Luminal polyethylene glycol alleviates intestinal preservation injury irrespective of molecular size

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Nonstandard abbreviations:

AMPK - AMP-activated protein kinase; FSS- Fluorescein sodium salt; FD4- fluorescein

isothiocyanate-dextran 4kDa; GC- Goblet cells; Iep - epithelial ion current; IPI - intestinal

preservation injury; ITx - intestinal transplantation; JNK - c-Jun NH2-terminal kinase;

MAPK - mitogen-activated protein kinase; PD - potential difference; PEG - polyethylene

glycol; Rep - epithelial electrical resistance; SMA - superior mesenteric artery; TJ - tight

junction; UW- University of Wisconsin;;   ZO-1 zonula occludens 1

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ABSTRACT

Intestinal preservation injury (IPI) and the resulting mucosal injury raise several serious

challenges early after intestinal transplantation (ITx). The current clinical approach using only

vascular perfusion allows the shortest preservation period among the abdominal organs. The

experimental addition of luminal polyethylene glycol (PEG) solutions has been repeatedly

suggested to alleviate preservation injury, improve graft quality and prolong the preservation

time. We investigated if the size of PEG in the solution influences the development of

intestinal preservation injury. Small intestines of Sprague Dawley rats were perfused with

University of Wisconsin solution. Group 1 underwent vascular perfusion only (clinical

control), group 2 received additional luminal PEG3350 Da, group 3 received luminal

PEG10000 Da whereas group 4 received luminal PEG20000 Da (n=8/group). Tissue samples

were obtained after 4h, 8h and 14h. We studied the tissue damage (Chiu/Park score, Goblet

cells, apoptosis, tight junctions), activation of c-Jun NH2-terminal kinase (JNK) and p38-

mitogen-activated protein kinase (MAPK) and performed Ussing chamber assessments.

Mucosal morphological and electrophysiological parameters were significantly improved in

the groups receiving luminal PEG. There was significantly less apoptotic activity in group 2,

3 and 4. Both MAPKs revealed an activation peak after 4h with group 3 showing lesser p38-

MAPK activation. PEG 20kDa interfered with protein immunodetection. The results indicate

that luminal solutions of PEG of medium and large molecular weight significantly delay the

onset and development of IPI. Hence, the present study provides further evidence that luminal

interventions may allow for longer cold storage intervals of the intestinal grafts.

INTRODUCTION

Intestinal transplantation (ITx) has evolved into an established therapy for complicated intestinal failure. Its outcomes are similar with those after pancreas and lung transplantation and approach those of other solid organ transplants (Grant et al., 2015; Abu-Elmagd et al., 2015). In spite of the growing experience and success, ITx still raises several serious challenges in the early post-transplant period (Oltean et al., 2006; Abu-Elmagd et al., 2012, Clouse et al, 2018). Many of these complications such as severe electrolyte unbalance, high stomal output or life-threatening infections may be related to the preservation-reperfusion injury and the resulting mucosal barrier injury (Siniscalchi et al, 2012; Oltean et al., 2012). The current clinical approach for intestinal preservation is based on in-situ vascular perfusion followed by static cold storage and allows for less than 10 hours of cold preservation (Abu-Elmagd et al., 2012). Prolonging cold storage beyond this limit may generate extensive mucosal loss that may favor life-threatening septic complications.

Luminal introduction of polyethylene glycol (PEG) 3350 solution has been proven effective in reducing intestinal preservation injury (IPI) (Oltean et al., 2010; Oltean et al., 2012, Oltean and Churchill, 2014). PEG-treated intestines revealed superior electrophysiological parameters and permeability and more mucus-filled Goblet cells. The exact mechanisms are unclear but it has been suggested that PEG coated the apical membrane, protected the tight junction (TJ) proteins from degradation by luminal proteases and maintained the intercellular cohesion. Similar observations have been made in models of chemically-induced colitis or sepsis after intracecal inoculation of Pseudomonas aeruginosa, with PEGs acting as a protective barrier between the intestinal epithelium and the luminal content (Wu et al., 2004; Videla et al., 2007).

PEGs may bind to cells and mucous layers changing key barrier function properties such as surface hydrophobicity and epithelial permeability. The physical and chemical properties of PEGs depend on molecular weight (Mw) and linear conformation and it is believed that the capacity of PEGs to form compact structures and the hydrophobic character increases with the molecular mass (Temenoff et al., 2002; Hauet and Eugene, 2008). These properties would recommend high Mw PEGs as more suitable for forming protective hydrogel films, yet beneficial effects have also been reported with PEGs with Mw ranging broadly between 400–20,000 Da (Valuckaite et al, 2013). Our own studies indicated protective effects against the IPI when medium Mw PEG was used (Oltean et al., 2010; Oltean et al., 2012; Oltean et al., 2015, Oltean et al., 2016). Hence, it remains unclear if and how the protective effects of PEG are related to its molecular size.

The current study aimed to directly compare the results of luminal preservation of rat intestines using solutions containing PEGs of medium (3350 Da and 10000Da) and high (20000 Da) MW. Resulting data indicated that both medium and high Mw PEGs have a significant ability to delay the development of IPI and to improve key epithelial variables compared with clinical controls undergoing vascular perfusion alone.

