Surgery 172 (2022) 1522–1528

Contents lists available at ScienceDirect

Surgery

journal homepage: www.elsevier.com/locate/surg

Presented at the Academic Surgical Congress 2022

Luminal administration of biliverdin ameliorates ischemia-reperfusion injury following intestinal transplant in rats

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ARTICLE INFO

Article history:
Accepted 27 July 2022
Available online 8 September 2022

ABSTRACT

Background: Intestinal grafts are susceptible to ischemia-reperfusion injury, resulting in the loss of mucosal barrier function and graft failure. Biliverdin is known to exert a variety of cytoprotective functions against oxidative tissue injury. Because the mucosal layer is the primary site of ischemia-reperfusion injury, mucosa-targeting strategies by luminal delivery of reagents might be beneficial. We tested whether intraluminal administration of biliverdin as an adjuvant to standard preservation solutions protected against ischemia-reperfusion injury.

Methods: Orthotopic syngeneic intestinal transplants were performed on Lewis rats after 6 hours of cold preservation. Saline containing biliverdin (10 μM) or without biliverdin was introduced into the lumen of the intestinal grafts immediately before cold preservation.

Results: Damage to the intestinal mucosa caused by ischemia-reperfusion injury resulted in severe morphological changes, including blunting of the villi and erosion, and led to significant loss of gut barrier function 3 hours after reperfusion. These changes to the mucosa were notably ameliorated by intraluminal administration of biliverdin. Biliverdin also effectively inhibited upregulation of messenger RNAs for interleukin-6, inducible nitric oxide synthase, and C-C motif chemokine 2. Additionally, biliverdin treatment prevented the loss of expression of claudin-1, a transmembrane, tight-junction barrier protein. The 14-day survival of recipients of biliverdin-treated grafts was significantly improved as compared with the recipients of saline-treated control grafts (83.3% vs 38.9%, $P = .030$).

Conclusion: This study demonstrated that luminally delivered biliverdin provides beneficial effects during the transplant of rat small intestinal grafts and could be an attractive therapeutic option in organ transplantation.

Introduction

Intestinal transplantation remains challenging because intestinal grafts often suffer ischemia-reperfusion (IR) injury, resulting in long-lasting intestinal impairment. Advanced IR injury can stimulate adaptive alloimmunity and promote acute or chronic rejection. Therefore, prevention of intestinal IR injury may significantly improve post-transplant long-term outcomes and patient care.

Bile pigments, including biliverdin (BV) and its metabolite bilirubin, are known to exert natural antioxidant properties and prevent organ damage after various pathological insults.[1–6] Protective effects of BV against transplantation-related IR injuries have been previously shown in a rat small intestinal transplant model,[7] a heart and kidney transplant model,[8] a lung transplant model,[9] and with vein grafts[10] after intraperitoneal administration of BV. In these studies, the targets of BV were primarily vascular endothelial cells. Vascular endothelial cells are easily damaged by the insults initiated during IR injury, resulting in a disturbance of the graft microcirculation and the subsequent activation of inflammatory cascades.[11]

Oral presentation at the 17th Annual Academic Surgical Congress. Orlando, FL, February 2, 2022.

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https://doi.org/10.1016/j.surg.2022.07.021

Previous laboratory, preclinical, and clinical studies have aimed to develop ideal solutions for organ perfusion and preservation that can be administered intravascularly.[12,13] We have focused on the novel intraluminal administration of therapeutics that prevent oxidative damage, as the intraluminal compartment of the intestine is also a target for intervention. The luminal membranes are the site of uptake for nutrients, water, and electrolytes in the small intestine. The mucosal epithelium that lines in lumen is very susceptible to IR injury, which can lead to a loss of mucosal barrier function and allow bacteria to translocate into the interstitial spaces and bloodstream. Luminal treatment during cold preservation has not yet been fully investigated in a clinical setting. Recently, our laboratory demonstrated that administration of hydrogen-[14] and carbon monoxide-releasing molecules[15] in the intestinal lumen can directly deliver these therapeutic gases through the luminal membrane to the enterocytes. Direct contact of these molecules with the mucosa may preserve the architecture of the intestinal villi.

In this study, we hypothesized that luminal administration of BV would reduce intestinal mucosal injury and consequently ameliorate IR injury in intestinal grafts. Luminal introduction of BV during cold storage may be an attractive approach to reduce IR injury due to its technical simplicity and safety.

