3 vs 14.3±0.35 µmol/g in freshly isolated tissues). After 24 h, ATP levels in all experimental groups dropped to one-third of FI values.

ATP/ADP ratio (figure 7-1b): Freshly isolated tissues had an ATP/ADP ratio of 1.4 ± 0.17, significantly different from control group following 4 h ($p<0.05$). Within 4 h cold ischemia, all amino acid treated groups exhibited significantly higher ATP/ADP ratio compared to Group 1 ($p<0.05$). Interestingly, Group 3 exhibited a higher ATP/ADP values compare to other groups after 12 h; the ratio was 1.33±0.3 in Group 3 compared to <0.8 in Groups 1, 2 and 4 ($p<0.05$). Within 24 h, all amino acid treated group showed significantly higher ratios compared to control group ($p<0.05$).

Total adenylates (TA, figure 7-1c): After 4 h, total adenylate values (TA=ATP+ADP+AMP) dropped from a freshly isolated (FI) value of 28.6±1.6 µmol/g to 18.1±1.9 in Group 1; Groups 2 and 3 exhibited increases compared to FI and Group 4 remained unchanged. Within 12 h, elevated TA values were observed only in Group 3

compared to control group ($p<0.05$). However, by 24 h cold storage, Groups 3 and 4 were significantly higher than only Group 1 (21.7 and 20.5 vs 12.8 μmol/g, respectively, $p<0.05$).

Lipid peroxidation and antioxidative capacity

Malondialdehyde (MDA, figure 7-2a): The extent of lipid peroxidation was determined by measuring tissue concentration of malondialdehyde. Trolox therapy in group 3 resulted in significantly decreased MDA levels throughout the entire 24 h cold storage ($p<0.01$). MDA levels tended to decrease gradually in almost all amino acid treated tissues during 24 h. After 12 h, Groups 2 and 3 exhibited significantly lower MDA levels compared to control group ($p<0.05$). Within 24 h, Groups 3 and 4 exhibited lower MDA levels than Group 1 ($p<0.05$).

Glutathione (GSH, figure 7-2b): Tissue GSH levels tended to increase in Group 3 during 24 h cold ischemic period. Trolox treated group exhibited markedly higher GSH levels throughout the entire 24 h. Surprisingly, after 24 h, GSH level in Group 3 was more than twofold greater than the corresponding values in other groups (10.6 in Group 3 vs 3.6-4.7 in Groups 1,2 and 3, $p<0.05$).

Mucosal permeability and electrophysiology

Manitol flux (figure 7-3a): Permeability of the mucosal barrier to mannitol in Group 1 (control) was more than twofold greater than in other groups ($p < 0.05$). Barrier function of the mucosa in Groups 2-4 was approximately equivalent after 12-h cold storage.

Short circuit current (figure 7-3b): Group 3 exhibited the highest value of Isc among the experimental Groups; the value was threefold greater than in the Groups 1 and 2 (17.6 in Group 3 vs 5.6 $\mu A/cm^2$ in Group 1 and 2, $p<0.05$). Isc level in Group 4 was significantly higher than Groups 1 and 2 values following 12 h cold storage.

Potential difference (figure 7-3c): After 12 h cold storage, the tissues of control group exhibited PD values less than 0.2 mV, indicative of severely injured tissue. Potential difference levels in Groups 3 and 4 were markedly higher than in Groups 1 and 2 ($p<0.05$), indicating a maintenance of ion-support processes across the mucosal membrane.

Histology

Histological alterations of small bowel graft following 12 and 24 h cold ischemia is summarized in table 7-1. After 12 h, all amino acid treated groups showed superior maintenance of mucosal integrity compared to control group (median Park's grade 2 for Groups 2-4 vs grade 6 for Group 1, $p<0.05$). After 24 h cold storage, the dominant grade of injury in Group 1 was 8 (severe damage including transepithelial and transmural infarction). However, amino acid treated groups showed consistently less mucosal injury compared to controls ($p<0.05$); median grade was 3.5, 2 and 3 in groups 2, 3 and 4, respectively (figure 7-4). Trolox treated tissues exhibited minimal damage including subepithelial edema in villous apex.

Discussion

Low temperature storage of small bowel in amino acid solution is significantly effective in suppressing lipid peroxidation during ischemia. However, this study has shown that oxidative stress that occurs during cold ischemia can be suppressed more effectively by administration of trolox to donor and preservation solution.

The period between harvesting a donor organ and transplantation into a recipient involves static cold storage, a period during which oxygen supply is halted. Energy levels within the tissue are soon depleted during ischemic storage because many energy consuming processes are still in operation even at hypothermia; consequently there is a

rapid decline in ATP (20). Small bowel is extremely susceptible to even brief period of ischemia (21). The epithelial permeability is largely maintained by the tight junctions close to the apical surface of intestinal epithelial cells. Tight junction consists of a number of proteins which are dynamic, energy requiring structures. Depleting cellular energy reserves results in an inability of epithelial cells to maintain this barrier. With the prolonged periods of hypoxia experienced during small bowel storage, functional and structural integrity rapidly decline with time, eventually leading to increases in trans-epithelial flux of potentially noxious macromolecules and enteric bacteria (22,23).

As ATP is consumed, lower energy adenylate forms (ADP and AMP) are produced. However, purine catabolism is not a 'dead end' pathway and AMP can be sequentially converted to a variety of metabolites including hypoxanthine, xanthine, uric acid, and urea (for excretion). Restoration of ATP and ADP stores is easily accomplished from AMP (via adenylate kinase and oxidative phosphorylation), however, once AMP is catabolized, high energy adenylate synthesis is not a simple process and either involves the purine 'salvage' pathway or de novo purine synthesis. Both of these anabolic pathways require significant amounts of energy and multiple substrates.

Small intestine is one of the richest sources of xanthine dehydrogenase-oxidase enzyme system, which is required for the OFR production (24). During ischemia, xanthine dehydrogenase is converted to xanthine oxidase leading to increased oxidation of hypoxanthine and subsequently, oxygen free radical production (25). Free radicals are highly unstable, highly reactive molecules which form in ischemic tissue upon reexposure to oxygen; this is due in part to the partial reduction of molecular oxygen typically catalyzed via two enzymes: NADPH peroxidase and xanthine oxidase (26-28). Another major OFRs production pathway is directly related to electron transport system

activity. As much as 4% of oxygen metabolized captures single electrons that leak out of the mitochondria during complex III activity and result in the generation of superoxide radicals (29). Superoxide can also be generated through the activation of phospholipase A_2 by oxygen radicals and production of arachidonic acid derivatives from lipid peroxidation which can further produce superoxide and hydroxyl radicals as intermediates (30).

Cytotoxicity of these oxygen metabolites (e.g. superoxide radical, hydroxide radical, hydrogen peroxide) manifests itself by inactivating enzymes, membrane transport proteins, damaging DNA and lysis of cell membranes due to lipid peroxidation (31,32). The result is compromise of cellular compartments and membranes which potentiates cellular dysfunction and eventually, death (33,34). Lipid peroxidation of membrane can also cause disruption of tight junctions and increase in transcellular permeability leading to bacterial translocation and endotoxinemia (35).

We already have demonstrated that luminal administration of nutrient-rich solution has been successful in higher maintenance of tissue energetics and augmenting endogenous antioxidant levels following ischemic assault which positively correlates with superior maintenance of mucosal barrier function (36,37). Due to the huge impact of oxidative stress during I/R on mucosal damage, in the present study, we tried to further inhibit oxidative damage to mucosal barrier during the period of cold preservation.

Vitamin E (alpha tocopherol) is a lipid-soluble compound which is known as chain-breaking antioxidant which intercalates in lipid membrane and stop peroxidative cascade, it is also effective scavenger of hydroxyl radicals (38). So, even small amount of vitamin E can reduce oxidative stress. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a water-soluble analogue of vitamin E which has been reported to

have greater free radical scavenging capacity (39). A major problem with vitamin E is that due to poor aqueous solubility, it is difficult to administer acutely by infusion, however, trolox can be given acutely to isolated organs to prevent immediate oxidative stress.

Antioxidant enzymes including superoxide dismutase (SOD), catalase and glutathione peroxidase protect tissues from reperfusion injury by counteracting oxygen free radicals (40). SOD is an enzyme that eliminates free radicals by converting superoxide anions into hydrogen peroxide ($O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$), which can then be removed by catalase and glutathione peroxidase (41). It has been shown that catalase, an enzyme that catalyzes the reduction of hydrogen peroxide to oxygen and water, attenuates I/R injury in various organs (42,43). The beneficial effect of antioxidants has been extensively investigated in reperfusion models, although less attention has been paid to the alterations during cold storage. Since small bowel is rich in xanthine oxidase, we hypothesized that adding simple antioxidants to preservation solution might have implications to improve the quality of the graft. Our approach through administration of SOD/catalase was not very successful. This might be as a result of low half-life of SOD, poor cellular uptake of the enzyme and/or low enzymatic activity at hypothermia.

In the present study, our attempts to supply adequate nutrients and antioxidants were successful by means of brief luminal perfusion with amino acid solution in combination with trolox. In terms of energetics, amino acid treated tissues far exceeds that of UW treated ones. However, trolox treated group exhibited even higher levels of ATP, ATP/ADP ratio, and total adenylates. ATP-to-ADP ratio is typically used to reflect

the quality of mitochondrial oxidative phosphorylation and energy charge values reflect the proportion of adenylates that are available for cellular work.

The effect of cold ischemia on the extent of oxidative damage was assessed by determining tissue malondialdehyde and glutathione levels. These indices of cellular damage have been related with the changes in overall bowel tissue energetics. Polyunsaturated fatty acids in the cell membranes are susceptible to oxidative damage and are broken to peroxidation product, malondialdehyde. Our results indicate that lipid peroxidation can prevented effectively through the administration of Trolox during cold storage. Cellular pools of GSH represent an intracellular defense mechanism against active oxygen compounds. Regeneration of cellular glutathione content requires ATP-derived energy. In UW solution treated tissues reduced glutathione was not adequately regenerated due to lack of substrate (i.e. amino acids) and available ATP. In this group, lower energetics was associated with an increase in lipid peroxidation, and a reduction in the overall antioxidant capacity of the graft during cold ischemia. Trolox administration provided a higher protection than SOD/catalase against oxidative damage, as indicated by the lower generation of MDA and greater tissue glutathione levels during cold storage.

Ultrastructural investigations have revealed alterations in the microvilli of apical epithelial within 5 min after the onset of ischemia (44). After 30 min of warm ischemia, the upper two thirds of villi are completely denuded of epithelial cells, whereas the crypts remain relatively intact (45). However, prolonged ischemic insult progressively leads to crypt infarction and eventually to infarction of the submucosa and muscularis layers. The crypt cells play a vital role in the reepithelialization of denuded villi once issues of limited oxygen and substrate delivery have been resolved on reperfusion. After 12 h and 24 h

cold ischemia, UW treated tissues showed major damage including crypt infarction and transmural infarction, while amino acid treated tissues showed minor changes including edema and extended subepithelial space. Moreover, administration of trolox decreased considerably the mucosal damage following 12 and 24 h cold ischemia. The correlation found between low energetic states and free radical production and mucosal damage during cold ischemia suggests strongly that oxidative stress is involved in the pathogenesis of ischemic injury.

Finally, to assess graft function after 12 h cold storage, electrophysiological parameters were evaluated by Ussing chamber study. In UW treated tissues, cold ischemia resulted in a significant increase in mucosal permeability as assessed by manitol flux measurement. All amino acid treated tissues exhibited almost normal gut barrier. However, transepithelial ion gradient was significantly enhanced by administering trolox to nutrient-rich preservation solution, again confirming the superior maintenance of polarity and structural integrity in this experimental group.

In conclusion, several mechanisms seem to be responsible for the development of ischemic damage in rat small bowel following cold preservation. The results of this study demonstrate that preservation of small bowel in the novel amino acid-rich solution with low doses of vitamin E analogue, trolox significantly increases the graft energetics and function and reduces peroxidative damage during cold storage leading to a greater graft quality. This effect was more pronounced than that observed when superoxidase dismutase/catalase was added. This combined strategy may have implications for the successful preservation and transplantation of SB in the clinic.