METHODS

Animals

Male Sprague Dawley rats (230-250 grams) were purchased from Charles River (Sulzfeld, Germany) and acclimatized for one week in the University Animal quarters, in 12 hours light-dark cycles, controlled temperature and pathogen free environment and receiving rat chow and water ad libitum. The animals were not fasted. The study followed the regulations outlined by NIH and the European Union and it was reviewed and approved by the local committee of the Swedish Animal Welfare Agency.

Surgery and sampling

Under 2% Isoflurane anesthesia, the abdominal cavity was opened through a midline incision and the infrarenal aorta was ligated above the bifurcation. Thereafter, a short aortic segment around the emergence of the superior mesenteric artery (SMA) was isolated. Aorta above SMA was then ligated and retrograde, in situ perfusion of the intestine through the infrarenal aorta was initiated within 20 seconds from ligation. The intestine was gently perfused with 8-10 ml ice-cold University of Wisconsin (UW) solution over 1-2 minutes until completely blood-free. The right atrium was cut to facilitate venous venting. After perfusion, the distal half of the small bowel (ileum) was cut out and placed in a Petri dish containing chilled UW solution. Intestines were randomly assigned to receive one of the luminal polyethylene-glycol solutions. Intestines without luminal treatment were used as controls. Graft ends were tightly ligated with 3-0 silk ligature and the intestines were stored in ice-chilled perfusion solution. After four, eight and fourteen hours of cold storage the luminal solution was removed and recovered and graft segments were resected and placed in 4% buffered formalin, snap frozen in liquid nitrogen or used for Ussing chamber experiments.

Intestinal tissue was also obtained from four normal, healthy animals anesthetized as above. The abdomen was opened and the ileum was rapidly resected (without perfusion). The

intestines were kept on ice and mounted in the Ussing chambers within 15 minutes from procurement to obtain the fresh tissue values. Tissue was also immediately placed in formalin or snap frozen (normal tissue).

Solutions and experimental groups

Ice cold University of Wisconsin (UW) solution (Viaspan®, Bristol Myers Squibb, Solna, Sweden) was used for the intestinal perfusion. Intestines perfused with UW but without any luminal treatment served as controls (group 1, n=8/time point). For intraluminal preservation we used an electrolyte solution containing 65 mmol/L sodium, 5,4 mmol/L potassium, 17 mmol/L bicarbonate, 53 mmol/L chloride and 13,5% of either PEG 3350 (group 2, n=8/time point), PEG 10000 (group 3, n=8/time point) or PEG 20000 (group 4, n=8/time point).The osmolarity was 239 mOsm/kg, 254 mOsm/kg and 249 mOsm/kg for the PEG 3350, PEG 10000 Da and PEG 20000 solution, respectively.

Grading the preservation injury

Full thickness tissue samples were formalin fixed, paraffin embedded, cut in 5 micron slides and stained with hematoxylin-eosin. At least six sections at three different levels were examined. The ischemic injury was scored independently by two blinded observers using the Chiu/Park score (Park et al, 1990).

Intestinal mucosal goblet cells

Goblet cells (GC) are found throughout the entire intestinal mucosa and produce glycoprotein-rich mucins that protect the enterocytes against luminal aggression and prevent bacteria from gaining direct access to the epithelium. In addition, GC have a significant contribution to the process of mucosal restitution after ischemia/reperfusion (Ikeda et al., 2002). Positive staining with Alcian blue indicates acidic mucins within goblet cells. Formalin fixed, paraffin embedded five-micron thick tissue slides were stained with Alcian blue (3%)

and counterstained with eosin. Positive cells from the villus-crypt junction to the villus tip were counted on seven different fields on at least two sections at high magnification (x40) by a single observer blinded to the study design.

Apoptosis

Apoptosis was studied using indirect immunohistochemistry for active (cleaved) caspase-3 using a Mach 3 polymer detection kit and a Warp Red Chromogen kit (Bio-Care Medical, CA) according to the manufacturer's instructions. Briefly, after deparaffinization, rehydration and antigen retrieval using citrate buffer (10 mM; pH 6.0), sections were blocked and then incubated with primary rabbit antibody against cleaved Caspase-3 (1:100, #D175, Cell Signaling Technology®, Danvers, MA) for one hour at room temperature. Sections were incubated with an anti-rabbit probe followed by an incubation with a rabbit alkaline phosphatase polymer, and then with a warp red chromogen, generating a positive staining in red. Nuclei were counterstained using Myers hematoxylin. Enterocytes positively labelled with cleaved caspase-3 were counted on ten random fields at high magnification (x40).

Tight junctions

Immunofluorescence was used to study tight junction protein zonula occludens-1 (ZO-1). In brief, after deparaffinization and re-hydration, 5 micron slides were incubated in 10mM citrate buffer (pH 6.0) in a pressure cooker at 95° C for 20 minutes for antigen retrieval. Slides were then cooled at room temperature, blocked with a species-specific serum then incubated overnight at 4°C with primary antibody against ZO-1, (1:100, Invitrogen, Stockholm, Sweden). Slides were then incubated with secondary antibody conjugated with Alexa 488 (1:200, Invitrogen), counterstained with 4'6'-diamidino-2-phenylindole (DAPI),

mounted with aqueous mounting medium and examined blindly under the fluorescence microscope.

