University of Alberta

Improving Small Bowel Preservation for Transplantation

By

David William Olson 

A thesis submitted to the Faculty of Graduate Studies and Research in partial Fulfillment of the requirements for the degree of Master of Science

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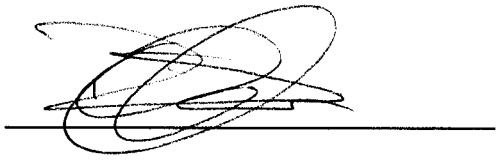

May 13, 2002

David William Olson

#206 9915-115 St.
Edmonton, Alberta
T5K 1S5

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled ***Improving Small Bowel Preservation for Transplantation***, submitted by **David William Olson** in partial fulfillment of the requirements for the degree of **Master of Science in Experimental Surgery.**

Dr. David Bigam (Co-Supervisor)

Dr. Richard Fedorak (Committee member)

Dr. Karen Madsen (Committee member)

Dr. Thomas Churchill (Co-Supervisor)

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Date of Completion

Abstract

Current SB preservation, comprised of vascular-delivered cytoprotective agents and hypothermia, fails to provide reliable graft storage for periods paralleling that of other transplanted intra-abdominal organs. This project addresses SB preservation injury using alternate cytoprotective solutions and/or techniques of administration.

Our initial experiment revealed that impermeant support, when added to simple crystalloid solutions, provided improved graft storage quality compared to the clinical standard, University of Wisconsin (UW) solution. The second study demonstrated that an appropriately selected buffering agent potentiates the benefits of glutamine-supplemented UW solution.

We then hypothesized that addition of a luminal-delivered solution would lead to improved preservation quality. Initially in a rat model, two luminal solutions containing glutamine plus 18 other amino acids, ± osmotic agent (lactobionate) and buffer (BES), were shown to provide significant improvements in energetics, barrier function and histology. Following this, we examined the simultaneous process of intestinal decontamination using Povidone-Iodine (PI). Each of the PI-treated groups however sustained greater levels of functional and morphologic injury compared to non PI-treated groups. The last of the luminal experiments addressed human SB preservation. Documenting improvement in functional and morphologic indices, this study again supported the use of luminal-delivered cytoprotective solutions as a simple and clinically-relevant addition to standard SB procurement techniques.

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Dedication

THIS THESIS IS DEDICATED TO MY FAMILY,
WILLIAM AND JUDY OLSON, DR. GREGORY AND LISA OLSON,
EDWARD AND SERENA OLSON, WHOSE NEVER-FAILING
SUPPORT CONTINUES TO ENRICH MY MEDICAL JOURNEY

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LIST OF ABBREVIATIONS

Adenosine Diphosphate	(ADP)
Adenosine Monophosphate	(AMP)
Adenosine Triphosphate	(ATP)
Ammonia	(NH_3)
Analysis of Variance	(ANOVA)
Arginine	(arg)
Aspartate	(asp)
N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid	(BES)
Bicarbonate	(HCO_3^-)
Calcium	(Ca^{2+})
Carbon dioxide	(CO_2)
Centimeter	(cm)
Chloride	(Cl^-)
Cysteine	(cys)
Cyclic Adenosine Monophosphate	(cAMP)
Ethylenediamine tetraacetic acid	(EDTA)
Electrical Charge	(EC)
Euro-Collins solution	(EC_s)
Glutamate	(glu)
Glutamine	(gln)
Glycine	(gly)
Gram	(g)
Histidine-Tyrptophan-Ketoglutarate	(HTK)

Hydrochloric acid	(HCl)
Hydrogen Phosphate	(HPO_4^{2-})
Hydroxyethyl Starch	(HES)
Intestinal Short-Circuit Current	(Isc)
Leucine	(leu)
Lysine	(lys)
Magnesium	(Mg^{2+})
Methionine	(met)
MilliSiemens	(mS)
Millimole	(mM)
Milliosmole	(mOsM)
Molecular Weight	(MW)
Nicotinamide Adenine Dinucleotide	(NADH)
Nitric Oxide	(NO)
Nuclear Magnetic Resonance	(NMR)
Oxygen Free Radical	(OFR)
Phosphate Buffered Saline	(PBS)
Potassium	(K^+)
Potassium Chloride	(KCl)
Potassium Hydroxide	(KOH)
Potential Difference	(PD)
Povidone-Iodine	(PI)
Proline	(pro)
Scanning Electron Microscopy	(SEM)
Serine	(ser)

Small Bowel	(SB)
Sodium	(Na^+)
Sodium Chloride	(NaCl)
Sodium Hydroxide	(NaOH)
Standard Error	(SE)
Threonine	(thr)
Total Adenylates	(TA)
Total Parenteral Nutrition	(TPN)
Transmission Electron Microscopy	(TEM)
Trichloroacetic acid	(TCA)
Tris-[hydroxymethyl]amino-methane	(Tris)
Tryptophan	(tryp)
University of Wisconsin solution	(UW)
University of Wisconsin solution + BES	(UWB)
University of Wisconsin solution + glutamine	(UWG)
University of Wisconsin solution + BES + glutamine	(UWBG)

Chapter I

Intestinal Failure: Clinical Problem, Management Options

Introduction

Intestinal failure is defined as the inability of the bowel to maintain nutrition, fluid and/or electrolyte homeostasis while on an enteral diet. If untreated, patients with this diagnosis develop variable but progressive degrees of malnutrition, acid/base disturbances, and dehydration. Up until the last 50-60 years, treatment options remained few, resulting in terminal prognosis for the majority of patients. The prevalence of intestinal failure continues to increase however, as advances in surgical techniques, post-operative care and nutritional support are made.

Intestinal failure affects both pediatric and adult populations (see Table 1-1). Etiologies are divided into mechanical and functional causes, with further division into the following sub-groups:

1) short bowel syndrome

2) defective intestinal motility

3) impaired enterocyte absorption

Short bowel syndrome, which results from extensive surgical resection, is by far the most common cause of intestinal failure in both the adult and pediatric populations. In adults, common causes of short bowel syndrome include mesenteric vascular accidents, inflammatory bowel disease (ie Crohn's), and locally invasive tumors involving the superior mesenteric artery. The pediatric population is also affected by mechanical etiologies of short bowel syndrome with gastroschisis, intestinal atresia, midgut volvulus and necrotizing enterocolitis being the most common. Although significantly less

frequent in overall occurrence, a number of functional disorders exist which similarly result in failure of the intestine to meet daily nutritional and fluid requirements. Some of these include radiation enteritis, microvillous inclusion disease and intestinal aganglionosis.

In order to understand intestinal failure, a concept of clinically-targeted intestinal structure and function is required. Human small bowel length varies substantially, measuring an average of 620 cm with a range of 300-850 cm (1,2). The residual length of bowel which results in clinical manifestations of intestinal failure is itself quite variable. A number of independent factors must be jointly considered including: a) extent and location of resection, b) presence/absence of the ileo-cecal valve and colon, as well as c) patient-specific factors. In practice, it is generally accepted that if the small bowel measures 200 cm or greater, it is likely that adequate nutrition can be maintained without requiring any form of supplementation (3). In order to remain independent of long-term parenteral nutrition however, studies have shown that a minimum of 70 cm of small intestine is required if the colon is present or 120 cm if the colon is absent (4). These numbers are controversial as some report up to 40% of patients, possessing less than 50 cm of small bowel in continuity with colon, being successfully weaned from parenteral nutrition (5). This fact introduces the next important factor; the presence of a colon in continuity with the residual small bowel.

The colon provides additional surface area for fluid absorption resulting in a lowered incidence of intractable diarrhea (6). It also provides an alternate metabolic site, where an additional 5-10% of caloric intake is provided by the metabolism of short-chain fatty acids (7). In addition to the importance of colonic continuity, the presence of an ileo-cecal valve provides multiple physiologic benefits. One of those benefits is to provide a barrier to colonic bacterial overgrowth within the remaining small bowel so as

not to exacerbate malabsorption. More importantly, the ileo-cecal valve provides an important physiologic delay to intestinal transit time. This "ileal brake" assists in maximizing contact time between enteric contents and the remaining absorptive mucosal surface.

Another consideration impacting the management of patients with intestinal failure is the site of intestinal resection. In its normal state, the native jejunum possesses a higher absorptive capacity then the ileum. However, following resection, residual jejunum has been shown to be far less likely to undergo structural and functional adaptation then the ileum (8).

In conclusion, it is apparent that individual assessments must be made in order to effectively treat patients with intestinal failure. It is through the initial study of the physiologic and anatomical capabilities of the small bowel that clinicians and researchers gain insight into improving treatment modalities.

Treatment Options

Medical Treatment

Intestinal failure is predominantly managed by non-surgical techniques. Control of diarrhea, replacement of fluid losses and maintenance of nutrition are paramount to successful management. Medical treatment of intestinal failure has improved dramatically over the past few decades as new pharmacological agents and methods of nutritional supply have been developed. Equally important to this is an improved understanding of the remaining bowel's ability to undergo both structural and functional changes following intestinal resection. This spontaneous process, referred to as intestinal adaptation, is now known to begin within 12-24 hours of resection and continues for a variable period lasting upwards of 1-2 years (5). Structurally, the

remaining bowel elongates and dilates. In addition, residual villus height and crypt depth increase, along with increases in both epithelial cell proliferation and brush-border enzyme activity (9). Along with these structural changes, slowing of both gastrointestinal transit time (10) and gastroduodenal emptying (11) occur.

As the intestinal adaptation period is lengthy and variable, management must be individualized. Following massive SB resection, most patients are unable to tolerate oral intake. As such, treatment largely focuses on maintenance of fluid, electrolyte, and acid/base status. Within 1-2 weeks on average, patients can usually initiate oral ingestion. Typically, only small volumes of isotonic fluids are tolerated, as diarrhea and abdominal pain are otherwise exacerbated in the presence of alternate enteral nutrition. It is now realized that enteral autonomy is best achieved through early enteral nutrition, as it acts as a potent mucosal growth stimulator (12).

A sub-group of the intestinal failure population fail to maintain a stable body weight on oral intake alone. Prior to 1968, the majority of these patients eventually succumbed to malnutrition and dehydration. Total parenteral nutrition (TPN), a treatment modality which revolutionized the management of intestinal failure became available at this time (13). This process, as its name suggests, provides total basal nutrient requirements via central venous access. The development of TPN, and more recently, the introduction of home parenteral nutrition programs, has enabled patients to survive a once fatal disease under reasonable conditions. In addition to the advantage of patients being allowed to remain largely outside the hospital setting, survival rates can range as high as 90% at 5 years. Despite its success, cost as well as potentially fatal complications do arise from chronic parenteral nutrition. These complications include: Development of cholestatic liver disease, loss of venous access, and line sepsis.

Compared to adults, liver disease tends to be more severe in the pediatric population, resulting in liver failure even within a few years of parenteral nutrition (14).

Surgical Treatment

Surgical approaches to intestinal failure generally remain reserved for those patients who fail medical management. These approaches can be divided into transplant and non-transplant procedures.

Non-transplant related procedures are aimed at optimizing intestinal function and/or prolonging intestinal transit time. A variety of functional procedures have been used including stricturoplasty for benign strictures, serosal patches for perforations, as well as tapering and lengthening procedures for dilated bowel loops (15). More intricate procedures aimed at optimizing intestinal functional status have proved largely anecdotal, as success rates remained low even in the hands of the inventor (16). Procedures aimed at prolonging intestinal transit time have also been investigated. These include: reversed intestinal segments (17), colonic interposition (18), and artificial sphincters (19). Again, these procedures remain largely unproven and are seldom used. As such, they are beyond the scope of this project and will not be discussed further.

The first report of experimental small bowel transplantation occurred in 1902 (20). At this time, Dr. Alexis Carrel showed that canine small bowel could remain viable when attached to the great vessels of the neck. The feasibility of small bowel transplantation only really became realized in 1959, when Lillehei showed that intestinal function could be maintained following allogeneic canine small bowel transplantation despite neural and lymphatic disruption (21). Following the success of early renal transplantation, Lillehei performed the first published human intestinal transplant at the University of Minnesota in 1967. Unfortunately, technical complications, graft rejection, and on-going poor survival rates, resulted in decreased clinical interest (22).

Furthermore, with the introduction of TPN in 1968, interest in small bowel transplantation for treatment of intestinal failure waned.

Improved survival in large animal models, largely as a result of combining new immunosuppressive agents (cyclosporine), began occurring in the early 1980's (23). However, combining cyclosporine in human clinical small bowel transplantation showed initial disappointing results (24-26). As a result, up until 1989, less than ten human small bowel transplants were performed annually throughout the world.

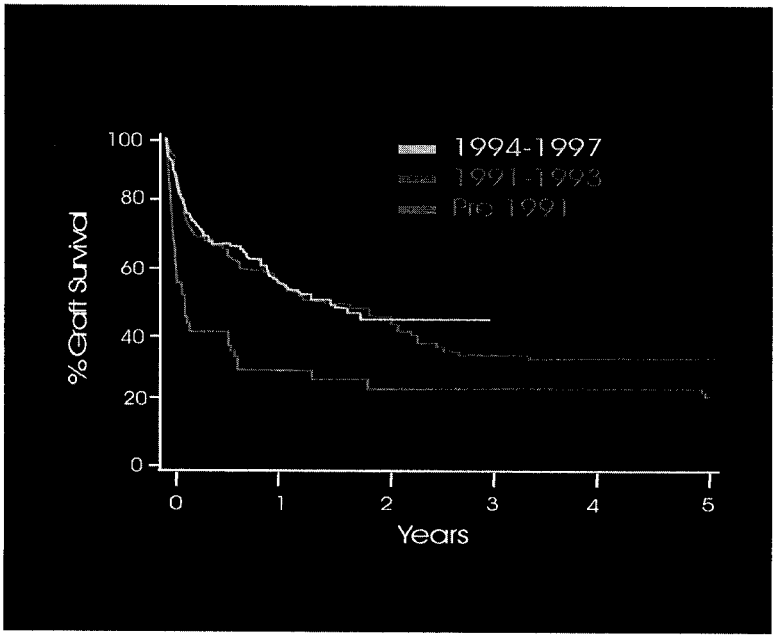
Current Global Status of Small Bowel Transplantation

Improved surgical techniques along with new immunosuppressive agents have led to steady improvements in small bowel graft and patient survival following small bowel transplantation. Worldwide intestinal transplantation results have been recently reviewed (27). From 33 intestinal transplant programs, a total of 273 transplants in 260 patients have been performed. Types of transplants included intestine only (n=113; 41%), intestine plus liver (n=130; 48%), and multi-visceral grafts including stomach, pancreas, intestine, and liver (n=30; 11%). Despite a reported mortality rate of 50%, 77% of the remaining patients (n=95) were off TPN. Registry data revealed that the number of small bowel transplants has continued to increase annually since 1990. More importantly, there was a consistent trend toward improvement in graft survival; Pre-1991 (~21% 5-yr), 1991-1993 (~33% 5-yr), and 1994-1997 (~46% 3-yr); see Figure 1-1. Overall, one-year patient and graft survival rates for small bowel transplantation are reported as approximately 70% and 60%, respectively (28). Current reported rates of patient survival on home parenteral nutrition are reported as approximately 85% at three years (29). This number indicates that most individuals do well on TPN. As a result, small bowel transplantation currently remains warranted only when standard therapies fail.

Table 1-1 Etiology of Intestinal Failure

Mechanical (Short Bowel)	Functional
Children	
Gastroschisis	Idiopathic pseudo-obstruction
Intestinal atresia	Microvillus inclusion disease
Malrotation/midgut volvulus	Intestinal aganglionosis
Necrotizing enterocolitis	Visceral myopathy
	Autoimmune enteritis
Adults	
Mesenteric ischemia	Crohn's disease
Volvulus	Radiation enteritis
Trauma	Idiopathic pseudo-obstruction
Desmoid tumor	
Gardner's disease/familial	
Polyposis	

Figure 1-1 Small Bowel Graft Survival vs Period of Transplant



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Chapter 2

Small bowel preservation injury:

Role of vascular-delivered preservation solutions

Background

Like other organs, the small bowel is subjected to variable lengths of ischemia during the transplant process. This insult is inevitable, as even immediate transplantation is necessarily accompanied by short periods of both warm and cold hypoxia. Clinically, the ability to extend storage periods further assists in maximization of donor pools and allows for identification of the most suitable recipient. To accomplish this, it has been suggested that organs would have to be effectively preserved for periods extending up to 24 hours (1). Extending storage periods for up to 24 h must be balanced with the time-related effects of this stress incurred by the organ. Intuitively, minimizing ischemic damage will result in healthier organs which are able to re-establish homeostasis and function more quickly following transplantation. This has been well documented as organs subjected to shorter ischemic periods possess better initial graft function (2-4) and lower incidences of complications in the immediate post-transplant setting (5). Regardless of the organ in question, the ability to effectively preserve both structural and functional status throughout clinically relevant periods of cold storage is integral to successful transplantation.

The effects of low-temperature storage have been thoroughly studied. Belzer and Southard summarized five key processes that necessarily affect tissues subjected to periods of low-temperature storage (6).

They include:

1) hypothermic-induced cellular swelling

2) intracellular acidosis

3) expansion of the interstitial space during reperfusion

4) formation of oxygen-free radicals

5) depletion of high-energy phosphate substrates

They went on to discuss the most probable causes for development of each of these five processes. Firstly, hypothermic-induced cellular swelling occurs due to suppression of the activity of the Na^+/K^+ ATPase, located on the basolateral membrane of individual cells. This is secondary to the effects of hypoxia (7), which leads to impairment of aerobic cellular metabolism and resultant loss of high-energy phosphates (ATP). With progressive depletion of high-energy phosphate pools, influx of Na+ (normally a relative impermeant in the presence of a functioning Na^+ pump) and water ensue. Additional explanations for cellular swelling include alterations in normal intracellular oncotic pressure. Normally, the oncotic pressure found within the cell is comprised of intracellular proteins and impermeable anions, measuring approximately 110-140 mOsm/kg (8). Supplying at least this level of impermeants would then theoretically retard fluid shifts.

Intracellular acidosis is thought to occur due to the cell's progressive reliance on anaerobic metabolism (glycolysis) which generates lactic acid and hydrogen ions. Alterations in intracellular pH can the lead to cell membrane damage, alteration of organelle structure and function (ie mitochondria), and perpetuation of lysosomal instability.

Interstitial space expansion can occur both at procurement (following initial vascular flush) and during reperfusion. The result of expansion of this space can

potentially compress capillaries resulting in non-reliable contact with the flush solution at the time of procurement or exacerbation of the cellular damage at the time of reperfusion.

Oxygen free-radicals (OFR), thought to be one of the key components of heightened graft injury following reperfusion, affect all transplanted organs. This has proved critical to reperfusion injury sustained by the small bowel as the intestine is rich in native xanthine dehydrogenase, a mucosal enzyme required for formation of OFR's. Lastly, ATP is required for energy consuming pathways that help to maintain the integrity of the cellular membrane including previously discussed energy-dependent pumps. All of the above explanations then point to the necessity for tailored solutions to maximize preservation.

Transplanted organs are variably affected by these five basic principles of low-temperature storage. This would imply that preservation techniques must be tailored to the individual organ. In addition to these principles, effective small bowel storage must also consider some of the unique characteristics of the intestinal graft. These include:

1) Intestinal mucosa is extremely sensitive to any ischemic insult (9-11). As with other organs, progression of damage occurs during cold storage (12).

2) There is chronic exposure to an external, non-sterile environment.

3) The mucosal surface is continually bathed by pancreatic, biliary, and other proteolytic enzymes. Each of these can exacerbate damage during storage (13).

Prior to exploring small bowel preservation techniques, the morphologic changes experienced by the intestinal graft during ischemic periods must be discussed. Early on, the majority of assessments were made through histologic analysis following trial periods of mesenteric ischemia in animal models.

The response of the small bowel to ischemic insults has largely been documented using warm ischemia models (14,15). Morphologic damage has been shown to be reproducible and time-related (16). Within minutes of the onset of ischemia, structural changes are noted to reliably begin at the villus tip. Although the epithelial cells themselves do not appreciably change in size, the basal intercellular space, located above the basement membrane, begins to swell. This swelling has been studied using electron microscopy, revealing the cause to be the formation of blebs within the epithelial cells which are eventually extruded into the lumen as well as the intercellular space (17). These cytoplasmic blebs, eventually loosen the epithelium from its attachment to the basal lamina. Providing the ischemic insult continues, a sub-epithelial space progressively develops from the tip to the base of the villi. This sub-epithelial space, referred to as Gruenhagen's space, is thought to be a manifestation of villus hypoxia, with secondary movement of fluid from both the vasculature (18) as well as the lumen (19). Eventually, the epithelial cells are extruded into the lumen, albeit attached and metabolically intact (20). As the morpholgic changes of the intestine are reproducible, grading methods have been developed to establish a standard process by which ischemic injury can be described. One of the most commonly used of these grading systems is the Park's classification of intestinal ischemia (see Table 2-1); (21).

Small Bowel Preservation Techniques:
Hypothermia and vascular-delivered preservation solutions

Early small bowel preservation models utilized hypothermia alone. Simple in application, the benefits of hypothermia are largely founded on the fact that tissues exposed to cooler temperatures have lowered oxygen demands. Lillehei et al documented similar rates of success in canine autotransplantation following 5 hours of hypothermic storage and 2 hours of warm ischemia (22). Even up to the 1980's, hypothermia as a sole means of intestinal preservation was being studied. In fact, Raju

et al reported a 67% five-day survival rate in a canine allotransplant model using bowel stored for 24 hours at 4°C (23). Closer review of their experimental methods however revealed that vascular and luminal-administered Ringer's Lactate had been simultaneously administered at the time of procurement. Ultimately, hypothermia alone has proven ineffective at preventing graft damage throughout longer periods of ischemia.

Intravascular Solutions

Apart from simple hypothermia alone, a variety of preservation techniques have also been tested. Hyperbaric oxygen (24), continuous perfusion models (25,26), and administration of various pharmacological support agents (27) have been used in an attempt to improve overall small bowel preservation quality. To date, the determinants for assessing the quality of preservation has been quite varied. The success of these various solutions are typically graded based on biochemical (28), histological parameters (17), and overall percentage graft-survival (29).

The most promising technique however employs intravascular delivery of cytoprotective agents. To date, the introduction of preservation solutions in combination with hypothermia has made the greatest contribution to the progress achieved in transplantation of solid organs. Single solutions now exist which provide reproducible tolerance to the effects of cold ischemia extending up to 24 h in liver and pancreas and up to 48 h in kidney (30-34).

Belzer and Southard's description of components thought to be necessary constituents of an ideal vascular preservation solution would suggest that the use of simple solutions would be inferior to more "tailored" solutions. In practice, no one solution has been unequivocally accepted for the preservation of small bowel. As such, a number of solutions have been formulated and used both in the laboratory as well as the clinical setting (see Table 2-2). Simple crystalloid solutions, including Normal Saline

and Ringer's Lactate, have been shown to be as effective as more intricate solutions (29,35). Conversely, Belzer and Southard, with the introduction of their University of Wisconsin solution (UW) in 1968, showed that organ preservation could be extended without jeopardizing graft structure and function (36). Originally marketed for the purpose of pancreatic preservation, UW solution has now become the "gold standard" preservation solution for the liver, kidney, and pancreas. Some studies have also shown that UW solution is far more effective than crystalloids for SB preservation. Thaler et al showed that no appreciable difference in overall survival existed regardless of solution used as long as ischemic time was limited to 30 minutes. However, differing from Schweizer et al (29) was the UW preserved group showing consistently better survival and histologic preservation on inter-group analysis out to 18 hours (37). Equally important was the fact that Normal-Saline was shown to be ineffective beyond six hours of preservation. This study suggested that there must be time related ischemic changes within the preserved bowel. If ischemic time was kept short, then it appeared that virtually any form of intravascular flush, crystalloid or not, could be used with equivalent results. Other proponents of UW performed biochemical analysis by measuring total adenine nucleotides and oxygen consumption as a predictor for small bowel survival (38). Segments stored in UW solution retained significantly higher total adenine nucleotide levels as well as greater capacity for oxygen utilization compared to those stored in either Euro-Collins or Normal Saline following 24 hours of storage.

Additions to vascular-administered preservation solutions

Research to date remains equally polarized with some supporting the use of simple crystalloids and some supporting the use of solutions like UW. The work of Ito et al addressed the fact that SB mucosal damage could be specifically targeted if additional substances, aimed specifically at the mucosa, were added to the preservation solution

(39). This group was one of the first to describe that provision of intra-arterial substrates had cell-specific effects within the intestinal mucosa. They documented that there was preferential uptake of vascular-delivered substances into crypt and junctional villus cells whereas luminal-administered substances showed preferential uptake into apical villus epithelial cells.

One substance receiving significant attention is glutamine, a non-essential amino acid which is the primary fuel source of the enterocyte (40-42). If glutamine is an important metabolic source, would inclusion of this substance help maintain the mucosa, especially in a cold ischemic model? Part of the answer came from studies that had shown that intestinal mucosal atrophy occurs in patients treated with non-glutamine supplemented parenteral nutrition (43,44). It was O'Dwyer et al who then documented a dose-response relationship between jejunal DNA and supply of glutamine-enriched parenteral nutrition (45). At a dose of 2g/100ml, mucosal atrophy was documented to be nearly completely prevented. Sasaki et al subsequently applied this finding to an intestinal preservation model showing that the addition of 2% glutamine to either UW solution or Normal Saline significantly improved function (determined by glucose transport level) and overall histologic damage (46). Along with the reduction in the level of injury incurred during cold storage, further reductions in reperfusion injury were also seen. Admittedly, they could not explain the exact mechanism by which glutamine had provided this protective effect. It was speculated that glutamine provides an important energy source during ischemia (assuming some degree of basal metabolism during hypoxia), or that glutathione production (an important antioxidant) had been stimulated. However, levels of this important antioxidant were not measured to support this. Lew et al also looked at the effect of glutamine on preservation and reperfusion of cold stored small bowel (47). Improved function (measured by glutaminase activity) and decreased

histologic damage was once again shown. Of note, similar to Sasaki et al, both intravascular and intraluminal flush had been performed.

Factors Affecting Intravascular Flushing

One remaining variable within the intravascular preservation model for small bowel exists which could be manipulated. The method by which intravascular preservation solution is applied may be important. Van Oosterhout et al investigated the effect of increased pressure during the vascular flushing procedure of the graft (48). They determined was that flushing pressures in excess of 35 cm H_2O were noted to be harmful to the jejunal graft. Histologic damage was markedly decreased at this pressure whereas 50 cm H_2O and 80 cm H_2O flushing pressures resulted in increasing amounts of edema and ischemic necrosis of the graft. Mueller et al looked at the effect of vascular washout, rewarming, pH, and preservation temperature on graft and recipient survival (49). They determined that a preservation temperature of 4°C resulted in slight improvement of overall survival compared to 8°C. A vascular washout just prior to reperfusion was noted to be detrimental to overall graft survival. A pH of 6.8 showed slight improvement in graft and recipient survival compared to a pH of 7.4. Interestingly, a period of re-warming prior to reperfusion significantly increased overall recipient survival.

Conclusion

An effective clinical preservation model is one that is both effective and practical. To date, the most effective small bowel preservation technique includes simple hypothermia and administration of vascular-delivered flush solutions. To date, UW and in some centers, Euro-Collins solution, remain the most commonly used vascular-administered cytoprotective solutions used for human small bowel preservation (50,51). This remains the case as the clinical confines of multiviscera procurement require the

use of a common vascular perfusate. And yet, acceptable cold storage times remain significantly shorter than that of other transplanted intra-abdominal organs. Clearly, steps to advance the quality and length of small bowel preservation must be continued. Equally important is the need for alternate methods and techniques to remain practical in their application. The following two chapters will address, via vascular-delivered cytoprotective solutions, two independent requirements for minimizing small bowel graft injuiry during cold storage:

1) The requirement for osmotic and oncotic impermeant agents
2) The maintenance of mucosal metabolism with exogenous glutamine and buffer.