Methods

Animals

Inbred male 200 to 250 g Lewis (LEW, RT.1[l]) rats were supplied from Japan SLC (Hamamatsu, Shizuoka, Japan) and housed in an animal facility equipped with laminar airflow at Okayama University. Animals were fed a standard diet and were given access to water ad libitum. All procedures were performed in compliance with guidelines from the Institutional Animal Care and Use Committee at Okayama University.

Reagents

Biliverdin hydrochloride (molecular weight 619.12; Funakoshi Inc, Tokyo, Japan) was dissolved in 0.2 *N* NaOH and adjusted to pH 7.4 with hydrochloric acid. Intestinal grafts were stored in either Ringer's lactate solution or University of Wisconsin (UW) solution (Belzer-UW; Astellas Pharma Inc, Tokyo, Japan).

Transplantation procedure

Orthotopic small intestinal transplants with caval venous drainage were performed as previously described.[14,16] After diluting in the BV in saline, 40 mL saline containing BV (10 μM) was administered to intestinal grafts by flushing the grafts.[9] Approximately 20 mL of the BV saline solution was then administered into the lumen of the intestine, and both sides of the grafts were clamped. The intestinal grafts were stored in Ringer's lactate solution for 6 hours at 4°C. After using Ringer's lactate solution for mechanistic investigations, we tested whether BV had similar effects when administered in Belzer-UW solution.

Experimental groups

Animals receiving intestinal grafts were either killed 3 hours after transplantation or followed for 14 days to assess survival. Autopsies were performed on all animals that died during the 14-day observation period after intestinal transplant. Animals in the sham group served as controls and received only the anesthetic without any surgical intervention before the removal of the intestine for analysis. The Sham group provided native intestine from treatment-naïve animals. The following 5 experimental groups were studied: (1) intestine from sham animals with intraluminal injection of saline during cold preservation, (2) transplanted intestinal grafts with intraluminal injection of saline before storage in Ringer's solution, (3) transplanted intestinal grafts with intraluminal injection of saline containing 10μM BV before storage in Ringer's solution, (4) transplanted intestinal grafts with intraluminal injection of saline before storage in UW solution, and (5) transplanted intestinal grafts with intraluminal injection of saline containing 10μM BV before storage in UW solution.

Histopathological analysis

Intestinal segments from each graft 3 hours after reperfusion or from the native small intestine in sham rats were collected and fixed in 10% buffered formalin, then embedded in paraffin, sectioned (4 μm thick), and stained with hematoxylin and eosin. A pathologist (I.T.) evaluated the degree of mucosal injury microscopically with the sample identities masked using a 0 to 9 summary score, in which grade 0 indicated healthy mucosa, whereas submucosal edema, erosions, and hemorrhages were each rated by grades 1 to 3.[17–19] At least 8 samples per animal were analyzed.

SYBR green 2-step real-time reverse transcriptase polymerase chain reaction

Messenger RNA (mRNA) levels for interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS), C-C motif chemokine 2 (CCL2), heme oxygenase-1 (HO-1), and β-actin were assessed using SYBR (Toyobo Co Ltd, Japan) green 2-step real-time reverse transcription PCR as previously described.[14] The levels of each mRNA were measured in tissue collected 3 hours after reperfusion. This time point was determined as a peak of proinflammatory activity post-transplant based on the previous studies with sequential analysis of mRNA levels.[20,21] The SYBR green PCR mixture was prepared with THUNDERBIRD SYBR qPCR Mix(R) (TOYOBO CO., LTD, Osaka, Japan) with specific primers.[15] The thermal cycling protocol consisted of 10 minutes at 95°C to activate the polymerase, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute using the StepOnePlus Real-time PCR system (Thermo Fisher Scientific, Waltham, MA).

Intestinal graft permeability

As an indicator of barrier integrity, intestinal permeability to fluorescein isothiocyanate-labeled dextran with an average molecular weight of 4 kDa was assessed using an everted intestine apparatus as previously described.[7,14,15] The fluorescent intensity of the content solution was measured using a fluorescence plate reader (Flexstation 3; Molecular Devices, San Jose, CA). The intestinal permeability to fluorescein isothiocyanate-labeled dextran was calculated as pmol/cm intestine/min.