Western blot analysis of the intestinal tissue

Frozen specimens were homogenized in a PE buffer (10 mM potassium phosphate buffer, pH 6.8 and 1mM EDTA) containing 10 mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate (CHAPS: Boehringer Mannheim, Mannheim, Germany) and protease inhibitor cocktail tablet Complete (Roche Diagnostics AB, Stockholm, Sweden). The homogenate was centrifuged (10,000 g for 10 min at 4ºC) and protein content in the supernatant was analyzed by the Bradford method. Samples (25μg) as well as a prestained molecular weight standard (SeeBlue, NOVEX, San Diego, CA) were diluted in SDS buffer and heated at 70ºC for 10 minutes before loaded on a NuPage 10% Bis-Tris gel using MOPS buffer (Invitrogen AB, Lidingö, Sweden). After electrophoresis, the proteins were transferred to a polyvinyldifluoride transfer membrane (Hybond, 0.45μm, RPN303F, Amersham, Buckinghamshire, UK) using the iBlot dry blotting system. The membranes were then washed with wash buffer (10mM phosphate, 2.7mM potassium chloride, 140mM NaCl pH 7.45, 0.1% (v/v) Tween 20), blocked in 0.2% (w/v) I-block reagent (Applied Biosystems, Bedford, MA) and incubated with primary antibody against p38 MAPK antibody (#9212, Cell Signaling Technology, Danvers, MA), Phospho-p38 MAPK (Thr180/Tyr182) (#4511, Cell Signaling Technology), JNK2 (#9258, Cell Signaling Technology), Phospho-SAPK-JNK (Thr183/Tyr185) (#4671, Cell Signaling Technology) and the loading control glyceraldehyde-3-phosphate dehydrogenase (GAPDH, IMG-5143A, Imgenex, San Diego, CA) overnight at 4°C. After repeated washings, a HRP-conjugated secondary antibody (#7074, Cell Signaling Technology) was applied for 1 h at room temperature and visualization was carried out using the WesternBright Quantum reagents (K-12042, Advansta Corporation, Menlo Park, CA).

The signal intensities of specific bands were detected and analyzed using a Chemidox XRS

cooled charge-couple device camera and the Quantity One software (BioRad Laboratories,

Hercules, CA). GAPDH was used as a control for equal loading and for each tested sample

the optical density (OD) of primarily antibody/GAPDH represent the results. Each time the

membrane was incubated with a new primary antibody, the previous antibody has been

removed with stripping buffer (Re-Blot Plus Mild Solution (10x), Millipore, Temecula, CA).

The gel was stained in 0.2% Coomassie blue to determine the efficiency of the protein

transfer.

Ussing chamber experiments

Several electrophysiologic parameters were studied by means of the Ussing chamber using

intestinal segments from at least four animals for each time point and group. The whole-

thickness rat small intestine was mounted in conventional Ussing chambers with the square

area of 0.29 cm^2 (Warner instruments, Hamden, CT, USA). After mounting, each half

chamber was filled with 5 mL Krebs solution with the following composition (in mM): 118.1

NaCl; 4.7 KCl; 2.5 $CaCl_2$; 1.2 $MgSO_4$; 1.0 NaH_2PO_4; 25 $NaHCO_3$ and 11.1 glucose. The

Krebs solution was maintained at 37°C and continuously oxygenated with 95% O_2 and 5%

CO_2 and stirred by a gas flow in the chambers. The potential difference (PD) was measured

with a pair of matched calomel electrodes (REF401, Radiometer analytical, Denmark). The

Ussing Pulse Method was used to determine the tissue's epithelial electrical resistance (Rep)

and the epithelial ion current (Iep) was received by using Ohm's law, where I=U/R

(current=voltage/resistance, *i.e.* Iep= PD/Rep) as previously described (Casselbrant et al.,

2009). In brief, the method is based on the concept that the epithelium consists of a capacitor

and resistor coupled in parallel. Separate trains of short current pulses induce a voltage

response in the tissue and charge the epithelial capacitor, which gradually is discharged when

the current ends. The epithelial voltage response, specifically, is received from the discharge curve, and by knowing the magnitude of the applied current the Rep can be calculated. The data were collected using an amplifier and specially constructed software developed in LabView (National Instruments, Austin, TX).

Up to six preparations could be retrieved from each intestinal segment after 4, 8 and 14 hours of preservation. The electrical parameters were measured over 20 minutes experiments (N=24-36 Ussing chambers/time point/group).

Transepithelial permeability

Fluorescein sodium salt (FSS; Mw-376kDa) (Sigma-Aldrich, Stockholm, Sweden) or fluorescein isothiocyanate-dextran (FD4; Mw-4000kDa) (Sigma-Aldrich) was added to the luminal chamber side to a final concentration of 1 and 2 mg/mL respectively. Samples from the serosal side of 0.2 mL were then collected at baseline and after 20 minutes and the concentration of each probe was measured by fluorescence at the excitation wavelength of 480 nm and the emission wavelength of 535 mm.

Statistical analysis

Statistical differences between independent groups were calculated using Kruskal-Wallis test followed by the Mann-Whitney U-test. Data are presented as means±standard deviation (SD) unless otherwise stated. GraphPad Prism6 (GraphPad Software, La Jolla, CA) was used and a *p*-value of less than 0.05 was considered significant.