Table 2-1 Park's Grade of Intestinal Tissue Ischemic Injury

Grade	*Description*
0	Normal mucosa
1	Subepithelial space at villus tip
2	Extended subepithelial space
3	Epithelial lifting along villus sides
4	Denuded villi
5	Loss of villus tissue
6	Crypt layer infarction
7	Transmucosal infarction
8	Transmural infarction

Table 2-2 Composition of Common Intravascular Preservation Solutions

	Normal Saline	Lactated Ringer's	UW	EC	HTK
Anions					
Bicarbonate				10	
Chloride	154	109		15	46
Lactate		28			
Phosphate			25	57.5	
Lactobionate			100		
Sulfate			5		
∝-ketoglutarate					1
Cations					
Calcium		1.5			0.015
Sodium	154	130	30	10	15
Potassium		4	125	115	10
Magnesium			5		4
Additional Osmotic Agents					
Hydroxyethyl starch			5g/l		
Raffinose			30		
Glucose				198	
Mannitol					30
Others					
Adenosine			5		
Glutathione			3		
Insulin (I/U)			40		
Allopurinol			1		
Histidine					198
Tryptophan					2
Antibiotics & Steroids					
Penicillin (I/U)			200,000		
Dexamethasone			16 mg/l		
Osmolality(mOsm)	280	273	320	365	300
pH	~5.5	6.3	7.4	7.4	7.3

Unless otherwise noted, all numbers in mM. UW – denotes the University of Wisconsin solution developed by F.O. Belzer and J.H. Southard. HTK – denotes the 'Histidine-Tryptophan-Ketoglutarate' based solution developed by Bretschneider. EC_s – denotes the 'Euro-Collins' solution developed by Collins.

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CHAPTER 3

The importance of impermeant support in small bowel preservation: A morphologic, metabolic, and functional study

Introduction

No one solution has repeatedly proven itself to be superior in the preservation of small bowel (SB) (1-3). As a result, preservation periods span only 6-10 hours with variable degrees of documented tissue injury (4). Most SB transplant programs continue to use University of Wisconsin (UW) solution as the vascular perfusate of choice. Despite its overall acceptance, others have noted equal or improved preservation with alternate solutions such as Normal Saline (5, 6). This suggests that SB may not adhere to all principles upon which UW was formulated. One key aspect of UW solution, namely oncotic and osmotic support, may prove critical to successful SB preservation. This is based on the fact that reproducible time-related histologic events occur during its storage. One of the earliest changes seen is the formation of sub-epithelial clefts (Gruënhagen's space). Development of these sub-epithelial clefts has been studied and attributed to fluid shifts into the intercellular space resulting in alteration of cell adhesion properties (7). Some suggest a luminal origin (8) while others point to fluid shifts from the intravascular space secondary to the effects of anoxia on capillary walls (9). Preventing Gruënhagen space formation may be the required step for overall improved SB preservation. The goal of this study was to determine if impermeant support is critical for prolonged SB preservation.

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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 either Sigma Chemical Company (Oakville, Canada) or BDH (Dorset, UK).

Surgical procedure and procurement of the small intestine: Rats were fasted overnight. Water was provided *ad libitum*, and cage floors raised to minimize coprophagy. At the time of laparotomy, rats were administered an interperitoneal dose of 65mg/250g pentobarbital (Somnotol; MTC Pharmaceuticals, Cambridge, Ontario, Canada), followed by inhalational halothane (1-2%) as required to achieve and 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 the individual preservation solution 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 then harvested. No intraluminal solution was administered at any time. The bowel was stored in 30 ml of ice-chilled preservation solution. A 1-2g tissue sample were taken at t=0, 1, 2, 4, 10 h. To arrest metabolic activity, samples were snap frozen in liquid nitrogen, and subsequently stored at -65°C until processed.

Composition of preservation solutions: Four groups, each comprised of four rats, were subjected to an isolated vascular flush using the solutions listed below. Each solution was adjusted to a pH of 7.4 using NaOH prior to administration and osmolality was measured using the freezing point depression method.

Group 1 - Normal Saline (0.9%) (154mM NaCl); 280 mOsM (±0.2%)

Group 2 - 154 mM NaCl + 5% Dextran (Mw=67,300 daltons); 300 mOsM (±0.3%)

Group 3 - 104 mM NaCl + 100mM Lactobionate + 5% Dextran; 400 mOsM (±0.4%)

Group 4 - UW – University of Wisconsin solution; 320 mOsM (±0.3%)

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 000g). Acid extracts were neutralized by the addition of 3 M KOH/ 0.4 M Tris/ 0.3M KCl and then recentrifuged (20min at 14 000g). Aliquots of the neutralized extracts were immediately used for ATP, ADP, and AMP assays. The remaining extract was frozen at –65°C. Metabolites were assayed enzymatically based on absorbance of NADH at 340nm, using an MRX microplate reader. Values are reported per gram wet weight. Assays were performed as described previously (10).

Ussing chamber study–in vitro electrical measurements: Ileal segments were taken at 4 h and 10 h cold storage, stripped of their serosa and muscular layers, and mounted in Ussing chambers. When mounted in these chambers, the mucosal and submucosal surfaces were perfused at 37°C with a recirculating solution containing bicarbonate Ringer's solution, with an ionic composition of: Na^+, 143 mM; K^+, 5 mM; Mg^{2+}, 1.1mM; Ca^{2+}, 1.25 mM; HCO_3^-, 25 mM; Cl^-, 123.7 mM; HPO_4^{2-}, 0.3 mM; and fructose, 20 mM with 95% O_2 and 5% CO_2, and pH=7.4. Transmural intestinal short-circuit current (Isc) were then measured over a surface area of 0.9 cm^2 (11).

Mannitol permeability: A Ussing chamber was used to measure the permeability of mannitol in each ileal specimen. 10 µCi [^{3}H]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 twenty minutes 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 (12).

Statistical analysis: Metabolite and permeability data were reported as means ± SE for each group where n=4. Statistical differences within each group was determined using ANOVA, followed by Dunnett's *post hoc* comparison test; $p<0.05$ was reported. Inter-group significance was assessed using an ANOVA test followed by Student-Newman-Keuls test; $p<0.05$ was reported.

RESULTS

Energetics

ATP (Fig. 3-1) Within 1h of storage, only Group 1 (154mM NaCl) showed a significant drop in ATP levels (1.70 μmol/g to 1.35 μmol/g, $p<0.05$). By two hours of preservation, Group 2 was the only group that lacked a significant decrease in ATP levels compared to freshly isolated specimens, 1.52 μmo/g and 1.70 μmol/g respectively. Beyond 4 hours, all groups exhibited a progressive decline in ATP levels. Transient increases in Group 2 and UW were noted but disappeared at subsequent time points. ATP levels were maintained at significantly higher levels in Group 3 by 10 hours of preservation compared to all other groups (0.84 vs 0.66, 0.62, 0.70 μmol/g respectively, $p<0.05$).

ATP/ADP ratio (Fig. 3-2) Compared to ATP alone, this ratio provides an additional parameter reflecting the potential for oxidative phosphorylation. Within 1 hour, all groups except for Group 3 exhibited significant declines compared to freshly isolated specimens. After 2 hours, storage in UW resulted in the lowest values of the experimental groups ($p<0.05$). Group 3 maintained greatest levels for each time point and even following 10 h storage was not significantly less than that of freshly isolated specimens.

Total Adenylates (TA) (Fig. 3-3) Total Adenylate levels were calculated based on the sum of ATP+ADP+AMP. All groups except those preserved with UW exhibited a progressive decline in total adenylates over the course of preservation. UW possessed consistently higher TA levels at each time point compared to all other groups. By 10 hours, Groups 2 and 3 compared for lowest TA levels measuring 1.62 μmol/g each; this value was less than Group 1 or UW (2.1 and 4.0 μmol/g respectively, $p<0.05$).

Energy Charge (Fig. 3-4) Energy Charge (EC) is calculated as [(ATP+ADP/2)/TA] and reflects the level of high-energy phosphates in relation to total adenylates (13). Group 1 and UW exhibited a significant decline within the first 1 h of storage, measuring 0.58 and 0.49 respectively ($p<0.05$). Both groups containing some form of osmotic impermeant (Groups 2 & 3) maintained levels similar to freshly isolated up until 4 hours. However, by 4 hours of preservation, only Group 3 (lactobionate and dextran) specimens had maintained levels close to that of the freshly isolated specimen (0.66). After 10 h, the most dramatic differences were observed. Specimens preserved with UW had the lowest energy charge, 0.34. Group 3 was the only group able to maintain a stable energy charge for the entire preservation period; the EC value after 10 h was 0.63.

Functional Assessment of Stored Tissue

Mannitol Flux (Fig. 3-5) After 4h storage, all groups, except UW solution, were < 55 nmol/cm^2/h ($p<0.05$). The impact of both osmotic and oncotic agents (lactobionate and dextran) was clearly apparent following 10 h storage; only Group 3 showed significantly lower permeability compared to both Normal Saline (Group1), and UW ($p<0.05$). Interestingly, values for the UW group were higher than all other groups at both 4 and 10 h storage despite the presence of its own osmotic and oncotic support.

Mucosal Short-Circuit (Isc)(μA/cm^2) (Fig. 3-6) Short-Circuit Current is the net effect of all ion activities across the mucosal layer; as the muscosal layer degenerates, the Isc

gradually falls to zero (a higher value indicates a greater degree of ion/electrical activity). By 4 h there was no significant difference between experimental groups and freshly isolated tissue, indicating a maintenance of ion flux across the mucosal membrane. However, by 10 h, Isc values were significantly higher in Group 3 compared to Group 1 and UW ($p<0.05$).

Scanning Electron Microscopy

Scanning EM of freshly isolated tissue depicted a typical compact and even villus pattern with intact villus tips and the absence of epithelial cell sloughing (Figure 3-7). Following 10 h cold storage, Group 1 exhibited a considerable degree of epithelial cell denudation resulting in large spaces between villi (with the presence of occasional columnar epithelial cells) and exposure of the lamina propria; (Figure 3-8A). This was in contrast to the morphology of Group 3, which showed a minor degree of apical epithelial cell denudation; villi remained tight and uniform with no exposure of the underlying lamina propria (Figure 3-8B). The presence of long, compact microvilli further demonstrated the integrity of the epithelial layer in Group 3 following 10 h storage (Figure 3-9); this plate depicts several epithelial cells which are just beginning to separate from adjacent cells yet still possessing an intact microvillus border. Group 2 showed extensive epithelial denudation; however, there were adjoining regions with only minor apical damage. The effect of UW solution was similar to Group 1 with evidence of considerable villus denudation and corresponding exposure of the lamina propria. Visibly different from Group 1, UW specimens possessed large cavernous spaces between the non-uniform villi, consistent with denuded crypts (Figure 3-10).

DISCUSSION

The success of SB transplantation continues to lag behind that of other transplanted intra-abdominal organs. One specific threat to successful transplantation of this unique organ is lack of reproducible preservation. To date, preservation protocols are predominantly dictated by the fact that SB is harvested as part of a multi-organ procurement procedure which utilizes a common vascular preservation solution. Currently, UW remains the gold standard vascular perfusate. Whether or not UW is the ideal preservation solution for SB is debatable as others have documented equivalent or better preservation with simple solutions such as Normal Saline (5,6). The fact that UW has not consistently proven itself to provide superior SB preservation suggests that this unique organ may not adhere to all principles upon which it was formulated.

Preventing hypothermic-induced cellular swelling has been recognized as one of the pre-requisites for successful organ preservation (14). This may be especially critical during cold storage of SB as one of the primary events resulting in morphologic damage is the formation of sub-epithelial clefts, referred to as Gruenhagen's space. To date, studies suggest that net fluid shifts from vascular and/or luminal origin are the inciting event (8,9). This leads to intercellular swelling, architectural distortion, and eventual lifting of intact, metabolically active epithelial cells from the lamina propria (15). If unchecked, progressive denudation of villi occurs, culminating in compromise of both primary functions of the SB; protective barrier and absorptive capacity.

Rules of fluid transport dictate that movement between intracellular and extracellular compartments are governed by hydrostatic and impermeant [oncotic + osmotic] forces. The period of cold storage alters the contribution of each of these forces to net fluid movement. Hydrostatic forces become minimized as blood flow ceases within the stored organ. Changes in oncotic and/or osmotic forces may

represent the greatest contributing factor to fluid shifts experienced during preservation. Since albumin provides approximately 75% of total plasma colloid osmotic pressure, oncotic support may be the primary contributor to overall impermeant support (16). Therefore, this study investigates the role of oncotic and supplemental osmotic support as the critical components required in preventing sub-epithelial cleft formation.

The original formulation of UW addressed the principle of hypothermic-induced cellular swelling by including both oncotic and osmotic agents. Oncotic support was provided by hydroxyethyl starch (HES), a polydisperse colloid with a weight average molecular weight of 450,000 daltons. Selection of this agent was presumably based on the fact that it possessed similar oncotic properties to human albumin (MW 69,000 daltons), the primary oncotic agent found in human plasma (16). This similarity is based on the number average molecular weight (69,000 daltons), a term more accurately representative of oncotic force, which includes both average molecular weight and the quantity of molecules at that average size (16). Osmotic support was provided by both lactobionate (100 mM) and raffinose (30 mM). Our primary end-point was not to test the efficacy of the specific individual oncotic and osmotic agents found in UW but rather the principle upon which they were based. We selected dextran (MW 67,300 daltons), a readily available, inexpensive oncotic agent. It was supplied at 5% to mirror the concentration of HES found in UW. Lactobionate was selected as the osmotic agent for this study and supplied in equal concentration to that found in UW.

As SB has been shown to undergo reproducible time-related histologic changes (17), analysis of morphology provided a reliable measure of preservation injury. It is well recognized that SB histologic injury typically occurs in a patchy pattern that can lead to a non-representative grade of tissue damage. Because of the potential limitation, scanning electron microscopy (SEM), capable of examining considerably greater tissue

area than light microscopy (5 mm vs 3-5 μm), was employed. Freshly isolated specimens revealed intact villus structures with no evidence of epithelial cell sloughing. Groups compared at 10 hours showed that SB flushed with a saline solution containing both selected impermeants (Group 3) had superior preservation of morphology compared to those stored in either UW or Group 1. Specimens from Group 3 sustained only minor epithelial denudation, which was isolated to the apex, while the remainder of the villus remained uniformly covered by intact epithelium. Upon closer examination of the villus apex, microvilli were noted to be long, compact, and most importantly, intact. Specimens stored in either UW or Group 1 developed severe denudation of the majority of the villus with evidence of large inter-villus cavernous spaces, consistent with loss of crypt cells. This is clinically relevant as the presence of crypt cells are necessary for epithelial cell regeneration upon reperfusion (18).

Maintenance of morphologic integrity was directly related to the ability of specimens to sustain both metabolic and functional parameters. Cellular energetics, as measured by high-energy phosphates, are directly related to the length of ischemic time. Numerous studies have shown that post-transplant function is positively correlated with the content of high-energy phosphates noted immediately after storage (19-21). All four groups followed this progressive decline in ATP levels over the time course of storage. However, by 10 hours, SB specimens preserved with both impermeants (dextran and lactobionate) retained significantly higher levels of ATP compared to UW and NaCl groups. Equally as important, significantly greater levels were also noted in ATP/ADP ratios and energy charge within this same group. In fact, Group 3 specimens were noted to maintain both ATP/ADP ratio and energy charge levels similar to freshly isolated specimens regardless of the time point measured. Interestingly, total adenylates (TA) was the only energetic parameter which remained significantly higher in

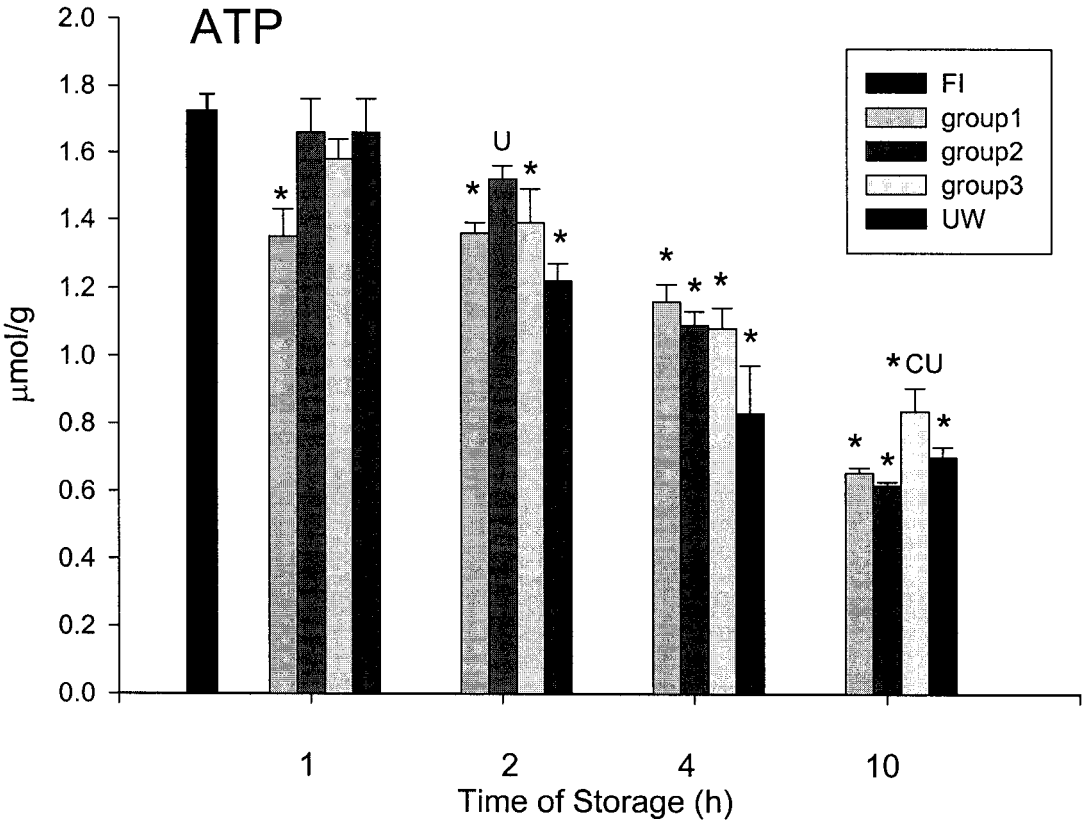
the UW group throughout storage. Based on other organ models, maintenance of this parameter has been viewed as a good predictor for the regeneration of high-energy phosphates (22). The fact that specimens preserved with UW solution had the highest TA levels of all experimental groups while exhibiting the most adverse morphologic damage suggests that maintenance of mucosal integrity may not only be energy-dependent but also substrate-specific. High TA levels can be explained by two agents normally found in UW which have known effects on purine catabolite balance. Allopurinol blocks purine catabolism at the level of xanthine oxidase (converting hypoxanthine to xanthine) resulting in the accumulation of lower energy purine catabolites and AMP. The presence of adenosine, as shown in other solid organs such as liver, has a similar effect on phosphorylated adenylate levels by reducing the decay of lower energy purine catabolites, thereby resulting in accumulation of AMP and hence TA pools.

There is little doubt that energy levels are directly related to the maintenance of barrier function in the SB. Specialized transcellular proteins provide extracellular binding latices for cell:cell adherence within the epithelial layer. These ligand:ligand interactions are reliant on energy-dependent processes involving classic tyrosine kinase-mediated phosphorylation and GTP-binding events in addition to several other classes of intracellular signaling cascades (Ca^{2+}, IP_3) [for review see 23]. Although the specific details of these mechanisms are clearly beyond the scope of the present study, impermeant support not only reduces morphologic injury but also improves cellular energetics and function in the SB.

In conclusion, the data presented in this study demonstrates that impermeant support is necessary for effective prolonged SB preservation. Clearly, the ability to reduce hypothermic-induced cellular swelling is a fundamental requirement for

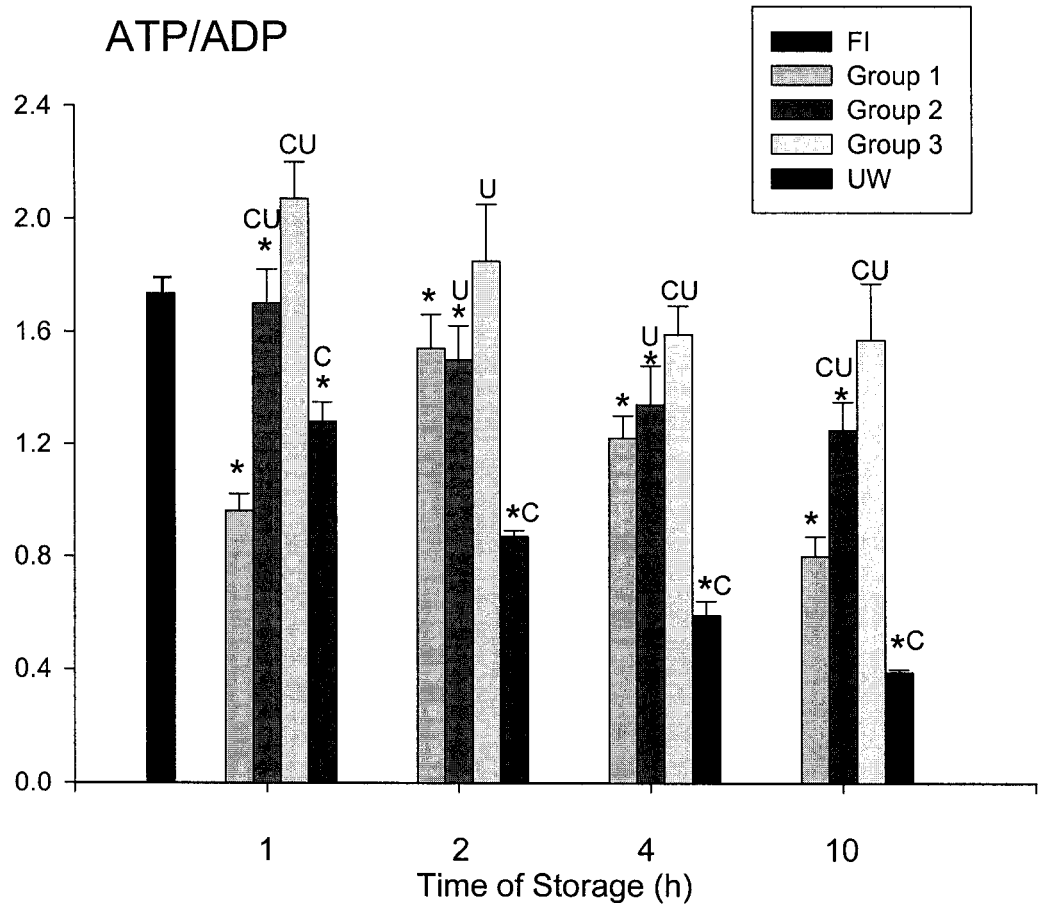
successful storage of this unique organ. Furthermore, direct evidence suggests that SB may not adhere to all previously established principles addressed by UW.

Figure 3-1 Effects of osmotic and oncotic agents on ATP levels throughout 10h cold storage.



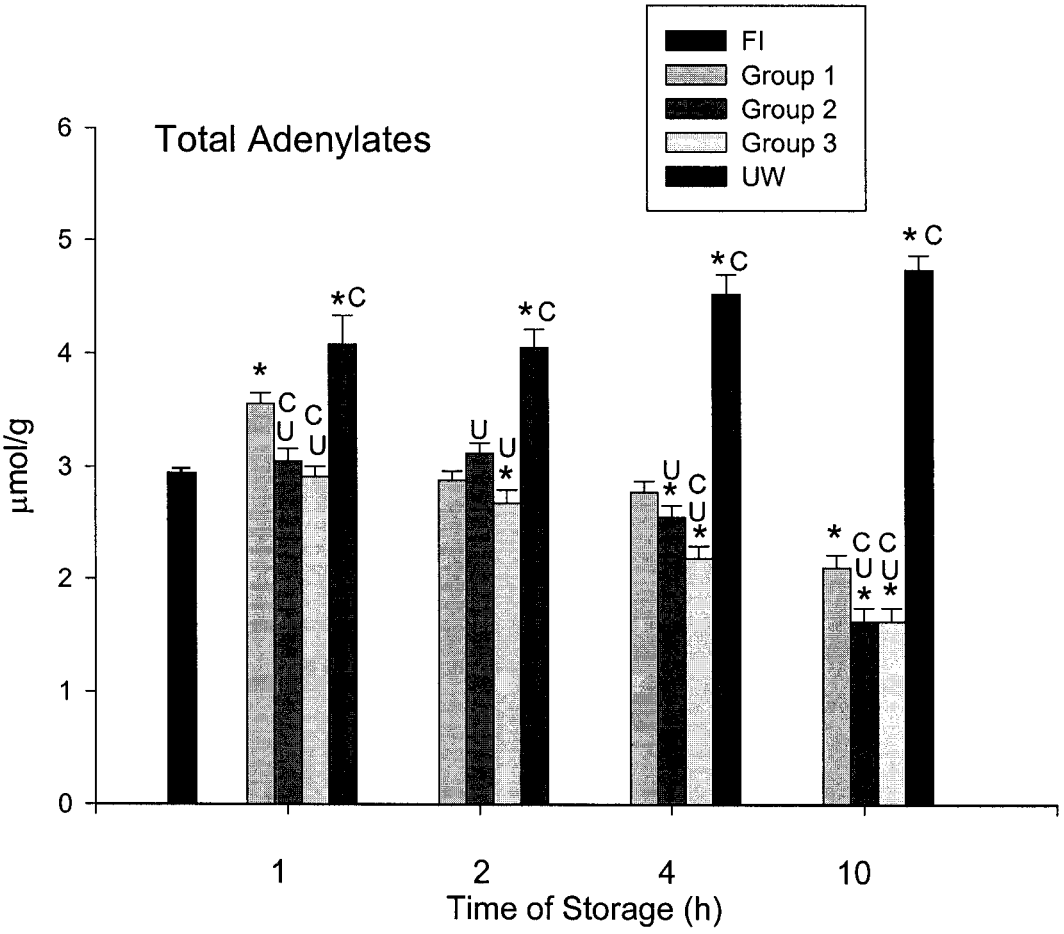
***,c,u- significantly different from freshly isolated, UW, and group 1 (normal saline) groups respectively; $p<0.05$.**

Figure 3-2 Effects of osmotic and oncotic agents on ATP/ADP levels throughout 10h of cold storage.



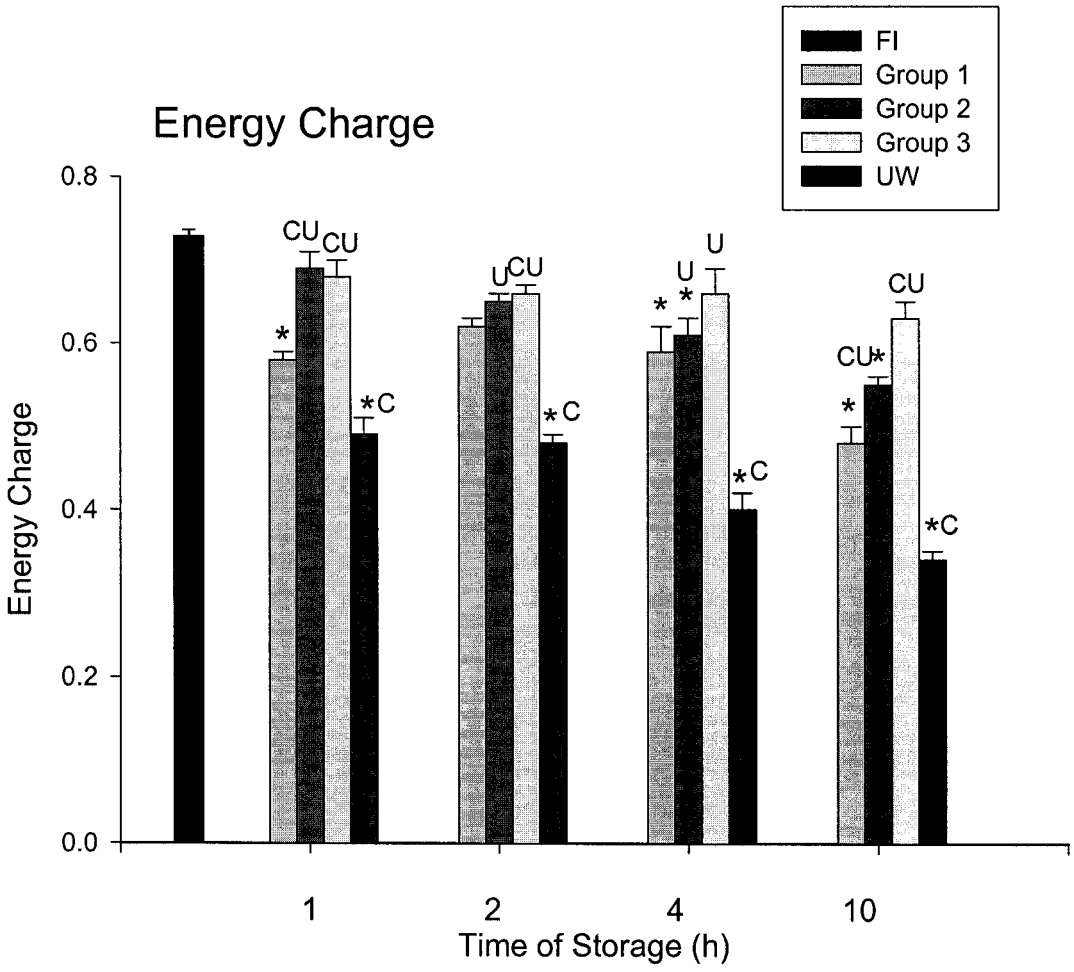
***,c,u- significantly different from freshly isolated, UW, and group 1 (normal saline) groups respectively; $p<0.05$.**

Figure 3-3 Effects of osmotic and oncotic agents on Total Adenylate (TA) levels throughout 10h cold storage.