Spectrophotometric assay for malondialdehyde

The concentration of malondialdehyde (MDA) in the homogenized graft intestinal tissues was measured using the Bioxytech MDA-586 kit (Oxis Research, Portland, OR) according to the manufacturer's protocol.[7,8]

Western blot

The protein was extracted using radio-immunoprecipitation assay buffer, which consisted of 50 mmol/L Tris-HCl (pH 8.0), 150

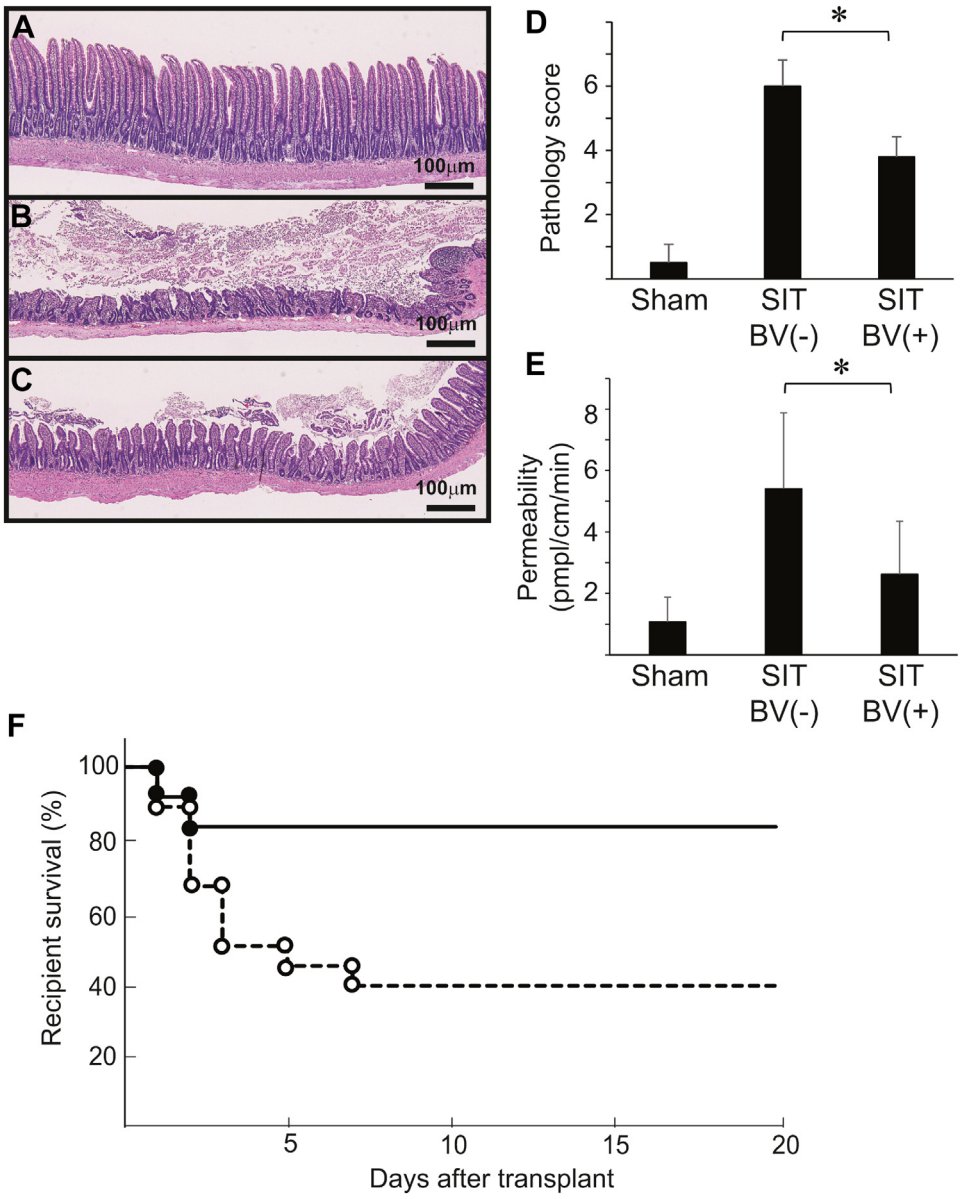

Figure 1. Assessment of graft morphology and function after cold storage in Ringer's solution with and without intraluminal biliverdin (BV), transplant, and reperfusion. Representative hematoxylin and eosin–stained sections of intestinal epithelium. (A) Sham/native, treatment-naive intestine. (B) Transplanted small intestine without intraluminal BV. (C) Transplanted small intestine with intraluminal BV. (D) Histopathological score. The degrees of graft mucosal changes are presented as a mean histopathological score of each animal. (E) Intestinal permeability. Gut permeability was determined by everted gut methods 3 hours after reperfusion. (F) Recipient survival. $P = .030$, Kaplan-Meier, log-rank test. *$P <$ .001, $n = 6$ for each experimental group. *BV*, biliverdin; *SIT*, small intestine transplant.