RESULTS

Histology

Hematoxillin-eosin

The histological alterations induced by the different preservation intervals are summarized in Figure 1. In brief, the microscopic examination revealed significant villus injury and subepithelial edema already after 4 h in intestines from group 1 while the intestines receiving luminal treatment showed incipient or no epithelial detachment. After 8 hours, the intestines in groups 2, 3 and 4 continued to show only discrete, incipient preservation injury regardless of the Mw of the PEG whereas the intestines in group 1 (vascular perfusion alone) showed extensive villus denudation, a mean injury grade 4.1 ± 1.9 and median of 4 (range 1-6.5). Prolonging the cold storage to 14 hours resulted in severe mucosal injury and frequent loss of villi in group 1 (mean 4.7 ± 1.4; median 4 (3-7) whereas the intestines in groups 2, 3 and 4 continued to compare favorably to the control intestines in group 1 and had a median injury grade of 1,5 (0-3), 1,15 (0-2.5) and 0,5 (0-2), respectively (p<0.001). There was no significant difference between the groups receiving luminal PEGs at any of the three time points studied.

Alcian blue

Normal intestines had a mean of 121 ± 32 positive cells/x40 field. Four hours of cold storage resulted in a marked, significant decrease in mucin-filled GC in group 1 but not in group 2, 3 and 4. Throughout the entire study period, mucin-filled Alcian blue positive GCs were more abundant in groups 2, 3 and 4 receiving luminal preservation compared with group 1 (vascular perfusion alone). At similar time points, no significant differences were noted between the groups receiving luminal PEGs. Abundant luminal mucus was noted in group 1 together with a GC depletion occurring preferentially at the apical portion of the villi. Moreover, swelling

of mucus-filled GC was also observed, earliest and most significant in group 1 (Fig. 1 B and 1D).

Apoptosis

In normal intestines, cells positive for active caspase-3 were very rare (one per high magnification field). In group 1, four hours of cold storage lead to marked increase in the amount of caspase-3 positive cells (77,5 ± 34 cells/field) whereas intestines in group 2, 3 and 4 had markedly less apoptotic activity (4,7 ± 5,1 cells, 5,5 ± 2 cells and 9,8 ±13 cells, respectively, $p<0.001$ vs. group 1). The apoptotic count figure remained significantly higher in group 1 after eight (48,7 ± 50 cells/field) and fourteen hours (134 ± 93 cells/field) of cold storage (Figure 1C).

Tight junctions

Normal intestines revealed a thin, continuous reticular fluorescent signal outlining the mucosal contour at the most apical part of the basolateral membrane. The signal was detected from the crypt bottom to the tip of the villus. The signal was absent in the distal third of the villus after 4 hours in group 1 (no luminal preservation) while groups 2, 3 and 4 showed essentially well-preserved ZO-1 expression along the villus axis (Fig 2). After eight hours of cold storage, ZO-1immunostaining receded to the lowest part of the villi and the crypts in group 1 (no luminal preservation) whereas the intestines receiving luminal preservation (groups 2, 3 and 4) revealed well maintained, albeit weaker intensity of the ZO-1 immunofluorescence signal along the entire villus and in the crypts together with frequent loss of staining at the villus tips. Fourteen hours of cold storage lead to massive tissue loss in group 1 and virtual absence of any ZO-1 immunostaining while the intestines receiving luminal solutions showed well preserved ZO-1 expression at least in the lower half in the villi and in the crypts, regardless of group. .

Mitogen activated protein kinase p38 and JNK

Phosphorylated p38-MAPK was found throughout the period of cold storage in groups 1 to 3 and in normal tissue. The maximal activation (highest ratio between phosphorylated and total p38) was found after 4 hours of preservation irrespective of the luminal treatment. At this point, intestines in group 3 had significantly lower p38-MAPK activation compared to group 1. Thereafter groups 1 to 3 revealed minimal p38-MAPK activation at levels found in normal, non-ischemic tissue. Phosphorylation of JNK occurred during cold storage with a maximal activation after 4 hours of preservation. Similarly, with p38, the levels of active, phosphorylated JNK were highest after 4 hours of cold storage; the levels of active JNK detected after 8 and 14 hours of cold storage returned to values found in normal, non-ischemic tissue (Fig. 3). Both p38-MAPK and JNK could also be detected in group 4 albeit the immunobands were fuzzy and poorly defined. Furthermore, phosphorylation of these proteins could not be detected at all. These detection problems were not noted in samples from groups 2 and 3 which were run simultaneously on the same gel, suggesting this phenomenon has been rather specific to the presence of PEG 20 kDa in the samples.

Electrical characteristics in the rat small intestine

Generally all the electrical parameters decreased from the baseline value to the end of the experiment (data not shown). After 4h preservation time both PD and Iep were significantly lower in group 1 (p-value$\leq$0.001) and group 2 (PD p-value$<$0.01) compared with group 3 and 4 as well as with fresh tissue. The Rep was lower in all groups compared to fresh tissue (p-value$<$0.05) and did not differ between groups.

After 8 and 14h preservation time, PD and Iep have decreased further in group 1 ($>$50%) undergoing vascular perfusion alone compared to group 3 and 4 (p-value $<$0.01). The Rep was

still high in group 3 and 4 after 8h but after 14h no differences were found between the groups (Figure 4).

In summary, the electrophysiological characteristics were frequently significantly improved by the luminal preservation but without a clear superiority of any of the PEG solutions used.