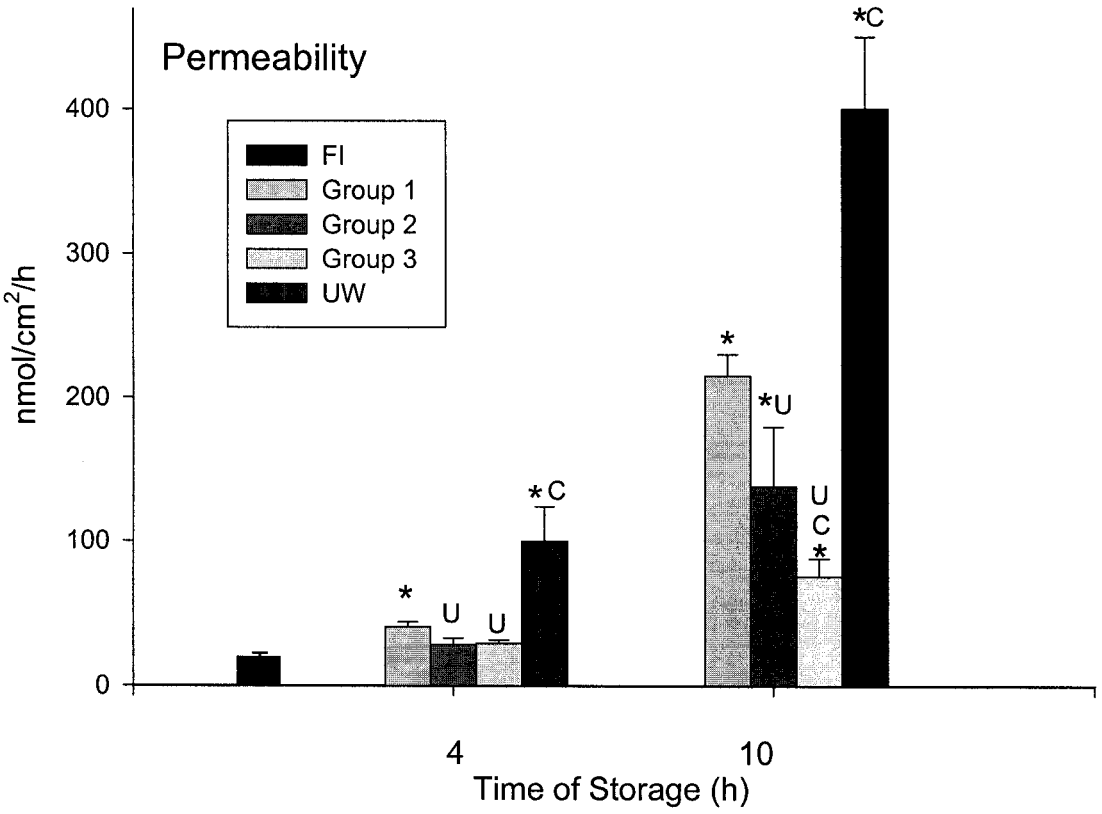
***,c,u- significantly different from freshly isolated, UW, and group 1 (normal saline) groups respectively; p<0.05.**

Figure 3-4 Effects of osmotic and oncotic agents on energy charge (EC) throughout 10h of cold storage.



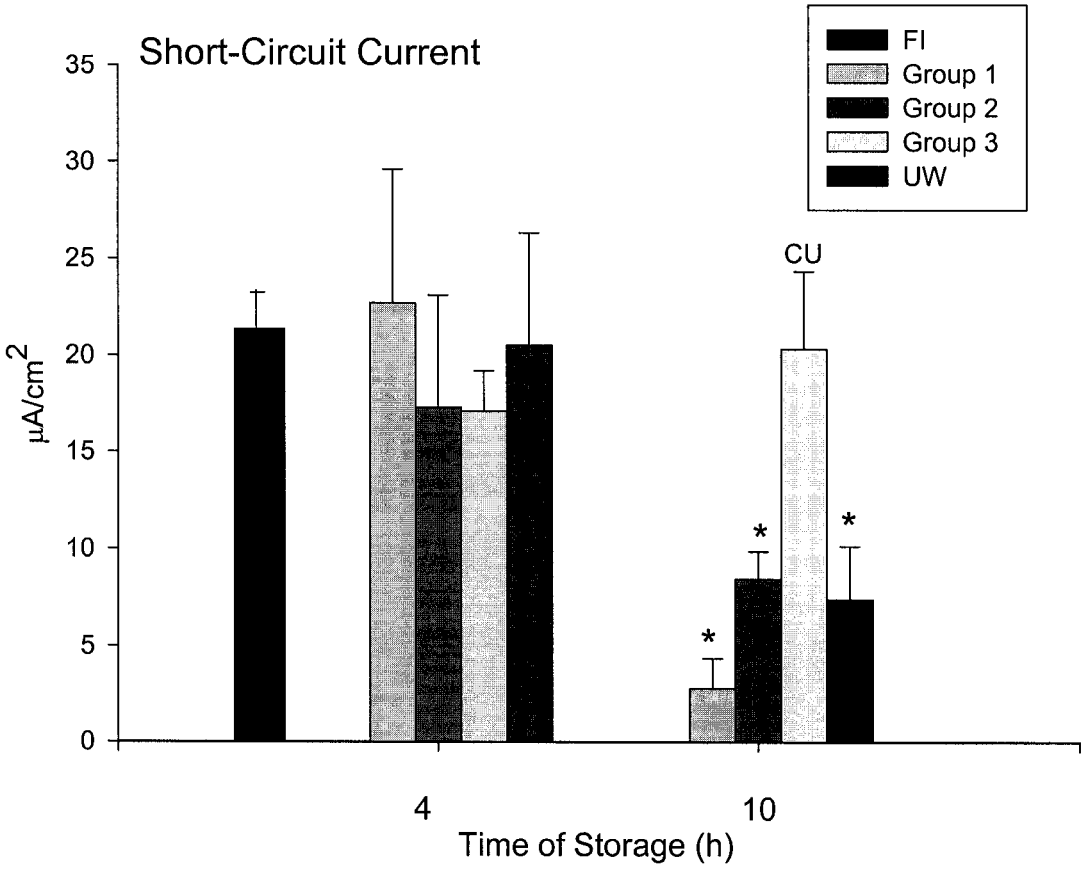
***,c,u- significantly different from freshly isolated, UW, and group 1 (normal saline) groups respectively; $p<0.05$.**

Figure 3-5 **Effects of osmotic and oncotic agents on mannitol permeability throughout 10h of cold storage.**



***,c,u- significantly different from freshly isolated, UW, and group 1 (normal saline) groups respectively; $p<0.05$.**

Figure 3-6 Effects of osmotic and oncotic agents on short-circuit current (Isc) throughout 10h of cold storage.



***,c,u- significantly different from freshly isolated, UW, and group 1 (normal saline) groups respectively; $p<0.05$.**

Figure 3-7 **Scanning electron micrograph of freshly isolated rat bowel. Bar, 50μm.**

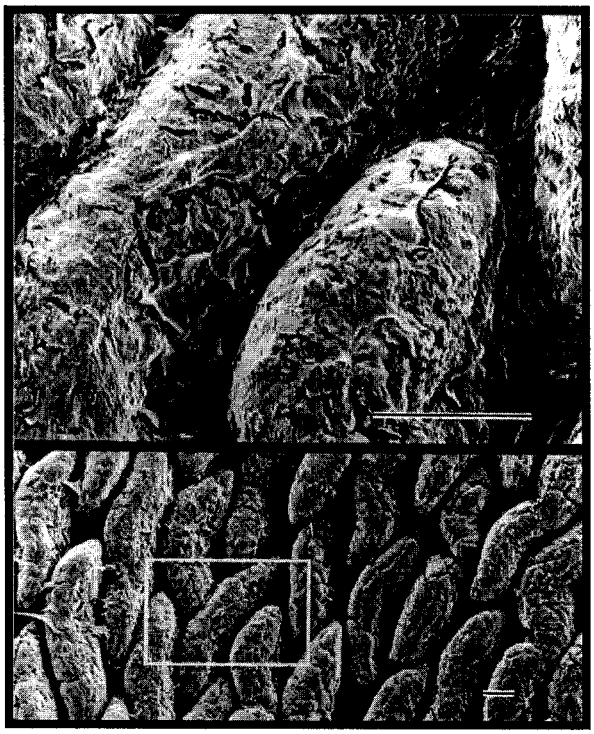


Figure 3-8 **Scanning electron micrographs of rat bowel from (A) group 1 and (B) group 3 after 10h cold storage. Bar 50 μm.**

A)

B)

Figure 3-9 **Scanning electron micrographs of columnar epithelial cells (lower frame) and intact microvilli (upper frame) from group 3 after 10h cold storage. Bar 2 μm.**

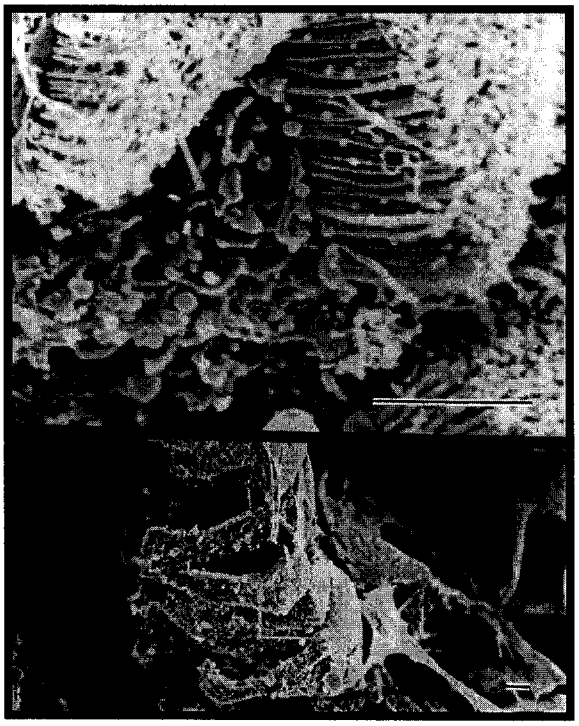
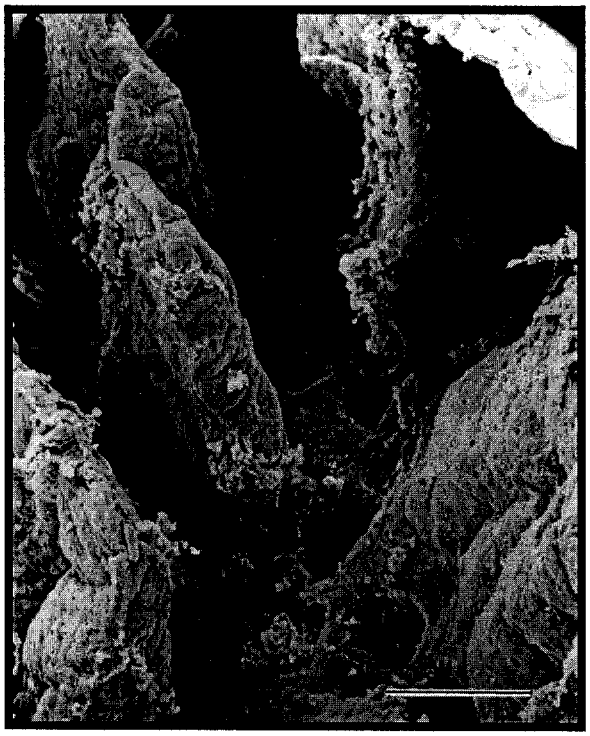


Figure 3-10 **Scanning electron micrograph of bowel stored in UW for 10h. Bar 50μm.**



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Chapter 4

Potentiating the Benefit of Vascular Supplied Glutamine During Small Bowel Storage: Importance of Buffering Agent.

INTRODUCTION

Currently, no one vascular solution has been accepted unequivocally for the preservation of small bowel (SB) for transplantation (1,2). Multiple studies have documented that a variety of solutions, including University of Wisconsin (UW), are incapable of preventing clinically acceptable degrees of morphologic injury beyond 6-10 hours of cold storage (3,4). In an attempt to address this, solutions formulated on satisfying organ-specific preservation requirements have been studied but remain problematic in their clinical application. This is due to the fact that most organs, including SB, are procured using a common vascular perfusate, as part of a multi-viscera procedure. Hence, the confines of multi-viscera procurement forces those interested in improving SB preservation to investigate supplementation of common perfusates without altering their effectiveness.

One such alteration has been the enrichment of UW, the current gold standard, with glutamine (gln), the primary fuel source of the enterocyte (5,6,7). When provided in supra-physiologic concentrations (2-4%), gln has been shown to improve the morphologic and functional integrity of SB throughout extended storage periods (8). Ultimately, gln addresses SB-specific preservation requirements while operating under the confines of a common vascular solution.

The effectiveness of exogenous vascular supplied gln has been clearly documented in both *in vitro* (9) and *in vivo* (10,11) organ systems. Additionally, use of

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this naturally occurring amino acid has not been shown to have detrimental effects on other organs. However, the metabolic consequences have not been studied for potential detrimental effects on both cellular function and morphology. Accumulation of potentially toxic end-products of gln catabolism including ammonia and organic acids can potentiate non-physiologic shifts in intracellular pH. As described in other organ models, these effects have functional repercussions on energy production, including altered enzyme kinetics mediated by direct pH inhibition and changes in phosphorylation status of key regulatory enzymes (12). Both heart (13) and liver (14) preservation models have successfully counteracted these effects through addition of buffering agents. It is our hypothesis that simultaneous addition of BES, a known buffering agent, will potentiate the effect of vascular supplied gln by addressing fundamental metabolic principles.

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 either Sigma Chemical (Oakville, Ontario, Canada), or BDH (Dorset, UK).

Surgical procedure and procurement of the small intestine: Rats were fasted overnight in cages. Water was provided *ad libitum*, and cage floors raised to minimize coprophagy. Prior to the time of laparotomy, rats were administered an interperitoneal dose of 65mg/250g pentobarbital (Somnotol; MTC Pharmaceuticals, Cambridge, Ontario, Canada), followed by inhalational halothane (1-2%) as required to achieve and 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 the individual preservation solution 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 then harvested. No intraluminal solution was administered. The bowel was stored in 30 ml of ice-chilled preservation solution identical to the vascular solution used in each group. A 1-2g tissue sample was taken at t= 4 and 10h. To arrest metabolic activity, samples were snap frozen in liquid nitrogen, and subsequently stored at -65°C until processed.

Composition of preservation solutions: Four groups of animals (n=4) were subjected to an isolated vascular flush with one of four solutions as detailed in Table 4-1. Solutions were adjusted to a pH of 7.4 using NaOH and gln containing solutions had 20 mM glucose added. Solutions were delivered according to the following groups (UW* denotes - modified University of Wisconsin solution):

UW	- UW*;
UWB	- UW* + 90 mM BES
UWG	- UW* + 137 mM gln;
UWBG	- UW* + 137 mM gln + 90 mM BES;

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 000g). Acid extracts were neutralized by the addition of 3 M KOH/ 0.4 M Tris/ 0.3M KCl and then recentrifuged (20min at 14 000g). Aliquots of neutralized extracts were immediately processed via standard enzyme-linked metabolite assays (15). Spectrophotometric analysis was then performed to measure the absorbance of NADH at 340nm, providing quantification of ATP, ADP, and AMP. Values are reported per gram wet weight.

Amino Acid Analysis: Samples (100 μl) were taken at t= 4 and 10h, weighed and extracted 1:20 w/v in 10% trichloroacetic acid (TCA) solution. Precipitated protein was removed by centrifugation (15 min at 20,000g). The supernatant was removed and mixed with diethylether. This process was repeated three times in order to completely remove residual TCA. Samples were then redisolved in 6N hydrochloric acid (HCl, 200 μl) containing 0.1% phenol and subsequently dried under vacuum and dissolved in Na-S buffer (800μl, Beckman). Aliquots (50μl) were analyzed on a Beckman System 6300 (post-column ninhydrin detection, Beckman Instruments Inc., San Ramon, Ca.) with System Gold (version 6.01) data analysis.

Histological Examination: 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 damaged bowel on two sections from each specimen (16). All samples were evaluated in a blinded fashion by two independent pathologists (LJ and JZ). Assessment of mitochondrial damage by transmission electron micrography (TEM) was also performed, providing an additional quantitative analysis of overall histologic damage. Ultrastructural integrity of randomly selected mitochondria from each specimen were scored according to the following criteria: Grade 1 –normal; linear and evenly spaced cristae, no swelling, Grade 2 –minor to moderate damage; patchy, non-uniform cristae, some cristeolysis and swelling, Grade 3 –severely damaged; cristae absent or located peripherally, swollen, and/or foci of crystalline calcium.

Functional Assessment: Ileal segments were taken at 4 h and 10 h cold storage, stripped of their serosa and muscular layers, and mounted in Ussing chambers. Once mounted, the mucosal and submucosal surfaces were perfused with a recirculating bicarbonate Ringer's solution, with an ionic composition of: Na^+, 143 mM; K^+, 5 mM;

Mg^{2+}, 1.1mM; Ca^{2+}, 1.25 mM; HCO_3^-, 25 mM; Cl^-, 123.7 mM; HPO_4^{2-}, 0.3 mM; fructose, 20 mM with 95% O_2 and 5% CO_2, at 37 °C, pH 7.4 (17). Permeability was measured via scintillation counter determining the flux of a radioactive substrate (10 μCi [^{3}H]mannitol, Dupont NET101) across individual ileal specimens. The spontaneous transepithelial potential difference (PD) and short-circuit current (I_{sc}) were determined for all segments, and tissue conductance was calculated from PD and I_{sc} according to Ohm's law (18).

Statistical analysis: Metabolite and permeability data were reported as means ± SE for each group. Statistical differences between groups was determined using ANOVA, followed by Dunnett's *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 4-1)

ATP: Compared to UW, no difference was noted at four hours between groups with either BES (UWB) or gln (UWG) alone; 0.9 μmol/g vs 1.0 and 1.0 μmol/g respectively. Unlike the UWB group, gln supplementation (UWG) did result in increased ATP levels at 10 h compared to UW alone; 0.7 μmol/g vs 0.4 μmol/g, $p<0.05$. When BES and gln were added in combination however (UWBG), ATP levels were significantly higher compared to UW as well as UWG groups. This was evident both at 4 h (1.24 μmol/g vs 0.9, 1.0 μmol/g respectively) and 10 h (1.0 μmol/g vs 0.4, 0.7 μmol/g respectively), $p<0.05$.

Total Adenylates: At 4 h, no significant difference was noted between UW and other groups containing BES and/or gln. This was noted throughout the storage period as 10 h values were not only similar but each of the UW, UWB, and UWG treated specimens exhibited decreases in TA levels; 2.4, 2.2, and 2.8 μmol/g, respectively. In direct contrast, UWBG specimens maintained consistency in overall TA levels throughout 10 h,

with only 4% variability noted between time-points. Furthermore, the UWBG group had better maintained this parameter compared to either UW or UWG (3.9 vs 2.4 and 2.8 μmol/g, $p<0.05$).

Intermediary metabolites and end products of glutamine catabolism (Table 4-2)

Glutamate (glu) and ammonia (NH_3), direct catabolic products of glutamine (gln) metabolism were included from detailed amino acid analysis. Two other amino acids, namely aspartate (asp) and glycine (gly), both intermediary metabolites in complete gln metabolism were also measured. At both four and 10 hours, groups containing glutamine possessed higher levels of glu and NH_3 compared to either UW or freshly isolated specimens. After 10 hours however, the level of both glu and NH_3 in the UWBG group (4.2 and 8.9 μmol/g) was significantly greater compared to UW, but more importantly, even UWG.

Histologic Injury (Table 4-3 and Figure 4-2)

Specimens were assessed for degree of morphologic damage using both light microscopy and TEM. Two independent pathologists analyzed specimens according to Park's classification of bowel damage using light microscopy. Histologic grading at 4h revealed no significant difference between UW, UWG, or UWBG. By 10 hours substantial damage was noted in the UW group (grade 8). The UWG sustained slightly, but not significantly less damage, of grade 7.5. Most important however was the injury grade of 5.5 noted in the UWBG group. This was significantly improved compared to either UW or UWG; $p<0.05$.

Ultrastructural Assessment by Transmission Electron Microscopy (TEM)

Assessment of Mitochondrial Structure (Table 4-4, Figure 4-3): *4 h* - Storage with UW solution resulted in moderate mitochondrial deterioration with the majority of mitochondria exhibiting grade 2 damage consisting of some cristeolysis, a minor degree

of swelling and non-uniform cristae occurring in a patchy appearance. The grade of mitochondrial damage increased from 1.06 in freshly isolated specimens to 1.95 ($p<0.05$) in UW after 4h. Although energetic parameters were similar after 4h storage, mitochondrial morphology in UWG and UWBG groups was significantly improved compared to UW. The inclusion of glutamine (UWG) reduced the mean grade to 1.68 ($p<0.05$) and the further addition of buffering agent in UWBG resulted in a grade of 1.23 ($p<0.05$). Although there was some damage evident compared to freshly isolated tissue, mitochondrial integrity was considerably improved in UWBG; mitochondria were even and uniform, with well-maintained cristae.

10 h - Storage with UW solution resulted in the majority of mitochondria assessed possessing grade 3 damage (mean grade = 2.50); this grade is indicative of severely vacuolated mitochondria with cristae located peripherally or absent, as well as an extreme swollen appearance with cavitations in the matrix [crystalline calcium foci were occasionally present]. This swollen and vacuolated appearance is indicative of water uptake and the cristeolysis event is typical of suppressed oxidative phosphorylation. The benefit of gln addition was not evident after 10 h storage; mean grading was 2.31 (not significantly different from UW grade). However, most notably was the significant improvement in mitochondrial grading with UWBG. Although there was some damage, the majority of assessed mitochondria were classified as grade 1. With a mean grade of 1.56, the UWBG was significantly better than UW and UWG, both with $p<0.05$.

Integrity of Microvilli and Intercellular Spaces (Figures 4-4, 4-5):

4h - Both UW and UWG exhibited similar patterns of damage. Intercellular spaces were even and uniform, resembling "train tracks" due to their consistent and parallel appearance. Other observations include the appearance of intact tight junctions. The microvilli, if present, were irregular, shortened and very loosely packed. The effect of

buffer plus glutamine (UWBG) was apparent with intact intercellular spaces and microvilli that were consistently maintained in a uniformly thick and full appearance.

10h Both UW and UWG exhibited inconsistent intercellular spaces with an irregular and uneven shape between adjacent cells; this is typically a direct consequence of edema. Treatment with UWBG vastly improved the ultrastructural integrity of the bowel. Microvilli were present and, although slightly less densely packed compared to fresh tissue, were of normal length. Tight junctions at the apical epithelial layer were visible and intact. Intercellular spaces maintained a normal "train-track" appearance indicating negligible edema. Overall, the integrity of the microvilli was nearly comparable to that of the fresh tissue

Functional Assessment of Stored Tissue (Figure 4-6)

Permeability: At 4h, glutamine-containing solutions (UWG, UWBG), possessed significantly lower degrees of permeability compared to UW (67,45 vs 153 nmol/cm^2/h, $p<0.05$). By 10 hours, the greatest degree of permeability was noted in the UW group, measuring 572 nmol/cm^2/h. Despite initial improvement seen in the UWG group at 4 hours, no significant difference was noted compared to UW at 10h. Specimens preserved with UWBG retained significantly less degrees of permeability compared to either UWG and UW at both 4 and 10h with 10h values measuring 169 vs 445, 572 nmol/cm^2/h, $p<0.05$).

Conductance: Conductance values are plotted as increases over freshly isolated values. Levels in UW (control) treated specimens increased most dramatically following 4 and 10h storage periods. By 4h of storage, both UWG and UWBG possessed significantly lower levels compared to UW (42, 40 vs 77 mS/cm^2, $p<0.05$). Although the presence of glutamine resulted in better maintenance of conductance, the addition of

buffering agent provided a protective effect as values in the UWBG group were improved compared to either UWG or UW (74 vs 170, 199 mS/cm^2, $p< 0.05$).

DISCUSSION

The non-physiologic stress of organ storage includes both hypothermic- and hypoxic-related components which contribute to a series of deleterious metabolic sequelae. Maintaining the delicate balance between energy production and consumption is critical during the period of storage since disruption leads to compromised cellular structure and function (19,20). Inclusion of exogenously supplied gln in supra-physiologic concentrations has been shown to provide metabolic support for intestinal mucosal cells, in part alleviating the energy imbalance precipitated by bouts of ischemia (8). As intestinal mucosal cells remain metabolically active during storage (21), toxic end products normally fed into the portal circulation for hepatic detoxification accumulate within the enterocyte contributing to non-physiologic alterations in intracellular pH. Ammonia is one of the primary end products, accounting for 38% of all nitrogen produced from gln catabolism during normothermia (6). Other compounds derived from gln metabolism which can further influence intracellular pH include organic substances such as malic, lactic, citric, succinic, α-ketoglutaric, pyruvic, and fumaric acids (22). As a result of pH sensitive regulatory control mechanisms, accumulation of such products will ultimately prevent sustained gln metabolism.

The maintenance of physiologic pH is recognized as one of the basic principles for sustaining viability during organ storage (23). Nuclear magnetic resonance studies (NMR) performed on heart (24), liver (25), and SB (26) models have shown that pH changes occur during bouts of ischemia. Heart (12,13) and liver (14) preservation models have documented that supplemental buffering agents provide an integral defense against non-physiologic pH shifts leading to improved quality of preservation,

however no equivalent data for SB exists. The protective benefit of buffer in heart and liver storage models has been purported to be due to maintenance of key regulatory enzymes in active phosphorylated states and simultaneous alleviation of pH-mediated enzyme inhibition. Although glycolysis provides the major pathway for energy production in both these organs during storage, SB amino acid catabolism follows similar principles of metabolic regulatory control.

Selecting an appropriate buffer is based on finding an agent with a pK value near the pH of the selected environment in which it will be used (27). BES, (N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid), a biological buffer with a pKa of 7.25 at 25°C (7.42 at 5°C) (24), is an ideal choice for SB undergoing extended cold storage. This is based on two important criteria. Firstly, standard acid/base titration curves show that maximum buffering capacity is achieved within a pH=pKa ± 1.0 (28). Therefore, a wide pH range (6.4-8.4), similar to that anticipated to occur during SB hypothermic storage, will be buffered effectively. Secondly, the effect of temperature on the pKa of a buffer ($\Delta pK/^{o}C$) is equally important in selecting an appropriate buffering agent. BES possesses a $\Delta pK/^{o}C$ (-0.016) (27) similar to that of the histidyl moieties of cellular proteins (-0.018) which play a primary role in the catalytic sites of enzymes (28). The importance of this is supported by the "alpha stat" hypothesis which states that the maintenance of pH relative to the shift in pKa of buffer will aid in the maintenance of net ionic charge of intracellular proteins and hence maintain function during cold exposure (29,30).