mmol/L NaCl, 1% Igepal CA-630 (Merck & Co., Inc, Rahway, NJ), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1 mmol/L ethylenediaminetetraacetic acid with Complete Mini Protease Inhibitor Cocktail (Merck). Thirty milligrams of powdered, frozen graft tissue were mixed with radio-immunoprecipitation assay buffer, homogenized, and centrifuged. The supernatant, which contained soluble cytosolic proteins, was used for protein assays and western blotting. The intestinal cytosolic proteins (10 μg) were separated using electrophoresis on 10% acrylamide-sodium dodecyl sulfate gels and transferred to Immobilon-P polyvinylidene difluoride membrane (0.45 μm; Merck). After blocking with 5% nonfat milk to prevent nonspecific binding, the membrane was incubated at 4°C overnight with primary polyclonal rabbit antibody against zonula occludens-1 (ZO-1; 1:1000 dilution, #61–7300; Thermo Fisher Scientific), polyclonal rabbit antibody against claudin-1 (CLDN-1; 1:1000 dilution, catalog no. ab15098; Abcam, Cambridge, UK), or monoclonal mouse antibody against β-actin (1:10 000 dilution, catalog no. A5441; Sigma-Aldrich, Burlington, MA). Antibodies against ZO-1 and CLDN-1 were diluted in Can Get Signal immunoreaction enhancer solution 1 (Toyobo, Osaka, Japan). After washing and incubation at room temperature for 1 hour with peroxidase-conjugated secondary antibody against rabbit IgG (1:10 000 dilution, 111–035–144; Jackson ImmunoResearch Laboratories, Inc, West Grove, PA) or mouse IgG (1:10 000 dilution, 115-036-020; Jackson ImmunoResearch Laboratories, Inc), the secondary antibodies were visualized with ECL Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, IL), and the signal was