Transepithelial permeability

The permeability of the smaller FSS-probe was higher than that of the larger FD4-probe and is shown in Figure 4D and E. As expected, the permeability of FSS-probe increased in relation to the preservation time. However after 4 hours, the level of FSS-probe was the same in the different groups as in the fresh tissue. Eight hours of cold preservation increased the FSS-permeation significantly in group 1 compared to fresh tissue (p-value=0.001) and group 3 (p<0.05), and after 14h in group 1 compared to fresh tissue (p-value=0.01). The level of the FSS-probe was also increased in group 2 to 4 after both 8 and 14h but not significant probably as a consequence of high intra-individually variations (Figure 4D).

The permeability of FD4 remained low and comparable with the fresh intestinal tissue irrespective of the preservation time and solution (Figure 4E)

DISCUSSION

Intestinal cold storage causes variable degrees of morphologic and functional breakdown of the mucosal barrier in a time-dependent fashion. The current results indicate that luminal solutions of medium and large Mw PEG significantly delay the onset and development of tissue changes typical for the intestinal preservation injury. These protective effects seem independent of the molecular size of the PEGs used.

PEGs display mucosal adhesion and can attach to a biological surface and change its electric charge depending on the molecular weight, linear conformation, and concentration of the polymer. Water molecules penetrate into the hydrophilic polymer film to form a hydrogen-bond network and results in a highly hydrated, adhesive polymer film (Temenoff et al., 2012). We previously reported that the development of the mucosal injury during intestinal cold storage can be delayed by the luminal introduction of a PEG 3,35 kDa solution with low sodium content (Oltean et al., 2010; Oltean et al., 2012). We ascribed much of the improvements observed in the intestines receiving additional luminal preservation to significant improvements in tight junction structures. Furthermore, we previously reported significant molecular and functional changes in the esophageal mucosa after one hour of incubation with trypsin and deoxycolic acid (Björkman et al., 2013). Therefore, we suggest that the protective hydrogel forming at the luminal interface may have limited the consequences of prolonged mucosal contact with the intestinal content rich in proteases and bile salts. This protection appears to be irrespective of the Mw of the PEGs used as several morphological and electrophysiologic parameters consistently reveal significant differences between treated and untreated intestines but not between the three types of solutions.

Intestinal ischemia leads to rapid mucus release and the crypt goblet cells are completely depleted in response to various stress signals (Grootjans et al., 2013). Damage to the

epithelial lining and the depletion of GC mucins in the small intestine may lead to an impaired mucosal barrier function and increased susceptibility to secondary enteric bacterial infection (Merga et al., 2014). Many hydrophilic materials have been demonstrated their nonfouling ability to reduce not only nonspecific protein adsorption, but also cell and bacteria adhesion. Luminal application of high- (15-20kDa) but not low Mw PEG (3,35 kDa) protected mice against otherwise lethal Pseudomonas aeruginosa. The protective mechanisms are unclear but it is likely that PEG acted as a physical barrier preventing the direct contact between P. aeruginosa and the enterocytes (Wu et al., 2004). The current results indicate not only maintained epithelial continuity but repleted GC in the undergoing luminal preservation. Hence, maintained mucus stocks and hydrogel coating at the apical surface may synergistically provide additional benefit to the physically intact epithelial lining and further strengthen the mucosal barrier.

Tight junctions are protein complexes essential in maintaining the epithelial morphologic and functional integrity. ZO-1 interacts with other TJ proteins such as occludin and claudins as well as the cytoskeleton and plays a central role in TJ regulation and the transepithelial permeability (Marchiando et al, 2010). Disruption of TJs and ensuing permeability changes occurs through several mechanisms after exposure to inflammatory mediators (Ivanov, 2012), oxidants (Cuzzocrea et al, 2000) or lytic enzymes (Björkman et al, 2013). We speculate that ZO-1 loss was due to the proteolytic action of luminal proteases which maintain a certain biologic activity at lower temperatures. Intestines receiving additional luminal preservation retained ZO-1 throughout the study as luminal PEGs may have diluted the luminal content, and interfered with the luminal proteases or limited the access of enzymes to the apical membrane and the junctional ring. These presumed interactions between PEGs and various epithelial biomolecules differ in structure and efficiency from the bio-pharmaceutic PEG-

ylation but are both plausible and possible as spontaneous binding to PEGs is well documented and currently used in laboratory medicine.

The difficulty to detect several proteins in the tissue homogenate of intestines receiving PEG 20kDa is remarkable and indirectly supports protein coating as a protective mechanism of PEGs in this setting. Although the luminal solution was evacuated before sampling, small volumes of PEG solution may have been retained inside the intestine. This may have contaminated the homogenates and later adsorbed various proteins on it, masking the binding sites for the monoclonal antibodies. It is well known that the polymer backbone of PEGs is a flexible moiety which can adsorb various proteins (Okutsu et al, 2011), and shield protein sites from recognition by the immune system, cellular receptors, or enzymes (Cunningham-Rundles et al, 1992). In fact, these properties are routinely used in transfusion medicine to rapidly remove unwanted antigens and require low concentrations of PEGs (Cheng et al, 2001). We speculate that our failure to detect several proteins in the homogenate of intestines receiving PEG 20kDa is due to a similar mechanism of unspecific binding by the largest PEG used. Whether smaller PEGs may have exerted similar effects and the extent of it remains unclear.

