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Luminal Administration of a Water-soluble Carbon Monoxide–releasing Molecule (CORM-3) Mitigates Ischemia/Reperfusion Injury in Rats Following Intestinal Transplantation

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Background. The protective effects of carbon monoxide (CO) against ischemia/reperfusion (IR) injury during organ transplantation have been extensively investigated. Likewise, CO-releasing molecules (CORMs) are known to exert a variety of pharmacological activities via liberation of controlled amounts of CO in organs. Therefore, we hypothesized that intraluminal administration of water-soluble CORM-3 during cold storage of intestinal grafts would provide protective effects against IR injury. **Methods.** Orthotopic syngeneic intestinal transplantation was performed in Lewis rats following 6 h of cold preservation in Ringer solution or University of Wisconsin solution. Saline containing CORM-3 (100 μmol/L) or its inactive counterpart (iCORM-3) was intraluminally introduced in the intestinal graft before cold preservation. **Results.** Histopathological analysis of untreated and iCORM-3–treated grafts revealed a similar erosion and blunting of the intestinal villi. These changes in the mucosa structure were significantly attenuated by intraluminal administration of CORM-3. Intestinal mucosa damage caused by IR injury led to considerable deterioration of gut barrier function 3 h postreperfusion. CORM-3 significantly inhibited upregulation of proinflammatory mRNA levels, ameliorated intestinal morphological changes, and improved graft blood flow and mucosal barrier function. Additionally, CORM-3–treated grafts increased recipient survival rates. Pharmacological blockade of soluble guanylyl cyclase activity significantly reversed the protective effects conferred by CORM-3, indicating that CO partially mediates its therapeutic actions via soluble guanylyl cyclase activation. **Conclusions.** Our study demonstrates that luminally delivered CORM-3 provides beneficial effects in cold-stored rat small intestinal grafts and could be an attractive therapeutic application of CO in the clinical setting of organ preservation and transplantation.

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INTRODUCTION

Carbon monoxide (CO) is one of the byproducts of heme degradation by heme oxygenase and is known to serve as a signaling molecule and exert a wide range of protective actions in various pathological conditions.[1-3] In the context of organ transplantation, several lines of evidence have indicated that inhaled CO gas at concentrations that circumvent its potential toxicity provides unexpected efficacy in transplant-induced ischemia/reperfusion (IR) injury and graft rejection via its anti-inflammatory, vasodilating, and antiapoptotic properties.[4-10]

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Intestinal transplants remain the most challenging and least frequently performed vascularized intra-abdominal transplantation procedures. This is because intestinal grafts often suffer IR injuries, resulting in prolonged intestinal dysfunction with consequent loss of intestinal barrier function leading to a potentially harmful bacterial translocation into the bloodstream. Therefore, prevention or minimizing intestinal IR injury may significantly improve post transplant patient care and the subsequent long-term outcome of transplanted recipients.

In previous studies using a rat experimental model, it was shown that inhalation of CO gas by the recipients prevents IR injury of the transplanted intestine, an effect that was attributed to the anti-inflammatory and antiapoptotic effects of CO.[11,12] These protective effects of CO have been confirmed in transplantation models of other organs, including the heart, liver, and kidney.[13-15] Despite these very promising results, the therapeutic benefits of CO gas administration have remained questionable because of its potential systemic toxicity when used at high concentrations but most importantly because of the difficulties in carrying and delivering CO to the target tissue in a controlled manner in vivo. To overcome these limitations, studies have been conducted by applying CO gas to organs ex vivo. More specifically, studies conducted in the context of organ preservation have reported that intestinal grafts stored in University of Wisconsin (UW) solution in which CO gas was bubbled during cold storage before transplantation improved barrier function, downregulated the expression of several proinflammatory mediators, and markedly increased recipient survival.[16] The efficacy of this strategic approach in which organs are preserved in a cold solution bubbled with CO gas has been reproduced in the rat model for kidney, lung, and vascular grafts[17-19] and for renal transplantation in pigs.[20]