Maintenance of high-energy phosphorylated compounds such as ATP have been correlated with minimization of cellular structural and functional damage incurred during cold storage (31). In addition, ATP levels are inversely proportional to the length of the preservation period (32). Therefore, maintenance of energetic parameters such as ATP

and Total Adenylates (TA= ATP + ADP + AMP) provide a marker of preservation quality. From this study the presence of buffer (BES), when added to UW, did not result in improvement of either ATP or TA levels at any point throughout the storage period; indicating that BES does not provide additional non-buffering effects. Although ATP levels were elevated at 10 h in the gln-supplemented group (UWG), similar findings were not found in TA levels when compared to UW alone. When BES and gln were combined significantly higher ATP and TA levels were found even at 10 h compared to either UW and more importantly the gln-supplemented group (UWG). Buffering of pH alterations is implicated as the mechanism of action when BES is combined with gln, the primary fuel source of the enterocyte.

To date, biochemical measures of SB metabolism incurred during preservation have largely been assessed by functional indices including glucose transport (33), and enzyme activity including maltase and glutaminase (34,35). To our knowledge, this is the first study that documents the effects of gln supplementation by measuring metabolites normally found during gln catabolism. The process of intestinal gln catabolism is a multi-step process resulting in ATP production via the TCA cycle or via provision of key elements to the nitrogen-carbon backbone of purines (35,36) (see Figure 4-7). The first step in this process is mediated by the phosphate-dependent, mitochondrial-based enzyme glutaminase (37), leading to production of glutamate (glu) and NH_3. As shown in Table 2, gln supplemented UW resulted in elevated levels of glu compared to UW alone (3.7 vs 2.4 μmol/g at 4h, 2.9 vs 2.1 μmol/g at 10h) suggesting a greater degree of gln metabolism. Compared to all other groups at 10h, BES + gln-enriched UW (UWBG) treatment resulted in the highest levels of both glu and NH_3, again supporting the requirement for a buffering agent. Upon initial deamination of gln, glu can proceed either through the TCA cycle via α-ketoglutarate or to purine synthesis via

transamination products aspartate (asp) and glycine (gly) (see Figure 4-7). Levels of these key intermediary metabolites provides a further reflection of overall gln catabolism since their production occurs because of the accumulation of glu. In addition to significantly greater levels of glu and NH_3, only UWBG preserved specimens exhibited significantly increased asp and gly levels. Since both asp and gly provide essential carbon/nitrogen components for purine synthesis, it is interesting that TA levels remained in excess of freshly isolated values throughout preservation in only the UWBG group. Surprisingly, TA values in only this group remained stable but more importantly were sustained at significantly higher levels throughout preservation compared to freshly isolated specimens. Since TA levels have historically provided an indirect measurement of purine levels, two explanations exist for their sustained levels. Original Belzer UW solution possesses allopurinol, an agent which inhibits catabolism of lower energy purine catabolites (ie hypoxanthine-xanthine) by inhibiting xanthine oxidase (38). Recognizing this, we purposely used a modified form of UW which lacked allopurinol which would theoretically remove this potential confounding variable from our study. Despite the omission of allopurinol TA levels remained significantly elevated. We subsequently postulate that there may actually be a degree of *de novo* purine synthesis, directly resulting from substrate (gly and asp) accumulation known to be required for its formation.

Energy levels are intimately related to the ability to sustain cellular morphologic integrity and barrier function during preservation. The epithelial permeability barrier is maintained largely by the tight junction region located close to the apical surface of intestinal epithelial cells. These tight junctions consist of a number of proteins which are dynamic, energy-requiring structures. At a mechanistic level, these proteins are modulated by alterations in pH (39), calcium concentration (40), cAMP levels (41),

temperature (42), and osmolarity (43). Furthermore, depletion of cellular ATP causes dilation of the tight junction at the ultra-structural level, a perturbation of the actin cytoskeleton, and an increase in epithelial permeability, resulting in an increase in transepithelial flux of macromolecules (44). This disruption of the permeability barrier has been shown to be caused by disassembly of the protein components of the tight junction, with 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. Although further details of these mechanisms are clearly beyond the scope of the present study, the beneficial effect of BES on both permeability and conductance when added to gln-enriched UW was far superior compared to gln-enriched UW or UW solutions alone. Similar findings were noted at both the gross histologic and ultra-structural level. Using Park's classification of histologic damage, only UWBG preserved specimens retained crypt cells, a pre-requisite for enterocyte regeneration (45,46). The ability to maintain mitochondrial integrity is a metabolic necessity, since glutaminase and other key enzymes of the TCA cycle and electron transport systems are located at this level (37). Deviations from physiologic pH have been shown to have direct effects on mitochondrial function and structure (46). In the current study, the inclusion of gln + BES resulted in a markedly lower degree of mitochondrial damage at each time-point; mean grade of 1.56 was achieved compared to a grade of 2.50 for the UW solution and 2.31 for UWG group, $p<0.05$ (see Table 4-4). Even the addition of gln alone (UWG) was unable to significantly reduce the degree of mitochondrial damage following 10 hours of storage; implying the combination of buffer and gln was requisite for improved mitochondrial structure and hence, function.

In conclusion, metabolism in a system devoid of hepatic detoxification, like that occurring during SB preservation, results in intracellular accumulation of pH active end products (ie ammonia). Accumulation of these products prevents sustained metabolism unless biochemical principles of organ storage such as prevention of non-physiologic pH shifts are adhered to. Significantly increased levels of energetics, gln-derived metabolites, functional and structural indices all implicate BES as requisite for sustained gln metabolism presumably via buffering effects. Lastly, enrichment of UW with gln and BES appears to be one method by which SB-specific preservation requirements can be addressed while working within the confines of multi-organ procurement.

Table 4-1 Composition of Preservation Solutions

	UW	UWB	UWG	UWBG
Lactobionate	100	100	100	100
Raffinose	30	30	30	5
Adenosine	5	5	5	5
MgSO4	5	5	5	5
KH_2PO4	25	25	25	---
KOH	100	100	100	100
Glutamine	---	---	137	137
BES	---	90	---	90
pH	7.4	7.4	7.4	7.4

Solution pH was adjusted at room temperature using NaOH. **G** – denotes addition of glutamine. **B** – denotes addition of buffering agent, BES. Unless otherwise noted, all numbers in mM.

Table 4-2 Levels of by-products of Glutamine metabolism during cold storage

	Glu	Asp	Gly	NH_3	Total AA
FI	2.3±0.4	1.2±0.1	0.8 ± 0.1	1.6±0.2	9.5±0.6
4h **UW**	2.4±0.4	1.5±0.3	1.3 ± 0.2*	4.1±0.3*	17.3±2.4*
UWG	*3.7±0.2*^{*c}	*2.0±0.1**	*1.6 ± 0.1**	*3.9±0.5**	*19.0±1.6**
UWBG	4.1±0.3^{*c}	2.1±0.3*	1.8 ± 0.4*	8.3±1.0^{*cg}	22.0±1.9*
10h **UW**	2.1±0.4	1.5±0.2	1.1 ± 0.2	5.0±0.3*	19.7±4.0*
UWG	2.9±0.2	1.7±0.1*	1.1 ± 0.1	6.5±0.4*	18.4±1.6*
UWBG	4.2±0.1^{*cg}	2.5±0.1^{*cg}	1.8 ± 0.2^{*cg}	8.9±0.9^{*cg}	23.4±1.0*

Values are µmol/g. Statistical significance is denoted at $p<0.05$. *-different from freshly isolated (FI) level; $p<0.05$. The following denote significant difference: **c**, versus UW; **g**, versus UWG, $p<0.05$.

Table 4-3 **Grade of Histologic Damage (Park's classification)**

	Time	Grade	Median
FI		0, 0, 0, 0	0
UW	4h	5, 5, 5, 7	5
	10h	8, 8, 8, 8	8
UWG	4h	2, 2, 4, 5 **c**	3
	10h	7, 7, 8, 8	7.5
UWBG	4h	4, 5, 6, 6	5.5
	10h	1, 5, 6, 6 **c,g**	5.5

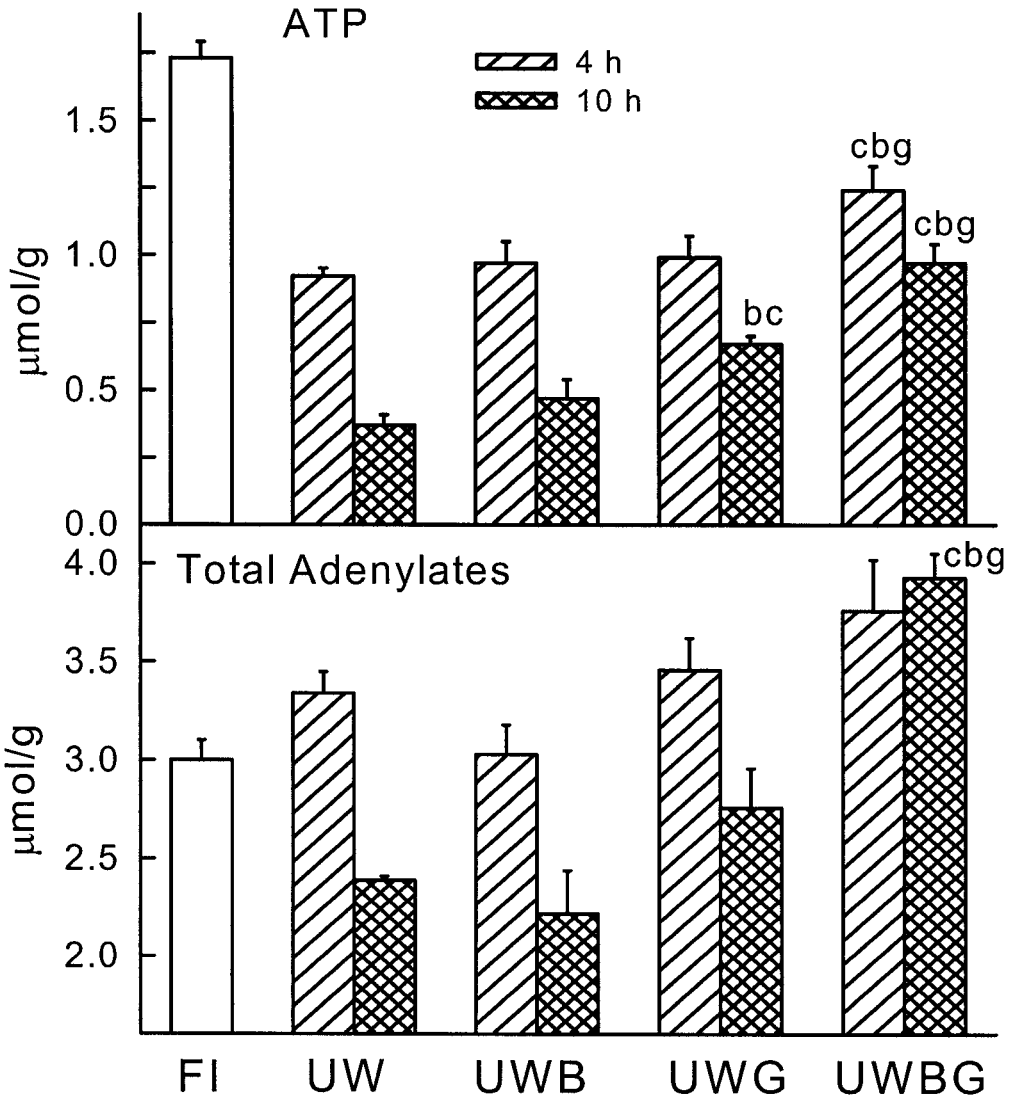
FI – denotes freshly isolated tissue. All experimental values were significantly higher than FI ($p<0.05$).
c – denotes significantly different from UW group; $p<0.05$. **g** – denotes significantly different from UWG group; $p<0.05$.

Table 4-4 Assessment of Mitochondrial Damage from TEM Micrographs

		Number of Mitochondria			Total # Graded	*Mean Grade*
		Grade 1	*Grade 2*	*Grade 3*		
Freshly Isolated		80	5	0	85	1.06 ± 0.03
4 h	**UW**	24	40	20	84	1.95 ± 0.08*
	UWG	36	40	9	85	1.68 ± 0.07*,**c**
	UWBG	67	15	2	84	1.23 ± 0.05*,**c,g**
10 h	**UW**	7	27	48	82	2.50 ± 0.07*
	UWG	14	35	42	91	2.31 ± 0.08*
	UWBG	47	28	10	85	1.56 ± 0.08*,**c,g**

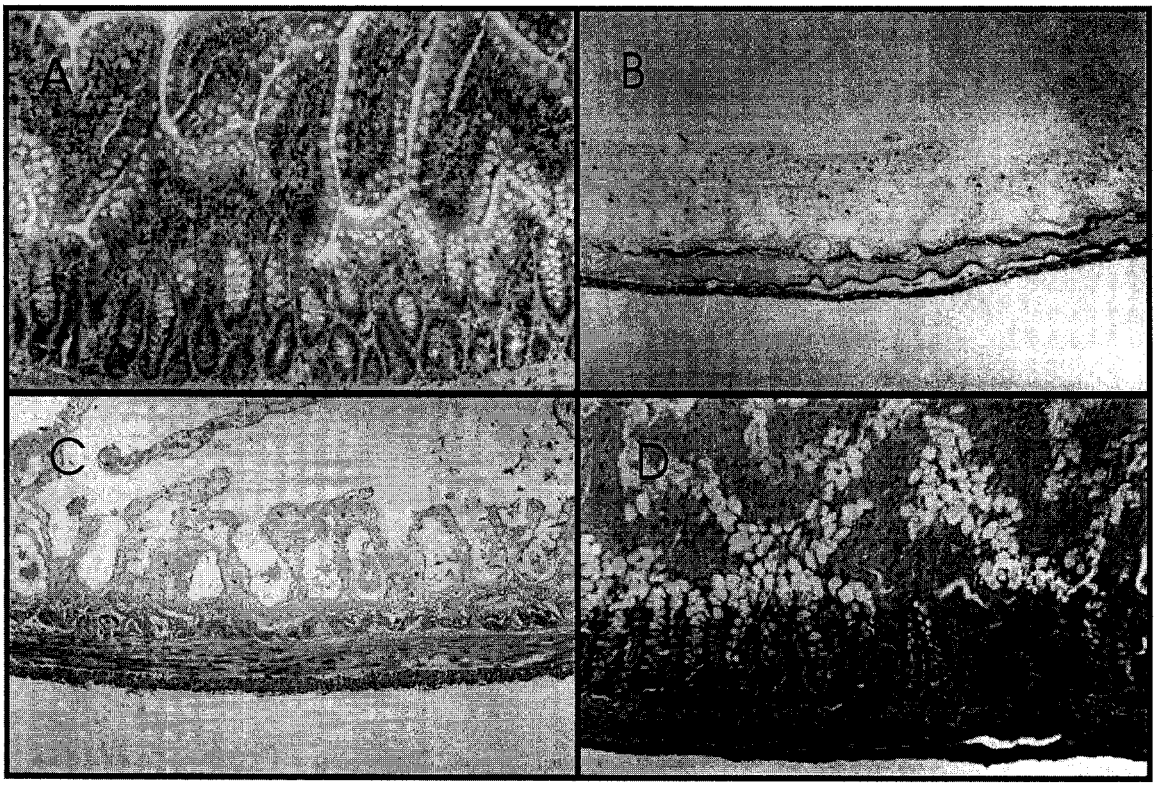
Assessed mitochondria were located in most apical region of the villus epithelium. Ultrastructural integrity of the mitochondria were assessed randomly and scored according to the following criteria: **grade 1** – normal: linear and evenly spaced cristae, no swelling; **grade 2** – minor to moderate damage: patchy, non-uniform cristae, some crysteolysis, swelling; **grade 3** – severely damaged: cristae absent or located peripherally, swollen, crystalline calcium foci. *-different from freshly isolated (FI) level; $p<0.05$. The following denote significant difference: **c**, versus UW; **g**, versus UWG, $p<0.05$.

Figure 4-1 Effect of supplemental glutamine and buffer on parameters of energetics in SB during 10h cold storage.



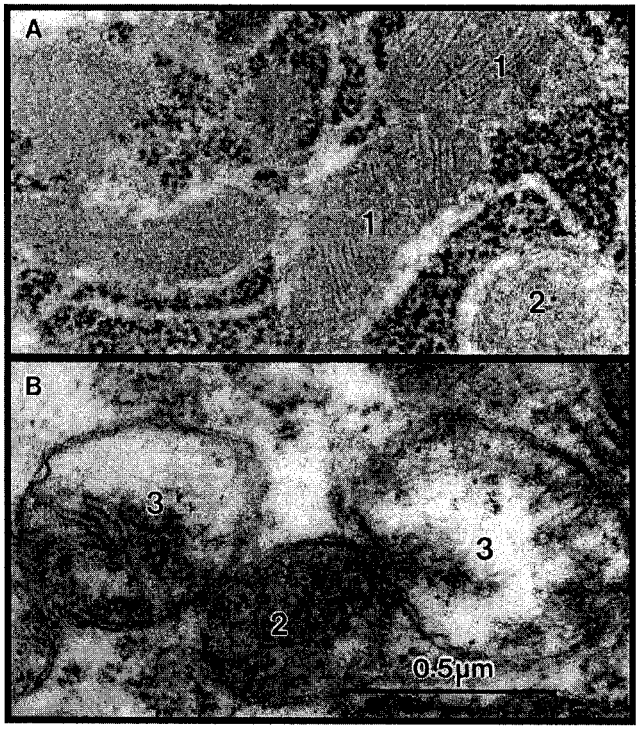
c, **b**, and **g**: significantly different from control (UW), UWB, and UWG groups, respectively ($p<0.05$). FI denotes freshly isolated tissue.

Figure 4-2 Histologic assessment of SB in A) freshly isolated tissue and in bowel stored for 10h in B) UW, C) UWG, and D) UWBG solutions



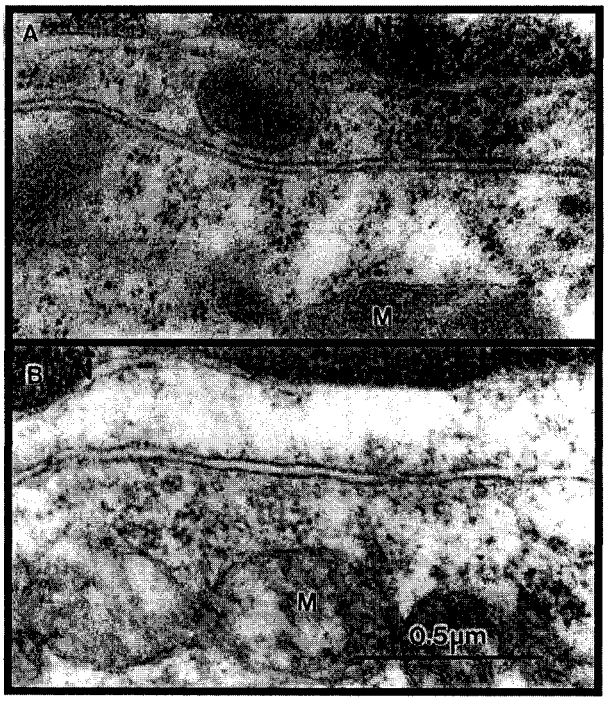
Median values of histologic damage according to Park's classification are: A) grade 0; B) grade 8; C) grade 7; D) grade 5. All plates were x10 magnification.

Figure 4-3 Ultrastructural assessment of mitochondria by TEM.



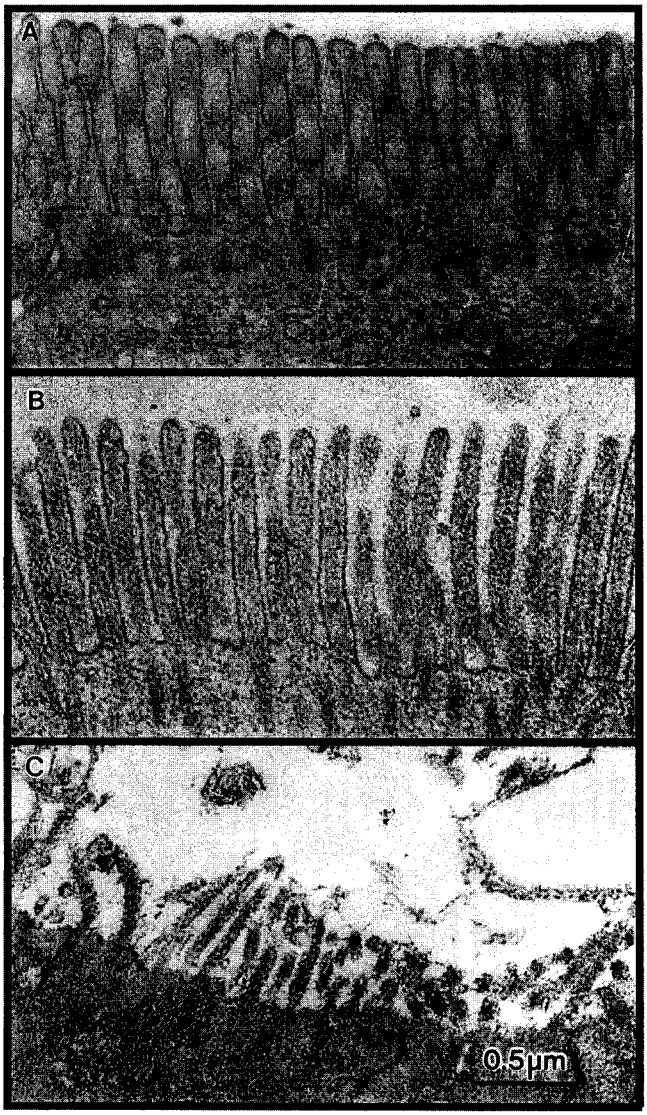
Representative examples of mitochondria with grades 1, 2, and 3. A) Mitochondria with minimal or no damage; photo taken from UWG after 4h. B) After 10h storage in UW, mitochondria have moderate to severe damage. Grading values are labeled on the photomicrographs.

Figure 4-4 Ultrastructural assessment of intercellular spaces by TEM.



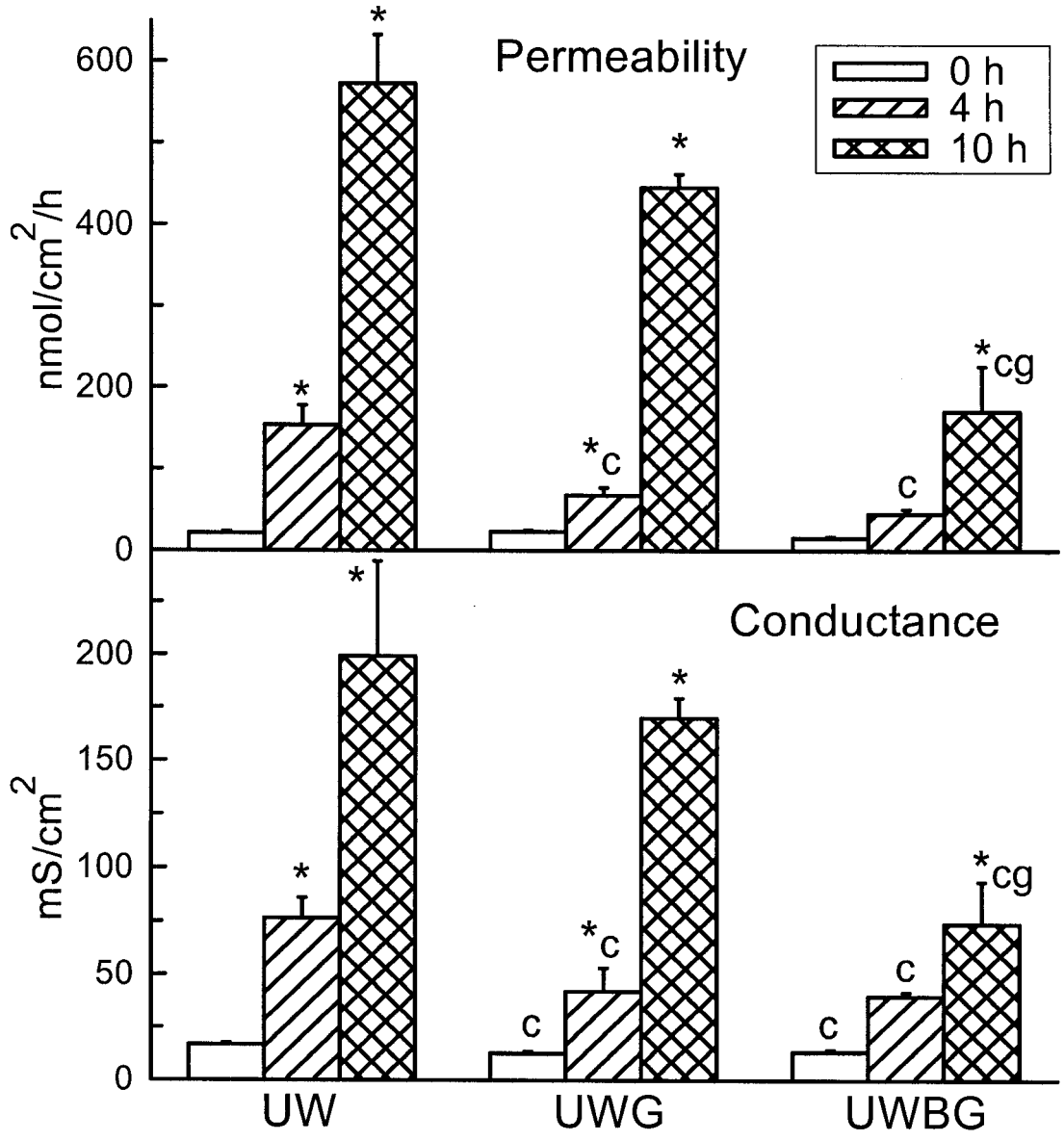
A) Typical intercellular junction with an even and parallel appearance resembling classical train tracks morphology; photo taken from UW after 4h. B) Intercellular junction from tissue stored in UW for 10h has an irregular, uneven pattern. N and M denote nucleus and mitochondrion, respectively.

Figure 4-5 Ultrastructural assessment of microvilli by TEM.



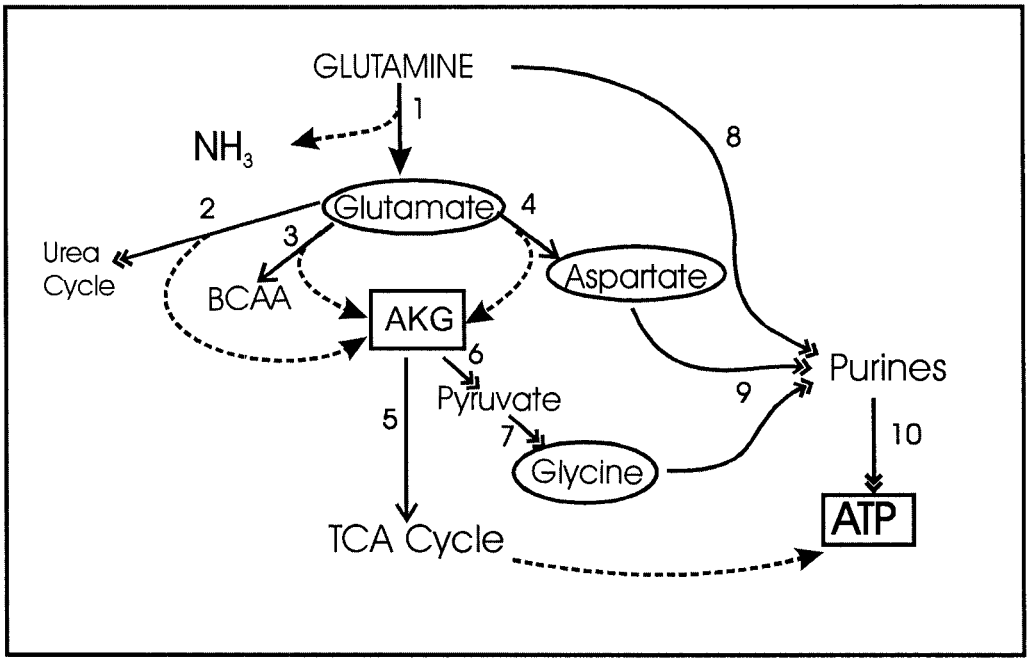
A) Microvilli from freshly isolated SB are long and have a tight, densely packed appearance. B) Microvilli from tissue stored in UWBG for 10h are long, but slightly less densely packed than freshly isolated tissue. C) Microvilli from tissue stored in UWG for 4h are extremely damaged and have degenerated into a short, intermittent appearance.