Increased enterocyte apoptosis is another central event during intestinal preservation and cleaved, active caspase-3 has been found already after one hour of cold storage (Salehi et al., 2007). Its exact cause is unclear but it has been suggested that ATP depletion during cold ischemia results in AMP-activated protein kinase (AMPK) phosphorylation and activation (Qi and Young, 2015). AMPK is a well-recognized cellular energy sensor that is activated by changes in adenine nucleotide concentrations and may initiate several cellular stress responses including apoptosis (Meisse et al., 2002). We found a significantly lower apoptosis rate in the mucosa of intestines different receiving luminal treatment compared with those undergoing vascular perfusion alone. It is s unlikely that the nutrient-free solution used in this study

influenced the energetic status of the enterocytes so other mechanisms than AMPK modulation may be involved.

Earlier reports indicated that high Mw PEGs (35kDa) may interfere with MAPK signaling during renal cold storage (Dutheil et al., 2006). Members of the MAPK super-family are activated by numerous types of cellular stressors such as hypoxia, heat stress or oxidative stress. Upon activation, MAPKs translocate into the nucleus and activate various transcription factors regulating apoptosis, cell survival or inflammation. JNK is considered to be part of the signaling cascade during apoptosis, whereas p38-MAPK has been linked to the pro-inflammatory response. Similar with previous reports, the present results indicate an early activation of both p38-MAPK and JNK during the intestinal cold storage followed by continuous signs of MAPK activation albeit at lower levels for the remaining time. Similarly with the report of Dutheil et al. we found that luminal PEG 10000 Da diminished p38-MAPK activation. Remarkably, a similar pattern of MAPK activation during intestinal preservation with an early activation and beyond 6 hours has been suggested earlier without a clear explanation (Salehi et al., 2007). We speculate that phosphorylated, activated MAPKs translocated into the nuclei during the first four to six hours of cold storage and were no longer found in the cytoplasmic fraction which was studied both herein and in the paper of Salehi (Salehi et al., 2007).

The present study has several limitations with the most significant being the use of a non-transplant model. A transplant model would have allowed a more complex assessment of the injury development after reperfusion and mucosal recovery as well as the impact on the transplant outcome. However, reperfusion is associated with extensive tissue destruction, massive tissue activation and inflammatory infiltration making mechanistic observations and inter-group comparisons difficult, at least in the early postreperfusion phase (Oltean et al., 2007). Therefore we believe the current setting allowed for detailed observations and

provided a wealth of valuable information for designing and evaluating novel strategies and solutions for luminal preservation.

The use of one single concentration and the use of only three molecular weights may be regarded as additional limitations. The choice of the PEG size has been made considering the ability of lower Mw PEGs to the readily penetrate the intestinal mucosa (Kim, 1996). PEGs with larger Mw (15-20 kDa) have already been shown to mitigate intestinal preservation injury (Valuckaite et al., 2013) and improve kidney endothelial cell survival, adenosine triphosphate (ATP) production, and activation of survival pathways (Giraud et al, 2018). Another limitation is the use of a less standardized volume of luminal solution as this has been shown important for the outcome of luminal preservation (Oltean et al., 2016). The small size of the rat intestine and the lack of any previous data in this species made difficult the choice of a certain filling volume. To ensure the consistency of the model, the same surgeons performed the experiments in a similar fashion throughout the study.

In conclusion, the current results indicate that luminal solutions of medium and large Mw PEG significantly delay the onset and development of tissue changes typical for the intestinal preservation injury. Hence, the present study provides further evidence that luminal interventions may allow for longer cold storage intervals of the intestinal grafts.

AUTHORS CONTRIBUTION

Participated in research design: Casselbrant and Oltean.

Conducted experiments: Casselbrant, Söfteland, Hellström, Malinauskas and Oltean

Contributed new reagents or analytic tools: Casselbrant, Hellström and Oltean

Performed data analysis: Casselbrant, Söfteland, Hellström, Malinauskas and Oltean

Wrote or contributed to the writing of the manuscript: Casselbrant, Söfteland, Hellström,

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FOOTNOTE

LEGEND TO FIGURES

Figure 1 The microscopic evaluation of the intestinal grafts. Preservation injury evaluated

using the Chiu/Park grading scale (panel A); summary of the goblet cell count using Alcian

Blue staining (panel B); enterocyte apoptosis quantified by caspase-3 positive cells (panel C)

in group 1 (no luminal preservation, white bar), group 2 (PEG 3350 Da, light grey bar), group

3 (PEG 10000 Da, dark grey bar) and group 4 (PEG 20000 Da, hatched bar); representative

microphotographs of Alcian blue staining showing severely damaged villus architecture,

goblet cell depletion and luminal mucus discharge in group 1 (D) and maintained villi and

mucus-repleted Goblet cells in group 2 (E), group 3 (F) and group 4 (G) after 14 hours of cold

preservation. Original magnification x100, scale bar represents 100 micrometer; * $p<0.05$, **

$p< 0.01$

Figure 2 Immunofluorescence for zonula occludens (ZO)-1. Microphotographs showing

zonula occludens (ZO)-1 expression (green) along the villus in the four studied groups at the

indicated time points. Images were viewed with x20 objective. Inset shows the area outlined

in the corresponding picture; nuclei stained in blue using 4'6'-diamidino-2-phenylindole

(DAPI); long bar – 75 μm, short bar 10 μm, * artifact..