Table 7-1. Histological grades of intestinal damage following 12 h and 24 h cold storage.

	Group	Grade	Median
	FI	0,0,0,0,0,0	0
12 h	1	4,5,7,8	6
	2	3,2,2,2,3,2	2[c]
	3	2,2,3,2,2,2	2[c]
	4	2,3,2,2,3,2	2[c]
24 h	1	5,6,8,8	7
	2	4,3,3,5,3,4	3.5[c]
	3	2,2,3,3,2,2	2[ca]
	4	2,3,3,2,3,3	3[c]

FI denotes freshly isolated tissue. All values after 12 or 24 h were significantly greater than FI ($p<0.05$). **c** and **a** - Significantly different compared to Groups 1 and 2, respectively, $p<0.05$.

Park's classification: 0 = normal mucosa; 1 = subepithelial space; 2 = extended subepithelial space; 3 = complete epithelial lifting; 4 = denuded villi; 5 = loss of villus tissue; 6 = crypt infarction; 7= transmucosal infarction; 8= transmural infarction.

Group 1, no luminal treatment then stored in UW solution; Group 2, 1 h perfusion with AA solution, then static storage in AA solution; Group 3, 1 h perfusion with AA solution + trolox, then static storage in AA solution containing trolox; Group 4, 1 h perfusion with AA solution + SOD/catalase, then static storage in AA solution containing SOD/catalase.

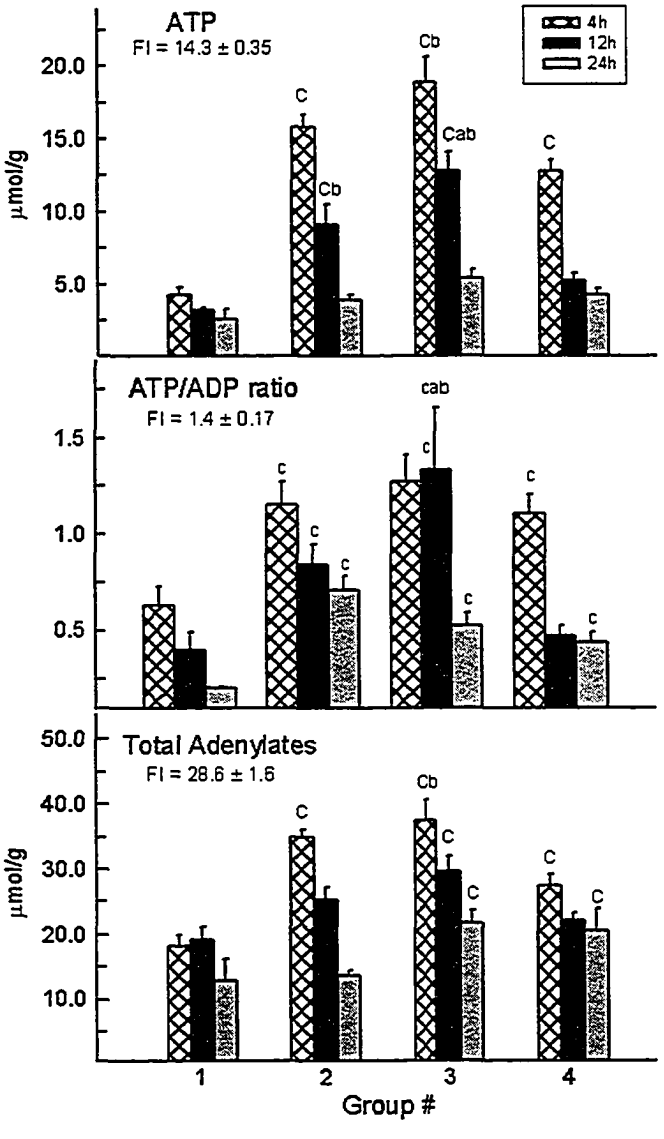

Figure 7-1. Effect of different preservation techniques on levels of ATP, ATP/ADP ratio and total adenylates during 24 h cold ischemia. **c**, **a**, and **b**, significantly different compared with group 1 (control), group 2, and group 4, respectively, $p<0.05$.

Group 1, no luminal treatment then stored in UW solution; Group 2, 1 h perfusion with AA solution, then static storage in AA solution; Group 3, 1 h perfusion with AA solution + trolox, then static storage in AA solution containing trolox; Group 4, 1 h perfusion with AA solution + SOD/catalase, then static storage in AA solution containing SOD/catalase.

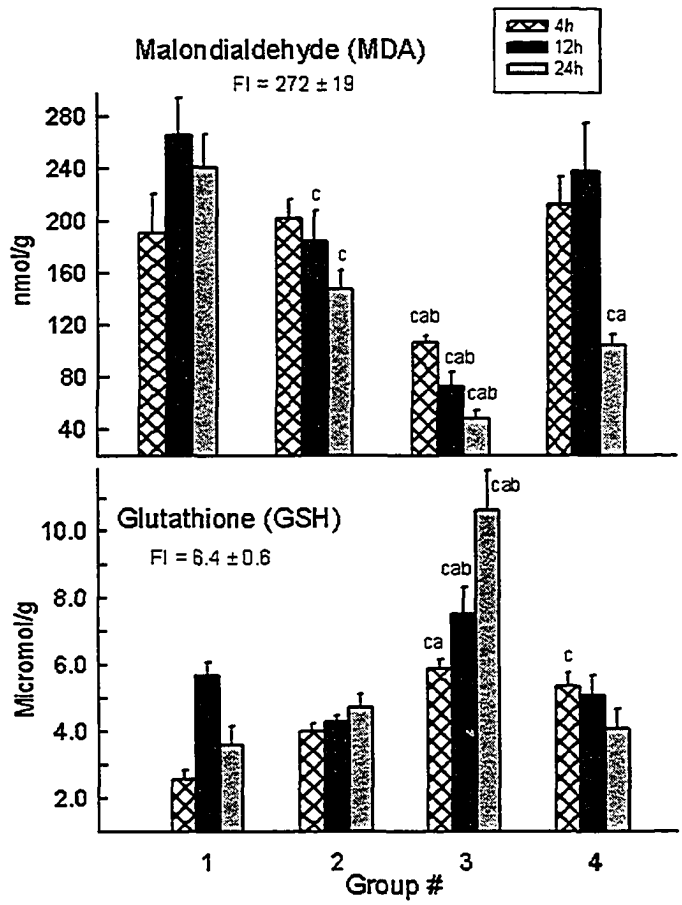

Figure 7-2. Effect of different preservation techniques on tissue levels of malondialdehyde (MDA) and glutathione (GSH) during 24 h cold ischemia. **c**, **a**, and **b**, significantly different compared with group 1 (control), group 2, and group 4, respectively, $p<0.05$.

Group 1, no luminal treatment then stored in UW solution; Group 2, 1 h perfusion with AA solution, then static storage in AA solution; Group 3, 1 h perfusion with AA solution + trolox, then static storage in AA solution containing trolox; Group 4, 1 h perfusion with AA solution + SOD/catalase, then static storage in AA solution containing SOD/catalase.

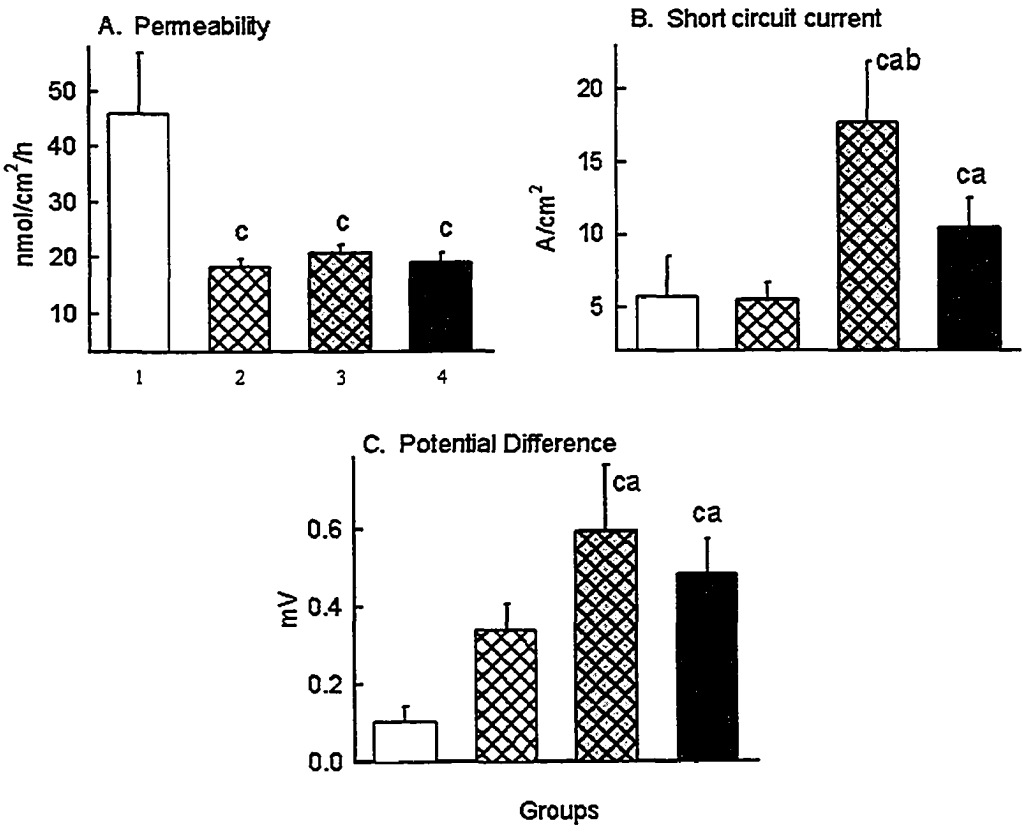

Figure 7-3. Effect of different preservation techniques on (A) mucosal permeability, (B) short circuit current and (C) potential difference after 12 h cold ischemia. **c**, **a**, and **b**, significantly different compared with group 1 (control), group 2, and group 4, respectively, $p<0.05$.

Group 1, no luminal treatment then stored in UW solution; Group 2, 1 h perfusion with AA solution, then static storage in AA solution; Group 3, 1 h perfusion with AA solution + trolox, then static storage in AA solution containing trolox; Group 4, 1 h perfusion with AA solution + SOD/catalase, then static storage in AA solution containing SOD/catalase.

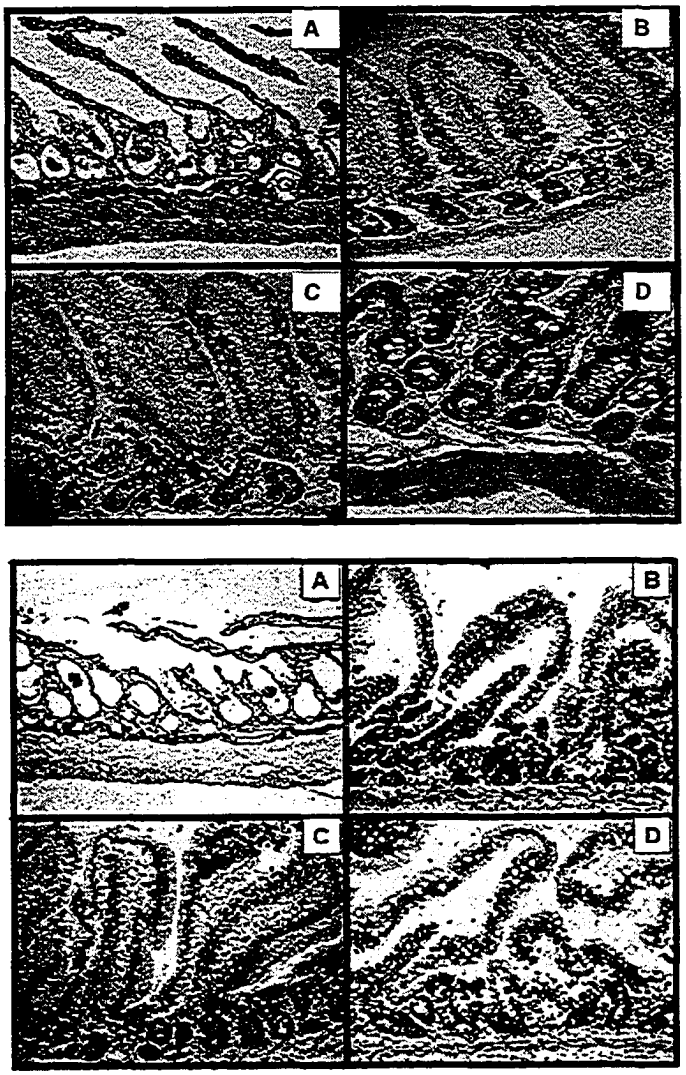

Figure 7-4. Histology of Small bowel after 12 h (upper panel) and 24 h (lower panel) cold preservation. (A) Group 1, no luminal treatment then stored in UW solution; (B) Group 2, 1 h perfusion with AA solution, then static storage in AA solution; (C) Group 3, 1 h perfusion with AA solution + trolox, then static storage in AA solution containing trolox; (D) Group 4, 1 h perfusion with AA solution + SOD/catalase, then static storage in AA solution containing SOD/catalase.