Figure 4-6 Effect of supplemental glutamine and buffer on mucosal barrier function in SB during 10h cold storage.



* Significantly different from initial (t=0h) value ($p<0.05$); c and g, significantly different from control (UW) and UWG groups, respectively, $p<0.05$

Figure 4-7 Intestinal mucosal glutamine catabolism.



Indicated reactions are catalyzed by the following enzymes:
1) phosphate dependent glutaminase; 2) pyrroline-5-carboxylate synthetase and ornithine aminotransferase; 3) Branched chain amino acid (BCAA) transaminase; 4) aspartate transaminase; 5) TCA cycle enzymes; 6)a-ketoglutarate (AKG) dehydrogenase; 7) NADP-linked malic enyzme, phosphoenolpyruvate carboxykinase/pyruvate kinase and oxaloacetate decarboxylase; 8) amido-phosphoribosyl-transferase catalyzes the initial step for de novo purine synthesis; 9) series of stepwise reactions utilizing glycine and aspartate for incorporation of C=C-N and N, respectively (formate and carbon dioxide contribute single C to the final purine product); 10) myokinase (adenylate kinase) to form ADP and oxidative phosphorylation.

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Chapter 5

Alternate Small Bowel Preservation Strategies: The Role of Luminal Preservation

Current small bowel procurement protocols including hypothermia and vascular-delivered cytoprotective agents remain unable to provide intestinal preservation for periods equivalent to that of other routinely transplanted intra-abdominal organs. Alternative methods aimed at minimizing preservation injury within the clinical confines of multi-viscera procurement are needed as intra-arterial UW solution fails to provide adequate graft protection beyond 6-10 hours of cold storage (1).

As early as 1967, evidence began accumulating that modification of the intraluminal contents could affect the response of intestinal mucosa to ischemic insults (2). The luminal environment is a potentially toxic one, rich in pancreatic and biliary secretions as well as bacteria. Bounous et al, in their study documenting hemorrhagic enteropathy in shocked animals, suggested that dilution of pancreatic enzymes reduced the level of "tryptic enteritis" seen in the mucosal layer, made vulnerable by decreased cellular energy levels (3). In addition to pancreatic enzymes, others suggested that release of lysosomal enzymes such as hydrolase from hypoxic-damaged lysosomes could also be diluted through application of a luminal flush solution (4).

During ischemic insults, reproducible, time-related morphologic changes occur beginning at the mucosal layer (5). As previously discussed, the first evidence of histologic damage is formation of a sub-epithelial space, referred to as Gruenhagen's space. This space has been suggested to occur secondary to fluid shifts of both vascular (6) and luminal (7) origin. Rules of fluid transport dictate that movement

between intracellular and extracellular compartments are governed by hydrostatic and impermeant [oncotic + osmotic] forces. The period of cold storage alters the contribution of each of these forces to net fluid shifts. Hydrostatic forces become minimized as blood flow ceases within the stored organ. Changes in oncotic and/or osmotic forces may represent the major contributing factors to fluid shifts experienced during preservation. As cellular hydration states participate in the regulation of cell function (8), study of the role of luminally delivered oncotic and osmotic support may also prove to be critical components required in preventing sub-epithelial cleft formation.

Along with barrier (9) and immuno-modulatory (10) functions, the primary role of the small bowel is to provide an important surface by which orally ingested nutrients are absorbed. Interestingly, epithelial cells have been shown to remain metabolically intact even during periods of cold storage (11). As such, intraluminal supply of substrate specific to the metabolic needs of the small bowel could theoretically prove integral to the advancement of small bowel preservation. Although no specific reason was noted, Zhu et al documented improved biochemical and histologic preservation in addition to overall improved survival if luminal flushing was added to the standard vascular flush (12). Others suspected that the presence of a solution in contact with the intestinal mucosa would provide a trophic stimulus to the small bowel, either through direct contact or systemic (secretagogue stimulus) routes (13). The work of Ito et al provided some of the greatest support for luminal delivery of nutrients (14). In this study, they showed that luminal substrate provided preferential metabolic support to the mature cells of the villus tips. Targeted nutrient supply was further suggested by showing that intravascular supply of substrate showed preferential supply to crypt and junctional epithelial cells.

In order to address nutrient supply via the intraluminal route during periods of cold storage, basic physiology of the small bowel must be discussed. Only then can one

concentrate on matching the metabolic needs of the bowel subjected to an environment of hypothermic ischemia.

Carbohydrate Metabolism

Sucrose, lactose and starches comprise the majority of carbohydrates ingested by the human diet. Although comprising a much smaller portion of ingested carbohydrates, a variety of other sources also exist. These include amylose, glycogen, alcohol, lactic acid, pyruvic acid, pectins, cellulose, and dextrins. Following ingestion, oral mastication mixes food particles with saliva, known to contain ptyalin (an ∝-amylase). This is secreted in large part by the parotid gland and results in hydrolysis of starch to maltose. Once transported to the stomach, the low native pH inactivates these salivary enzymes. Following movement into the duodenum, pancreatic enzymes including ∝-amylase result in further hydrolysis. The remaining small bowel then sees a predominance of maltose and other small glucose polymers. As the enteric contents proceed along the bowel lumen, mucosal enterocytes potentiate further carbohydrate metabolism. This is made possible by four enzymes; lactase, sucrase, maltase, and ∝-dextrinase, all collectively referred to as disaccharidases. These four enzymes are located within the brush border of the villi facilitating conversion of carbohydrates from disaccharides to monosaccharides, the final byproduct which is easily absorbed into the enterocyte and subsequent portal venous system. The actual absorption of monosaccharides into the enterocyte is largely energy (ATP) and Na^+ dependent. Movement of glucose into the portal system is then via a non-Na^+ dependent carrier. Fructose is the only monosaccharide that is absorbed by a process of facilitated diffusion. This is a carrier-mediated process independent of Na^+.

The inclusion of carbohydrates and their potential to improve graft quality was studied early in the course of small bowel preservation. This was largely due to the fact

that glucose was originally deemed the primary fuel source of the enterocyte (15). Chiu et al looked at the protective effect of intraluminal glucose during periods of warm ischemia using a canine model (16). Following 60 minutes of warm ischemia, significant improvements in both structural (grade of histologic damage) and biochemical (mucosal ATP levels) end-points were documented in intestinal specimens that had been luminally treated with 10% glucose solution. Those specimens treated with the glucose solution were also noted to have significantly higher lactate levels, suggesting that glycolytic pathways had been fueled and anaerobic metabolism ultimately better supported. As hypothermia and ischemia are necessarily incurred during small bowel storage, reliance on anaerobic metabolism seemed implied. Kokudo et al also investigated the role of glucose during cold ischemia (17). Based on electrophysiologic function they showed that mucosal function was better preserved with a glucose storage solution. The optimal glucose concentration reported was 2.5%. Further support was made for luminal-administered glucose as other studies documented overall changes in amino acid absorption with minimal to no change to glucose absorption following transplant. Oishi et al used a brush border membrane vesicle model to show that the number of glutamine transporters were decreased post-transplant whereas glucose absorption remained unchanged (18). As well, the effect of extrinsic denervation, an inevitable result of small bowel procurement, also left glucose absorption unchanged whereas glutamine absorption was significantly decreased (19). Weber et al, did show that an intraluminal glucose concentration of 56mM stimulated mucosal protein synthesis in both fed and fasted states using a rat model (20).

The majority of initial studies supporting glucose as the primary respiratory fuel of the enterocyte were performed in vitro. Mucosal tissue subjected to this was determined to exhibit significantly increased rates of glycolysis, presumably the result of inadequate

tissue oxygenation (21). Later studies performed in vivo did not support this level of glucose metabolism. In fact, glutamine and ketone bodies, rather than glucose, were determined to be the principal source of intestinal energy supply (22). In fact, glucose only provided 6% of total splanchnic CO_2 production.

Protein Metabolism

Human metabolism of ingested protein is broken down into gastric and intestinal phases. Once proteins are ingested into the stomach two different enzymes, called pepsins, are released by chief cells and activated within the acidic environment. These enzymes are called Pepsinogen A or I and Pepsinogen C or II. Once activated they hydrolyze internal peptide bonds of proteins resulting in smaller peptide fragments. Along with the activity of pepsins A and C, the acidic pH of the stomach also assists in protein breakdown. This is based on the effect of denaturing of proteins seen within an acidic environment.

The intestinal phase is subdivided into luminal, brush border, and intracellular phases. Once within the lumen of the duodenum, proteins come in contact with pancreatic secretions. All pancreatic secretions are released in an inactive form (referred to as zymogens). These pancreatic products are broken down into two groups: endoproteases and exopeptidases. There are three forms of endoproteases: trypsinogen, chymotrypsinogen, and proelastase. There are two forms of exopeptidases: procarboxypeptidase A and B. Activation of these pancreatic zymogens occurs on contact with a duodenal, brush border produced enzyme referred to as enteropeptidase (also known as enterokinase). The only substrate for enteropeptidase is trypsinogen. Upon contact with enteropeptidase, trypsinogen is activated to trypsin that in turn can perpetuate further activation of itself via an auto-catalytic process as well as activation of remaining pancreatic secretions. Activated enzymes now result in

protein metabolism via substrate-specificities. This results in a mixture of oligopeptides and free amino acids which subsequently come in contact with the brush border of the enterocyte. The brush border phase is important in two respects; free amino acids are absorbed directly into the enterocyte via distinct classes of transport carriers whereas oligopeptides undergo further breakdown via brush-border enzymes (aminopeptidase, endopeptidase, and dipeptidase). Each carrier protein transports a class of amino acids (neutrals, cationics, aromatics, amino acid amides) rather than a specific amino acid. These transport systems are loosely divided into Na^+-dependent and Na^+-independent groups (23). Glutamine is one example of an amino acid utilizing this Na^+ gradient. It is absorbed by the carrier protein designated System B. Located on the brush border membrane, a Na^+ ion attaches to this carrier causing a conformational change. This results in greater affinity for glutamine and as a result both Na^+ and glutamine are moved to the intracellular space. Aspartate and glutamate on the other hand, undergo absorption via a separate transport carrier which is still dependent on a Na^+ gradient. A list of transport systems including their amino acid specificities are included in Table 5-1. The end product of protein within the enterocyte is either further degradation by intracellular peptidases, conversion into other amino acids, or incorporation into cellular proteins.

Glutamine has been determined to be the principal fuel source of the small intestine (24-27). Supply of this non-essential amino acid has now overtaken glucose as the most commonly studied nutrient given to intestinal specimens undergoing periods of cold storage. Further work by Windmueller and Spaeth documented that approximately 39% of total splanchnic CO_2 production comes from oxidation of luminal-supplied glutamine, glutamate, and aspartate (28). An additional 38% of total respired intestinal CO_2 was derived from arterial glutamine. Jungas et al confirmed similar rates of amino

acid usage in human intestine (29). Apart from glutamine, absorption of other vascular-supplied amino acids was found to be negligible (30). It was the work of Wu that further defined mucosal amino acid catabolism. Through quantification of the percentage of absorption and utilization of individual amino acids, this lab was able to show that the greatest contributing route of amino acid administration was indeed via the lumen. In addition, this study showed that not all amino acids absorbed by the small intestine entered the portal circulation directly. Some are required for mucosal metabolic needs while others serve a synthetic component (ie synthesis of proteins and other critical molecules) see Table 5-2. Through characterization of amino acid specific absorption rates, it is possible to tailor luminal-delivered solutions to provide energy and support other cellular housekeeping roles required by the small bowel during the critical period of cold storage.

Overall, there remains a relative paucity of research looking at manipulation of intraluminal perfusates for the sole purpose of small bowel preservation. As most protein and carbohydrate metabolic data have been determined using normothermic models, direct application to models of hypothermic ischemia is subject to potential discrepancies. Whether through dilution of potentially toxic enteric contents, provision of impermeant support, or supply of nutrients to otherwise metabolically active cells, luminal flush solutions may provide an important link to overall improved small bowel preservation. The following three chapters look to address the role of luminal-delivered solutions for the purpose of improving small bowel preservation quality.

Table 5-1 Amino acid transport systems in the brush border membrane of the small intestine.

Transport System	Substrates	Dependent on Na^+ gradient	Involvement of other ions
B	Dipolar α-amino acids	Yes	None
$B^{0,+}$	Dipolar α-amino acids Basic amino acids, Cysteine	Yes	None
$b^{0,+}$	Dipolar α-amino acids Basic amino acids, Cysteine	No	None
y^+	Basic amino acids	No	None
IMINO	Imino Acids	Yes	Cl^-
β	β-amino acids	Yes	Cl^-
X_{AG}^-	Acidic Amino Acids	Yes	K^+

Table 5-2 Important Roles of Intestinal Amino Acids

Amino Acid	*Action*
Polyamine precursor ornithine is derived from Arg, Gln, Pro	Essential to proliferation, differentiation, epithelial cell repair
Arg is precursor of intestinal NO (16% of total body NO)	Regulates intestinal blood flow, integrity, secretion, epithelial cell migration.
Glutathione precursors glu (non gln-derived glu), gly, cys	Defends intestinal mucosa against toxic & peroxidative damage
Thr, Lys, Leu, Met, Trp, Ser, Gly	Implicated in major protein synthetic role

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Chapter 6

Defining the role of a tailored luminal solution for extended small bowel preservation.

INTRODUCTION

The small bowel (SB), like all transplanted organs, is susceptible to cellular injury sustained during periods of cold storage. Within minutes of ischemia onset, morphologic changes begin at the mucosal level, the layer most sensitive to a hypoxic insult (1). As storage time increases, progressive compromise and eventual loss of the mucosa occurs, resulting in violation of its barrier function (2) and absorptive capacity (3). The SB graft simultaneously becomes susceptible to bacterial translocation (4,5) and increased antigenic stimulus. This may ultimately result in a combination of coincident sepsis and rejection, each significantly affecting current outcomes in SB transplantation (6). The ability to maintain mucosal morphology and function throughout clinically relevant storage periods may provide another important step toward successful preservation of this unique organ.

Currently, SB storage protocols involve isolated vascular administration of preservation solutions. To date, no one solution, including the "gold standard", University of Wisconsin (UW) solution, can reliably prevent mucosal injury for extended periods of storage (7,8). The SB is unique however, in that it possesses an additional route by which cytoprotective agents can be administered. The lumen normally provides an important route for absorption of nutrients, water, and electrolytes. Mucosal epithelial

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cells at the villus apex, known to remain metabolically active during storage (9), have been shown to preferentially absorb nutrients from the lumen rather than vasculature (10). Therefore, luminal administration of preservation solutions, formulated on physiologic requirements may target the exact site of early storage injury, namely the mucosa.

This study was designed to test the hypothesis that luminal administration of a preservation solution, formulated on SB metabolic requirements, will maintain energy levels, barrier function, and morphology of the mucosal epithelial surface during SB graft 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. Water was provided *ad libitum*, and cage floors raised to minimize coprophagy. At the time of laparotomy, rats were administered an interperitoneal dose of 65mg/250g pentobarbital (Somnotol; MTC Pharmaceuticals, Cambridge, Ontario, Canada), followed by inhalational halothane (1-2%) as required to achieve and 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 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 (ligament of Treitz to ileocecal junction)

were harvested in continuity. The proximal jejunum was immediately cannulated and twenty millilitres (based on 1x volume/weight calculation) of individual preservation solution (Table 6-1) subsequently flushed via the lumen. Enteric effluent was allowed to freely exit the distal ileum until clear (approximately 12-13 ml of 20 ml). The terminal ileum was then ligated with 3-0 silk and the remaining volume (7-8 ml) infused allowing for mild distention as well as equal distribution of the solution throughout the entire length of the harvested graft. The bowel was then stored in 30 ml of ice-chilled preservation solution identical to that used to flush the lumen. A 1-2 g tissue sample was taken at t= 1, 2, 4, 10, 24 h. Tissue samples were taken beginning at the jejunal end of the graft. To arrest metabolic activity, samples were snap frozen in liquid nitrogen, and subsequently stored at -65°C until processed.

Composition of preservation solutions (Table 6-1) Four groups of animals (n=4) received intravascular UW solution followed by one of four luminal solutions ($UW_{(l)}$, UWG, AA1, AA2). These were compared to a control group, treated only with vascular UW solution, $UW_{(v)}$.

Sample Preparation and Metabolite Assay for Energetics: Frozen SB samples were weighed and then extracted 1:5 w/v in 6% perchloric acid containing 1 mM EDTA. The precipitated protein was removed by centrifugation (20 min at 20,000 g). Acid extracts were neutralized by the addition of 3 M KOH/0.4 M Tris/0.3 M KCl and then recentrifuged (20min at 20,000 g). Aliquots of the 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, and AMP. Values are reported in μmol/ gram wet weight.

Histological Examination: 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 the Park's histologic classification for damaged bowel on two sections from each specimen (12). All samples were evaluated in a blinded fashion by two independent pathologists (LJ and JZ).

Functional Assessment– In Vitro electrical measurements: Ileal segments were taken at 4 h and 10 h cold storage, stripped of their serosa and muscular layers, and mounted in Ussing chambers. Once mounted, the mucosal and submucosal surfaces were perfused with a recirculating solution containing bicarbonate Ringer's solution, with an ionic composition of: Na^+, 143 mM; K^+, 5 mM; Mg^{2+}, 1.1mM; Ca^{2+}, 1.25 mM; HCO_3^-, 25 mM; Cl^-, 123.7 mM; HPO_4^{2-}, 0.3 mM; and fructose, 20 mM with 95% O_2 and 5% CO_2, at 37 °C, and pH=7.4 (13). Permeability was measured via scintillation counter determining the flux of a radioactive substrate (10 μCi [^{3}H]mannitol, Dupont, NET101) across individual ileal specimens. The spontaneous transepithelial potential difference (PD and short-circuit current (I_{sc}) were determined for all segments, and tissue conductance was calculated from PD and I_{sc} according to Ohm's law (14).

Statistical analysis. Energetics and permeability data were reported as means ± SE for each group. Statistical differences within groups was determined using ANOVA, followed by Dunnett's *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 6-1A): Specimens preserved with either vascular UW alone ($UW_{(v)}$) or luminal UW ($UW_{(l)}$) possessed significantly lower levels of ATP throughout 10 h when compared to all other luminal groups (UWG, AA1, and AA2). Tissue treated with UWG, AA1, or AA2 retained significantly higher ATP levels compared to control $UW_{(v)}$ at 10 h

(0.9, 1.0, 0.9 vs 0.4 μmol/g; $p<0.05$). By 24 hours, no significant difference was noted between any luminal group and control; range of 0.1-0.3 μmol/g vs 0.2 μmol/g, $p<0.05$

Total Adenylates (TA) (Fig. 6-1B): All luminal treated groups with the exception of $UW_{(l)}$ maintained significantly higher TA levels compared to vascular UW treatment alone throughout 4 h. At 10 h, only the AA1 group possessed levels greater than either $UW_{(v)}$ or $UW_{(l)}$, 3.3 μmol/g vs 2.4, 2.6 μmol/g, $p<0.05$. By 24 h however, the $UW_{(l)}$ treated group result in the greatest TA level at 24h, with a value of 1.4 μmol/g.

Energy Charge (EC) (Fig. 6-1C): Over the first 10 h of storage, patterns of EC values paralleled ATP/ADP ratios, with the 3 amino acid containing solutions [UWG, AA1, AA2] exhibiting much higher values than control $UW_{(v)}$ and $UW_{(l)}$ groups. EC values after 10 h were equivalent to the earlier (1-2h) time points for $UW_{(v)}$ for these three experimental groups. Even after 24h, UWG, AA1, AA2 groups remained significantly greater than control; values ranged from 0.40 to 0.56 compared to 0.30, respectively ($p<0.05$).

Functional Assessment of Stored Tissue

Permeability (Fig. 6-2A) (in nmol/cm^2/h): At both time points (4 and 10 h), specimens preserved with a luminal solution possessed significantly lower degree of permeability. At 10 hours, tissues treated with either AA1 or AA2 solutions retained permeability values significantly lower when compared to $UW_{(v)}$, $UW_{(l)}$, and UWG (67, 22 vs 438, 146 and 123 nmol/cm^2/h, respectively; $p<0.05$). Of note, at 10 h, AA2 treated specimens was the only group which retained barrier function equivalent to that of freshly isolated tissue (22 nmol/cm^2/h.

Conductance (Fig. 6-2B) (in mS/cm^2): Conductance values are plotted as increases over freshly isolated values. Levels in $UW_{(v)}$ treated tissues rose dramatically compared to all other experimental groups with values of 59 and 243 mS/cm^2 at 4 and 10 h, respectively. Although luminal flushing had a marked effect independent of solution

composition when compared to $UW_{(v)}$, AA2 treatment possessed the lowest conductance values of any group; with only a 2.4 mS/cm^2 increase above freshly isolated values (15.8 mS/cm^2).

Mucosal Short-Circuit Current (Isc) (Fig. 6-2C): Isc is plotted as the deviation from freshly isolated values. The three amino acid containing luminal solutions, (UWG, AA1, AA2) exhibited a significantly greater degree of maintenance compared to both $UW_{(v)}$ and $UW_{(l)}$ groups. Paralleling permeability and conductance findings at 4 at 10 h, treatment with AA2 resulted in superior maintenance of Isc compared to all other groups; $p<0.05$.

Light Microscopy Histology (Table 6-2, Figures 6-3, 6-4, 6-5)

By 10h storage, complete loss of the mucosal layer and transmural infarction was noted in the clinically relevant control, $UW_{(v)}$ (median grade 8); Fig. 6-3b. Regardless of luminal solution administered, the degree of damage was significantly decreased; median values after 10h storage ranged from 2 to 5.5 ($p<0.05$). The smallest degree of morphologic damage occurred with AA2 treatment; median grade 2 (Fig. 6-3d). Even after 24 h of cold storage, the protective effect of amino acid solutions, AA1 and AA2, remained evident, with each possessing median grades of 4.

Correlation values were determined between Park's grade of histologic damage and a) ATP, b) Isc, and c) Permeability (Figure 6-5). A linear correlation was found for both ATP and Isc with correlation r values of 0.908 and 0.963 respectively. A value of 0.996 was noted for Permeability.

DISCUSSION

The small bowel (SB), unlike other transplanted organs, remains in perpetual contact with the toxic environment found within the intestinal lumen. Normally, potentially toxic contents including microorganisms remain confined to the lumen by the

mucosal epithelial surface. This protective mucosal barrier however is extremely sensitive to even minor alterations in regional blood flow and oxygen supply. As shown to occur during shock and other low flow states (15,16), the mucosa is the bowel layer most sensitive to hypoxia, undergoing structural damage within minutes of insult (17). When exposed to prolonged periods of hypoxia, as experienced during clinical graft storage, continued compromise and eventual loss of this barrier is inevitable. As this occurs, direct contact with luminal microorganisms results, placing the SB transplant recipient at risk for bacterial translocation (4,5) and resultant antigenic stimulus. The ability to reliably prevent mucosal injury may provide an integral step for successful SB preservation and transplantation.

The SB possesses both a vascular and luminal route by which fluids, nutrients, and other substrates can be absorbed. Animal models (18,19), detailing physiologic rates of nutrient uptake have documented that the lumen, rather than the vasculature, is the route by which the majority of the body's nutrient absorption takes place. The importance of luminal supply is further suggested as metabolic support for specific portions of the mucosal layer itself has been shown to occur from use of this route (10). Mature epithelium, located at the apex of the villus, preferentially absorb the bulk of its nutrient support from the lumen whereas crypt cells are supported in large part by nutrients from the vasculature.

Currently, the role that luminal-delivered preservation solutions play in SB graft storage has not been thoroughly investigated. Luminal flushing with simple crystalloid solution has been shown to maintain mucosal function compared to vascular flushing alone (20). This was originally postulated to occur by dilution of resident enteric contents, comprised of feces and other cytotoxic agents including bacterial endotoxins as well as biliary and pancreatic secretions (21,22). However, this beneficial effect was

absent by 12 hours of storage and was noted to be at the expense of histologic integrity. In addition to potential diluting benefits, supplementing a luminal solution with gln, the primary fuel source of the enterocyte, was shown to improve mucosal structure and function when compared to similar vascular treatment alone during storage as well as a pseudo-reperfusion period (10). Even though the importance of a luminal-supplied preservation solution has been suggested by such studies, the composition required for such an agent has not been fully elucidated. Therefore, based on known physiologic SB nutrient requirements (18) and associated *in vivo* luminal absorption rates (19), each solution tested in this study was composed in an attempt to further assess and ultimately tailor the composition of a luminal-supplied solution which could sustain both energy and non-energy related processes of SB during cold storage.

As noted initially, administration of any of the four luminal-delivered solutions resulted in improvement of functional indices as well as degree of histologic damage incurred when compared to the clinical control, UW(v). This beneficial effect again points to the possibility of non-specific dilution of cytotoxic substances located within the lumen. Energy levels however, in the form of ATP, remained consistently higher in specimens treated with only UWG, AA1, or AA2 solutions for up to a clinically relevant period of 10 hours. The important difference in each of these three solutions was the presence of gln, ranging from 35-137 mmol/l. As the SB mucosa remains metabolically active during periods of hypothermic-hypoxia (9), elevated ATP levels implicate continued gln absorption and utilization. In the absence of gln supply, as seen in the luminal UW group, rapid declines in ATP levels occurred, further demonstrating that luminally-delivered nutritional support can significantly improve SB cellular energy stores even during periods of hypothermic-hypoxia. By 24 hours however, ATP levels in each of the four luminal treated groups, regardless of the presence or absence of glutamine,

were unchanged from that of the clinical standard, $UW_{(v)}$. With respects to energetics alone, the cumulative effects of persistent hypothermia and hypoxia eventually negate the benefit of using such a tailored solution for small bowel preservation beyond 10 hours of cold storage.

Energy levels are directly related to the maintenance of barrier function in the SB graft (1). This barrier is maintained largely by the tight junction region located close to the apical surface of the intestinal epithelial cells. Agents which deplete cellular ATP lead to dilation of the tight junction by perturbation of the actin cytoskeleton, ultimately increasing both epithelial permeability and transepithelial flux of macromolecules (23). Importantly, this disassembly is reversible; repletion of cells with ATP leads to re-assembly of the tight junctions and restored epithelial barrier function. Despite similar energy profiles in UWG, AA1, and AA2 groups for up to 10h storage, significant differences persisted in both barrier function and grade of morphologic injury. This suggested that other components found only in AA1 and AA2 solutions provided support for other non-energy related cellular processes. Both AA1 and AA2 solutions were comprised of 18 additional amino acids known to be absorbed only by the luminal route (18). Included among these were arginine (arg), proline (pro), glycine (gly), cysteine (cys), threonine (thr), lysine (lys), leucine (leu), methionine (met), and serine (ser); all of which have been noted to play key roles in a variety of cellular 'housekeeping' processes. For example; arg, glu and pro are precursors of ornithine, known to be required for epithelial cell proliferation and repair (24). In addition, defense of the intestinal mucosa against toxic and peroxidative damage is made possible due to production of glutathione, dependent on the precursors glu, gly and cys (25). Arg, a precursor of nitric oxide (NO), has been shown to play an integral role in the regulation of intestinal blood flow (26). Other amino acids including thr, lys, met, tryp, ser and gly,

have also been implicated in major protein synthetic roles (27). The contributing role of each individual amino acid during storage is clearly beyond the scope of this paper. However, when combined, these non-energy related amino acids provide an important adjunctive role in maintaining overall mucosal viability during periods of hypothermic-hypoxia.