Figure 3. Immunoblotting using antibodies against the phosphorylated and total forms of p38-

MAPK and JNK. Representative western blot bands (top panel) and the relative activity

(expressed as the ratio of phosphorylated form/total form) of p38-MAPK (lower left panel)

and JNK (lower right panel) in intestines undergoing vascular perfusion only (group 1, white

bar) and in intestines receiving additional luminal preservation with PEG 3350 Da (group 2,

light grey bar) or PEG 10000 Da (group 3, dark grey bar). In group 4 (PEG 20000 Da) no

ratio could be calculated due to impossibility to detect any phosphorylated proteins (see

Results section). ** p< 0.01 probably because the antibody could not bind to the PEG20-

coated enterocyte. ** p< 0.01

Figure 4. The functional assessment of the intestinal grafts using Ussing chamber

experiments. The effect of various luminal solutions on potential difference (panel A),

epithelial electrical resistance (panel B), epithelial electrical current (panel C), permeability

for fluorescein sodium salt (FSS) (panel D) and permeability for fluorescein-Dextran 4 kDa

(FD4) (panel E) after 4 h, 8h and 14h of cold storage. Values are presented as differences

between values in fresh tissue (FT, black bar) and intestines in group 1 (no luminal

preservation, white bar), group 2 (PEG 3350 Da, light grey bar), group 3 (PEG 10000 Da,