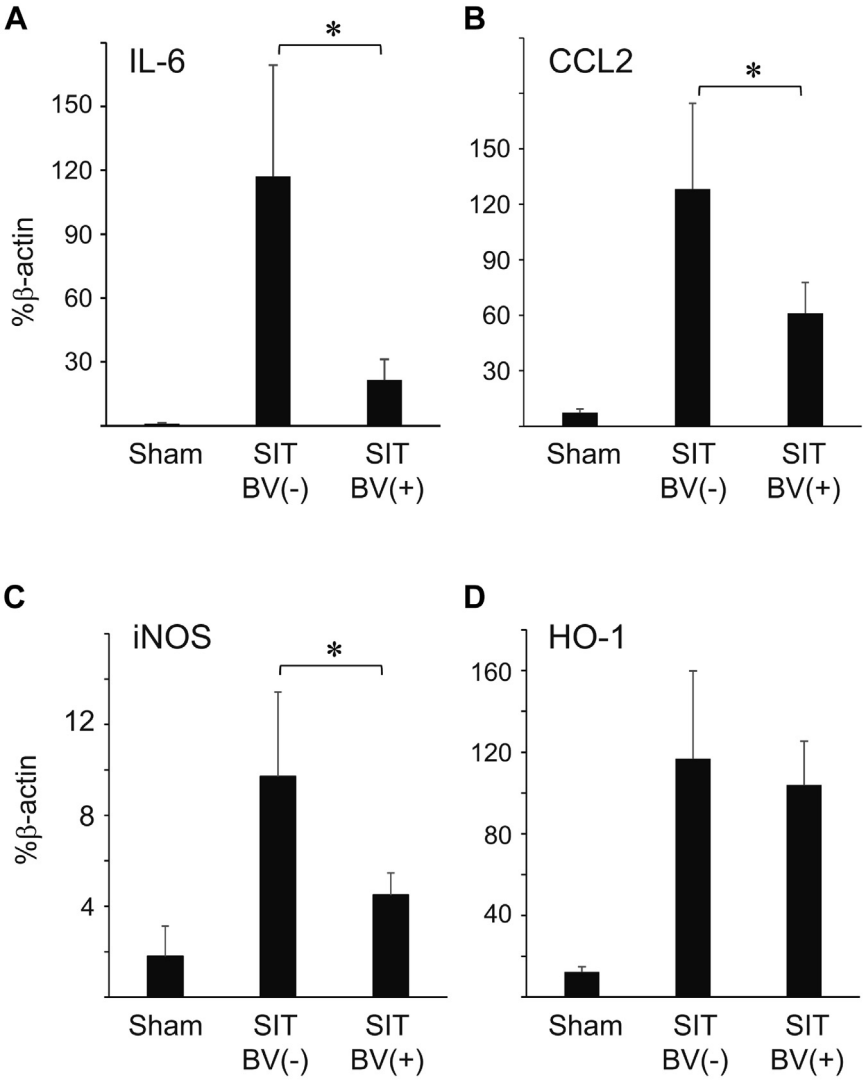

Figure 2. Realtime SYBR green reverse transcription polymerase chain reaction analysis of proinflammatory mediators. (A) Interleukin-6. (B) C-C motif chemokine 2. (C) Inducible nitric oxide synthase. (D) Heme oxygenase 1. $N = 6$ per group. *$P < .001$ versus saline-treated recipients. *BV*, biliverdin; *CCL2*, C-C motif chemokine 2; *HO-1*, heme oxygenase 1; *IL*, interleukin; *iNOS*, inducible nitric oxide synthase; *SIT*, small intestine transplant.

detected with WSE-6100 LuminoGraph I (ATTO Technology, Inc, Amherst, NY).

Statistical analysis

The results are expressed as mean ± SEM. Statistical analysis was performed using the unpaired Student's *t* test or analysis of variance, where appropriate. For the survival study, Kaplan-Meier curves were generated, and log-rank tests were performed.

Results

Biliverdin mitigates morphological changes in the graft tissue after intestinal transplantation

Cold storage and reperfusion resulted in significant pathological changes in the intestinal grafts. The mean histopathological score obtained from the blinded evaluation of the native intestine (sham) was 0.5 ± 0.6. The pathological changes in the untreated intestinal grafts 3 hours after reperfusion included significant epithelial loss associated with villous congestion and erosion. The mean pathology score for the untreated grafts was 6.0 ± 0.8. In contrast, treatment with BV before cold storage mitigated these pathological changes 3 hours after reperfusion, as indicated by a significant decrease in the pathology score to 3.8 ± 0.6 ($P < .001$; Figure 1, *A–D*).

Biliverdin mitigates postreperfusion intestinal permeability and improves recipient survival after transplantation

To correlate the morphological changes in the intestinal epithelium associated with IR injury after transplantation with intestinal function, gut barrier function was determined by measuring intestinal permeability 3 hours postreperfusion ($n = 4$ for each group). Although the permeability of native intestine was 1.1 ± 0.5 pmoL/cm·min, the permeability of grafts stored in intraluminal saline deteriorated considerably to 5.41 ± 1.4 pmoL/cm·min. This decline in permeability was almost completely abolished by luminal injection with BV-containing saline as indicated by an intestinal permeability of 2.62 ± 1.0 pmoL/cm·min (Figure 1, *E*).

Only 38.9% (7/18) of the recipient animals with grafts stored with intraluminal saline before transplant survived >14 days. On the other hand, 83.3% (10/12) of the recipients of intestinal grafts treated with intraluminal BV survived >14 days ($P = .030$) (Figure 1, *F*). All deaths were caused by intestinal IR injury. This manifested as dehydration or sepsis due to compromised mucosal barrier function in recipients that died 2 to 3 days after transplant and as malabsorption or anastomotic complications >4 days after transplant. No deaths were due to technical failure of the transplant procedure.

Intraluminal administration of BV reduces up-regulation of pro-inflammatory mediators after cold storage and transplantation

The effects of intraluminal administration of BV on the expression of inflammatory mediators in intestinal tissue were also examined. Three hours after reperfusion, IL-6 mRNA showed an approximately 130-fold increase in the grafts treated with saline as compared with treatment-naïve intestine (sham group). Intraluminal BV treatment significantly reduced this upregulation to 20% (Figure 2, *A*). Similarly, CCL2 (Figure 2, *B*) and iNOS (Figure 2, *C*) mRNAs were significantly upregulated in saline-treated grafts as compared with the intestines of naïve rats. The upregulation of both CCL2 and iNOS was significantly mitigated by intraluminal BV administration. The HO-1 mRNA was also upregulated 3 hours after reperfusion as compared with sham controls. The BV treatment did not alter HO-1 mRNA upregulation, however (Figure 2, *D*).