As an alternative strategy to the use of CO gas and to achieve a more controlled delivery of CO in vivo and ex vivo, small molecules based on metal carbonyl complexes capable of releasing CO, termed CORMs, have been developed as potential pharmaceutical agents for a more practical and possibly safer way of administering CO as a therapeutic.[21,22] Among the various CORMs identified and tested so far, tricarbonylchloro-glycinate ruthenium (II) (Ru(CO)3Cl-glycinate) (CORM-3) has been extensively studied in a variety of in vivo experimental models. This compound, which represents the first transition metal carbonyl soluble in aqueous solutions, was shown to exert important pharmacological activities via the liberation of controlled amounts of CO, including anti-ischemic, anti-inflammatory, and antibacterial effects.[23,24]

The small intestine is a unique organ because it possesses both vascular and luminal routes through which preservation solutions can be administered. Apart from the vessel walls, the layers of epithelial cells forming the mucosa and covering the inner part of the lumen are also a site highly susceptible to IR injury and, thus, a potential therapeutic target. Therefore, the current study was undertaken to explore if mucosa-targeted exogenous delivery of CO using graft intraluminal administration of CORM-3 during cold preservation could ameliorate intestinal IR injury.

MATERIALS AND METHODS

Animals

Inbred male 200 to 250-g Lewis (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

One mg of water-soluble CORM-3 (molecular weight 294.61, Sigma-Aldrich Japan Inc, Tokyo, Japan) was dissolved in 1-mL saline and stored at –20 °C until the experiments. An inactive counterpart of CORM-3 (iCORM-3) was prepared by incubating CORM-3 in a phosphate-buffered solution (pH 7.4) at room temperature for 2 d to liberate CO gas from the molecule[24,25] and was used as negative control in our experiments. Stock solutions of CORM-3 or iCORM-3 were prepared in 34-mL saline (100 μmol/L, final concentration) and administered into the intestinal lumen before placing the graft into the preservation solutions. UW solutions (Belzer-UW, Astellas Pharma Inc Tokyo, Japan) were used for cold preservation of the intestinal grafts, as they are approved clinically in humans. Additional experiments were conducted to determine a possible involvement of soluble guanylyl cyclase (sGC) in the mechanism of action of CORM-3 because CO liberated from this compound has been shown to activate sGC in vascular tissues (Nakao et al[18] and Nakao et al[19]). For this purpose, an inhibitor of sGC, 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ; Research And Diagnostic Systems, Inc, Minneapolis, MN), was injected in combination with CORM-3 in the intestinal lumen before cold preservation (final concentration of ODQ of 10 μM).

Transplantation Procedure

Orthotopic small intestinal transplantation with caval venous drainage was performed as previously described.[26,27] Approximately 20 mL of experimental solution was administered into the lumen of the intestine, and both sides of the grafts were clamped. The intestinal grafts were stored in Ringer lactate solution for 6 h at 4 °C. In addition to using Ringer lactate solution for mechanistic investigations, we then performed additional studies on CORM-3 that were supplemented to Belzer-UW solutions and, therefore, more relevant for a possible translation into the clinical practice.

Experimental Groups

Animals receiving intestinal grafts were either followed for 14 d to assess the survival rate or killed 3 h after transplantation. Based on previously published data, we chose the 3 h as a time point to assess the mRNA levels of proinflammatory mediators in the collected intestinal tissues.[11] Animals in the sham group served as control and received only the anesthetic without any surgical intervention (naive animals) before the removal of the intestine for analysis. Six experimental groups were studied: (1) intestine from sham animals with intraluminal

injection of saline during cold preservation; (2) intestine from sham animals with intraluminal injection of saline plus ODQ; (3) transplanted intestinal grafts (3 h postreperfusion) with intraluminal injection of saline; (4) transplanted intestinal grafts of saline containing iCORM-3; (5) transplanted intestinal grafts of saline containing CORM-3; and (6) transplanted intestinal grafts of saline containing CORM plus ODQ.

Histopathological Analysis

Three hours after reperfusion, intestinal segments (4-μm thick) were first collected and fixed in 10% buffered formalin, then embedded in paraffin, and finally stained with hematoxylin and eosin. A pathologist (I.T.) evaluated the degree of mucosal injury microscopically in a blinded fashion based on a 0 to 9 summary score: grade 0 indicated healthy mucosa, whereas submucosal edema, erosions, and hemorrhages were each rated by grades 1 to 3.[28-30] All slides, at least 8 samples per animal, were analyzed.