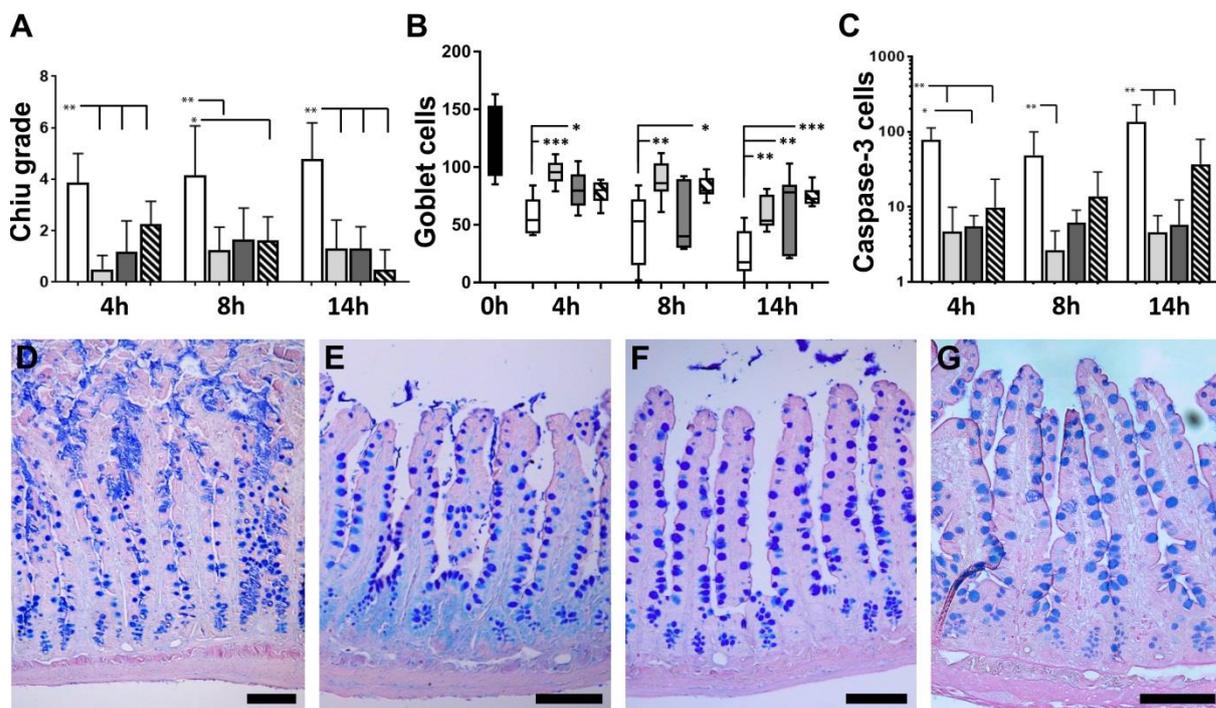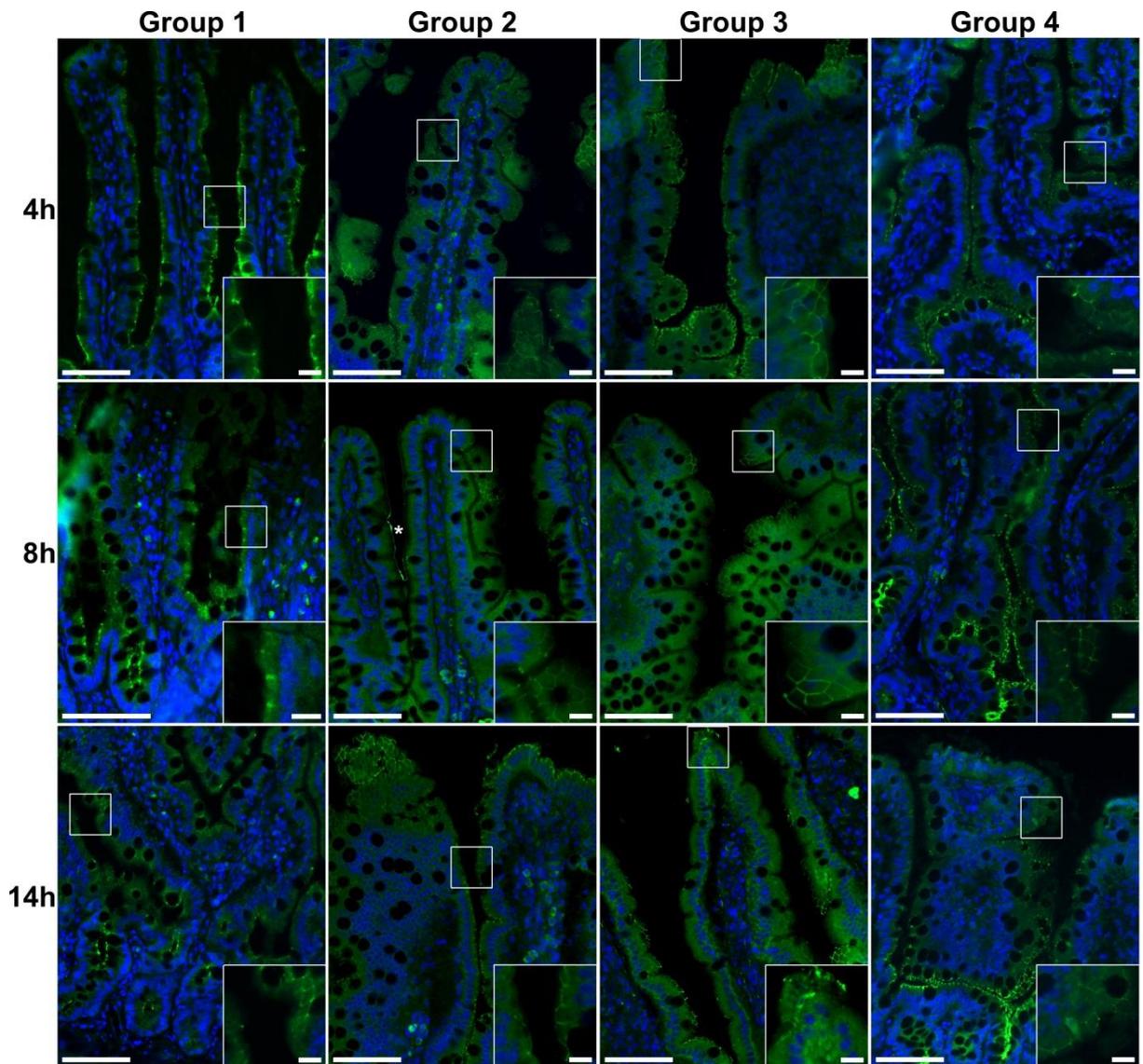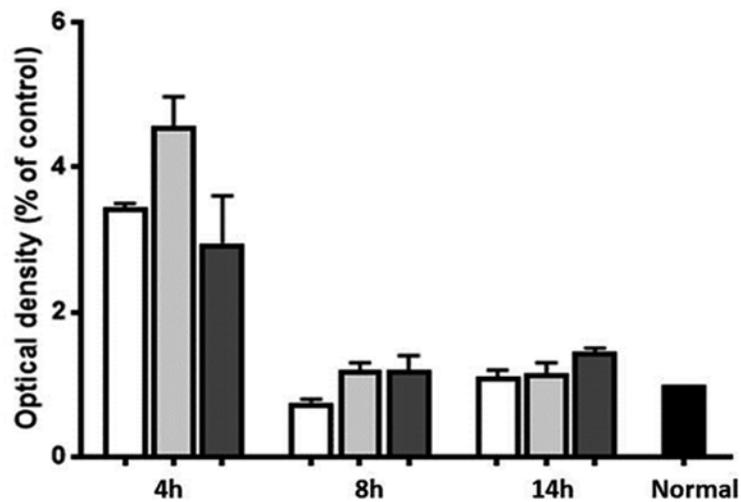dark grey bar) and group 4 (PEG 20000 Da, hatched bar); * p<0.05, ** p< 0.01
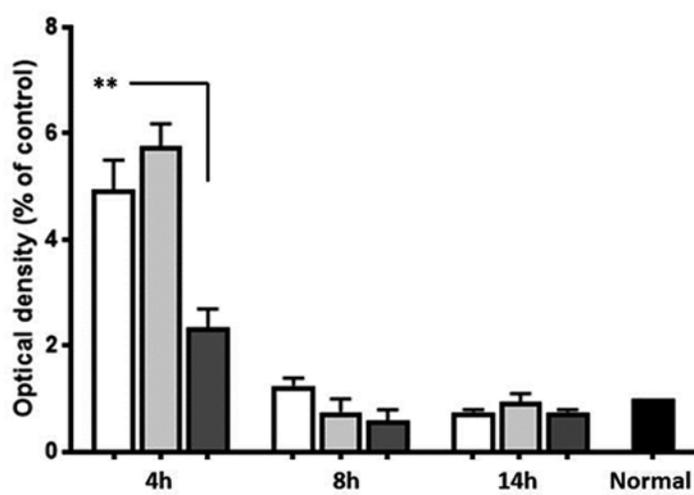
Figure 1

Figure 2

p38 MAPK

Phospho-p38 MAPK

JNK2

Phospho-SAPK-JNK

GAPDH

4h	8h	14h	4h	8h	14h	4h	8h	14h	4h	8h	14h
Group 1			Group 2			Group 3			Group 4		

Phospho-p38 MAPK/p38 MAPK

Phospho-SAPK-JNK/JNK2

Figure 4