Intraluminal administration of BV reduced lipid peroxidation of the intestinal graft tissue

The MDA, an end-product of lipid peroxidation used to assess oxidative stress, was low in the treatment-naïve intestinal tissue (0.17 ± 0.04 μM/mg protein) and was significantly increased in the saline-treated graft tissues 3 hours after reperfusion (0.32 ± 0.07 μM/mg). The BV treatment significantly reduced tissue MDA levels to 0.23 ± 0.06 μM/mg ($P = .036$) (Figure 3, *A*).

Intraluminal BV-containing saline maintained tight junction barrier-related proteins

Tight junction proteins ZO-1 and CLDN-1 primarily regulate the barrier function of intestinal epithelium. The expression of these proteins was reduced in saline-treated intestine grafts 3 hours after reperfusion (Figure 3, *B*). Intraluminal BV administration did not alter ZO-1 expression (Figure 3, *B*). However, BV treatment mitigated the downregulation of the transmembrane protein CLDN-1 (Figure 3, *B*).

Intraluminal administration of BV protected intestinal grafts from IR injury after cold preservation in Belzer-UW solution

The Belzer-UW solution is commonly used for cold storage before transplant in clinical rather than experimental settings. Therefore, we tested whether intraluminal administration of BV before cold preservation had similar effects when grafts were stored in Belzer-UW solution to support the clinical applicability of our findings. Histopathological analysis revealed massive epithelial loss and bleeding in untreated grafts 3 hours after reperfusion. These drastic changes in mucosa morphology were substantially mitigated by intraluminal treatment with BV (Figure 4, *A*–*D*). The mean tissue pathology score of the intestine grafts stored without intraluminal BV was 6.67 ± 0.58, whereas the mean score for tissues treated with BV was 3.67 ± 0.58 (Figure 4, *E*). Similarly, intraluminal BV treatment protected the mucosal barrier function of the intestinal epithelium in the grafts. Three hours after reperfusion, the permeability of the grafts treated with saline and stored in BV in Belzer-UW solution was 2.96 ± 1.19 pmoL/cm·min, whereas intraluminal injection of BV significantly blocked deterioration of barrier function as indicated by a permeability of 0.68 ± 0.21 pmoL/cm·min.

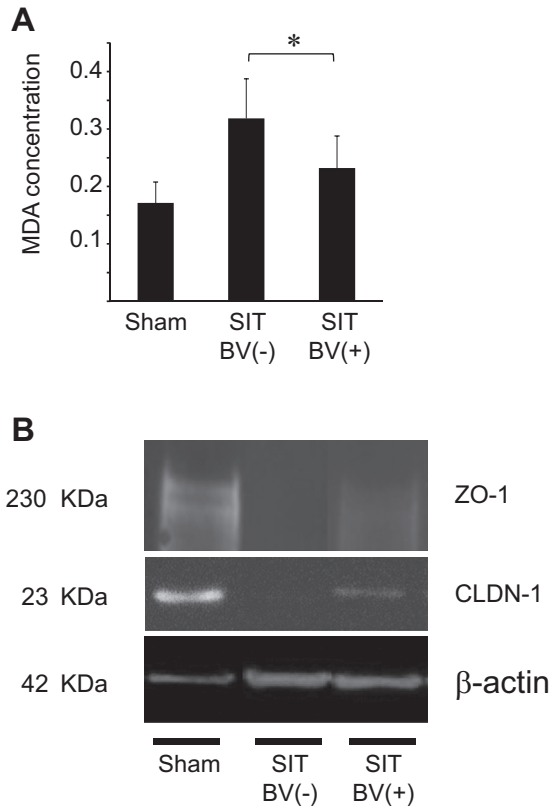

Figure 3. Tissue malondialdehyde levels as an indicator of oxidative stress. Western blot revealed that biliverdin treatment regulated loss of barrier-related protein claudin-1 but not zonula occludens-1. $N = 6$ *$P = .036$. *BV*, biliverdin; *CLDN-1*, claudin-1; *MDA*, malondialdehyde; *SIT*; small intestine transplant; *ZO-1*, zonula occludens-1.

Interestingly, although cold preservation resulted in only mucosal edema (Figure 4, *B*), a significant increase in intestinal permeability was observed (Figure 4, *F*). The permeability of the intestine with 6 hours cold storage without reperfusion was 2.85 ± 0.97, indicating that cold storage caused deterioration of mucosal barrier function independent of IR injury during warming (Figure 4, *F*).