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Chapter 8

The influence of short-term fasting on the quality of small bowel graft preservation*

Introduction

Major obstacles to the development of reliable and safe small bowel transplantation (SBT) are primarily those of bacterial infection and allograft rejection; 67% of patient mortality is due to sepsis (55%) or rejection (12%) (5). Following transplantation, sepsis is the direct consequence of bacterial translocation across an injured small bowel (SB) graft (1,11). In clinical small bowel transplantation many factors including the quality of the donor organ, storage time and preservation method can affect graft function and ultimately recipient survival.

No single preservation solution has proven truly effective for SB and equivalent results can be achieved with simple crystalloid solutions (Normal Saline) or with complex solutions such as UW solution (31,21). Over the past few years, our lab has developed a novel amino acid-rich solution which is specifically tailored to the metabolic requirements of SB. We have demonstrated a superior preservation of energetics and mucosal integrity via a strategy of intraluminal administration of a nutrient-rich solution at the time of organ procurement (16,24,30).

One factor which has not received much attention is the nutritional status of the donor. The bowel is an organ that is sensitive to caloric and nutrient restriction, and starvation may deplete adenylates pool and peptides such as glutathione. It has also been shown that this mechanism may decrease the tissues ability to tolerate the

*A version of this chapter has been published in Cryobiology 2005; 50 (1): 83-92 (Salehi P, Churchill TA).

additional stress of cold ischemic storage (14,39). Additionally, it appears that the bowel is remarkably sensitive to alterations in its microenvironment and that interference with either the pattern of feeding, the quality or quantity of food may result in significant disturbances in intestinal structure and function (20). No data are available concerning the effects of nutritional status on the quality of the bowel following cold storage. Moreover, it is questionable whether past experiments which have been often performed on fasted animals have not been influenced by reduced nutritional status. In this study, the quality of SB grafts from fasted and non-fasted animals was assessed throughout cold storage over 24 h.

Materials and Methods

Male Sprague-Dawley rats (200-300 g) were obtained from the University of Alberta and used as bowel donors. All experiments were conducted in accordance with the regulations and policies of the Canadian Council on Animal Care. Chemical agents were AR-grade and were purchased from Sigma Chemical (Oakville, Canada).

Experimental groups: Three groups of animals (n=6) were fasted and three groups were non-fasted. All animals were flushed vascularly with UW solution and then treated luminally in the following group designations:

Fasted: **UWV** – no luminal treatment;

UWL – UW solution;

AAL – AA solution;

Non-fasted: **UWV** – no luminal treatment;

UWL – UW solution;

AAL – AA solution.

Surgical procedure and procurement of the small intestine: Rats in the *non-fasted* groups were fed standard laboratory diets and animals in the *fasted* groups had food withheld for 12-14 h overnight in cages with raised floors to minimize coprophagy. Water was provided *ad libitum*. Prior to laparotomy, rats received an intraperitoneal dose of pentobarbital (65mg/250g; Somnotol; MTC Pharmaceuticals, Cambridge, Ontario, Canada), followed by inhalational halothane (0.5-2%) to maintain anesthesia. A midline laparotomy was performed and the aorta exposed infrarenally and at the level of the celiac trunk. The supraceliac aorta was then clamped and 10 ml of modified University of Wisconsin (UW) solution (equivalent to ~0.5 ml/g tissue) was administered via the infrarenal aorta. The suprahepatic vena cava was transected to facilitate the outflow of both blood and perfusate. The entire jejunum and ileum was subsequently harvested. In the groups treated luminally, UW or amino acid (AA) solution (20 ml; equivalent to ~1.0 ml/g tissue) was then placed into the lumen of the bowel allowing the effluent to exit uninhibited. Each end was immediately ligated with 3-0 silk, leaving the bowel filled without turgor with the same solution. The bowel was then stored in an additional 40 ml of each respective solution and stored on ice in a 4°C incubator. Whole thickness tissue samples (1-2 g) were taken at 0, 4, 12 and 24 hours post vascular flush. Before freezing the samples, fecal contents were removed by saline flush through the segment. To arrest metabolic activity, samples were snap frozen in liquid nitrogen, and subsequently stored at –65°C until processed.

Composition of Solutions: Modified UW solution contained: lactobionic acid (100 mM), raffinose (30 mM), KOH (100mM), NaOH (15 mM), KH_2PO_4 (25 mM), $MgSO_4$ (5 mM), adenosine (5 mM), allopurinol (1mM), dextran (67.3 kdal; 5%), pH 7.4. AA solution contained: lactobionate (20 mM), adenosine (5 mM), allopurinol (1 mM), dextran (67.3

kdal; 5%), BES [N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] (15 mM), glutamine (35 mM), glucose/glutamate/ aspartate (20 mM each), arginine/glycine/valine/asparagine/lysine/threonine/serine (10 mM each), methionine/ornithine/leucine/isoleucine/histidine/proline/cysteine (5 mM each), tyrosine/ tryptophan (1 mM each), hydroxybutyrate (3 mM), Trolox (1 mM), 3-aminobenzamide (1 mM), pH 7.4.

Sample Preparation and Metabolite Assay: Frozen SB samples were weighed and then extracted 1:5 w/v in perchloric acid containing 1mM EDTA. The precipitated protein was removed by centrifugation (20 min at 20,000xg). Acid extracts were neutralized by the addition of 3 M KOH/ 0.4 M Tris/ 0.3M KCl and then recentrifuged (20min at 14,000xg). Aliquots of neutralized extracts were immediately processed via standard enzyme-linked metabolite assays (12). Spectrophotometric analysis was then performed to measure the absorbance of NADH at 340 nm, providing quantification of ATP, ADP, AMP. Values are reported as µmol per gram protein. Protein was measured according to Lowry et al (22).

Malondialdehyde (MDA): Frozen tissue (100 mg) was homogenized 1:10 in phosphate-buffered saline. The homogenate was then processed and fluorescence was compared to standard amounts of MDA according to Yagi (23,36).

Glutathione (GSH): Frozen tissues (100 mg) were homogenized 1:5 w/v in 6% perchloric acid containing 1mM EDTA. Precipitated protein was removed by centrifugation (20 min, 14,000xg). Acid extracts were neutralized with 3 M KOH/0.3 M imidazole and recentrifuged (20 min, 14,000xg). Extracts were analyzed based on fluorescence and compared to standard amounts of reduced glutathione according to Fernandez-Checa and Kaplowitz (15).

Histology: Full-thickness biopsies were fixed in 10% buffered formalin solution, embedded in paraffin, cut to 5 μm, and stained with hematoxylin and eosin. Histologic damage was assessed using Park's histologic classification of intestinal tissue injury (25).

Statistical analysis: Metabolite data were reported as means ± SE for each group. Statistical differences between groups were determined using analysis of variance, followed by Tukey's Honest Significant Difference (HSD) *post hoc* comparison test; $p<0.05$ was reported. Histology scores were compared using the Kruskal-Wallis test; $p<0.05$ was reported.

Results

Energetics (Figure 8-1)

ATP: Overall, there were no major differences between fasted and non-fasted state in each particular experimental group. During ischemic period, ATP levels tended to decrease in all experimental groups in both fasted and non-fasted conditions. ATP levels in AAL groups were significantly higher than the corresponding values in the two other groups throughout storage. After 12 h, in fasted groups, AA treated tissues ATP level was more than 2-fold greater than the corresponding values in control and UWL groups (12.6 vs 2.2 and 5.6 μmol/g protein, $p<0.05$). Even after 24 h cold storage, ATP levels in the fasted AAL group was higher than control and UWL group (5.5 vs 1.4 and 3.8 μmol/g protein, $p<0.05$); the same pattern was observed in non-fasted groups.

Total Adenylates: There were no differences between fasted and non-fasted groups. TA values dropped significantly by 12 h storage in UWV groups and by 24 h in both UWL and AAL groups. Interestingly, after 24 h, TA levels in AAL groups were significantly

reduced compared to corresponding UWL values; despite considerably higher values for the AAL groups in all other parameters of energy metabolism.

ATP/ADP: ATP/ADP ratios were consistently and markedly superior in both AAL groups compared to UWV or UWL groups with no differences between fasted and non-fasted animals. Of particular interest, even after 24 h storage, ATP/ADP ratios were significantly greater than 4 h values in both UWV and UWL groups.

Energy Charge (EC): Energy Charge values paralleled those of ATP. The only time-point that exhibited a different response between fasted and non-fasted animals was after 24 h in the AAL groups. ATP and EC values after 24 h in the AAL groups showed significant differences compared to UWL in non-fasted animals, whereas values were not significantly different for fasted animals.

End-products of carbohydrate and amino acid metabolism (Figure 8-2)

Lactate: Within 4 h, levels of this anaerobic end product were slightly elevated compared with 0 h in all experimental groups. No important difference was observed between fasted and non-fasted conditions in any group. Values between 12-24 h, in both UWV and UWL groups (both fasted and non-fasted) were significantly higher than AAL groups ($p<0.05$); presumably indicating a greater reliance of these tissues on anerobic pathways for energy production.

Ammonia: The by-product of amino acid metabolism, ammonia, tended to increase gradually in all groups during ischemia. After 24 h, AA treated groups showed significantly higher ammonia levels compared to UWL groups and UWV (non-fasted) ($p<0.05$); this effect was not as apparent in the fasted groups.

Peroxidative injury and antioxidant levels (Figure 8-3)

Malondialdehyde (MDA): In both UWV and UWL groups, fasting increased MDA levels after 12 and 24 h storage ($P<0.05$), thus reflecting a shift towards a more oxidized state during fasting; values ranged from 60 to 245 nmol/g protein. The effect of fasting on MDA levels was only observed after 12 h storage in AAL groups. Interestingly, overall, AA treated tissues exhibited a considerable and significant reduction in levels of this by product of lipid peroxidation compared to the UW treated tissues (both UWV and UWL). After 12 h ischemia, MDA levels in AAL groups ranged from 10-30 nmol/g protein whereas corresponding values in the UW treated groups (UWV and UWL) ranged from 60-180 nmol/g protein; an approximate 6 fold reduction in peroxidative injury.

Reduced glutathione (GSH): GSH levels in all fed and fasted groups tended to decrease as period of ischemia progressed. The effect of fasting on GSH levels was more apparent after 12-24 h storage; values were generally lower in fasted animals compared to their non-fasted counterparts. This effect was most prominent in the AAL treated tissues; after 24 h, levels in non-fasted AAL treated tissue was 3 fold greater than in fasted AA treated tissue (5.0 vs 1.5 μmol/g protein, $p<0.05$).