Intestinal Blood Flow Measurements

Intestinal microvascular blood flow was monitored 3 h after reperfusion using a laser Doppler flowmeter (BLF 21D; Transonic Systems, Ithaca, NY) placed on the mucosal surface of the graft intestine adjacent to the mesenteric border. A technical assistant (or a member of the laboratory) without knowledge of the experimental groups being analyzed repeated this measurement 3 times each for the proximal, middle, and distal portions of the intestinal grafts (9 measurements per animal).

SYBR Green 2-Step Real-time Reverse Transcriptase Polymerase Chain Reaction

The mRNA levels for interleukin (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 Green 2-step real-time reverse transcription polymerase chain reaction (PCR) as previously described.[27] SYBR green PCR mixture was prepared with THUNDERBIRD SYBR qPCR Mix(R) (TOYOBO Co Ltd, Osaka Japan) with specific primers (Table 1). The thermal cycling protocol consisted of 10 min at 95 °C to activate the polymerase, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 1 min on StepOnePlu Real-Time PCR (Thermo Fisher Scientific, Waltham, MA).

Intestinal Graft Permeability

As an indicator of barrier integrity, the intestinal permeability to fluorescein isothiocyanate-labeled dextran with a molecular weight of 4 kDa was assessed using an everted intestine apparatus as previously described.[27,31] The fluorescent intensity of the content solution was measured using a fluorescence plate reader (Flexstation 3, Molecular Devices). The intestinal permeability to fluorescein isothiocyanate-labeled dextran with a molecular weight of 4 kDa was calculated as pmol/cm intestine/min.

Measurement of Tissue's cGMP

Frozen flushed intestinal full-thickness segments from each experimental group were homogenized. The corresponding tissue's cGMP was analyzed by using a cGMP detection kit (Cyclic GMP Complete, Enzo Life Sciences Inc, NY). Approximately 40 mg of powdered frozen graft tissues were extracted in 10 volumes of 0.1 M HCl buffer and completed ELISA. The sample was also subjected to protein concentration by using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). The results were expressed as picomolar per microgram of protein for the tissue levels.

Statistical Analysis

All the results are expressed as means ± SEM. Statistical analysis was performed using the unpaired Student *t* test or ANOVA where appropriate. For survival study, Kaplan-Meier curves and log-rank test were performed. A probability level of $P < 0.05$ was considered statistically significant.

RESULTS

Blood CO-Hemoglobin Levels Do Not Change in Recipients After Transplant of Intestinal Graft Treated With CORM-3

To determine whether CO liberated from CORM-3 in the intestinal lumen during the cold preservation phase is ultimately found in the recipient's blood circulation, CO-hemoglobin levels were measured early after transplantation as previously described.[11] CO-hemoglobin levels in animals receiving the intestinal grafts before reperfusion were 0.8% and did not change significantly at 10, 20, and 30 min after reperfusion (data not shown).

CORM-3 Mitigates Graft Tissue Injury After Intestinal Transplantation

The histopathological scores obtained from blinded evaluation of the naive intestine (sham) with saline and saline/ODQ were 0.20 and 0.33. Pathological changes of the intestinal grafts 3 h after reperfusion included significant epithelial loss associated with villous congestion

TABLE 1.

Primer summary

	Forward	Reverse	Size (bp)
β-Actin	5′-AACCCTAAGGCCAACCGTGAA-3′	5′- TCCAGGCTGTGTTGTCCCTG-3′	96
IL-6	5′-TCCTACCCCAACTTCCAATGCTC-3′	5′-GGCTAAGGACCAAGACCATCCAA-3′	79
CCL2	5′-AGCCAACTCTCACTGAAGC-3′	5′-ACCTGCTGCTACTCATTCAC-3′	158
iNOS	5′-GGAGAGATTTTTCACGACACC-3′C	5′-TGCAAGTCCAAATTATGCATGG-3′	73
HO-1	5′-CACAAAGACCAGAGTCCCTCACAG-3′	5′-ACCGTGGCAGTGGGAATTT-3′	187

Bp, base pair; CCL2, C-C motif chemokine 2; HO-1, heme oxygenase-1; IL, interleukin; iNOS, inducible nitric oxide synthase.

and erosion, as seen in the untreated grafts (5.71 ± 1.06) and iCORM-3–treated grafts (5.50 ± 0.96). In contrast, CORM-3 mitigated these pathological changes at the same time points as indicated by a significant decrease in the score to 3.33 ± 1.52. The protective effect mediated by CORM-3 was reversed by ODQ (score = 6.33 ± 0.58), an inhibitor of sGC (Figure 1A–G).