Belzer and Southard have previously suggested that an effective preservation solution must also contain agents aimed at minimizing hypothermic-induced swelling as well as non-physiologic pH deviations (28). Fluid shifts from both the lumen (29) as well as the surrounding vessels (30) have been jointly implicated as the cause of early sub-epithelial clefting (Gruenhagen's space), exacerbating disruption of the mucosa from the underlying submucosal layer. A minimum osmotic force of 110-140 mOsm/kg has been determined to counteract such cellular swelling. The addition of lactobionate to the AA2 group alone could explain, at least in part, the superior maintenance of histology at 10 hours when compared to the AA1 group (grade 2 vs grade 3.5). Addition of a buffering agent, known to counteract non-physiologic pH changes incurred during storage (31,32), likely also contributed to maintenance of functional parameters including mannitol permeability, conductance and short-circuit current remaining unchanged from values of freshly isolated tissue in only the AA2 group.

In summary, contact with the potentially toxic luminal environment occurs during cold storage as well as in the post-transplant period. Graft injury incurred during storage reliably begins with loss of mucosal barrier function and morphologic integrity leading to failure of this natural barrier to isolate the sterile internal from the non-sterile external environments. Regardless of composition, vascular solutions have been unable to provide support for this critical barrier during clinically relevant periods of storage. Based on the *in vitro* preservation data presented, this study strongly implicates the

requirement for using a luminal-delivered solution, formulated on physiologic nutrient requirements supporting both energy and non-energy related cellular processes, to minimize mucosal epithelial injury incurred during storage. Ultimately, follow-up transplant studies will address the anticipated relationship between reduction of preservation injury and superior clinical outcomes.

Table 6-1 Composition of Preservation Solutions

	UW	UWG	AA1	AA2
Lactobionate	100	100	---	100
Raffinose	30	30	---	---
Adenosine	5	5	---	5
MgSO4	5	5	---	---
KH2PO4	25	25	---	---
KOH	100	100	---	---
BES	---	---	---	90
NaCl	---	---	80	---
Glutamine	---	137	35	35
Glucose	---	20	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	---	---	10	5
Ornithine	---	---	10	5
Leucine	---	---	10	5
Isoleucine	---	---	10	5
Histidine	---	---	10	5
Cysteine	---	---	10	5
Proline	---	---	10	5
OH-butyrate	---	---	3	3
Tyrosine	---	---	1	1
Tryptophan	---	---	1	1

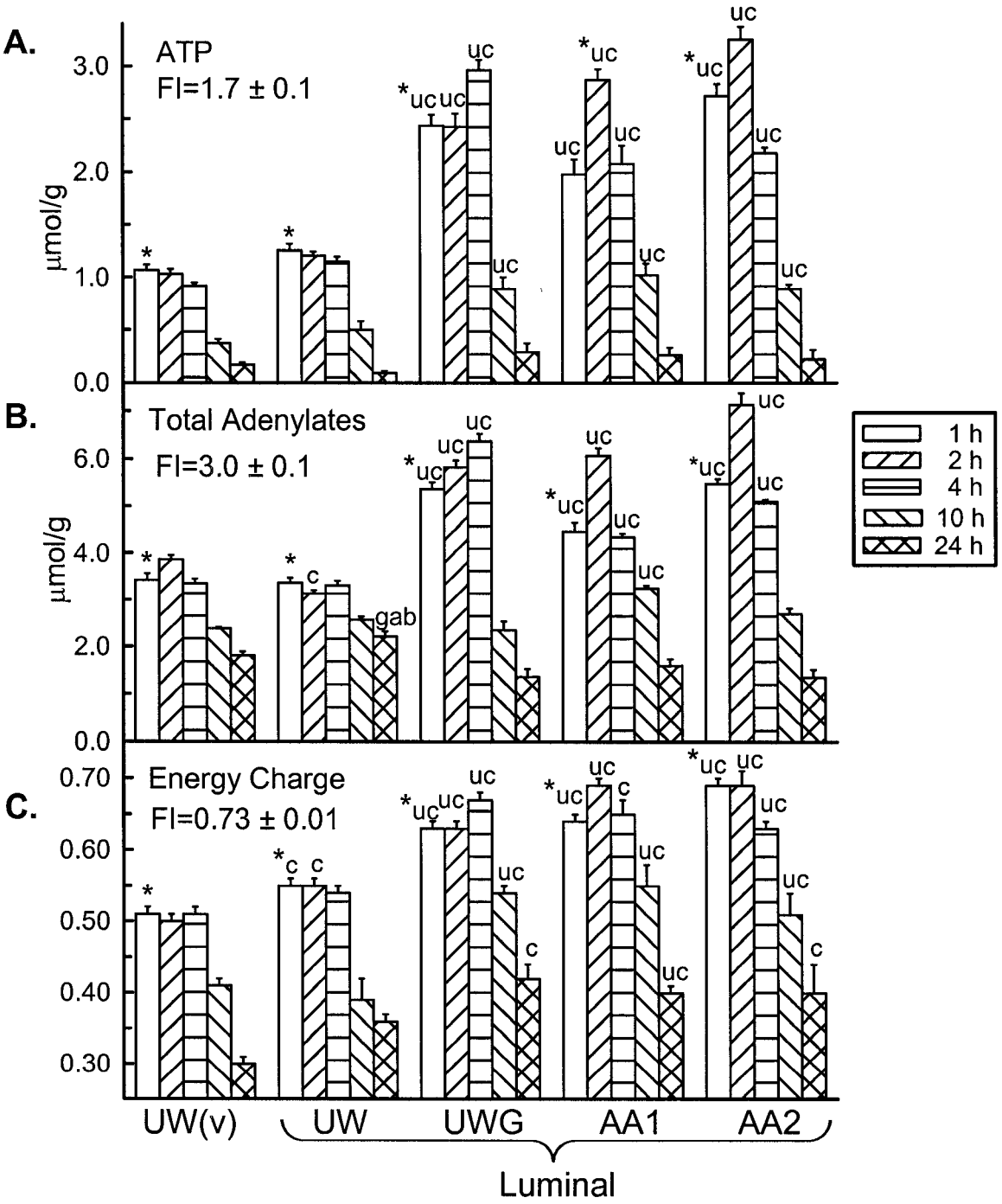
Values in mM/litre. The pH of solutions was adjusted to 7.40 ± 0.01 using NaOH. UW –denotes modified University of Wisconsin solution; G –denotes the presence of glutamine. AA –denotes a mixture of amino acids based on physiological requirements.

Table 6-2 Histological Grading by Park's Classification

Solutions *Vascular*	*Luminal*	Time	Grading	Median	Significance *FI*	*UW(v)*	*UW(l)*	*UWG*	*AA1*
----------	FI 0	0, 0, 0	0 0, 0, 0, 1						
UW	----	4 h	5, 5, 5, 7	5	+				
		10 h	8, 8, 8, 8	8	+				
UW	UW	4 h	0, 0, 1, 1	0.5	-	+			
		10 h	2, 4, 7, 7	5.5	+	+			
		24 h	6, 6, 6, 7	6	+				
	UWG	4 h	2, 2, 2, 3	2	-	+	+		
		10 h	3, 4, 6, 8	5	+	+	-		
		24 h	3, 7, 7, 8	7	+		-		
	AA1	4 h	2, 2, 3, 3	2.5	-	+	+	-	
		10 h	3, 3, 4, 6	3.5	+	+	-	-	
		24 h	3, 4, 4, 6	4	+		+	+	
	AA2	4 h	0, 0, 1, 3	0.5	-	+	-	+	+
		10 h	1, 2, 2, 3	2	-	+	+	+	+
		24 h	4, 4, 4, 5	4	+		+	+	-

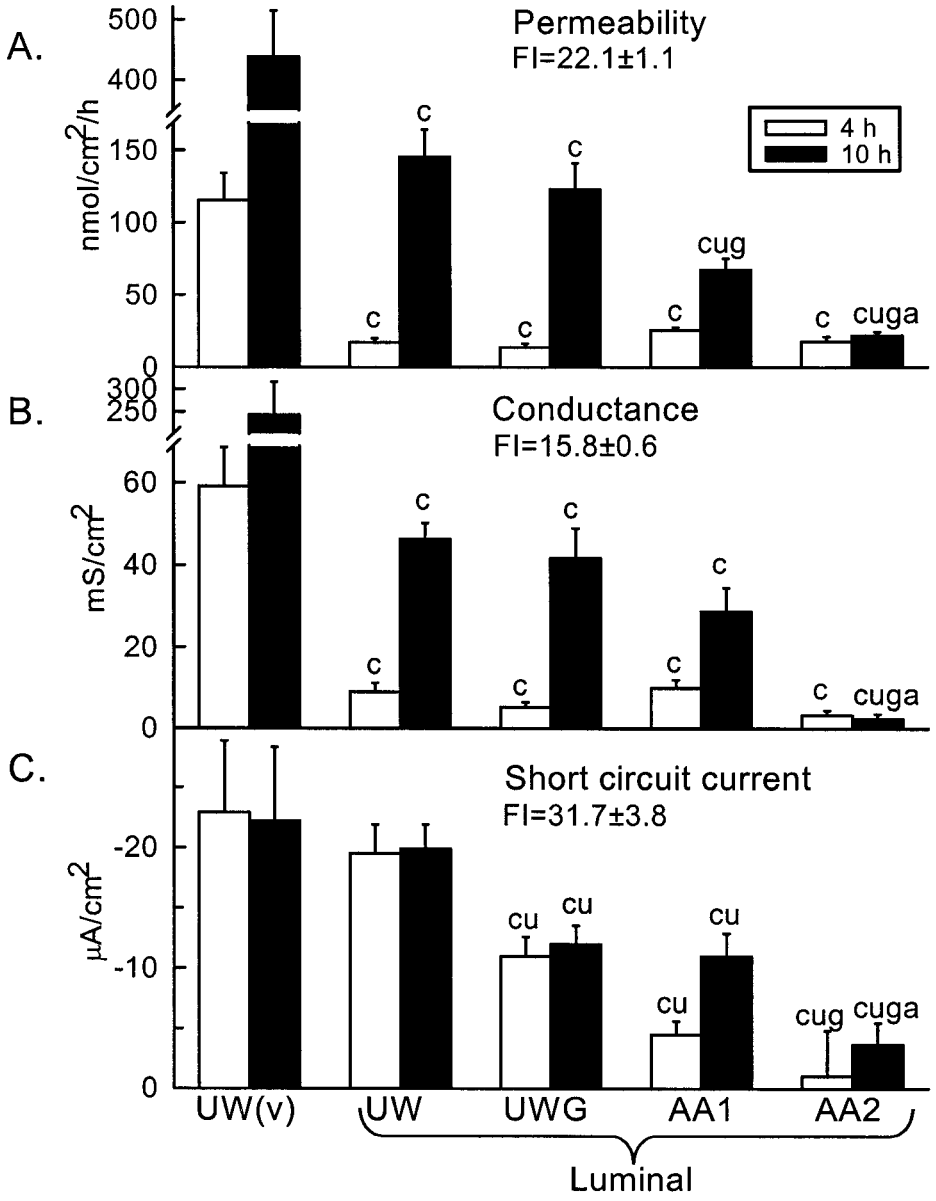
FI –denotes freshly isolated tissue. **+**, denotes statistical significance, $p<0.05$.

Figure 6-1 Effects of various luminal flush solutions on A) ATP, B) Total Adenylates and C) Energy Charge throughout 24h cold storage.



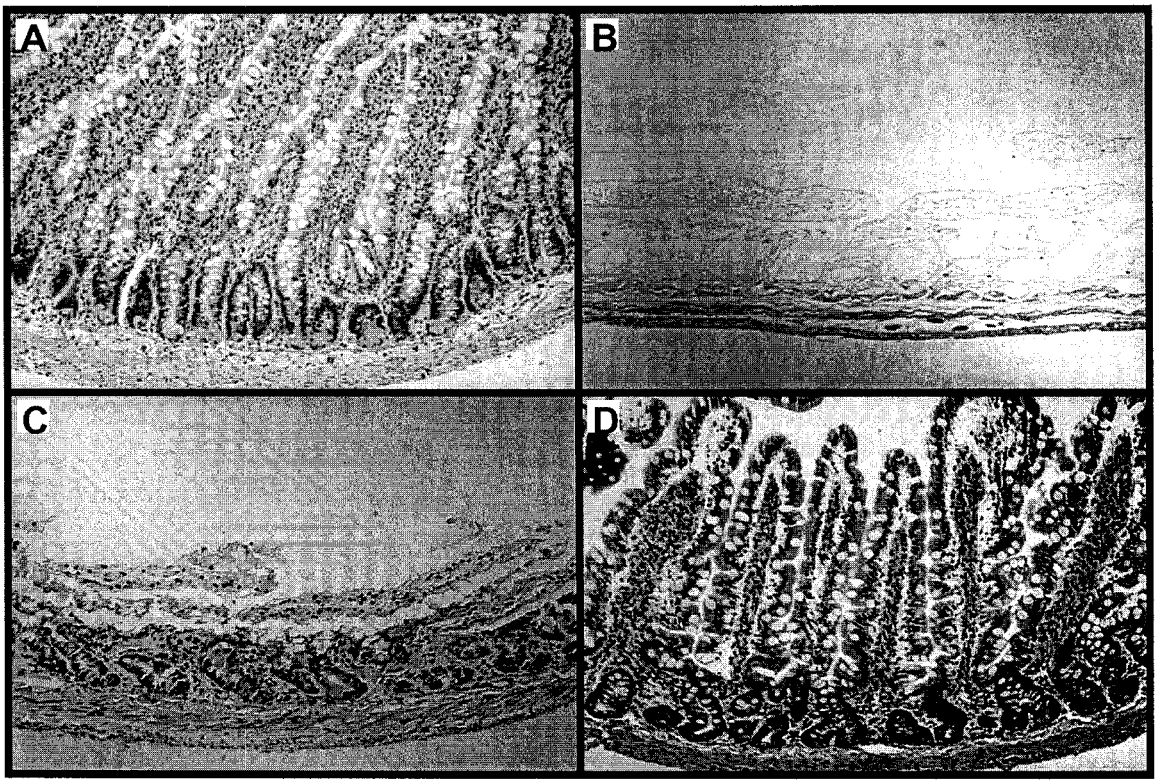
*- significantly different from freshly isolated (FI) values; $p<0.05$; c,u- significantly different from control ($UW_{(v)}$) and the $UW_{(l)}$ group, respectively; $p<0.05$

Figure 6-2 Effects of various luminal flush solutions on A)Permeability, B) Conductance and C) Short-Circuit Current after 4h and 10h cold storage.



Values are presented as differences from freshly isolated (FI) tissue values. c,u,g,a-significantly different from control ($UW_{(v)}$), $UW_{(l)}$, UWG, and AA1 groups respectively; $p<0.05$.

Figure 6-3 Light microscopy pictures of rat SB (5 μm, Hematoxylin and Eosin staining).



A) freshly isolated bowel (Park's grade 0), B) 10h $UW_{(V)}$ (Parks' grade 8), C) 10h UWG (Park's grade 5) and D) 10h AA2 (Park's grade 3) solutions. All pictures at x10.

Figure 6-4 **Light microscopy picture of bowel stored with AA2 solution for 24h (Park's grade 4). X10.**

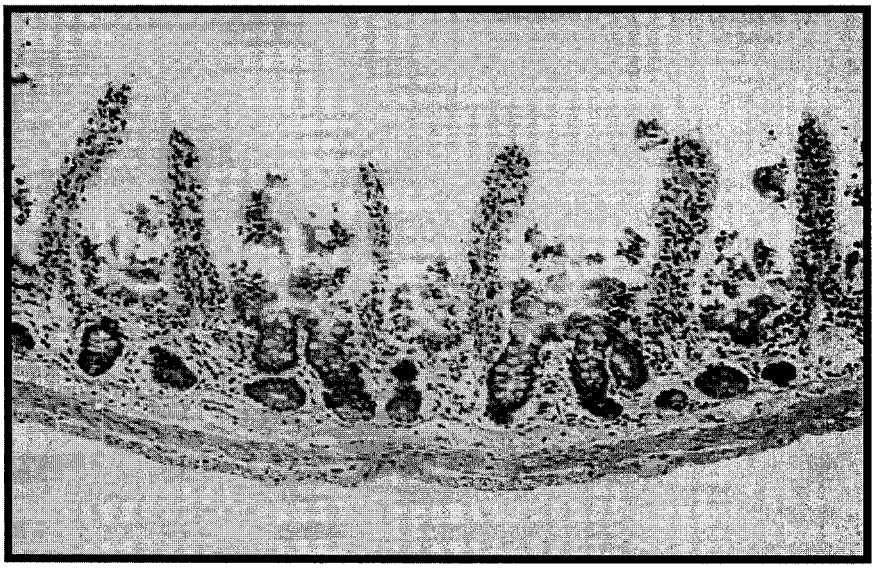
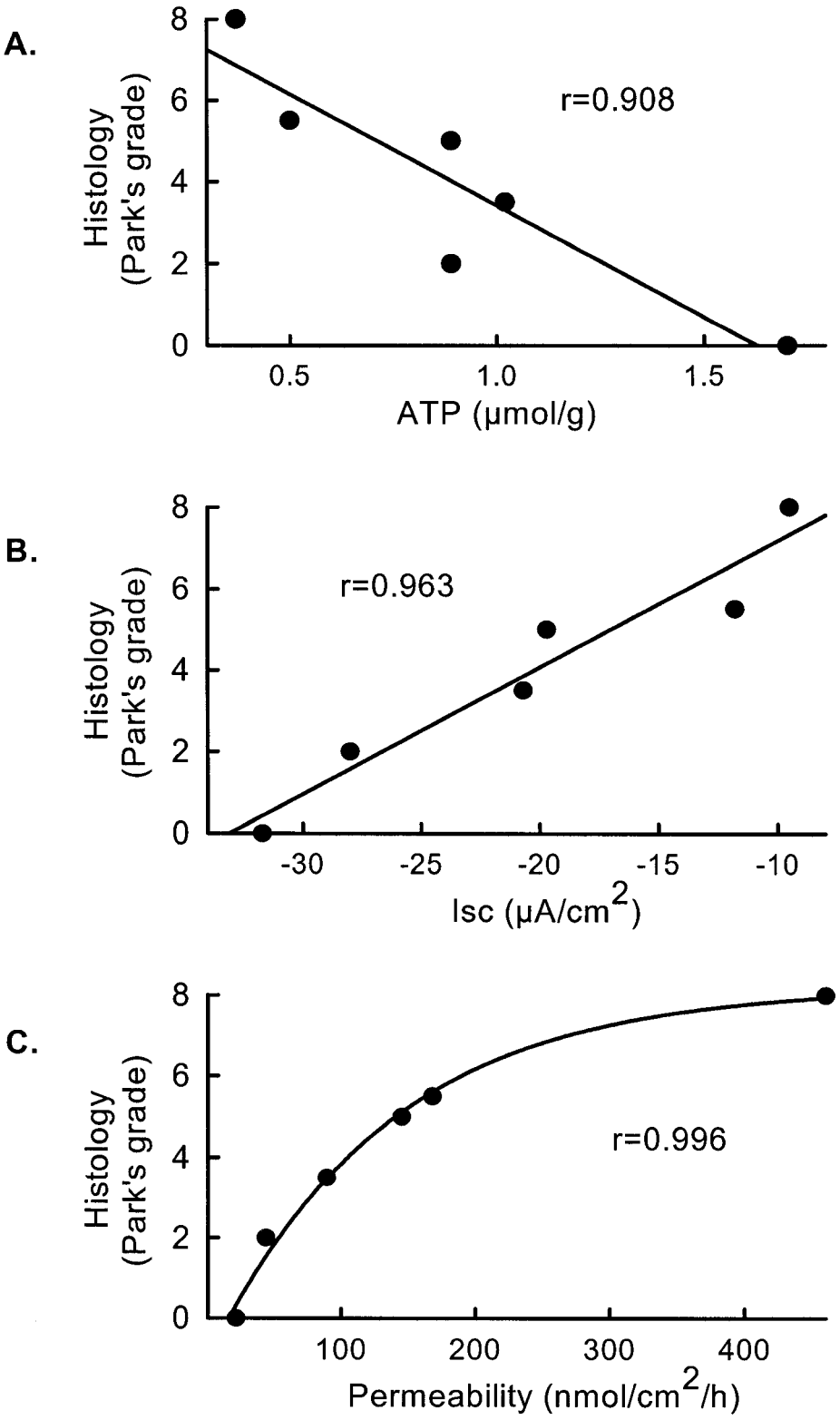


Figure 6-5 Correlation (r) values between median Park's grade of histologic damage and A) ATP, B) Isc and C) Permeability.



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Chapter 7

Intestinal Decontamination Using Povidone-Iodine Compromises Small Bowel Storage Quality

Introduction

The body remains in relative isolation from contents found within the enteric lumen because of the protective barrier provided by the small bowel (SB) mucosal layer (1). This layer however is extremely sensitive to a wide variety of non-physiologic insults, all of which result in varying degrees of enterocyte damage and resultant barrier compromise. Once mucosal damage develops, bacterial translocation (BT), a process by which bacteria and their products migrate from the intestinal lumen to sterile extraintestinal sites, can occur. Normally, systemic effects remain absent, as a functioning immune system rapidly eliminates the infectious threat. However, when sufficient mucosal damage and/or alteration of the hosts immune system exists, systemic effects, namely septic episodes, will result. As this describes the usual setting following SB transplantation, it is understandable why sepsis remains one of the most common post-operative complications (2).

Bacterial translocation has been shown to routinely occur following SB transplantation (3,4). Because of its clinical significance, strategies need to be developed to limit its prevalence. Even at procurement, two strategies exist. The first is to maintain the morphologic and functional integrity of the mucosal layer. As with other transplanted organs, this is done via administration of preservation solutions in

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conjunction with minimization of ischemic storage times. Compared to the current SB preservation standard, University of Wisconsin (UW) solution, we have previously documented that the addition of a luminal administered amino-acid rich (AA) preservation solution results in significantly less mucosal damage and improved barrier function during similar storage periods (5). Despite the noted improvements in overall graft quality, absolute arrest of ischemic damage is not currently possible. Therefore, translocation of bacteria remains a clinical threat.

Intestinal decontamination, a process which reduces the level of enteric bacteria, is an alternate strategy. Currently, clinical whole pancreas transplantation routinely institutes steps addressing the potentially toxic enteric lumen. At procurement, many transplant programs administer Povidone-Iodine (PI) within the duodenal stump for the purpose of duodenal decontamination (6,7). Interestingly, clinical duodenal stump complications are rarely seen following pancreas transplantation.

Since the transplanted SB is similarly non-sterile, use of an agent such as PI may provide a necessary link toward limiting bacterial translocation. The purpose of this study was to investigate the combined effect of luminal delivered PI and AA solution on SB graft quality throughout clinically relevant storage periods.

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. Water was provided *ad libitum*, and cage floors were raised to minimize coprophagy. Just 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 University of Wisconsin (UW) solution 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. Individual solution combinations were then placed into the lumen of the bowel allowing the effluent to exit uninhibited. Groups treated with PI remained in mucosal contact with this solution for a two-minute period, followed by flushing with the amino-acid rich solution. Each end was immediately ligated with 3-0 silk, leaving the bowel mildly distended with the same amino-acid rich solution. Two centimeters of terminal ileum were immediately removed from each specimen for measurement of bacterial counts (see Quantification of Bacterial Contents below). The bowel was then stored in 30 ml of the amino acid-rich (AA) preservation solution and stored at 4°C. A 1-2 g tissue sample was taken at time= 0 (immediately following flush), 4, and 10 hours. To arrest metabolic activity, samples were snap frozen in liquid nitrogen, and subsequently stored at -65°C until processed.

Composition of groups: Four groups, each with n=4, were luminally flushed with AA solution ± 10% Povidone-Iodine (PI) pre-treatment.

Group 1 – 1 x v/w* AA solution (20 ml)

Group 2 – 1 x v/w* 10% Povidone-Iodine (20 ml) + 1 x v/w* AA solution (20 ml)

Group 3 – 5 x v/w* AA solution (100 ml)

Group 4 – 1 x v/w* 10% Povidone-Iodine (20 ml) + 5 x v/w* AA solution (100 ml)

*- volume/weight

Quantification of Bacterial Contents:

Terminal ileum specimens were manually homogenized in 2.5 ml of sterile Dulbecco's phosphate buffered saline (PBS) and diluted in same. Enteric bacteria present were enumerated by triplicate plating onto Brain Heart Infusion plates (Difco #0037-03-0) and supplemented with 0.5% w/v yeast extract (Difco #0127-01), 0.05% w/v L-cysteine (Sigma #C-1276), 50 μg/ml Hemin (Sigma #H-1652) and 1 mg/ml vitamin K. All plates were incubated overnight at 37°C. Bacterial colonies were counted and expressed as colony forming units (CFU) per gram of homogenized tissue.

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 (8). Spectrophotometric analysis was then performed to measure the absorbance of NADH at 340 nm, providing quantification of ATP, ADP, and AMP. Values are reported per gram protein.

Functional Assessment: Ileal segments were taken at 4 h and 10 h cold storage, stripped of their serosa and muscular layers, and mounted in Ussing chambers. Once mounted, the mucosal and submucosal surfaces were perfused with a recirculating solution of bicarbonate Ringer's solution, with an ionic composition of: Na^+, 143 mM; K^+, 5 mM; Mg^{2+}, 1.1mM; Ca^{2+}, 1.25 mM; HCO_3^-, 25 mM; Cl^-, 123.7 mM; HPO_4^{2-}, 0.3 mM; and fructose, 20 mM with 95% O_2 and 5% CO_2, at 37°C, pH 7.4 (9). Permeability was measured via scintillation counter determining the flux of a radioactive substrate (10 μCi [^{3}H]mannitol, Dupont, NET101) across individual intestinal specimens. The spontaneous transepithelial potential difference (PD) and short-circuit current (I_{sc}) were determined for

all segments, and tissue conductance was calculated from PD and I_{sc} according to Ohm's law (10).

Statistical analysis: Metabolite and permeability data were reported as means ± SE for each group. Statistical differences between groups was determined using analysis of variance, followed by Tukey's 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 (Fig 7-1A): At each time point, PI-treated specimens (Groups 2 & 4) retained significantly lower levels of ATP compared to their non-PI treated counterparts (Groups 1 & 3), $p<0.05$. Even at 10 h of cold storage, Groups 2 & 4 remained 1.9-3.2 μmol/g protein lower than Groups 1 and 3; each with $p<0.05$. Furthermore, when compared to Group 1, significantly higher values were also noted when additional volume of AA solution (Group 3) was administered. This was evident at both 0 hours (19.1 vs 16.7 μmol/g protein) and 4 hours (14.9 vs 9.7 μmol/g protein), but not at 10 h, $p<0.05$.

Total Adenylates (TA) (Fig 7-1B): Luminal administration of PI resulted in consistently lower TA levels at each time point compared to non-PI treated specimens. Despite time-related reductions in all groups by 10 h, Groups 1 & 3 retained significantly higher levels compared to Groups 2 & 4; 22, 18.8 μmol/g protein vs 7.8, 13.2 μmol/g protein; each with $p<0.05$. In addition, following PI administration, higher levels of TA were found in specimens treated with larger volumes of the AA solution. Compared to Group 2, Group 4 possessed higher levels at all time points including 10 h, with levels measuring 7.8 and 13.2 μmol/g protein respectively, $p<0.05$.

Functional Assessment of Stored Tissue

Permeability (Fig 7-2A): When compared to freshly isolated specimens, only Group 2 specimens were noted to have any significant increase in the level of permeability after 4 h of cold storage. By 10 h, both PI treated groups (Groups 2 and 4) showed significantly higher levels of permeability compared to their non-PI counterparts, measuring 203, 66 vs 46, 44 nmol/cm^2/h respectively, $p<0.05$. In PI treated groups (2 and 4), an increased volume of AA solution following PI exposure resulted in significantly improved permeability (44 vs 66 nmol/cm^2/h, $p<0.05$).