Histology (Table 8-1, Figure 8-4)

The histologic changes produced by ischemia in all fasted and non-fasted groups are summarized in table 1. Throughout the entire 24 h cold ischemia, mucosal integrity was markedly superior in luminally treated tissues (with either UW or AA solution) in both fasted and non-fasted states. Within 12 h, loss of villus tissue and crypt infarction were obvious in control groups, UWV (median Park's grades 5 and 6 in fasted and non-fasted tissues, respectively). After 12 h, luminally UW (UWL) treated tissues exhibited minor changes including subepithelial space at villus tips and epithelial lifting along villus sides

in some tissues (grades 2 and 1 in fasted and non-fasted rats, respectively). Although AA treated tissues showed minor changes in fasted state (grade 2), normal mucosa was observed in non-fasted group (grade 0) ($P<0.05$). After 24 h, both luminal treatment groups, UWL and AAL, demonstrated substantial improvement in mucosal morphology compared to control groups (UWV, fasted and non-fasted) which exhibited severe injury including transmucosal and transmural infarction. In the UWV groups, non-fasted animals showed more severe damage (5/6 specimens exhibited transmucosal infarction; median grade 7.5), whereas injury in the fasted animals was somewhat improved (only 3/6 exhibited grade 7 or greater; median grade 5.5).

Discussion

Successful small bowel transplantation is dependent upon many factors, one of which is the quality of the donor organ. The ability of transplanted small bowel to rapidly resume normal metabolism following the period of cold storage is essential to achieving good early graft function (37). Several studies on liver have demonstrated that nutritional status of an animal affects the tolerance of the liver to various noxious agents (7). Fasting prior to exposure to hypoxia has been shown to increase hepatocellular injury and increase the rate of loss of liver cell viability. Moreover, microvascular injury has been reported to be greater in cold stored rat livers obtained from fasted animals. These studies suggested that the depletion of liver glycogen (as a source of ATP) and a reduced capacity to withstand oxidative stress are the primary causative factors for increased hepatic sensitivity to ischemia/reperfusion injury; ATP and glutathione (reduced form) were consistently lower in fasted animals [6]. Based on these data, we hypothesized that donor nutritional status would affect the quality of the graft throughout

hypothermic storage. The data reported in the current communication support this hypothesis.

The integrity of the epithelial layer is maintained mainly by the tight junction region close to the apical surface of intestinal epithelial cells. These tight junctions act as a barrier that divides the plasma membrane into apical and basolateral domains and is of utmost importance to prevent permeation of potentially noxious substances, including endotoxins and bacteria (4,17). Tight junctions consist of a number of proteins that are dynamic, energy-requiring structures. These proteins are modulated by alterations in pH (18), temperature (13), and osmolarity (34), among other factors. Agents that deplete cellular ATP also cause a dilation of the tight junction at the ultra-structural level, a perturbation of the actin cytoskeleton, an increase in epithelial permeability, and an increase in transepithelial flux of macromolecules (12). This has the potential to result in bacterial translocation, elevated inflammatory response and the potential for life-threatening infection (32,38). Hence, proper functioning of tight junctions is essential to maintain normal physiologic processes in the gut. Another important to the preservation of the small bowel is the removal of enteric contents prior to cold storage which aids in the elimination of bile acids, digestive enzymes and bacterial endotoxins (8). Supplementation with essential nutrients further increases the benefit of luminal flushing prior to standard storage techniques. The nutrient-rich preservation solution employed in this study has been specifically tailored to the physiological amino acid requirements of the SB. The composition of amino acids in the solution addresses both metabolic (energy production) and synthetic (synthesis of important molecules) aspects of intestinal metabolism (2,35). When combined in a solution, these amino acids play an important adjunctive role in not only preserving the integrity of epithelial tight junctions

but also in maintaining overall mucosal viability during conditions of hypothermic hypoxia and ischemia-reperfusion. In the current study, the intraluminal administration of amino acids resulted in superior parameters of energy metabolism [ATP, ATP/ADP and energy charge values]. This positive effect on intestinal metabolism was independent of nutritional status; fasted and non-fasted animals demonstrated identical response profiles with respect to energy metabolism.

The intestine is extremely susceptible to OFR-mediated injury due to an abundance of xanthine oxidase (XO) in mucosal epithelial cells and an inherent sensitivity of intestinal mucosal cells to ischemic injury (27). In pathologic states such as ischemia, xanthine dehydrogenase is converted to XO leading to increased oxidation of hypoxanthine and a concomitant generation of superoxide radical. Elevated rates of purine catabolism during ischemia (as ATP is consumed), thereby producing lower energy purines (ADP, AMP, IMP, adenosine, etc.) result in oxygen radical production even during cold storage (26). Glutathione is one of the most important endogenous buffering systems against antioxidative stress and is essential for normal cell function and proliferation (3). Not only does glutathione exist in a reduced form (GSH) and dimeric oxidized form (GSSG), membrane proteins in addition to specific enzyme systems undergo thioltransferase-mediated thiolation (and non-specific thiolation) to yield protein-SSG products. It has been proposed that these protein thiolation products possess reduced activity profiles and are a deleterious consequence of oxidative stress. Of particular consequence is the interference of protein disulfides when the enzyme proteins affected are directly involved with redox metabolism; this is of primary import for a system where redox balance is shifting due to oxidative stress. Hence, for successful organ preservation not only is it important to maintain elevated GSH levels (and lower

GSSG) for direct glutathione inactivation of potentially toxic oxidative agents/by-products, an elevated GSH pool ensures that secondary protein thiolation reactions are kept to a minimum (10).

Glutamine, a precursor of glutathione is able to enter the cell and studies have documented elevated glutathione levels in organs of glutamine-treated animals subjected to ischemia/reperfusion (19). This leads to the question of the role of an amino acid-rich luminal solution that contains all three precursor amino acids (glutamate, cysteine, glycine). Our lab has previously documented substantial increases in intestinal glutathione levels afforded by preservation solutions containing all three precursor amino acids throughout cold storage and ischemia-reperfusion; sometimes as high as 2.8 fold that of untreated tissue (29). In this study, both endogenous antioxidant levels (GSH) and lipid peroxidation (MDA) in bowel tissues were significantly affected by donor nutritional status. Glutathione levels in non-fasted tissues treated with AA solution were markedly higher than other groups, supplying further evidence that luminal delivery of a nutrient-rich solution facilitates intracellular antioxidant augmentation (33). Although AA treatment did not improve GSH levels in fasted animals in this experiment, it must be noted that levels of peroxidation by-product, malondialdehyde, were ~10 fold lower than untreated groups; presumably indicating that antioxidant status is a function of antioxidant capacity (GSH) and oxidative injury (MDA). Oxidative injury was generally lower in non-fasted animals; this effect was more prominent in non-AA treated tissues. Treatment of SB with the AA solution (containing antioxidant, Trolox) clearly had a dominant effect between treatment groups at reducing MDA levels; however, even after 12 h storage MDA levels were significantly lower in non-fasted animals in the AA treatment group.

In the current communication, the effect of reduced ATP and greater oxidative stress culminated in greater alterations in mucosal morphology. Histologic damage was greater (i.e. crypt infarction) in non-fasted rats than fasted ones in control groups (UWV). Since no intraluminal flushing was applied, greater epithelial injury was evident compared to tissues that were flushed luminally; presumably this was due to the presence of feces and other cytotoxic agents including bacterial endotoxins and biliary and pancreatic secretions (9). All tissues flushed luminally, exhibited superior mucosal morphology; nutrient-treated tissues demonstrated not only superior energetics and reduced oxidative stress, but also morphology was maintained at near normal architecture, this protective effect was most prominent after 12 h storage.

In the clinic, the bowel is typically harvested from a non-fasted donor, however the longer the period that the patient is without enteral nutrition prior to organ harvest, the greater the impact on intestinal oxidative stress. This study demonstrates the importance of clearing the enteric contents from the lumen prior to prolonged cold storage. Secondly, the beneficial effect of a luminally administered nutrient-rich solution supplemented with exogenous antioxidant dramatically improves the quality of intestinal preservation throughout cold storage. Institution of this technique and nutrient-rich solution clinically would require no alterations to existing harvesting protocols, luminal administration of the preservation solution would simply take place on the 'back table' and would require only rudimentary equipment and skills.

Table 8-1. Histology of Small Bowel Following Cold Storage.

		Park's Classification			
		12 h		24 h	
		Grade	median	Grade	median
Fasted	**UWV**	1,1,5,5,6,8	5.0	1,2,4,7,8,8	5.5
	UWL	1,1,2,2,3,4	2.0 [c]	1,1,1,3,3,4	2.0 [c]
	AAL	0,1,1,3,3,4	2.0 [c]	1,1,2,2,3,4	2.0 [c]
Non-Fasted	**UWV**	4,5,6,6,7,8	6.0	4,7,7,8,8,8	7.5
	UWL	0,1,1,1,2,3	1.0 [c]	1,1,1,2,3,3	1.5 [c]
	AAL	0,0,0,0,0,1	0.0 [c,f]	0,0,1,2,3,3	1.5 [c]

c -denotes significantly different from UWV (control) group; $P<0.05$.

f -denotes significantly different from corresponding fasted group; $P<0.05$.

UWV, no luminal treatment; UWL, luminal UW solution; AAL, luminal AA solution.

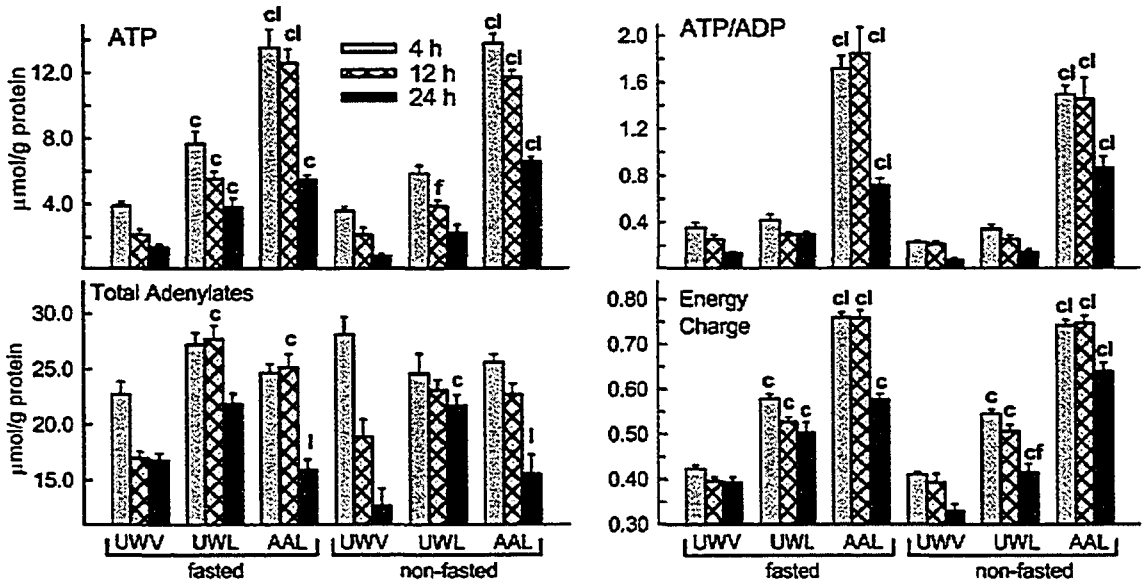

Figure 8-1. Effect of nutritional status on ATP, Total Adenylates (ATP+ADP+AMP), ATP/ADP and energy charge [EC= (ATP+ADP/2)/(ATP+ADP+AMP)] values. ***c,l,f*** - Significantly different compared to UWV (control), UWL, corresponding fasted group, respectively; $P<0.05$.

UWV, no luminal treatment; UWL, luminal UW solution; AAL, luminal AA solution.