CORM-3 Ameliorates Intestinal Permeability Disruption and Improves Recipient Survival After Transplantation

To correlate the changes in epithelial damage associated with IR injury after transplantation, gut barrier function was determined by measuring intestinal permeability at 3 h postreperfusion (n = 4 for each group). Although

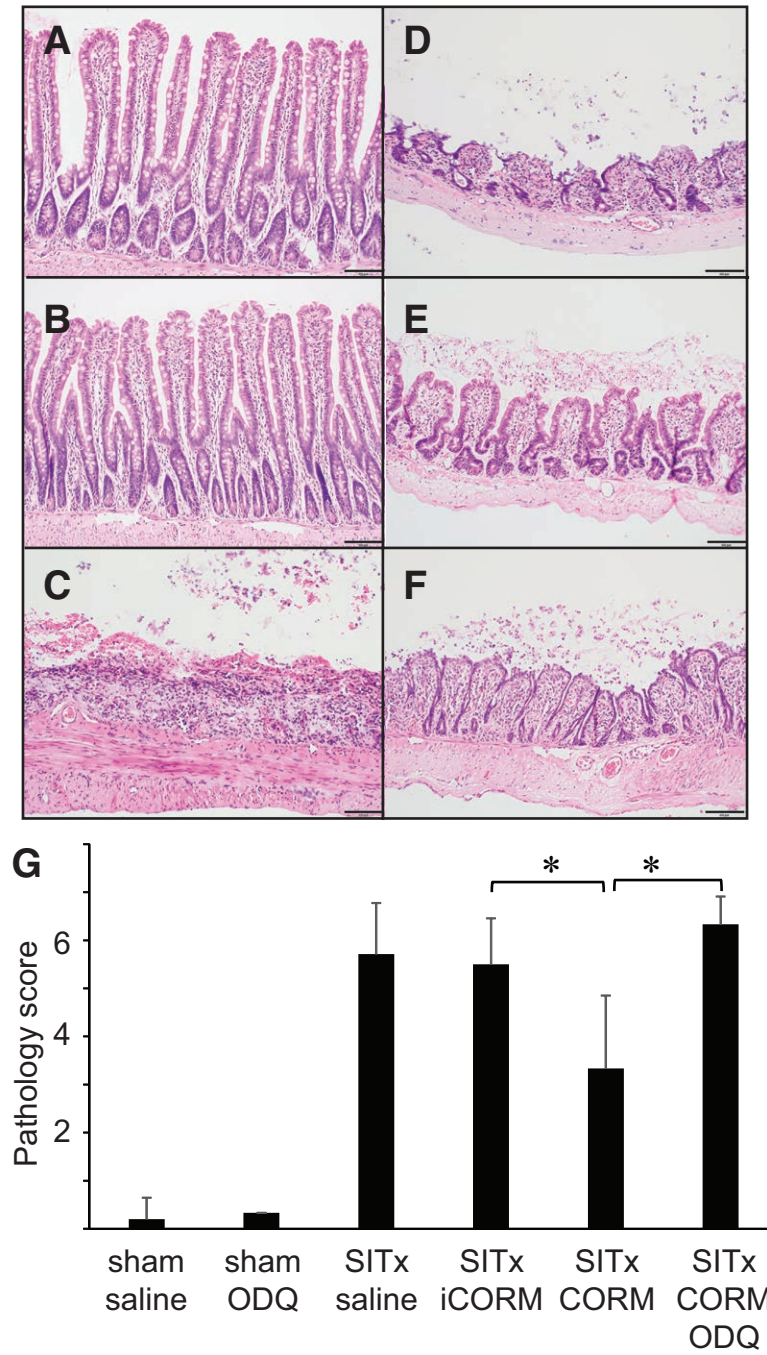

FIGURE 1. Carbon monoxide–releasing molecule 3 (CORM-3) mitigates graft tissue injury after intestinal transplantation. Representative histopathological images of the intestine harvested 3 h after reperfusion (size marker: 100 μm) are shown. A, Intestine of sham animal (n = 5). B, Intestine of sham animal treated with intraluminal 1H- [1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ) (10 μM) (n = 4). C, Untreated intestinal graft (n = 7). D, Intestinal graft treated with intraluminal inactive counterpart of CORM-3 (iCORM) (n = 7). E, Intestinal graft treated with intraluminal CORM-3 (n = 8). F, Intestinal graft treated with intraluminal CORM-3 supplemented with ODQ (n = 6). G, The degree of graft mucosal damage is presented as a histopathological score of each animal. As shown, CORM-3 treatment significantly ameliorated histopathological changes 3 h after reperfusion (*$P < 0.05$). SITx, small intestinal transplantation.

the permeability of naive intestine was 1.12 ± 0.28 pmoL/cm·min, permeability of grafts containing saline (Group 3) and grafts treated with iCORM-3 (Group 4) considerably deteriorated to 5.63 ± 1.30 pmoL/cm·min and 4.58 ± 0.70 pmoL/cm·min, respectively; however, this decline in permeability was almost abolished by luminal injection