Discussion

This study demonstrated that intraluminal BV treatment during cold storage effectively mitigates IR-induced mucosal injury in intestinal grafts. The interaction of BV with the mucosa significantly improved the structural status of the graft mucosa as compared with the control grafts in each experiment. The current study supplemented previous work on prevention of IR injuries by our group and challenged us to integrate multiple therapies to prevent IR injury and improve post-transplant outcomes.[22] Amelioration of IR injury with BV may improve both acute and long-term graft function and thereby reduce graft rejection. Administering BV intraluminally to excised grafts may be easy to apply in clinical scenarios.

Biliverdin is a water-soluble bile pigment formed as a byproduct of heme breakdown through the heme oxygenase system.[23] BV is

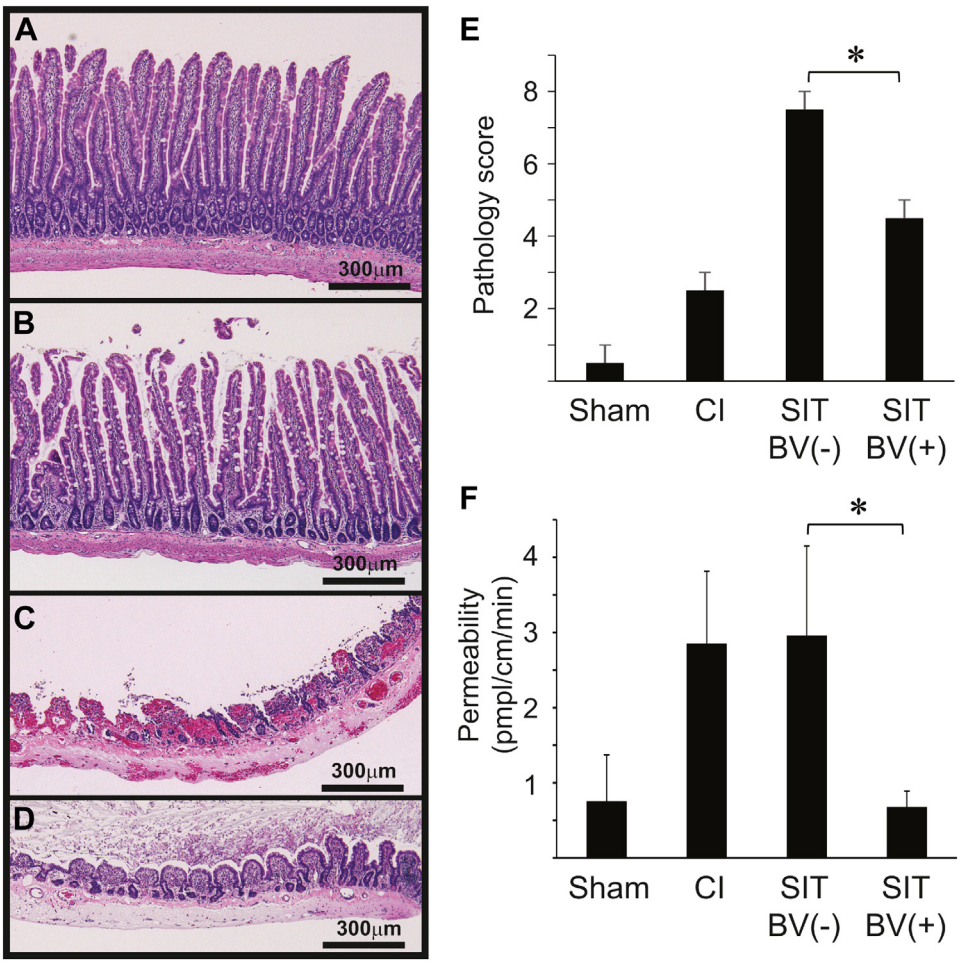

Figure 4. Assessment of graft damage when stored in the University of Wisconsin (UW) solution with and without intraluminal biliverdin (BV). Representative hematoxylin and eosin–stained sections of intestinal epithelium. (A) Sham/native intestine. (B) Intestine stored in UW solution at 4°C for 6 hours but not transplanted. (C) Transplanted small intestine without intraluminal BV treatment. (D) Transplanted small intestine with treated with intraluminal BV. (E) Mean histopathological score to quantitate the degree of graft mucosal changes. $N = 6$ per experimental group. (F) Intestinal permeability was measured after storage in UW solution for 6 hours. $N = 8$ per experimental group. *BV*, biliverdin; *CI*, control intestine; *SIT*, small intestine transplant.