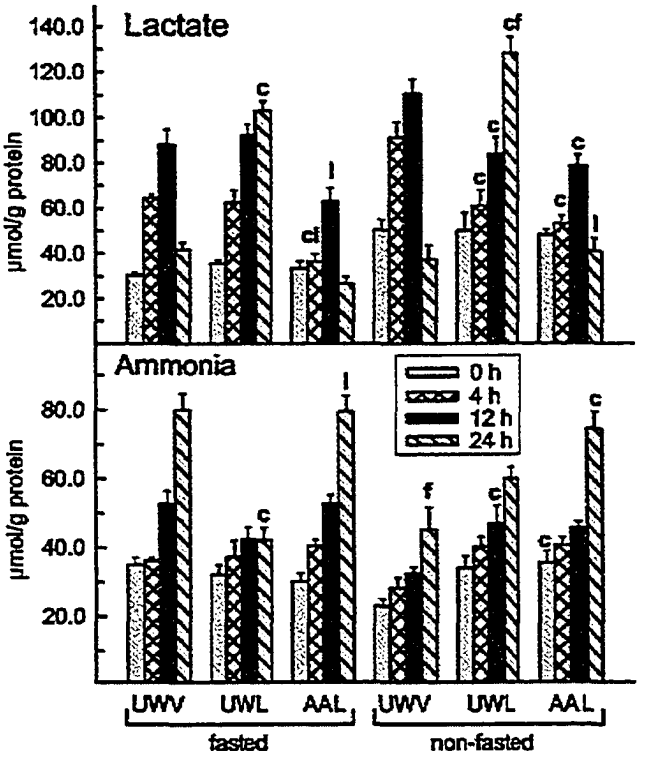

Figure 8-2. Effect of nutritional status on endproducts, lactate and ammonia. ***c,l,f*** - Significantly different compared to UWV (control), UWL, corresponding fasted group, respectively; $P<0.05$.

UWV, no luminal treatment; UWL, luminal UW solution; AAL, luminal AA solution.

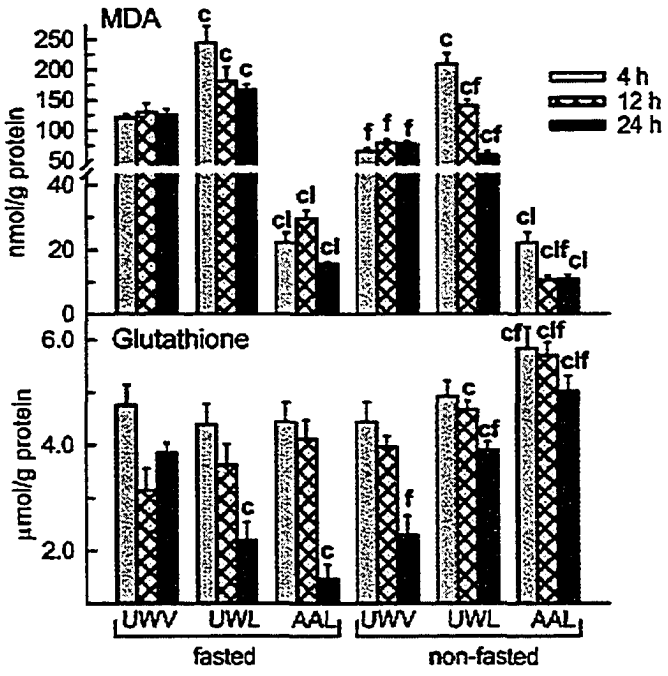

Figure 8-3. Effect of nutritional status on levels of malondialdehyde (MDA) and glutathione (reduced form, GSH). ***c,l,f***-Significantly different compared to UWV (control), UWL, corresponding fasted group, respectively; $P<0.05$.

UWV, no luminal treatment; UWL, luminal UW solution; AAL, luminal AA solution.

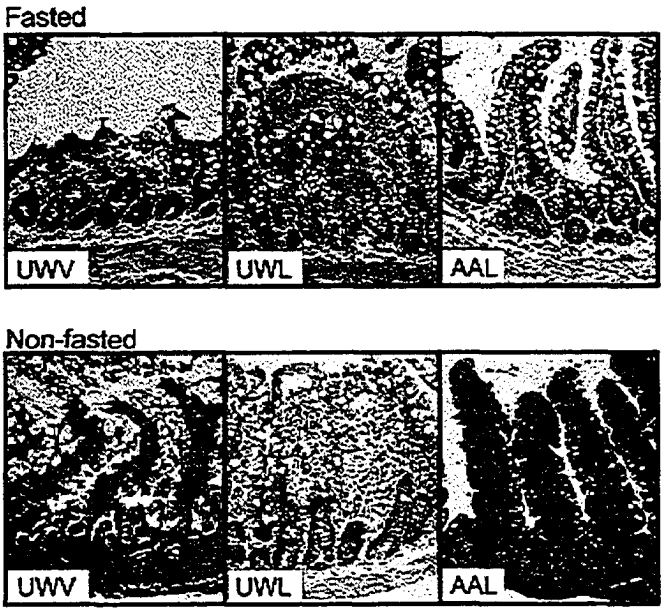

Figure 8-4. Histologic injury following 12 h cold storage; median grades shown. 10X; hematoxylin & eosin staining.

Grade of injury is as follows. Fasted – UWV, grade 5; UWL, grade 2; AAL, grade 2.

Non-fasted – UWV, grade 6; UWL, grade 1; AAL, grade 0.

UWV, no luminal treatment; UWL, luminal UW solution; AAL, luminal AA solution.

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Chapter 9

Nutrient-related issues affecting successful experimental orthotopic small bowel transplantation*

Introduction

Small bowel transplantation (SBT) as a life-saving treatment for patients with end-stage intestinal failure has become closer to a reality in clinical practice. Despite the progress in clinical management, the outcome after SBT is still not comparable to that of other organs (kidney, liver, heart, and pancreas) (1). Among the problems limiting the clinical application of small bowel transplantation is our inability to preserve the graft successfully prior to implantation. Unfortunately, the mucosal epithelium is extremely susceptible to even brief periods of ischemia (2). Therefore, graft viability related to preservation is one of the most important factors in successful transplantation. At the present time, donor and recipient procedures must be undertaken simultaneously in the same hospital. Improving small bowel preservation techniques/solutions would facilitate recovery of intestinal absorptive function, reduce episodes of sepsis and permit the harvesting of organs from remote locations, thus expanding the donor pool. Considering these facts, the development of a simple and effective method for small bowel preservation is essential in achieving success in the clinical arena.

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* A version of this chapter has been accepted for publication in *Transplantation* 2005 (Salehi P, Zhu LF, Churchill TA).

The University of Wisconsin (UW) solution has been established as one of the best organ preservation solutions for solid organ storage (3,4). However, the optimal solution for intestinal preservation still is controversial (5-7). No single preservation solution has proven truly effective for SB and equivalent results can be achieved with simple crystalloid solutions (Normal Saline) or with complex solutions such as UW solution (8,9). Thus, it is by default that the current clinical standard for SB consists of a vascular flush with UW solution as part of multi-visceral organ procurement.

Over the past several years, our lab has developed a novel amino acid-rich solution which is specifically tailored to the physiological requirements of SB. We have demonstrated that the strategy of intraluminal administration of a nutrient-rich solution at the time of organ procurement/harvest is able to preserve the metabolic and morphologic integrity of whole small bowel grafts in a number of *in vitro* and *in vivo* models (10-12).

The present communication presents data describing the effectiveness of an intraluminal nutrient-rich preservation solution in the final experimental test-bed of small animal transplantation. Orthotopic transplantation of the whole small bowel (transplanted ileum + jejunum placed in its normal anatomical site) was performed after a clinically relevant period of static cold storage (6 h); energetics, oxidative stress, neutrophil recruitment and graft viability were assessed.

Material and Methods

Male syngeneic inbred Lewis rats (200-400 g) were obtained from the University of Alberta and used as bowel donors and recipients. Donor animals were 65 ± 7 g lighter than recipients. All experiments were conducted in accordance with the regulations and

policies of the Canadian Council on Animal Care. Chemical agents were AR-grade and were purchased from Sigma Chemical (Oakville, Canada).

Anesthesia and pre-operative care: Rats were fed standard laboratory diets and food was withheld from the donors and recipients 10-12 h before surgery in cages with raised floors to minimize coprophagy. Water was provided *ad libitum*. All operations were carried out with animals under anesthesia with 1-3 % isoflurane (Baxter Corporation, Toronto, ON) and O_2. Ophthalmic ointment was applied to the eyes. Core body temperature was monitored and a warming lamp/heating pad was used to ensure constant body temperature throughout the operation.

Surgery: *Graft preparation*- With the rat properly heated, 5 ml of warm lactated Ringer's solution was given intravenously. Under microsurgical microscope, the donor abdomen was opened and the entire small intestine was isolated on a pedicle containing an aortic cuff (with the superior mesenteric artery attached) and the portal vein. The bowel was divided 1 cm below the duodenal-jejunal ligament and 1 cm above the ileo-cecal valve. Heparin (250 U) was administered IV to the donor to provide systemic anticoagulation. The aorta was then ligated above and below the origin of the superior mesenteric artery and the artery was flushed with 2-4 ml of the University of Wisconsin solution (Barr Laboratories, Inc. Pomona, NY, USA). After the bowel was blanched, the portal vein was transected close to its bifurcation and allowed to drain the blood forced out by the arterial infusion. The bowel was quickly removed by cutting the mesentery along its root. Organs in Group 1 did not receive any luminal solution. Experimental groups included bowels isolated from randomized animals (n=5 in each group) which were flushed luminally (40 ml; equivalent to ~2.0 ml/g tissue) with either UW solution or an amino acid-based (AA) solution; effluent was permitted to exit the distal end uninhibited. Luminal pressures were

maintained <40 cm water. Once thoroughly flushed, each end was immediately ligated with 3-0 silk, leaving the bowel filled without turgor. Manipulation of the intestine was kept to a minimum. Time of harvest operation was 46 ± 2 min. Grafts were then stored in 30 ml of the respective flush solution on ice in a 4°C incubator for 6 h.

Experimental groups: Animals were assigned to the following groups based on luminal solution received: **Group 1** – none;

Group 2 – UW solution;

Group 3 – AA solution.

Composition of the AA solution: AA solution contained: lactobionate (20 mM), adenosine (5 mM), allopurinol (1 mM), dextran (67.3 kdal; 5%), BES [N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] (15 mM), glutamine (35 mM), glucose/glutamate/aspartate (20 mM each), arginine/glycine/valine/asparagines/lysine/ threonine/serine (10 mM each), methionine/ornithine/leucine/isoleucine/histidine/proline/ cysteine (5 mM each), tyrosine/ tryptophan (1 mM each), hydroxybutyrate (3 mM), Trolox (1 mM), 3-aminobenzamide (1 mM), pH 7.4, 320 mOsmol/L.

Recipient Surgery- A one-stage orthotopic transplant was performed using the modified method described by Zhong et al [13]. Briefly, keeping the rat warmed and hydrated with 5 ml of Ringer's solution, the recipient rat was opened through a mid-line incision. The infrarenal aorta was isolated, as was the infra-renal inferior vena cava (IVC). The superior mesenteric artery and portal vein were isolated, ligated and divided. The native small bowel from the duodenal-jejunal ligament to the ileo-cecal valve was then removed from the abdominal cavity. Immediately prior to vascular anastomoses of the donor graft, 3 ml of lactated Ringer's solution was used to clear the preservation solution within the vascular bed; in order not to exacerbate any mucosal injury incurred during storage,

bowel lumens were not flushed to remove preservation solution in Groups 2 and 3. Reconstruction of the artery and vein was accomplished through end to side anastomoses of the graft portal vein to the isolated infra-renal IVC and the aortic cuff to the isolated infra-renal aorta. During vascular anastomoses, the graft was kept cold by application of gauze sponges that were moistened with 4°C Ringer's solution. Monofilament 10-0 nylon sutures were used for the vascular anastomoses. The venous clamps were released first, followed by arterial clamp; time of surgery until reperfusion was 24 ± 5 min. The anastomotic sites were compressed lightly with a dry sponge for 1-2 minutes after reperfusion. The graft was then placed into the recipient's abdomen to avoid unnecessary manipulation. Primary anastomoses of the two graft ends were performed using 6-0 silk interrupted sutures over a piece of dry macaroni noodle as a luminal stent. The abdomen was closed using 4-0 polyglycolic acid sutures. All animals received an additional 10-18 ml of warm Ringer's solution intravenously through the penile vein using a 25 gauge needle for blood pressure maintenance during the course of surgery. Total time of transplant operation was 70 ± 4 min.