with CORM-3-containing saline as shown by an intestinal permeability of 1.62 ± 0.53 pmoL/cm·min (Figure 2A). As observed for the tissue pathological scores, the protection exerted by CORM-3 was reversed by selective inhibition of sGC activity with ODQ as indicated by a deterioration in permeability (4.54 ± 0.77 pmoL/cm·min) similar to grafts treated with the inactive compound.

It is interesting to note that only 37.5% (6/16) of the recipientanimals transplanted survived >14 d if the grafts were previously stored in cold solution after luminal injection with iCORM-3; however, 83.3% (10/12) of the recipients survived >14 d when the intestine grafts were injected with CORM-3 ($P < 0.05$, Kaplan-Meyer, log-rank test) (Figure 2B).

Intraluminal Administration of CORM-3 Reduces Upregulation of Proinflammatory Mediators Mediated by Cold Storage and Transplantation

In the grafts previously treated with saline or iCORM-3 and collected 3 h after IR induced by transplantation, IL-6 mRNA levels showed approximately a 60-fold increase compared with the naive intestine (sham group). Interestingly, CORM-3–treated grafts significantly reduced IL-6 upregulation by 75% (Figure 3B). Similarly, the mRNA expression of iNOS (Figure 3B) and CCL2 (Figure 3C) was markedly enhanced in untreated or iCORM-3–treated grafts, whereas they were greatly attenuated in intestine previously treated with the intraluminal injection of CORM-3. HO-1 mRNA was also upregulated 3 h after reperfusion compared with controls. CORM-3 treatment significantly attenuated HO-1 mRNA upregulation to ≈30% of the value of untreated grafts (Figure 3D) (n = 8 for each group).