immediately reduced to form bilirubin by BV reductase, which is present in large functional excess in all mammalian tissue.[24] A remarkable bilirubin–BV cycling mechanism has been proposed wherein bilirubin reacts with oxyradicals, specifically generating BV, which is then reduced back to bilirubin by reduced nicotinamide adenine dinucleotide phosphate/BV reductase.[25] Previous studies have shown that exogenous BV is able to downregulate the expression of adhesion molecules and inhibit the aggregation of white blood cells, thereby reducing the expression of proinflammatory cytokines and chemotactic factors, ultimately mitigating inflammation.[26] The cytoprotective effects of BV are mediated by downregulating the nuclear factor (NF)-κB/iNOS pathway and activating the NF erythroid 2-related factor (Nrf) 2/HO-1 pathway.[27] Biliverdin can also inhibit lipopolysaccharide-induced complement component 5a receptor 1 and decrease tumor necrosis factor-α and IL-6 expression via the mechanistic target of rapamycin pathway in macrophages, thereby preventing an inflammatory response.[28] Furthermore, BV has been shown to damper the complement cascade reaction.[29]

Bile plays an important role in maintaining intestinal barrier function to prevent the invasion of enteric bacteria into the underlying tissues.[30] Multiple studies showed that the absence of bile in the gut, as seen in animals with either bile duct obstruction or bile diversion, leads to mucosal injury, as demonstrated by morphological changes including villous atrophy, villous edema, lacteal canal dilatation, increased intestinal permeability, and increased translocation of bacteria from the gut to other organs. Although the role of bile and bile acids in bacterial translocation is not fully understood, the most important action of bile acids is their inhibition of bacterial overgrowth in the intestine.[31,32] There is decreased but persistent metabolism in the intestinal mucosa during cold storage, as attested by decreases in cellular energy charge during cold storage. Intestinal mucosal injury is further promoted by reperfusion due to excessive generation of reactive oxygen species, leading to impaired mucosal barrier function. Furthermore, the release of proinflammatory mediators from the intestinal graft results in systemic inflammatory responses in distant organs. Direct contact of the mucosa with BV during cold storage may be beneficial in preventing these adverse events initiated by cold storage and IR. Although further toxicological studies will be required before BV can be employed as a drug in the clinical setting, there is increasing evidence that BV has potential as a therapeutic in its purified form. A preliminary clinical report detailed the effectiveness of Goou and Yutan, a traditional Chinese drug containing BV, for the treatment of chronic liver disease.[33]

Most experiments in this study were performed using saline for intraluminal injection and a Ringer's lactate solution for cold preservation, consistent with the experimental protocols used in

previous studies.[7,14,34,35] The use of Ringer's lactate solution for intestinal perfusion and storage may greatly limit the clinical relevance of this study, which was the rationale for performing validation experiments in UW solution. The use of Ringer's solution, which include antioxidants or metabolically inert substances and are supplemented into other commonly used and commercially available preservation solutions, was important to assess the effects of BV without any interference of additional ingredients. Experiments to further test the protective effects of BV and provide the foundation for a clinical trial are warranted, and we are currently exploring testing in large animal transplantation models.

There were several limitations to our study. First, we analyzed the intestinal graft recipients for only a short time. However, previous studies have demonstrated that the severity of intestinal graft injury at 3 hours after reperfusion closely correlates with long-term outcomes.[7,36] Therefore, the evaluation of graft integrity at a single, early time point is likely to yield valuable information when evaluating potential therapeutics. Second, we treated with 10 μM BV based on the previous reports.[9] Future experiments to determine the minimal and most effective dose of BV in this setting are absolutely warranted. Finally, isografting is artificial and rarely happens in clinical transplantation. However, syngeneic transplantation is considered an ideal experimental model for studying IR injury because it allows isolation of factors involved in IR injury from factors involved in alloimmune reactions.[14,20,36,37]

In conclusion, this study provided the first evidence that intraluminal BV treatment during cold ischemia may prevent intestinal graft IR injury by protection of gut barrier function. Administering BV intraluminally to excised grafts and retention of the BV in the graft during cold storage may be a straightforward methodology that translates easily to clinical practice.

Funding/Support

This study was supported by a grant from the JSPS KAKENHI, Grant/Award Number: 20K22953.

Conflict of interest/Disclosure

The authors have no conflicts of interests or disclosures to report.

Acknowledgments

The authors thank Shannon Wyszomierski for editing the manuscript.

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