Postoperative care: Postoperatively, the animals recovered in a warm nursery incubator for 24 to 48 hours and received 5% sugar water *ad libitum* for the first 24 h followed by normal rat chow. Subcutaneous injection of 10 ml warm 0.9% saline was given after operation. Additionally, continuous intravenous fluid infusion of a 1:1 solution of lactated Ringer's:5% dextrose was maintained through the jugular vein for the first 4 h with a rate of 2.2 ml/h followed by 20 h at a rate of 1.1 ml/h. A single dose of 0.05 mg/kg buprenorphine was provided IP for analgesia.

Tissue sampling and assessments: All recipients rats were monitored closely throughout the first 12 h and then daily; all animals were sacrificed on the day 14th

postoperative day. Tissue biopsies (300-400 mg) were taken for analysis at: t=0 min (6 h cold storage), 35 min, 3 d, 7 d, and 14 d after transplantation. The first two samples of tissue from distal ileum were taken immediately following 6 h storage and 35 min after reperfusion (immediately prior to anastomosis of the lumen and wound closure); care was taken to only sample 2-4 cm segments in order not to significantly affect the remaining length. Animals were recovered and after 3, 7, 14 d, animals underwent laparotomies for sampling of the bowel. Samples were snap frozen in liquid nitrogen, and subsequently stored at –65°C until processed. Freshly isolated (FI) tissue was obtained from the recipient native bowel to approximate 'normal' *in vivo* metabolite/enzyme levels and histology.

Sample Preparation and Metabolite Assay: Frozen SB samples were processed and extracted in acid as previously described (14). Aliquots of extracts were analyzed via standard spectrophotometric enzyme-linked metabolite assays, providing quantification of ATP, ADP, AMP (14). Values are reported as µmol per gram protein. Protein was measured according to Lowry et al (15).

Malondialdehyde (MDA): Frozen tissue (100 mg) was processed and fluorescence was compared to standard amounts of MDA as previously described (16,17).

Glutathione (GSH): Frozen tissue (80-100 mg) was processed and extracted in acid. The extract was then analyzed and fluorescence was compared to standard amounts of reduced glutathione as previously described (18).

Myeloperoxidase (MPO) activity: Frozen tissue (50 mg) was processed and MPO activity was measured spectrophotometrically according to Krawisz et al (19).

Histology: Full-thickness samples were fixed in 10% buffered formalin, embedded in paraffin, cut to 5-µm thick, and stained with hematoxylin and eosin. Histologic damage

was assessed using Park's histologic classification of intestinal injury (20) by two histopathologists (GS and LJ) who were 'blinded' to group designations.

Statistical analysis: Metabolite data were reported as means ± SE for each group. Statistical differences between groups were determined using analysis of variance, followed by Tukey's Honest Significant Difference (HSD) *post hoc* comparison test; $p<0.05$ was reported. Histology scores were compared using the Kruskal-Wallis test; $p<0.05$ was reported. Survival was calculated by the method of Kaplan-Meier. The impact of different treatments on animal survival was evaluated using the log-rank test.

Results

Survival: After orthotopic small bowel transplantation following 6 h cold preservation, the 7-day survival rates were 0% (0/5) in Groups 1 and 2 (treated with either UW vascular or UW luminal) and 100% (5/5) in AA-treated rats (Group 3) (Figure 9-1). In all Groups 1 and 2 rats, intraluminal hemorrhage of the graft occurred immediately after 35 min reperfusion (Figure 9-2) and most of these died within the first 4 h following transplantation and all died within 12 h. Autopsy of dead rats showed hemorrhagic necrotic bowels with intact vascular anastomotic pedicles. The cause of death was attributed to acute graft failure leading to massive fluid loss, as a result of poor graft preservation. In contrast, in Group 3, all grafts became pinkish following reperfusion with a near-normal appearance with slight congestion and all animals survived 24 h post transplant. All animals in Group 3 exhibited good peripheral circulation, solid bowel movements with no diarrhea and a return to normal appearance and activity after 24 h. In Group 3, one animal died on day 9 after transplantation; histologic assessment of this graft after day 7 revealed grade 4 damage. Fourteen-day survival rate in Group 3 was 80% (4/5), $P<0.01$.

Energetics: *ATP (Figure 9-3a).* After 6 h cold ischemia, ATP levels were significantly higher in luminally-treated tissues compared to the Group 1 (5.2 and 4.8 in Groups 3 and 2 vs 3.1 μmol/g in Group 1, $p<0.05$). After 35 min reperfusion, ATP levels increased 30-60% in all experimental groups; being significantly higher in Group 3 compared to Groups 1 and 2 ($p<0.05$). After 3 days post-transplantation, ATP levels in Group 3 tissues resumed values that were not different from freshly isolated tissues.

Energy Charge (Figure 9-3b). Energy charge (EC= [ATP+ADP/2]/TA) value for FI specimens was 0.69. After 6 h storage, EC values dropped 51% in Group 1 (0.34), 41% in Group 2 (0.41) and 35% in Group 3 (0.45, $P<0.05$ compared to Group 1). After 35 min reperfusion, EC values increased to values not significantly different from FI tissues in all groups.

Lipid Peroxidation: *Malondialdehyde (MDA; Figure 9-4a).* The level of MDA in freshly isolated tissues was 286 ± 24 nmol/g. After 6 h cold ischemia, Groups 1 and 2 exhibited 4.2- and 3.3-fold increases in MDA levels; Group 3 did not show an increase throughout the entire experimental period ($p<0.05$, compared to Groups 1 and 2). After 35 min, Group 3 exhibited significantly lower MDA levels compared to Groups 1 and 2 ($p<0.05$).

Antioxidant: *Reduced Glutathione (GSH, Figure 9-4b).* Glutathione level was 4.4 ± 0.3 μmol/g in freshly isolated tissues. In Group 1, GSH levels dropped significantly after 6 h cold ischemia and remained low during reperfusion period. Groups 2 and 3 showed significantly higher GSH values after 6 h ischemia and 35 min reperfusion compared to Group 1 ($p<0.05$). In Group 3, GSH levels dropped slightly over the first 7 days after transplant but resumed normal values by 14 days.

Neutrophil recruitment: *Myeloperoxidase activity (MPO; Figure 9-4c).* MPO activity was 9.2 ± 1.1 U/g protein in freshly isolated tissues. After cold ischemia and subsequent

reperfusion, MPO activity increased dramatically in Groups 1 and 2; by 35 min, MPO values were 38 and 44 U/g, respectively. In Group 3, MPO activity was significantly lower after 6 h cold storage and during the entire reperfusion period; MPO values were 39% of Group 1 after 35 min reperfusion ($p<0.05$).

Histology (Table 9-1, Figure 9-5): After 6 h cold storage, AA-treated tissues exhibited near-normal intestinal architecture while, Groups 1 and 2 exhibited varying degrees of subepithelial clefting. After 35 min reperfusion, histologic integrity of the mucosal layer was markedly superior in Group 3 (Park's median grade 3). Although Groups 1 and 2 grafts were highly damaged from preservation and reperfusion injuries, specimens in these Groups showed various levels of injury including complete epithelial lifting, denuded villi and even in some grafts loss of villus tissue (median grades 5 in both Groups 1 and 2, $p<0.05$ compared to Group 3). After 3 days, most biopsies indicated complete regeneration of the mucosa (median grades of 0 at 3 d and 7 d), although there was some damage still present in 1-2 specimens. By 14 days, all surviving animals exhibited complete regeneration of the grafts.

Discussion

Major obstacles to the development of reliable and safe SB transplantation are largely those of bacterial infection and control of allograft rejection; 67% of patient mortality is due to sepsis (55%) or rejection (12%) (21,22). Encouraging new data indicate that post-transplant survival is steadily improving. Intestinal Transplant Registry data (up to 2001-2002) indicate that 2.5 year graft survival is 52% & 66% for intestinal and multi-visceral transplants. Recently, even better graft survival has been reported at one center; 71% 3 year survival for isolated intestinal transplants (23). These improvements are due primarily to increased surgeon/center experience and better

medical management (diagnosis of rejection & immunosuppression). Never the less, SB injury, whether due to preservation injury before transplantation or upon reperfusion after transplantation, may lead to a loss of intestinal barrier function and subsequent bacterial translocation with a dramatic increase in inflammation and the potential for life-threatening infection (24-26).

Despite the benefit of UW for preservation of other intra-abdominal organs (primarily liver, pancreas) (27,28), the maximum clinical storage time for SB remains relatively brief (6-8 hours); furthermore graft quality is often compromised. No single preservation solution has proven effective for SB and equivalent results can be achieved with simple crystalloid solutions (Normal Saline) or with complex solutions such as UW solution. Thus, it is by default that the current clinical standard for SB consists of a vascular flush with UW solution as part of multi-visceral organ procurement.

Luminal flushing even with a simple crystalloid solution has been shown to improve the metabolic and structural integrity of the mucosal layer over varying periods of static storage compared to vascular flushing alone. This has been postulated to occur by dilution of resident enteric contents, which contain feces and cytotoxic agents, including bacteria, endotoxins and biliary/pancreatic secretions (29). Supplementation of the luminal solution with essential nutrients, such as amino acids, further increases the benefit of luminal flushing. The nutrient-rich preservation solution employed in this study has been specifically tailored to the physiological amino acid requirements of the SB. The composition of amino acids in the solution cater to both metabolic (energy production) and synthetic (synthesis of critical molecules) aspects of intestinal metabolism (30-33). Preservation and reperfusion studies have indicated excellent protective effects with this luminal preservation solution in both in vitro and in vivo

models of ischemia and reperfusion (10-12, 34-38). Furthermore, we have previously demonstrated that a brief 1 h period of oxygenated perfusion with our nutrient-rich solution is superior than with static storage alone or perfusion with UW solution (10,37). Presumably, boosting oxygen tensions in an aqueous nutrient-rich solution permits maximal utilization of the oxidative substrates during the initial period of entry into cold storage; these benefits extend to periods of prolonged storage (10). In future, due to issues of clinical applicability, a brief oxygenation period may be replaced with simple pre-oxygenation of the nutrient-rich preservation solution; however, this strategy remains to be tested.

In the current study, the intraluminal administration of amino acids resulted in full recovery of high energy adenylates within the first 3 days post transplant; energy charge ratios resumed freshly isolated values within 35 min reperfusion. Measured indices of oxidative stress (malondialdehyde, MDA and reduced glutathione) indicated superior control of oxidative injury following reperfusion. This overall reduction in energetic and oxidative stress may have been responsible for the marked reduction in neutrophil recruitment in nutrient-treated grafts. The provision of a higher quality small bowel graft with a decreased stimulus to ischemia/reperfusion injury may be a pivotal advance in avoiding the inflammation, rejection, sepsis cascade after intestinal transplantation (24-26).

Short heterotopic segments of small bowel survive cold storage up to 12 h (39). Sakai et al. have reported successful results in heterotopic and segmental transplant model after 24 h two-layer method preservation using perfluorocarbon and UW solution (40-42). Since a segmental graft undergoes classic rejection in hosts, this model has been typically used for immunological studies (43). However, limitations of these models

are that only 15-25 cm of jejunum or ileum (or less) has been used rather than the entire small bowel. The entire length of the small bowel in the size of rats used for this study is 50 ± 0.8 cm (n=48; unpublished data) from pyloric valve to ileal-cecal valve (duodenum + jejunum + ileum); with the duodenum measuring ~10 cm in these animals, total ileum + jejunum length is 40 cm. It is difficult to interpret the results from other groups using the orthotopic model, since complete surgical details with respect to length and proportion of jejunum/ileum transplanted has not been clearly outlined (44). Recent studies have overshadowed the usefulness of segmental heterotopic transplantion with respect to graft rejection and overall viability. Larger grafts typically undergo greater rejection than shorter segments (45). Furthermore, modifications of segmental intestinal transplants may be more convenient with greater graft and animal survival, however, deviation from clinical practice raises the question of direct clinical applicability in these types of studies (46). Orthotopic and heterotopic whole small bowel transplants have been directly compared, with heterotopic grafts exhibiting greater rejection injury, poorer contractility and overall reduced viability (47,48). Intraluminal nutrients and secretions have been hypothesized to play a potential role in modulating immune status (delaying graft rejection) and improving recovery from transplantation in the orthotopic model.