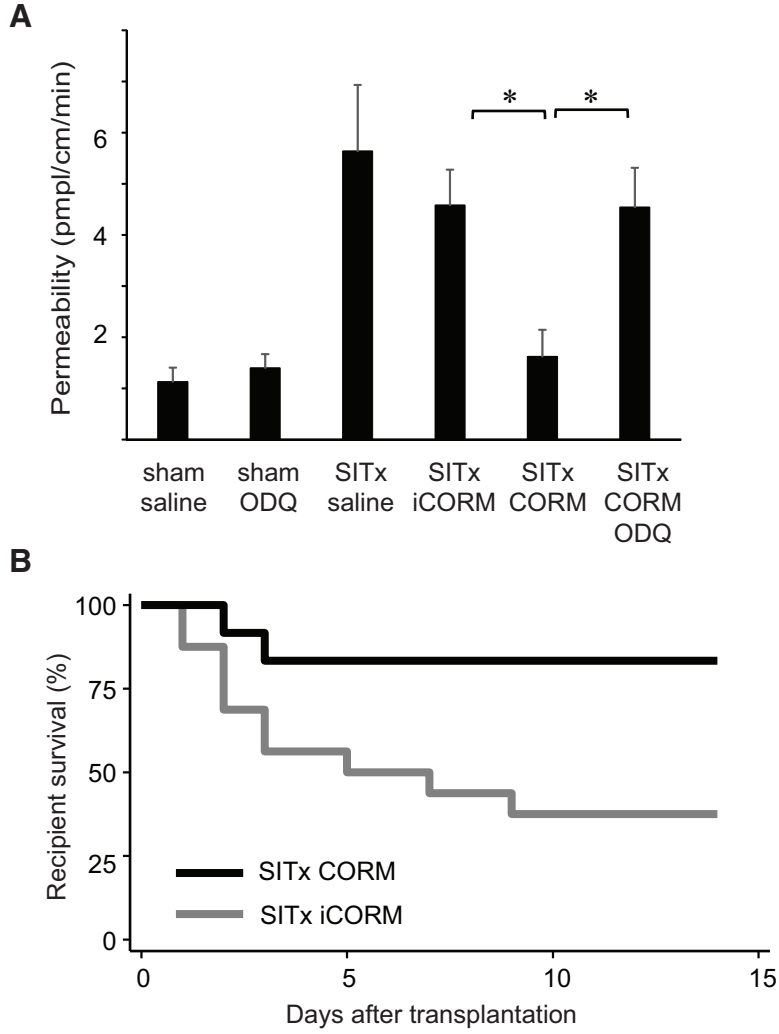

FIGURE 2. Carbon monoxide–releasing molecule 3 (CORM-3) improves graft permeability and survival following intestinal transplantation. A, The effect of different treatments on intestinal permeability is shown. Untreated (sham) and inactive counterpart of CORM-3 (iCORM-3)–treated intestinal grafts at 3 h after transplant revealed a significant impairment of intestinal wall permeability. Intraluminal CORM-3 administration significantly improved graft wall permeability. Addition of 1H- [1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ), a soluble guanylyl cyclase (sGC) inhibitor, reversed the protective effects provided by CORM-3 (n = 4–8, $^*P < 0.05$). B, Recipient survival after transplantation. Cold preservation of intestinal grafts with iCORM-3 for 6 h in Ringer solution resulted in intestinal graft dysfunction, and the overall survival rate at 14 d was 37.5% (6/16). In contrast, recipient survival was significantly enhanced to 83.3% (10/12) in grafts previously stored with intraluminal CORM-3 treatment ($P < 0.05$, Kaplan-Meyer, log-rank test). SITx, small intestinal transplantation.