Conductance (Fig 7-2B): At both 4 and 10 h time-points, Groups 2 and 4 were noted to have significantly higher levels of conductance compared to their non-PI equivalents. By 10 h, the effect of PI was most evident as levels measured 85.5 mS/cm^2 in Group 2 and 53.6 mS/cm^2 in Group 4 compared to 36.4 mS/cm^2 in Group 1 and 22.7 mS/cm^2 in Group 3, $p<0.05$. Once again, the effects of PI exposure were decreased following a 5 x v/w flush (53.6 mS/cm^2) compared to a 1 x v/w flush (85.5 mS/cm^2), $p<0.05$.

Short Circuit Current (Fig 7-2C): Paralleling permeability and conductance values at 10 h, a 5 x v/w flush following PI exposure resulted in significantly better short circuit current values compared to a 1 x v/w flush; -26.1 vs –11.6 μA/cm^2, $p<0.05$.

Bacteria Quantification (Table 7-1 and 7-2): The level of intestinal decontamination achieved with PI was noted to be concentration dependent. Following a luminal contact period of two minutes, total bacteria counts measured 1.3 x 10^7 CFU/g tissue when treated with 0.1% or 1% PI vs 3.6 x 10^5 CFU/g tissue when using 10% PI solution; $p<0.05$. The average total bacterial concentration in freshly isolated specimens measured 4.75 x 10^7 CFU/g tissue. Luminal treatment with either 10% PI, 1 x v/w AA (Group 1), and 5 x v/w AA (Group 3) all resulted in significant reductions in total bacterial counts compared to levels found in freshly isolated specimens. No significant

difference was noted between these groups however, with total bacterial counts measuring 3.6×10^5, 3.8×10^5, and 3.7×10^5 CFU/g tissue respectively.

Histology (Table 7-3): Both PI-treated groups (Group 2 and 4) possessed median grade histologic damage equal to or more than grade 5 (5.5, 5 respectively). No significant difference was noted in the level of histologic damage present at 10h however as Group 1 and 3 possessed median scores of 3 and 4 respectively.

Discussion

It has previously been shown that bacterial translocation routinely occurs following SB transplantation (2, 3). A number of factors incurred throughout the transplantation process jointly contribute to its occurrence. First of all, the SB is not sterile. Depending on the anatomic location, bacterial concentrations can range from a low of 10^2-10^3/g tissue in the proximal jejunum to as high as 10^8-10^{10}/g tissue in the terminal ileum (11). At procurement, surgical manipulation of the graft necessarily results in denervation and division of lymphatics; each contributing to altered motility, a known risk factor for bacterial overgrowth (12). Cold-storage related injury is inevitable, as the effects of hypothermia and ischemia yield reproducible levels of graft injury beginning at the mucosal level (13). Once transplanted, the SB graft is susceptible to further morphologic damage due to the effects of reperfusion injury and rejection. Finally, this all occurs in the face of potent immunosuppression, inhibiting the recipient from mounting an appropriate immune response.

Strategies aimed at reducing bacterial translocation can theoretically be targeted at each of these steps in the SB transplant process. This paper focused on combining two independent strategies which could be instituted at the time of graft procurement. The first of these is to address cold-storage related graft injury. When compared to the clinical standard, vascular administered University of Wisconsin (UW) solution alone, we

have previously documented that mucosal damage can be significantly reduced with addition of a luminal administered amino-acid rich (AA) solution (5). As absolute arrest of mucosal ischemic damage is not possible, we combined this step with an alternate strategy aimed at reducing the concentration of luminal bacteria. Intestinal decontamination, as this is known, has been routinely applied prior to elective colorectal procedures for decades. With use of either antibiotic prophylaxis and/or mechanical purgative agents, the incidence of anastomotic dehiscence (14) and post-operative wound infections (15) have both been reduced significantly. This strategy has also been used in the field of SB transplantation but remains controversial. While some have documented benefit (16,17), others have shown that intestinal decontamination does not appear to reduce the rate of bacterial translocation (18, 19). One other routinely transplanted intra-abdominal organ does address intestinal antisepsis. At the time of pancreas procurement, a number of programs administer Povidone-Iodine (Betadine) within the duodenal lumen (6,7). Interestingly, few clinical duodenal complications are reported post-operatively. In this context, use of an agent such as PI at the time of SB procurement may provide a necessary link to reduced levels of BT.

Povidone-Iodine is a commercially available, inexpensive antiseptic agent. Its microbicidal spectrum is broad as it is active against both aerobic and anaerobic bacteria as well as fungi, protozoa, and viruses (20). It is typically supplied as a 10% solution (pH 4.5); comprised of 90% water, 8.5% polyvinylpyrrolidone, and 1 percent iodine (21). Free iodine, the active component, travels complexed to a carrier, polyvinylpyrrolidone. This hydrophilic polymer carrier has a natural affinity for cell membranes. Once in contact with bacterial cell membranes, iodine dissociates from its carrier, disrupting and ultimately killing the organism by inactivating respiratory enzymes through amino acid oxidation.

At the outset, we could not find studies which assessed the quality of duodenal or small bowel decontamination using PI. In fact, the only available data documenting the local effects of this agent in a hollow viscus was in colonic decontamination. These studies revealed effective decontamination (22), but interestingly showed that variable degrees of mucosal damage were incurred depending on the duration of mucosal contact. This mucosal damage was highly variable, ranging from erythema and excess mucous production within five minutes of contact (23) to frank desquamation as early as 30 minutes after treatment (24). Since the threshold for colonic and SB bacterial translocation has been shown to be different (25), the first step in this study was to determine an appropriate concentration to be administered as well as an allowable time of mucosal contact. Knowing that PI's antimicrobial effect occurs within seconds of contact (26), and that colonic mucosal damage was noted as early as 5 minutes after treatment (23), we selected a contact period of two minutes. Bacterial counts were subsequently measured using varying PI concentrations. As shown in Table 7-2, the lowest attainable bacterial concentration following two minutes of contact was found when using a 10% solution.

The intrinsic barrier capacity of the mucosal layer is determined not just by the physical presence but also the configuration of the epithelia which comprise it. Organized cell-cell adhesion, provided principally by the tight junctions located at the apicolateral portion of the epithelial wall, allows for maintenance of functional integrity (27). As a result, selective permeability, a state which refers to the ability of a membrane to allow only passage of specific substrate across it, exists. Ultrastructurally, these tight junctions are comprised of a number of dynamic, energy-requiring proteins. In the presence of diminished ATP levels (28), or in conjuction with changes in pH (29), calcium concentration (30), cAMP levels (31), temperature (32), and/or osmolarity (33),

these proteins become functionally altered. This leads to loss of the selectively permeable nature of this layer, allowing for increased transcellular and paracellular macromolecule and microbial flux (27). At a mechanistic level, assessment of these modulators would provide indirect evidence for altered tight junction function and ultimately mucosal barrier dysfunction.

In our study, luminal PI contact did result in mucosal biochemical alteration. Even with only two minutes of contact, PI-treated specimens were found to have significantly lower levels of high energy phosphorylated compounds compared to their non-PI treated counterparts (Figure 7-1). This remained evident throughout the 10 hour storage period in both ATP and total adenylate values. Further evidence of the detrimental effect was suggested by the fact that the group which received a higher volume of AA solution (5 x v/w) following PI treatment (Group 4) possessed higher total adenylate values then Group 3 (PI + 1x v/w AA flush) throughout the study period. Since these groups differed only in the volume of AA solution administered, protective dilution from the toxic effects of PI was most likely. A number of PI-specific mechanisms could have contributed to this altered biochemistry. The first of these is the effects of non-physiologic pH (approx. 4.5). Heart and liver storage models have documented that preservation quality and ATP levels are improved if steps are taken to defend against such pH shifts (34, 35, 36). Biochemical viability was likely further altered because free iodine is known to have additional oxidizing effects on other cellular molecules. These include sulfhydryl compounds, peptides, proteins, vitamin C, cytosine, lipids, and other enzymes (37).

Maintenance of high-energy phosphorylated compounds such as ATP have been correlated with minimization of cellular functional damage incurred during cold storage (38). Functional assessment in this study similarly revealed that mucosal integrity and

ion function remained significantly better in specimens not treated with PI. Throughout the study period, PI treatment resulted in 1.5 to 4.4 times greater permeability and 1.4 to 32 times lower ion activity (Short circuit current- Fig. 7-2C).

Lastly, and most importantly, the adverse effects noted following PI treatment were not counter balanced by additional reduction in total bacterial counts. In fact, equivalent decontamination was obtained using the luminal delivered AA preservation solution. It could be argued that a two minute contact time was not of sufficient duration to allow for PI's maximum bactericidal effect. The results of this study however show that SB mucosal damage is potentiated following even short duration contact with luminal delivered PI. Since maintenance of mucosal integrity is the best defense against bacterial translocation, our data suggests that routine use of Povidone-Iodine should not be included in SB procurement protocols. Furthermore, as the average duodenal bacterial count is significantly lower than that seen in the ileal segments studied, the risk/benefit ratio would strongly argue against its routine use during pancreas procurement.

Table 7-1 Total bacterial counts vs Povidone-Iodine concentration

% PI Concentration	Mean Total CFU/g tissue ($x10^7$)	SEM
Fresh Tissue	4.75	± 0.82
0.1	1.25	± 0.23
1.0	1.26	± 0.09
10	0.0359 *	± 0.001

reported as mean colony forming units per gram tissue (CFU/g tissue) ± standard error of the mean. *- significantly lower than 0.1% and 1.0% PI solutions; $p<0.05$.

Table 7-2 Total bacterial counts vs Specific Luminal flush

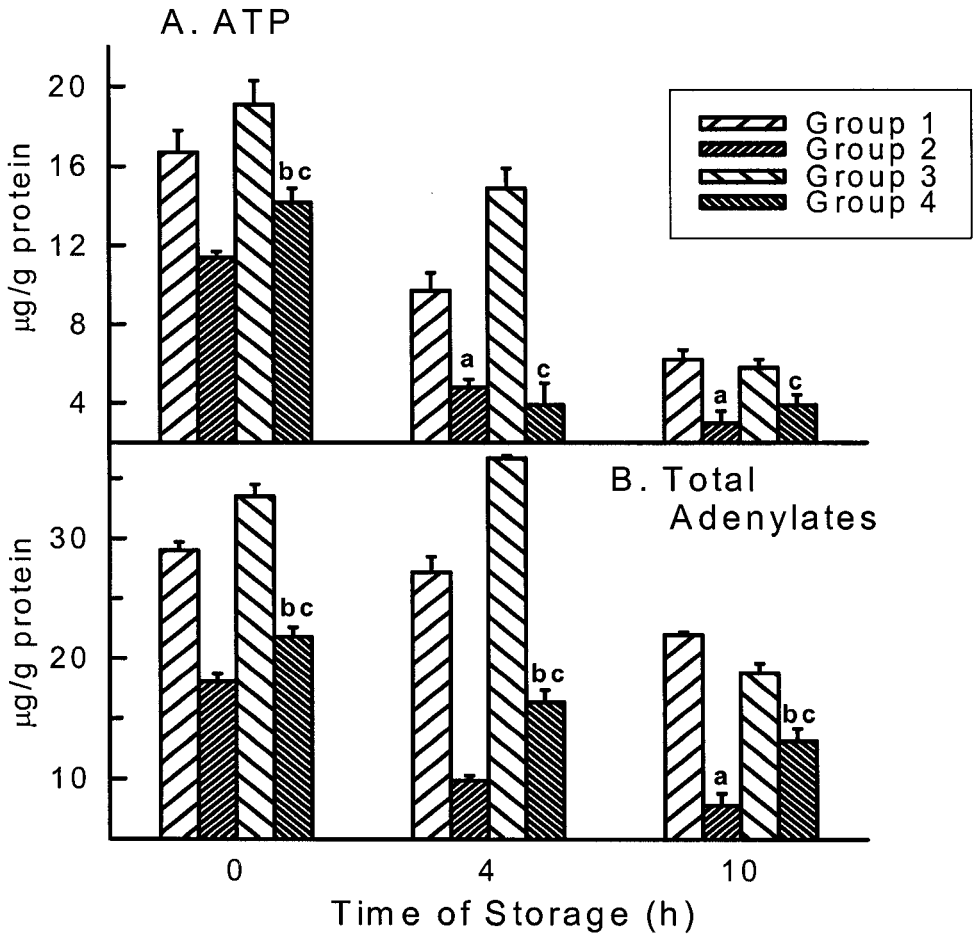
% PI Concentration	Mean Total CFU/g tissue ($x10^5$)	SEM
Fresh Tissue	475	± 0.82
1x AA	3.81 *	± 0.95
5x AA	3.69 *	± 0.81
10% PI	3.59 *	± 0.001

reported as mean colony forming units per gram tissue (CFU/g tissue) ± standard error of the mean. *- significantly lower than freshly isolated tissue; $p<0.05$.

Table 7-3 Grade of Histologic Damage (Park's classification)

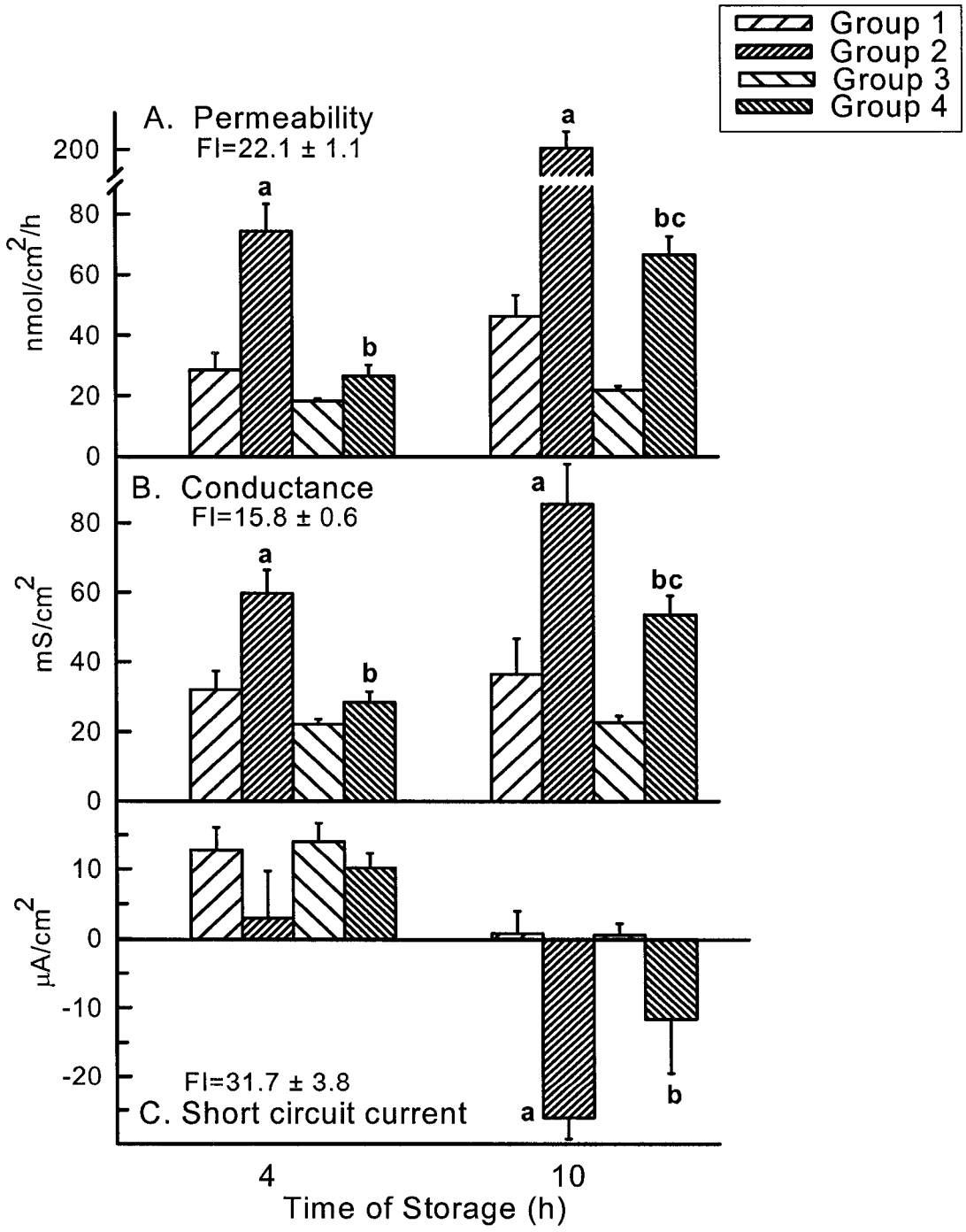
	Grade	Median	Maximum
Group 1	2,3,3,4	3	4
Group 2	2,4,7,7	5.5	7
Group 3	3,3,5,5	4	5
Group 4	4,5,5,5	5	5

Figure 7-1 Effect of Luminal-Administered Povidone-Iodine on Cellular Energetics



a,b,c- significantly less than group 1, 2, and 3 respectively; $p<0.05$.

Figure 7-2 Effect of Luminal-Administered Povidone-Iodine on A) Permeability, B) Conductance and c) Short Circuit Current



a,b,c- significantly less than group 1, 2, and 3 respectively; $p<0.05$.

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Chapter 8

Human small bowel storage: The role for luminal preservation solutions

Introduction:

Although improvements have been noted more recently, rejection, infection, preservation and reperfusion injury continue to be obstacles to successful small bowel (SB) transplantation (1). Early postoperative complications, including endotoxemia, bacterial translocation, and stimulation of the recipient's immune response have been attributed to preservation injury (2). Like all transplanted organs, the SB is susceptible to cellular injury sustained during the obligatory period of cold storage. To date, the upper limit of allowable storage time falls short of other transplanted organs, including the liver, kidney, and pancreas. This discrepancy has been attributed to both the organ-specific effects of hypothermic hypoxia, but equally to the lack of a single preservation solution which can reproducibly limit these same effects. Currently, University of Wisconsin solution (UW), the agent used for procurement of other transplanted intra-abdominal organs, remains the most commonly used SB preservation perfusate. Despite a variety of other solutions, including simple crystalloids, which have yielded similar SB preservation results in animal models (3,4), the clinical confines of multi-viscera procurement necessitate use of UW solution as the common vascular perfusate.

Fortunately, the SB possesses another potential route by which cytoprotective solutions can be administered. Using a rodent model, we have previously documented

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A version of this chapter has been submitted to *Transplantation.*

that use of luminal-administered preservation solutions in addition to UW vascular flush results in significantly improved SB structural and functional integrity throughout clinically relevant periods of cold storage (12 hours) (5). Findings like these cannot be automatically applied to the clinical setting as the documented benefit may be nullified by species-specific physiologic differences. As such, this study was performed to test for similar improvements in human SB storage quality when using luminal-delivered preservation solutions.

Materials and Methods:

All experiments were approved by the University of Alberta Human Research Ethics Board. Intestinal specimens were acquired from nine multi-organ donors after obtaining informed consent from the family for both organ donation and research. SB procurement was performed by the first author in all cases at the time of a standardized multi-visceral retrieval procedure, comprised of *in situ* arrest and retrograde aortic flush using UW solution. At no time did the retrieval of the SB compromise the procurement of any organ used for clinical transplantation.

Surgical procurement of the small intestine:

Potential small bowel donors were screened for any antecedent intestinal pathology via family discussions as well as chart review. Those patients with a history of any previously diagnosed chronic intestinal pathology (ie inflammatory bowel disease) were immediately refused for study. No pre-retrieval steps were taken to prepare the small intestine (ie luminal antibiotics or luminal flushing).

Operatively, small bowel specimens were obtained at the time of a standard multiviscera procurement procedure (6). Following aortic clamping, 3-4 liters of cooled University of Wisconsin (UW) solution was subsequently administered via retrograde aortic *in situ* flushing. The small bowel mesentery was then divided at its base.

Following this, the small intestine was divided proximally at the ligament of Treitz and distally at the level of the ileocecal junction with a surgical stapler, removed en bloc, and placed in a basin containing ice cold UW solution. Three individual segments measuring 40 cm each were serially divided beginning at the ileal end of the graft. The jejunal (proximal) end of the individual segment was then cannulated and a total of 400 milliliters (based on volume of a cylinder = $\pi r^2 h$; where r^2= radius squared= 3.0 cm on average, and h= height= 40 cm) of individual preservation solution was subsequently flushed via the lumen. Each bag of individual luminal-administered preservation solution was hung from a height of 90 cm above the level of the dissecting table, so as to provide sufficient pressure to evacuate luminal contents without further manual manipulation of the intestinal specimen. Enteric effluent was allowed to freely exit the distal end of the graft (approximately 325-350ml of 400ml). The ileal (distal) end of the segment was then ligated with 3-0 silk and the remaining volume (50-75ml) infused allowing for mild distention and equal distribution of the solution throughout the entire length of the harvested segment. The specimen was then stored in 500 ml of ice-chilled preservation solution identical to that used to flush the lumen. A 1-2 g tissue sample was taken at t= 0 (immediately following flushing), 4, 8, 12, 24h. Tissue samples were then taken serially, beginning at the jejunal end of the intestinal segment. To arrest metabolic activity, samples were immediately snap frozen in liquid nitrogen, and stored at -65°C until processed.

Composition of preservation solutions (Table 8-1 and reference 7) Initially, each group received intravascular UW solution (7) at the time of the multivisceral procurement. Group 1, $UW_{(v)}$, received no additional preservation solution whereas Groups 2 and 3 were subsequently administered one of two luminal-delivered solutions; $UW_{(l)}$- University of Wisconsin solution, $AA_{(l)}$- amino acid-enriched.

Sample Preparation and Metabolite Assay for Energetics: Specimens were then weighed and extracted 1:5 w/v in 6% perchloric acid containing 1 mM EDTA. The precipitated protein was removed by centrifugation (20 min at 20,000 g). Acid extracts were neutralized by the addition of 3 M KOH/0.4 M Tris/0.3 M KCl and then re-centrifuged (20min at 20,000 g). Aliquots of the neutralized extracts were immediately processed via standard enzyme-linked metabolite assays (8). Spectrophotometric analysis was then performed to measure the absorbance of NADH at 340 nm, providing quantification of ATP, ADP, and AMP. Values are reported in μmol/ gram protein.

Histological Examination: 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 the Park's histologic classification for damaged bowel on two sections from each specimen (9). All samples were evaluated in a blinded fashion by two independent pathologists (LJ and JZ).

Functional Assessment– in vitro measurements: Ileal segments were taken at 0, 8, 12, and 24h cold storage, stripped of their serosa and muscular layers, and mounted in Ussing chambers. Once mounted, the mucosal and submucosal surfaces were perfused with a recirculating solution containing bicarbonate Ringer's solution, with an ionic composition of: Na^+, 143 mM; K^+, 5 mM; Mg^{2+}, 1.1mM; Ca^{2+}, 1.25 mM; HCO_3^-, 25 mM; Cl^-, 123.7 mM; HPO_4^{2-}, 0.3 mM; and fructose, 20 mM with 95% O_2 and 5% CO_2, at 37 °C, and pH=7.4 (10). Permeability was measured via scintillation counter determining the flux of a radioactive substrate (10 μCi [^{3}H]mannitol, Dupont, NET101) across individual ileal specimens.

Statistics: Energetics and permeability data were reported as means ± SE for each group. Statistical difference between groups was determined using ANOVA, followed by Dunnett's *post hoc* comparison test; $p<0.05$ was reported. Histology scores were

compared using the Kruskal-Wallis test followed by Mann-Whitney test for pairwise comparison; $p < 0.05$ was reported.

Results

Donor Data: SB specimens were obtained from nine individual multi-organ donors. The average donor age was 39 years (range 17-61). Seven donors died from head trauma: three due to a motor vehicle collision, two from a fall, and two suffered an intracerebral bleed. An additional donor died from an upper airway obstruction while the final patient had hanged himself.

All donors received vasopressors at some point during their hospital stay. At the time of organ retrieval, four of the nine donors required no pressor support. Of the remaining five, four were on dopamine (ranging from 3 to 20 μg/kg/min), two were on norepinephrine (ranging from 0.05 to 6 μg/min) and one was on 6 μg/min of epinephrine.

Energetics;

ATP (Figure 8-1): ATP values at 0 h ranged from 19.7μmol/g protein in Group 1 to 25.7 and 28.4 μmol/g protein in Groups 2 and 3 respectively. Although not significantly different, each of the groups sustained time-related reductions in ATP levels throughout the 24 h storage period. By 12 hours, ATP levels were 44-75% of initial values; measuring 14.8, 12.6, and 14.3 μmol/g protein, for Groups 1-3 respectively. At 24 h, luminal treated specimens (Groups 2 and 3) possessed similar ATP levels to that of the clinical standard ($UW_{(V)}$): 11.5 and 10.2 μmol/g protein vs 11.4 μmol/g protein respectively.

Total Adenylates (Figure 8-2): Each of the three treatment groups possessed similar TA values at 0 h; measuring 45.6, 56.0, and 52.8 μmol/g protein for Groups 1, 2, and 3 respectively. Regardless of the preservation method used, no significant difference was noted at each time point throughout the first 12 h of cold storage. At 24 h, UW luminal

treated specimens (Group 2) retained higher levels when compared to the AA luminal treated group; 52.4 µmol/g protein vs 35.8 µmol/g protein (AA), $p<0.05$. However, there was no significant difference when compared to the control group, $UW_{(V)}$.

Functional Assessment of Stored Tissue:

Permeability (Figure 8- 3): 0 h permeability values were similar for Groups 1, 2, and 3; measuring 8.7, 8.1, and 8.4 nmol/cm^2/h respectively. Following 8 h of storage, each of the luminal treated groups (Groups 2 and 3) were found to possess significantly lower levels of mannitol flux when compared vascular UW alone: 12.6 and 12.2 nmol/cm^2/h vs 28.0 nmol/cm^2/h respectively, each $p<0.05$. Although no significant difference was noted between groups 2 and 3 at any time point, each of these groups retained significantly lower permeability values compared to the UW vascular group throughout the entire storage period including 24 h values of 56.9 nmol/cm^2/h (group 1) vs 30.5 and 34.0 nmol/cm^2/h (group 2 and 3); each $p<0.05$.

Structural Assessment of Stored Tissue:

Histologic damage (Table 8-2, Figures 8-4 to 8-6): Mucosal injury was evident within 4 hours of cold storage. Compared to Group 1, the median grade of injury was found to be significantly lower in group 2 (median grade 0), consistent with normal appearing villus; $p<0.05$. Although not significantly different, a greater number of specimens in group 3 retained normal mucosal architecture compared to that in group 1 at this same time period. By 8h, all groups possessed similar levels of injury, each showing epithelial lifting along the sides of the majority of villi (Park's grade 1.0-2.5). After 12 h of cold storage, macroscopic evidence of patchy desquamation was noted in only group 1, where the only Park's scores of grade 4 (denuded villi) were noted (see Figure 8-4). Furthermore, dissections during preparation for permeability studies revealed consistently less friable mucosal layers in each of the luminal treated specimens. This

macroscopic finding correlated with group 2 specimens possessing significantly lower levels of morphologic damage when compared to group 1 at 12h: 1.0 vs 3.0; $p<0.05$ (see Figures 8-5 and 8-6). At the end of the storage period, only specimens in group 2 possessed median grades of damage below 2, whereas groups 1 and 3 were each noted to have median grades of 3.0.

Discussion:

Preservation injury continues to be an important obstacle to successful SB transplantation. As shown in other organ models, the ability to minimize injury during periods of cold storage leads to improved outcomes and broader applications of the individual transplant procedure (11). Currently, the combination of vascular flushed cytoprotective agents and hypothermia remains unable to provide reproducible preservation of the SB for periods paralleling that of other routinely transplanted intra-abdominal organs. Longer storage times however have been reported using more complicated techniques including hyperbaric oxygenation (12,13) and continuous perfusion models (14,15). Acceptance of these more complicated preservation techniques has not been wide-spread. The goal of this paper therefore was to show that luminal-delivered preservation solutions can provide an uncomplicated method for reproducibly improving SB preservation in a clinically applicable model.