Despite the usefulness of small animal transplants in assessing physiological and functional aspects of small bowel preservation and transplantation, issues of overall technical complexity and high mortality rates limit wide use of this model. Microsurgical expertise is the main cause of early mortality and it has been demonstrated surgical experience is a very important factor to succeed (49). In our study, there were no technical failures due to issues of vessel patency at anastomotic sites, thrombosis, or significant blood loss (>5%). In our experience, animals that receive total jejunum/ileum

transplants following 4-6 h storage suffer from massive fluid loss and unless remedied will not survive overnight (>12 h). Our results are similar to Fishbein et al. who transplanted the entire jejunoileum following 4 h storage in UW solution; survival in syngeneic Lewis rats was 33% after 6 days (no animal survived past 9 days) (50). Hypovolemic shock leading to issues of hydration and electrolyte imbalance has been proposed as the leading cause of mortality in the first 24 h (13). In the present study, we managed this issue properly by fluid replacement both during and after surgery. An equivalent of at least 100% blood volume (estimated for a 330 g rat; assuming 50 ml/kg blood volume) was provided via venous injection throughout the surgery; this was presumably required to alleviate alterations in vascular tone. During the first 24 h post transplant, another two blood volumes were provided via continuous intravenous infusion. Although these volumes are high, in the clinic, patient management would include vasopressor support peri-operatively in addition to the provision of adequate electrolytes/fluids. In preliminary testing, without the continuous infusion of fluids over the first 12 h post-operative period, there was a 100% incidence of irreversible hypovolemic shock presumably leading to cardiovascular collapse and/or arrhythmia. In animals receiving grafts treated with UW solution (either vascular flush alone or luminal flush), fluid management became secondary to poor graft quality. This was observed within several minutes following reperfusion as large regions of hemorrhage resulted in rapid fluid loss into the intraluminal space; all animals treated with UW solution died within 12 h. In contrast, grafts stored in the nutrient-rich solution, exhibited only minor congestion in the serosal/mucosal layers with no fluid collecting in the lumen; as long as vascular pressure was maintained there was consistent recovery from the operation over the first 24 h. Throughout the recovery period, all animals that survived the operation

exhibited good peripheral circulation, solid bowel movements with no diarrhea and a return to normal appearance and activity. The small bowel has a significant regenerative capacity and it has been shown that recovery of a normal intact epithelium occurs within 2-7 days after significant ischemic insult in the rat (51,52). Interestingly, normal mucosal architecture was observed in 60-80% of animals in the nutrient-treated group after the 3rd day post-transplant; all animals had normal mucosal architecture by 14 days. Despite the slight elevation in neutrophils recruited to the grafts even after 14 days, pharmacologic anti-inflammatory agents should be able to control this minor, yet persistent indication of localized inflammation.

Differences in post-operative survival between this study (notably in grafts only flushed vascularly with UW solution) and clinical outcomes are probably a result of several important constraints of an experimental setting: inherent species differences, and post-operative fluid/electrolyte maintenance. Tolerance to ischemic insult in rats is poorer than in larger animal models (pigs) or humans; histologically, maximum injury following extended storage is exhibited by Park's grade 5-6 (villus disruption & crypt infarction) in large animal models and human tissue compared to grade 7-8 (complete transmucosal & transmural infarction) in rodent bowel (11, 12). In addition to species-specific differences, other factors unique to an experimental study are difficult to control; particularly maintenance of post-operative electrolyte balance. Clearly, the clinical setting is stringently monitored and regulated whereas in an experimental setting, electrolyte balance is maintained via temporary electrolyte infusion either with bolus saline injections or IV infusion over the short-term. Complications of maintaining intravenous lines over the entire recovery period and animals removing IV lines is a constraint of this experimental setting not encountered with human recipients. Although

a shorter storage time (or hetertopic model) could have been selected to ensure survival in all experimental groups, rigorous testing of our novel solution necessitated an extended period of cold storage resulting in extensive intestinal injury. Our experimental design was intended to demonstrate a 'proof of principle' with respect to testing an experimental preservation solution and should be interpreted as such, without direct extrapolation to the clinical setting. Clearly, further studies particularly in a large animal pre-clinical model are required prior to direct translation to human tissue and outcomes in the clinic.

The use of amino acids in organ preservation has been tested experimentally in multiple organ systems [liver (53,54), heart (55), lung (56), pancreas (57)]. The supplementation of several key amino acids in preservation solution has generally focused on glycine, arginine, histidine as membrane stabilizers, nitric oxide donor, buffering agents although combined amino acid treatments has also focused on anti-proteolytic effects. The formulation of our amino acid-rich preservation solution is based on the in vivo requirements established specifically for the maintenance of intestinal metabolism (58). Our research over the past several years (10-12, 34-38) focusing on amino acids and intestinal preservation supports the involvement of enhanced ATP production (from gln, glu, asp) (30, 59) and antioxidant augmentation (due to the presence of precursor amino acids, gly, cys, glu/gln) (33) during storage, although there are theoretical grounds for the inclusion of antioxidant (Trolox; 60), xanthine oxidase inhibitor (allopurinol) (28), DNA repair enzyme, PARP inhibitor (3-aminobenzamide; 61), adenosine (purine substrate), precursors of growth-promoting polyamines (gln, arg, pro) (32), nitric oxide donor (arginine) (62). Although the requirement of each component has not been established on an individual basis, we have attempted to build on the work of

others in the field to develop an effective intestinal specific organ preservation solution. The results of the current study suggest that we are closer to the end goal of effective preserving small bowel for transplantation. Further investigation may demonstrate further benefit of this nutrient-rich solution when supplied enterally to the recipient in order to facilitate regeneration of their new organ.

In summary, the data presented in this study demonstrates that the strategy of administering a luminal flush with a nutrient-rich preservation solution improves overall graft viability when tested in a small animal model of orthotopic whole small bowel transplantation. This study represents an important test-bed of experimental proof for the beneficial effects of an intraluminal preservation solution tailored to the small bowel.

Table 9-1. Histologic grades of intestinal damage over 14 days post transplant.

Group	Time (reperfusion)	Grade	Median
Group 1	0 min	1,2,2,5,6	2
	35 min	4,5,5,6,7	5
Group 2	0 min	0,0,0,2,3	0
	35 min	5,5,5,5,6	5
Group 3	0 min	0,0,0,0,3	0
	35 min	3,3,3,3,4	3^{c}
	Day 3	0,0,0,0,4	0
	Day 7	0,0,0,2,4	0
	Day 14	0,0,0,0	0

c - Significantly different compared to Groups 1 and 2; $p<0.05$. Park's classification: 0 = normal mucosa; 1 = subepithelial space; 2 = extended subepithelial space; 3 = complete epithelial lifting; 4 = denuded villi; 5 = loss of villus tissue; 6 = crypt infarction; 7= transmucosal infarction; 8= transmural infarction.

Group 1, no luminal treatment; Group 2, treated with luminal UW solution; Group 3, treated with luminal AA solution.

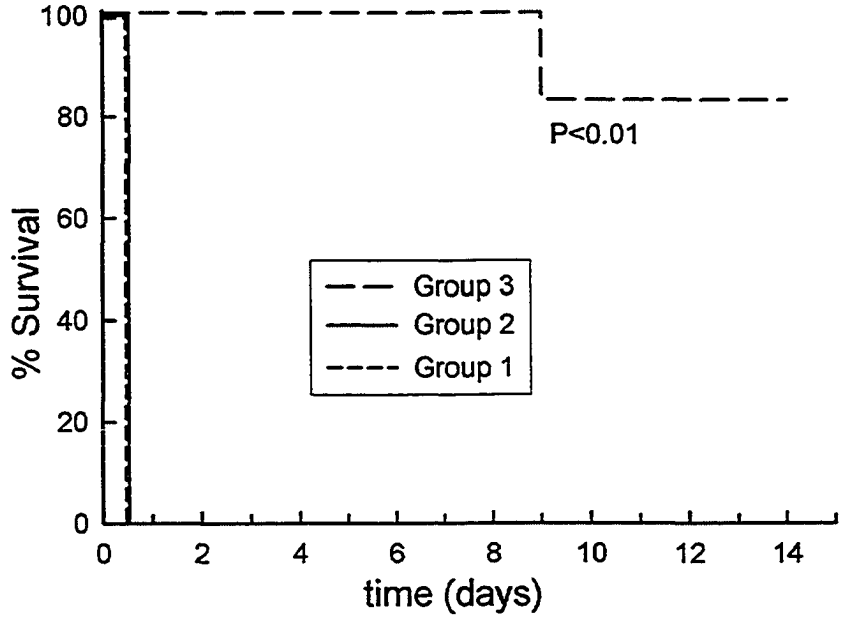

Figure 9-1. Survival rates after orthotopic small bowel transplantation.

Group 1, no luminal treatment; Group 2, treated with luminal UW solution; Group 3, treated with luminal AA solution.

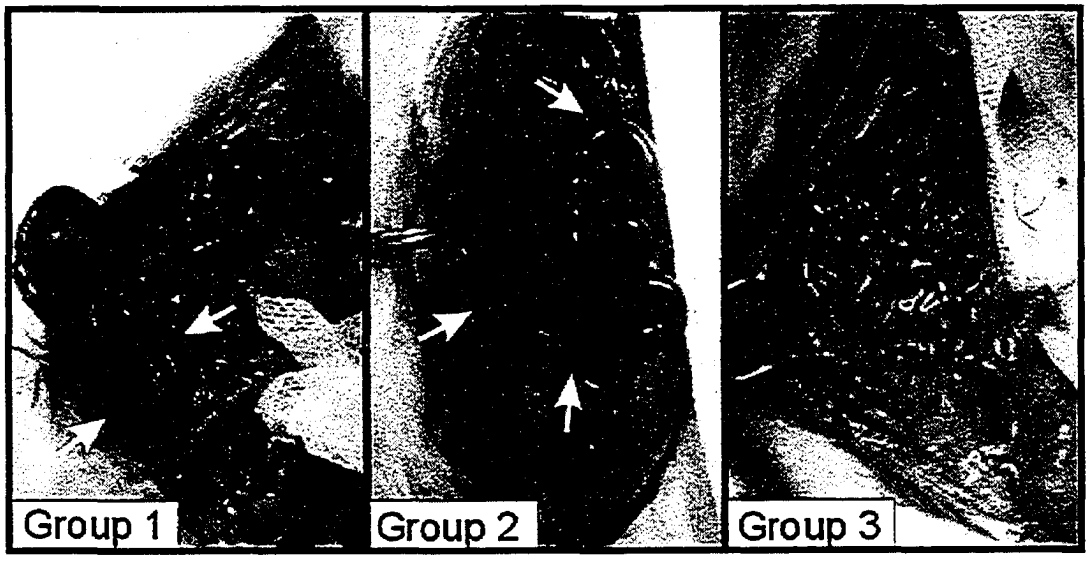

Figure 9-2. Gross appearance of small bowel following 35 min reperfusion post transplant. Intraluminal hemorrhage was prominent in Groups 1 & 2; indicated with arrows.

Group 1, no luminal treatment; Group 2, treated with luminal UW solution; Group 3, treated with luminal AA solution.

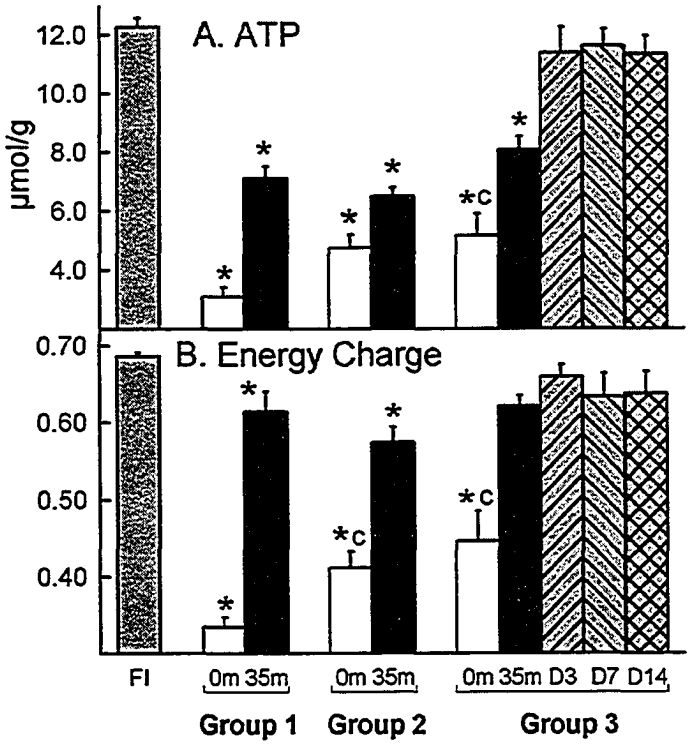

Figure 9-3. Effect of preservation solutions on levels of A) ATP and B) energy charge following transplantation. *, **c** -significantly different compared with freshly isolated tissues (FI) and group 1 (control), respectively, $p<0.05$.

Group 1, no luminal treatment; Group 2, treated with luminal UW solution; Group 3, treated with luminal AA solution.

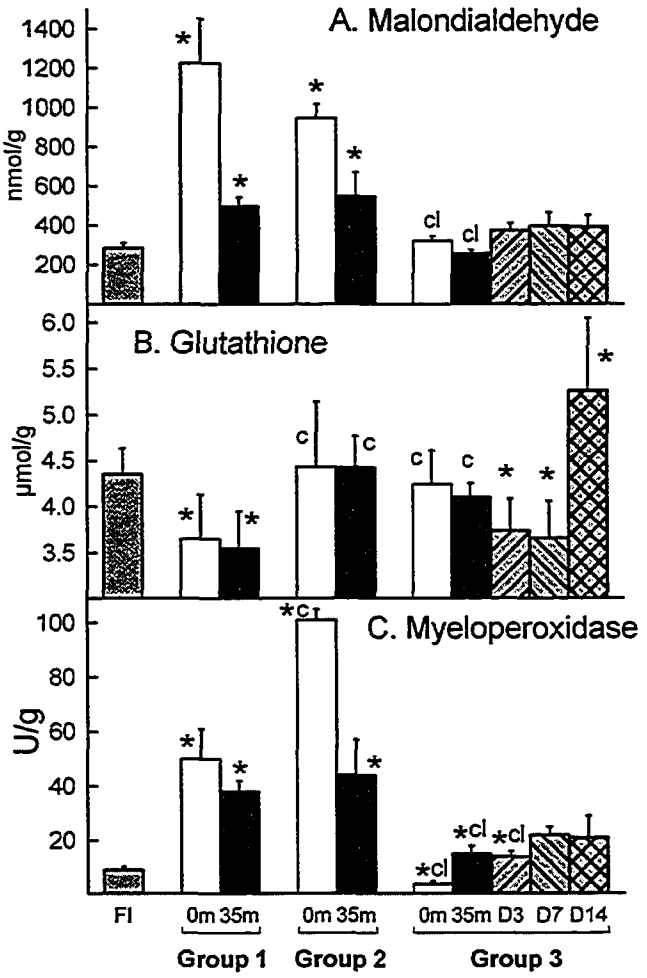

Figure 9-4. Effect of preservation solutions on levels of A) malondialdehyde (MDA), B) Glutathione, and C) Myeloperoxidase activity following transplantation. *, **c**, **I** - significantly different compared with freshly isolated tissues (FI), group 1 (control) and group 2, respectively, $p<0.05$.

Group 1, no luminal treatment; Group 2, treated with luminal UW solution; Group 3, treated with luminal AA solution.

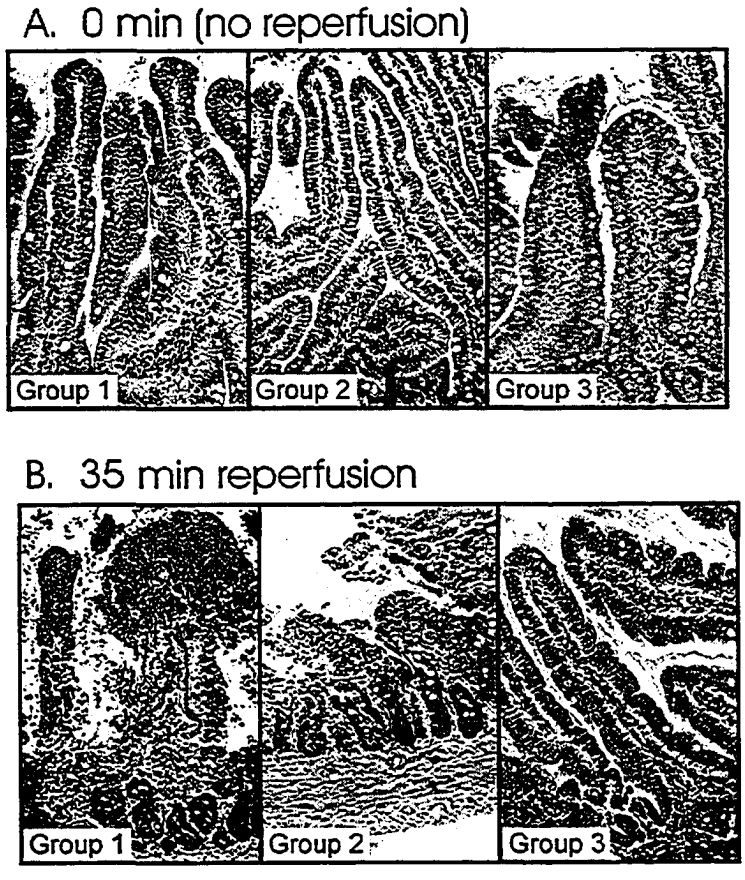

Figure 9-5. Histology of small bowel following: A) 6 h cold storage (t=0 min reperfusion). Blood flow was not yet established. B) 35 min reperfusion. 5 μm sections. Hematoxylin and eosin staining. 10X

Group 1, no luminal treatment; Group 2, treated with luminal UW solution; Group 3, treated with luminal AA solution.

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Chapter 10

Conclusion

The intestine is more difficult to transplant than other solid organs due to its strong expression of histocompatibility antigens (large numbers of resident leukocytes) and colonization with microorganisms. Despite the considerable improvements in surgical techniques and perioperative care, ischemia/reperfusion–induced small bowel injury remains a significant cause of early morbidity and mortality after small bowel transplantation.

Bowels that have been selected for transplantation are generally flushed vascularly with University of Wisconsin solution and hypothermically preserved to decrease their metabolic rate and energy requirement until implantation in the recipient. Currently, the period of cold ischemic storage is kept as short as possible. Although hypothermia is essential for organ storage, it is associated with a series of events such as cellular energy reserve depletion, oxidative stress, neutrophil recruitment, intracellular calcium overload, and induction of cell death that may induce upregulation of molecules on the cell surface membrane and the release of proinflammatory mediators that will eventually activate recipient leukocytes after reperfusion.

The preservation solution of choice for a small bowel graft is still controversial. Over the past decade, numerous studies have been performed to optimize the technique of small bowel preservation. Many different strategies for the prevention and treatment of ischemia/reperfusion injury have been introduced into experimental area and have translated into a reduction in the incidence of severe ischemia/reperfusion injury. Graft

injury incurred during storage begins with loss of mucosal barrier function and morphologic integrity, leading to failure of this natural barrier to isolate the sterile internal from the nonsterile external environment. Regardless of composition, vascular solutions have been unable to provide support for this critical barrier during clinically relevant periods of storage. Based on the abundant *in vitro/in vivo* preservation data, the requirement to use a luminal-delivered solution is strongly suggested. Also, based on the significant role of the selected amino-acids (i.e. gluthamine, glycine, arginine...) on mucosal protection, regeneration and proliferation, in this series of experiments, the effect of the nutrient-rich solution, recently developed in our lab, during small bowel cold storage were investigated.

In the first study, it was shown that the technique of hypothermic luminal perfusion with the oxygenated gold standard UW solution could provide better graft quality compared to the clinical standard, vascular flush with UW solution. However, physical injury incurred as a direct result of continuous mechanical perfusion (even at a slow rate); mucosal integrity was markedly superior with only a brief 1 h period of luminal perfusion. Considering this finding, a similar strategy was applied to examine the effect of oxygenated amino acid-rich solution on mucosal integrity in the continuous perfusion system. Our attempt to supply adequate oxygen and nutrients to the mucosal surface via continuous luminal perfusion at hypothermia was successful in terms of maintenance of metabolic homeostasis. However, mucosal integrity was again markedly superior with only a brief 1 h period of perfusion, presumably due to limited mechanical and oxidative stress.

As mucosal layer remains metabolically intact during storage, in a separate set of experiments, we examined the role of oxygen by combining two proven preservation

strategies involving perfluorocarbon (PFC) and our novel luminal amino acid-rich solution. There was a definite advantage of luminal UW solution in combination with PFC over control specimens. However, the same graft quality to PFC-treated was observed in tissues preserved with oxygenated amino acid-based solution. Despite marked differences in oxygen solubility of PFC and aqueous solutions, energy metabolism and its resultant effect on mucosal barrier and electrophysiology appeared to be equivalent in our small animal model of organ preservation.

In the next study, we moved further and examined the role of our novel nutrient-rich solution upon reperfusion. Apart from higher energetic values, AA-mediated protection during IR resulted in reduced neutrophil infiltration suggesting a weaker inflammatory response. Barrier function and electrophysiology parameters exhibited a clear pattern of mucosal preservation in AA-treated tissues; histology supported these findings. The only issue was that malondialdehyde level, an index of lipid peroxidation, was equally high in all experimental groups. Due to the huge impact of oxidative stress during I/R on mucosal damage, in another study, we tried to further inhibit oxidative damage to mucosal barrier during the period of cold preservation by addition of known antioxidants to our proven preservation solution. Our data demonstrated that preservation of small bowel in the amino acid-rich solution with low doses of vitamin E analogue, trolox significantly increased the graft energetics and function and reduced peroxidative damage during cold storage leading to a greater graft quality. This effect was more pronounced than that observed when superoxidase dismutase/catalase was added.

One factor which had not received much attention was the effect of nutritional status of the donor on graft quality. Moreover, it was questionable whether past experiments which had been often performed on fasted animals had not been influenced by reduced

nutritional status. Therefore, in an experiment, the quality of SB grafts from fasted and non-fasted animals was assessed throughout cold storage over 24 h. Surprisingly, poorer donor nutritional status did not affect energetics throughout storage, but caused mucosal injury as a result of increased oxidative stress, even after a brief period of donor fasting (12-14 h).

In the last experiment, as a final test-bed of experimental proof for our preservation solution, we found that the strategy of administering a luminal flush with nutrient-rich preservation solution markedly improved overall graft viability when tested in a small animal model of orthotopic whole small bowel transplantation after 6 hour cold preservation.

Better understanding of the mechanisms of ischemia/reperfusion injury, improvement in the technique of small bowel preservation, and introduction of this new preservation solution specifically developed for the bowels have helped to reduce the incidence of ischemia/reperfusion injury in experimental era. The results obtained from these series of experiments give an encouraging basis for further work in small bowel preservation. In the future, application of the nutrient-rich solution and the development of new agents and their application in prospective clinical trials are expected to prevent the occurrence of this potentially devastating complication and to further improve the success of intestinal transplantation.

Hopefully, this project will provide new insights into the mechanisms of injury and reveal potential new strategies to improve small bowel preservation and transplantation.