The SB is unique in that it possesses an alternate route by which cytoprotective solutions can be easily administered. The enteric lumen and surrounding mucosal layer, in its normal physiologic state, provides an effective route for absorption of a wide variety of nutrients, fluids, and pharmacologic agents. Human (16) and animal (9) intestinal cold ischemia models have each documented that the mucosal layer, specifically the mucosa lining the villus tip, is the initial site of injury. Damage to this area has been suggested to occur because of hypoxic-related reductions in high-energy phosphates (ATP), known to

be integral to mucosal barrier (17) and absorptive function (18). Even during periods of cold storage, the mucosal layer remains metabolically intact and capable of absorption (19). Through the work of Ito et al, it has been documented that epithelial cells along the villus tip preferentially absorb luminal substrate (20) Because of this, it is theoretically possible for luminal-delivered cytoprotective solutions to directly target the site of initial ischemic damage.

In contrast to the higher levels of ATP which we noted in rat intestinal specimens treated with luminal perfusates, no significant differences were noted in any of the group's biochemical indices (ATP, Total Adenylates) in this study. Although similar time-related reductions in ATP levels were noted in each group, the presence of intestinal nutrients (ie glutamine) in the AA luminal solution did not prevent the decay of ATP to lower level energy phosphates any better than the group treated with isolated vascular UW solution (Group 1). Interestingly, despite similar biochemical indices, significantly better barrier function was noted in both luminal-treated groups throughout the 24h study period. Functional indices, including mannitol permeability, reflect the levels of energetics present within the epithelium tested. This is based on the fact that ATP depletion leads to progressive dilation of tight junctions, ultimately leading to increased permeability and transepithelial flux of macromolecules (21). The discrepancy of the biochemical and functional data in this study can in part be explained by the fact that cellular energetic measurements were based on full thickness bowel specimens. Since the human bowel is considerably thicker then that of the rat, actual differences in mucosal ATP values may have been nullified by greater protein contributions from other intestinal layers. As such, ATP and TA levels may have been more reflective of the permeability findings had only mucosal samples been used.

The fact that permeability values in both luminal treated groups remained significantly lower than those specimens treated with vascular UW solution alone suggests that a common benefit was present secondary to the luminal flushing procedure itself. This beneficial effect points to the likelihood of non-specific dilution of potentially cytotoxic substances normally found within the lumen; including pancreatic enzymes, biliary secretions and bacteria (22) and activated lysosomal enzymes (23). In addition to dilutional effects, further non-specific benefits of luminal flushing may have also included the impermeant (osmotic and oncotic) support supplied by each of the individual solutions. This is based on the fact that the first evidence of SB histologic injury occurs with formation of a sub-epithelial space at the villus tip. Gruenhagen's space, as this is referred, has been suggested to occur in part due to fluid shifts of luminal origin (24). As the UW and AA solutions possessed similar osmotic agents (lactobionate) and osmolality (320 mOsM), both provided basal impermeant support throughout the storage period.

The ability to maintain the structural integrity of the mucosal layer is the common denominator by which SB preservation techniques are measured. Although some reports have been published (25-28), the direct effects of cold storage injury on human small bowel has not been thoroughly investigated. To our knowledge, only one other study has specifically looked at the effects of ischemic injury incurred by human bowel during clinically relevant periods of cold storage (29). Similar time-related grades of morphologic damage were noted in the UW vascular group in this study. By 12 h of storage, epithelial detachment and villus denudation was noted. Compared to our findings in the rat study, significantly lower levels of damage were noted in the human bowel for equivalent periods of cold storage. Because of the similarities in morphologic damage documented by this study and Tesi et al, it appears that the human bowel may

be intrinsically more resistant to ischemic injury than smaller animal models like the rat. However, if damage remains lower than that found in animal models, it may be that histologic assessments are not the "gold standard" by which human SB preservation quality should be measured. As noted in some reperfusion studies, electrophysiologic and biochemical markers recover before that of morphologic markers (30). In the face of progressive hypoxia and inevitable alteration in aerobic metabolism, it would seem probable that the reverse would occur during cold storage; namely electrophysiologic and biochemical changes being noted before structural damage. Therefore, differences in functional indices (ie permeability values) as noted between luminal treated groups and the $UW_{(v)}$ group in this study may be equally as important when the average grade of histologic damage reveals the mucosa to be altered but still present.

The opportunity to perform basic-science transplant research on human tissue is the exception rather than the rule. Although animals provide readily available study models, discoveries made using them must be tempered by the fact that the findings may not be automatically transferable to the human setting. This study reaffirms that point as the grade of morphologic damage remained significantly lower than that found in the rat model for equivalent periods of cold storage. Despite those differences, this study again points to the benefit of luminally-administered cytoprotective agents. Although not conclusive, use of UW solution for this purpose appears to provide improvement in functional and morphologic indices of human SB graft subjected to clinically relevant periods of storage. Furthermore, application remains simple, resulting in no greater burden to the already technically demanding nature of the multi-viscera procurement procedure. At a cost of approximately $320.00 per litre (Canadian), luminal UW flushing would translate to an additional $2000.00 (based on average SB length of 650cm) to the procurement procedure. Overall, this cost is negligible compared to the

entire transplant procedure, and as such, points to a viable addition to standard SB procurement.

Table 8-1 Composition of AA Solution.

	AA
Lactobionate	20
Adenosine	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
OH-butyrate	3
Tyrosine	1
Tryptophan	1

AA –denotes a mixture of amino acids based on physiological requirements. The pH of the AA solution was adjusted to 7.40 ± 0.01 using NaOH. Osmolality is 320 mOsM. All numbers in mM/liter.

Table 8-2 **Grade of Histologic Damage**

	Time	Grade	Median	
$UW_{(v)}$	4h	3,3,1,2,1,3,1,0,2	2	
	8h	1,2,2,3,2,3,2,2,2	2	
	12h	2,3,3,4,4,2,2,3,4	3	
	24h	3,3,2,3,3,2,2	3	
$UW_{(l)}$	4h	0,1,0,0,1,1,0,0,2	0	**u**
	8h	3,3,1,2,3,0,0,0,2	2	
	12h	0,0,3,3,0,2,2,1,1	1	**u**
	24h	1,3,1,3,2,1,2	2	**a**
$AA_{(l)}$	4h	0,0,0,3,2,2	1	
	8h	3,3,2,0,3,0	2.5	
	12h	1,3,1,2,3,3	2.5	
	24h	3,3,3,3,3,3	3	

u – denotes significantly different from group 1; $p<0.05$. **a** – denotes significantly different from group 3; $p<0.05$.

Figure 8-1 Effect of preservation solutions and route of administration on cellular energetics (ATP).

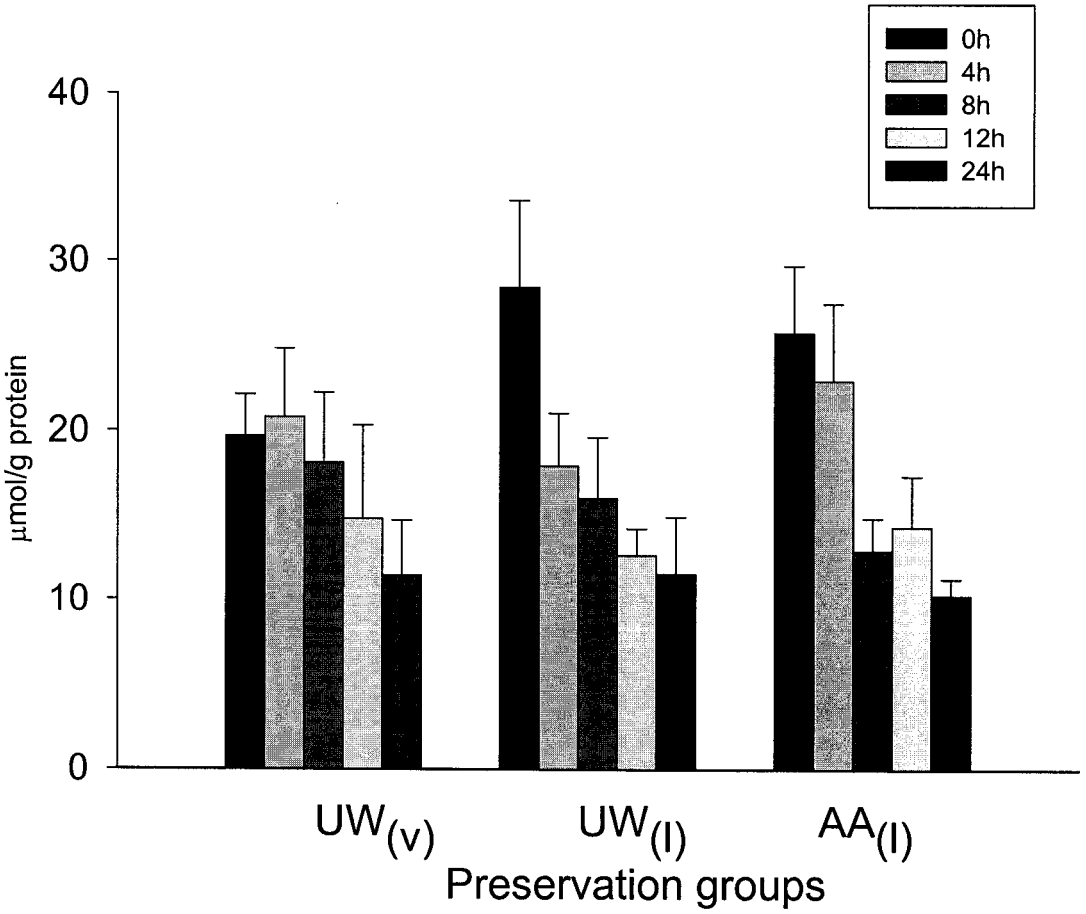
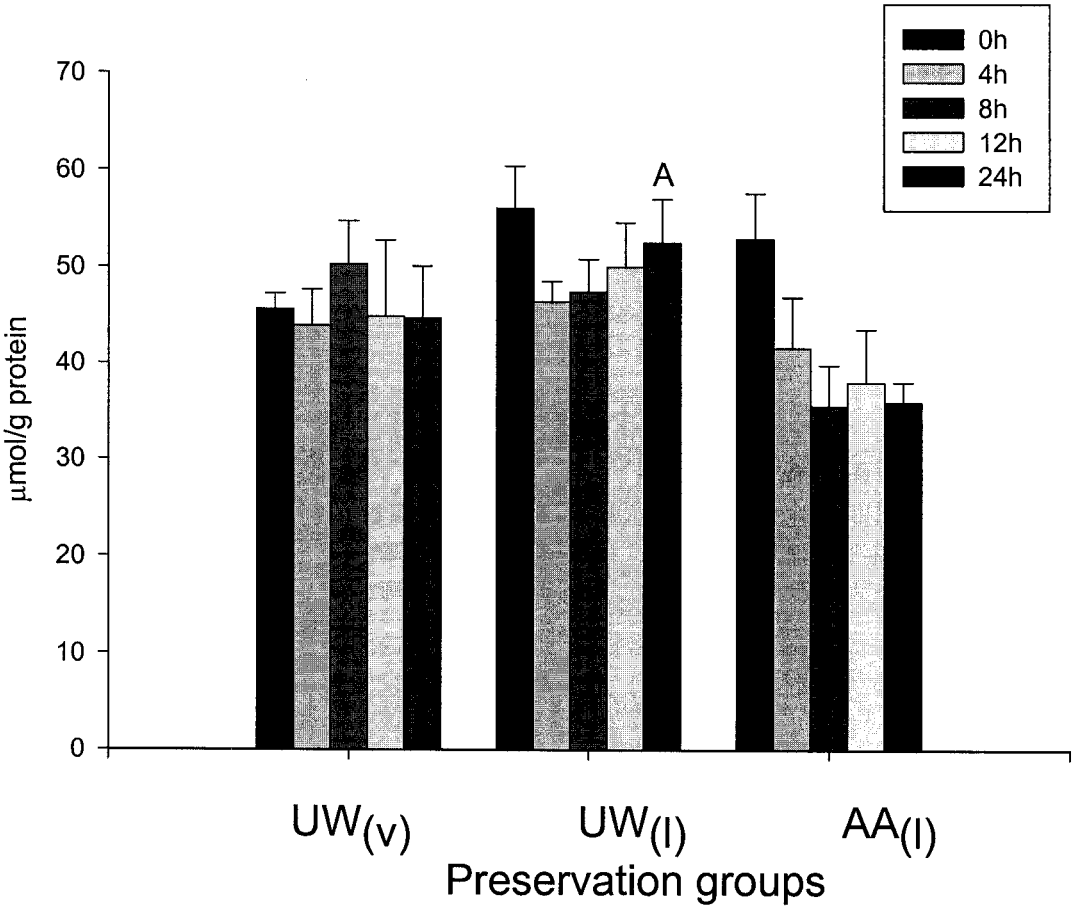
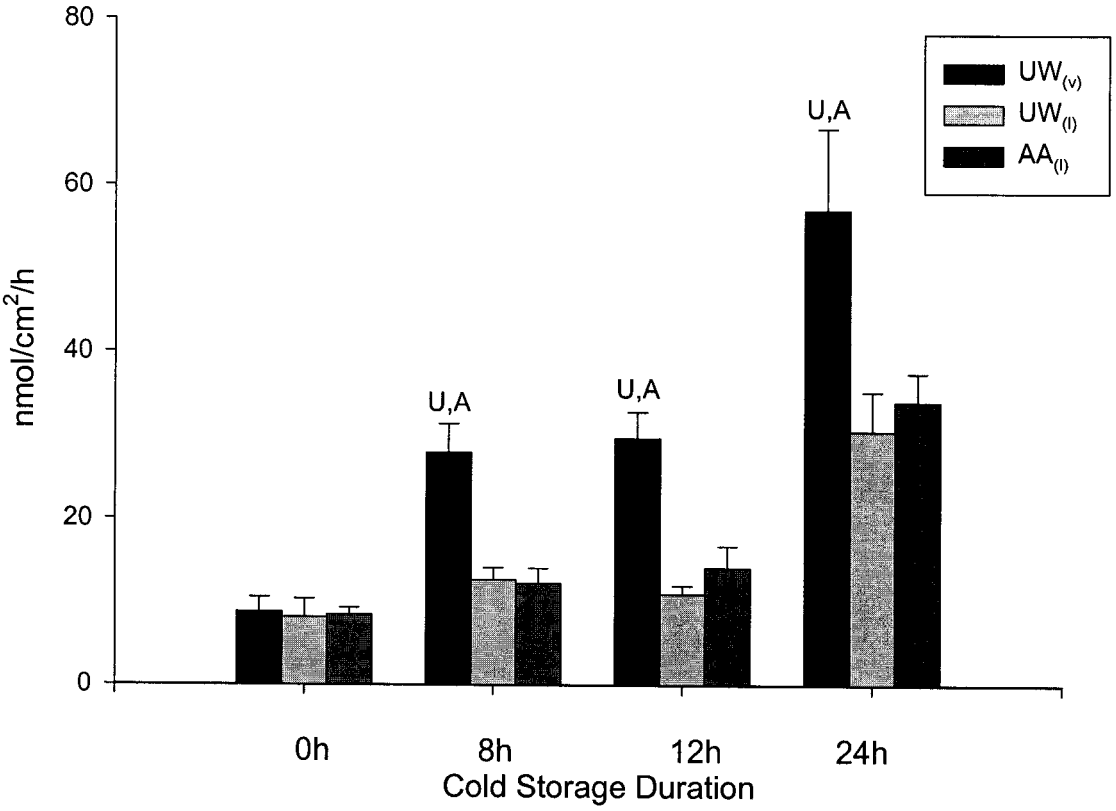


Figure 8-2 Effect of preservation solutions and route of administration on Total adenylates (TA).



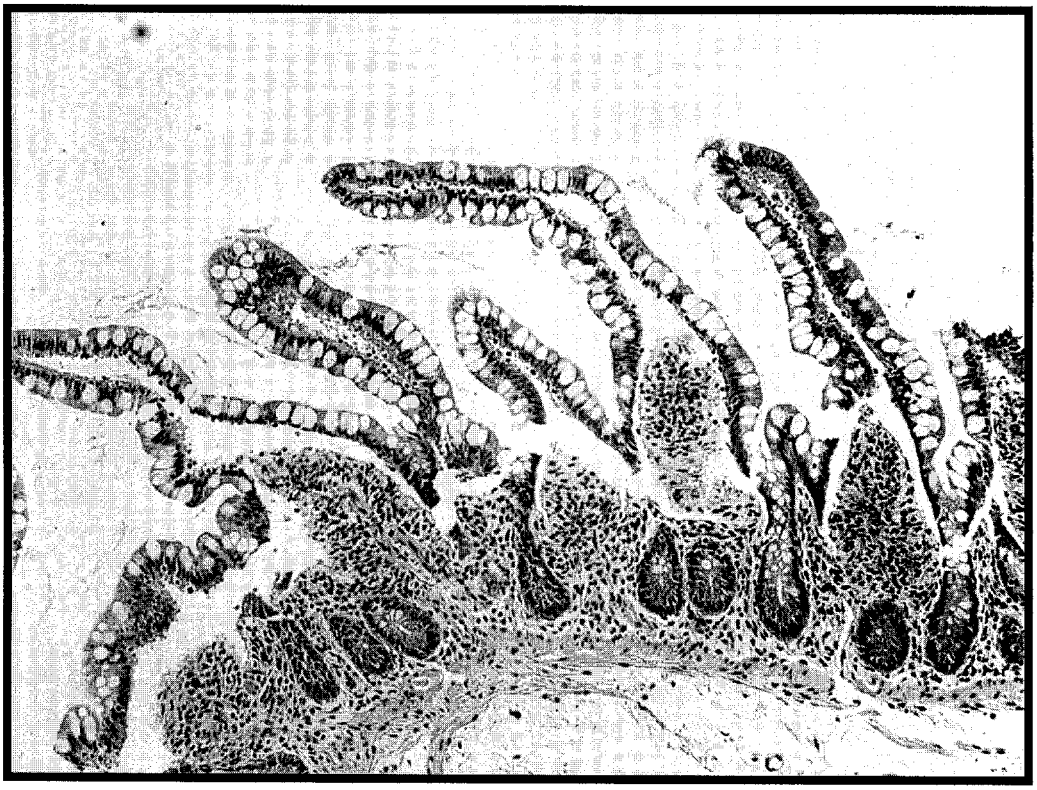
A- significantly greater than $AA_{(I)}$ group; $p<0.05$.

Figure 8-3 Effect of preservation solutions and route of administration on Mannitol permeability.



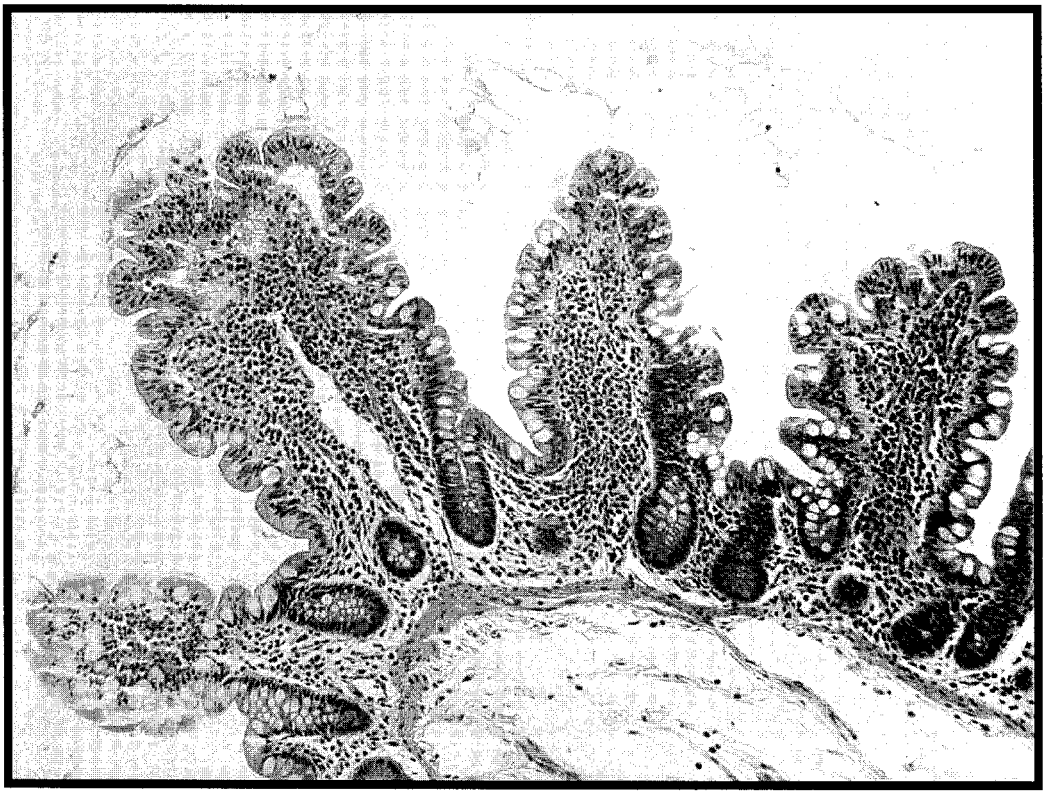
U,A- significantly greater than $UW_{(l)}$ and $AA_{(l)}$ groups respectively; $p<0.05$.

Figure 8-4: Light microscopy picture of Group 1 ($UW_{(v)}$) SB stored for 12h



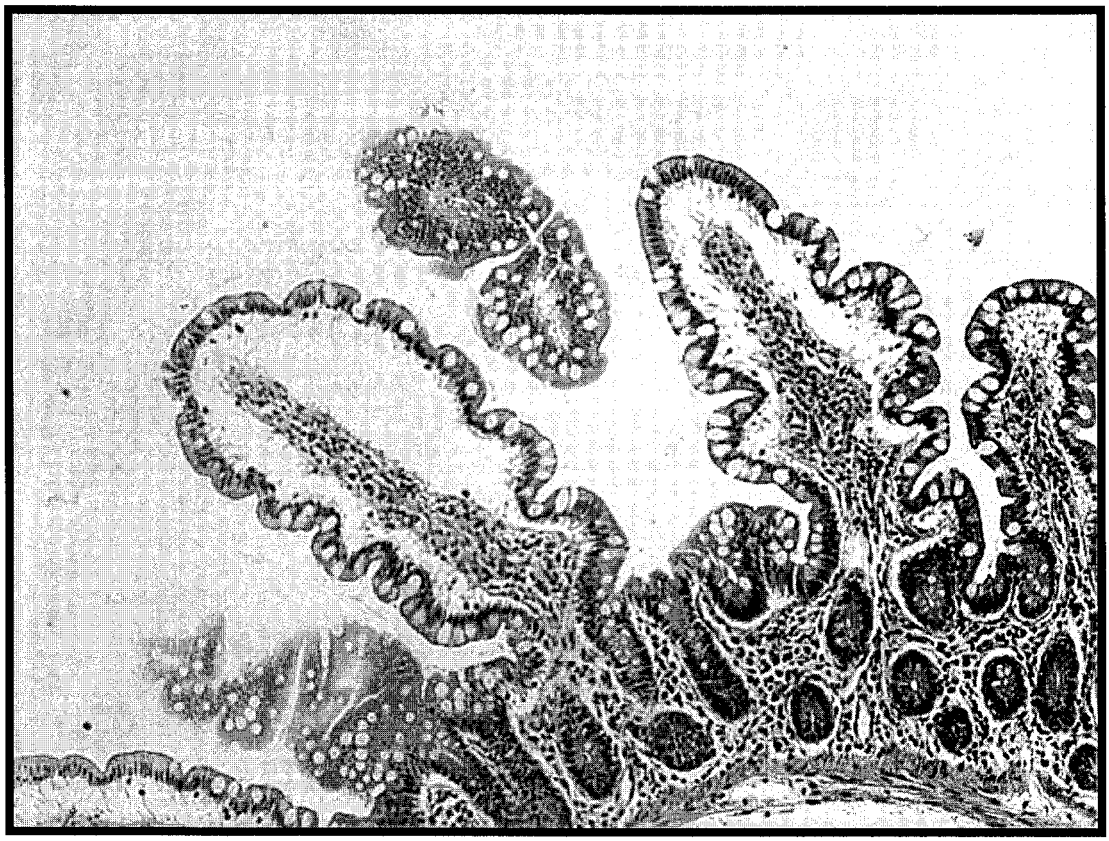
-median Park's grade 3. x10 magnification.

Figure 8-5: Light microscopy picture of Group 2 ($UW_{(l)}$) SB stored for 12h



-median Park's grade 1. x10 magnification.

Figure 8-6: Light microscopy picture of Group 3 ($AA_{(I)}$) SB stored for 12h



-median Park's grade 2.5. x10 magnification.

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Chapter 9

Conclusions

Despite new immunosuppressive agents and refinements in surgical techniques, the success of SB transplantation continues to lag behind that of other routinely transplanted organs. A number of factors jointly contribute to this on-going lack of success. These include preservation and reperfusion injury, as well as episodes of rejection and infection. Each of these obstacles are intimately related, as mucosal alteration, occurring secondary to preservation and reperfusion injury, has been shown to increase the risk for both bacterial translocation (infection) and non-specific antigenic stimulus (rejection). Because of their inter-dependence, the focus of our research was to minimize the first of these obstacles; namely graft injury incurred during cold storage. Despite a variety of alternate preservation models, the most clinically applicable model for SB storage remains simple hypothermia and delivery of cytoprotective agents. In the context of this clinical model, cytoprotective agents are usually administered via vascular flush. The SB is unique however, in that it possesses both a vascular and luminal route by which cytoprotective agents can be administered. As such, the preceding chapters have looked at further development of both vascular- and luminal-delivered preservation solutions.

In the first study, it was shown that addition of impermeants (both osmotic and oncotic) to simple crystalloid solutions could provide better SB graft quality when compared to the current clinical standard, University of Wisconsin solution. This re-iterated the fact that no one vascular-delivered solution is unequivocally accepted for SB preservation. More importantly however, the findings provided evidence that impermeant support was a basal requirement for maintaining the mucosal layer during extended cold storage. As SB is typically obtained at the time of multi-visceral

procurement which relies on a common vascular perfusate, use of such a solution was not clinically applicable and therefore not pursued any further.

As the mucosal layer remains metabolically intact during storage, the second paper looked at supplementing UW solutions with glutamine, the primary fuel source of the enterocyte. Initial literature review showed that glutamine-supplemented solutions provided improved SB graft quality. Equally important however, was that we could not find any sources that documented a deleterious effect in other organ transplant models when supplying supra-physiologic glutamine concentrations. This implied that glutamine could potentially be added to standard preservation solutions used during multi-visceral procedures. In the face of continued metabolic activity in a non-perfused organ, non-physiologic pH shifts are expected. Therefore, through selection of a buffering agent (in this case BES) which possessed peak activity at the temperature experienced during cold storage, we showed that glutamine metabolism could be further potentiated. As buffers have also been documented to provide cellular protection in other transplant models, it is clinically realistic to supplement standard UW solutions with both glutamine and buffering agent if SB is to be obtained at the time of multi-viscera procurement.

The remaining chapters addressed luminal-delivery of cytoprotective solutions. Each of these papers looked at harnessing the intrinsic absorptive capacity of the mucosal surface. Initially in a rat model, then followed by replication using human tissue, it was determined that luminal delivery of preservation solutions did provide improved functional and morphologic support throughout cold storage periods of 12-24 hours. The method by which improvement was seen is most likely multifactorial. Firstly, the enteric lumen is a potentially toxic environment, rich in bacteria and other macromolecules. Simple non-specific dilution of enteric contents was one of the primary benefits noted. In addition, luminal delivery of an amino-acid rich solution (AA) provided

significantly higher levels of high energy phosphates throughout the storage period in the rat model. This was not born out in human SB storage, although equally improved functional status was noted in specimens treated luminally with either UW solution or AA solution. This pointed to the likelihood that luminal preservation solutions, like those vascularly delivered, actually provided basal impermeant support, thereby retarding morphologic damage.

In the end, our primary focus was to develop a reproducible 12 hour SB preservation model. By doing so, this would bring the accepted time of SB cold storage in parallel with that of other routinely transplanted intra-abdominal organs. It was equally important to make this model clinically applicable within the field of multivisceral procurement procedures. Ultimately, luminal-delivered preservation solutions provide a simple method of maintaining the functional and morphologic integrity of the SB graft stored for periods up to 24 hours in small animal models, and up to 12 hours in humans.