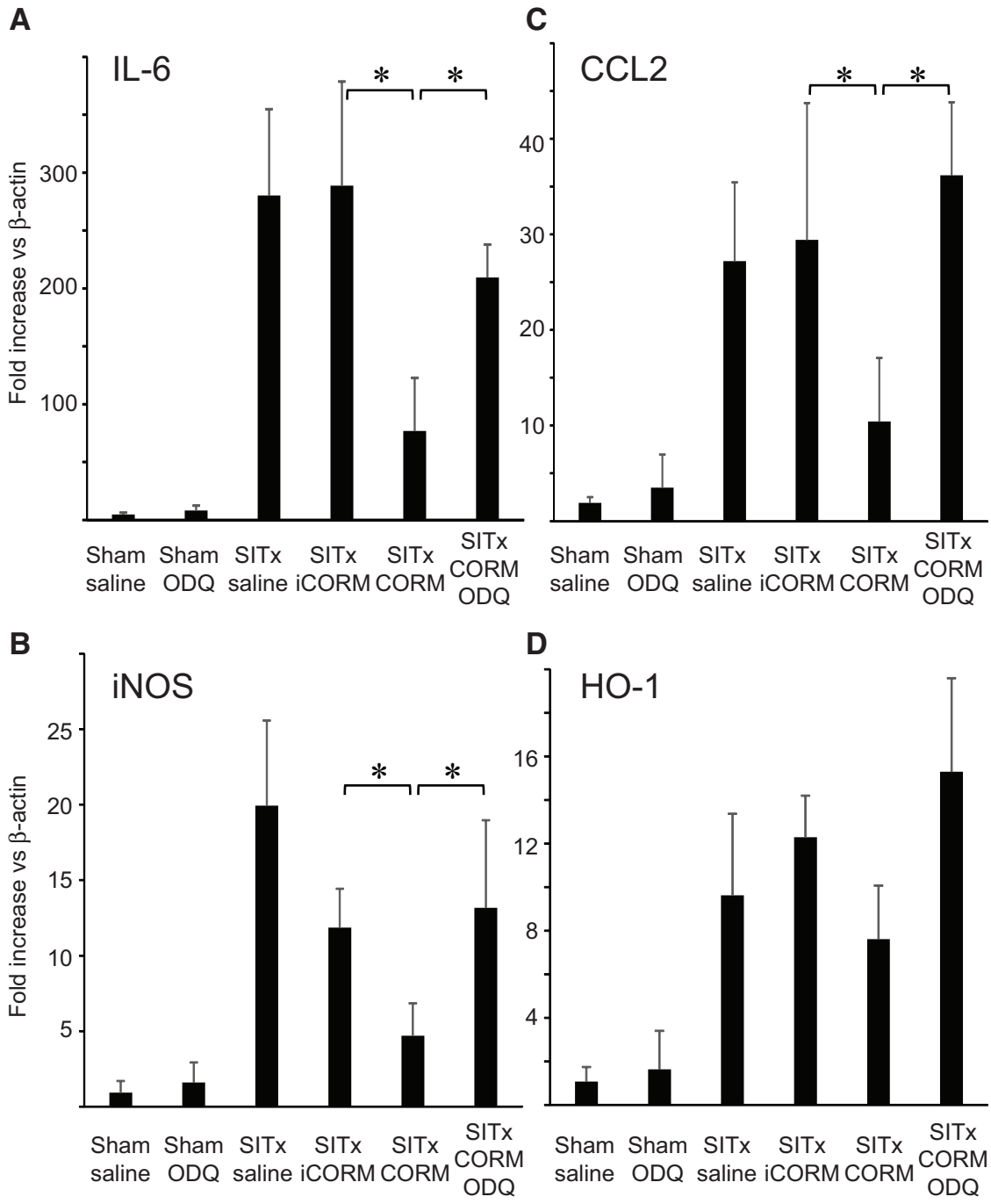

FIGURE 3. Intraluminal administration of carbon monoxide–releasing molecule 3 (CORM-3) reduces upregulation of proinflammatory mediators mediated by cold storage and transplantation. mRNA of proinflammatory markers, such as interleukin (IL)-6 (A), inducible nitric oxide synthase (iNOS) (B), and C-C motif chemokine 2 (CCL2) (C) as well as heme oxygenase-1 (HO-1) expression (D) were upregulated at 3 h after reperfusion in the grafts stored in Ringer solution or in the presence of inactive counterpart of CORM-3 (iCORM-3). These upregulations were significantly mitigated by intraluminal CORM-3 treatment (n = 8 for each group, $^*P < 0.05$). The effects of CORM-3 were reversed by addition of the sGC inhibitor 1H- [1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ). SITx, small intestinal transplantation.

Luminal Administration of CORM-3 Leads to Increased Mucosal Blood Flow and Activation of Intestinal sGC

Blood flow of the intestinal marginal arteries did not differ in the intestine of sham groups and the transplanted intestine regardless of the presence of intraluminal CORM-3. Blood flow in these groups ranged between 114 and 128 laser doppler unit (LDU) (data not shown). When considering the mucosal side, blood flow in the naive intestine (sham animals) detected by Doppler flowmeter was 28.3 ± 4.11 LDU. Predictably, and in association with the data on impaired intestinal permeability, graft microcirculation in the mucosa was significantly reduced as indicated by a decrease in blood flow (18.6 ± 2.0 LDU) 3 h after reperfusion in the intestine previously treated intraluminally with iCORM-3; however, mucosal microcirculation was significantly improved (blood flow, 22.6 ± 3.8 LDU) in the grafts preserved after luminal administration with CORM-3 (Figure 4A). Because sGC activation is one of the main contributors in the regulation of blood flow, we

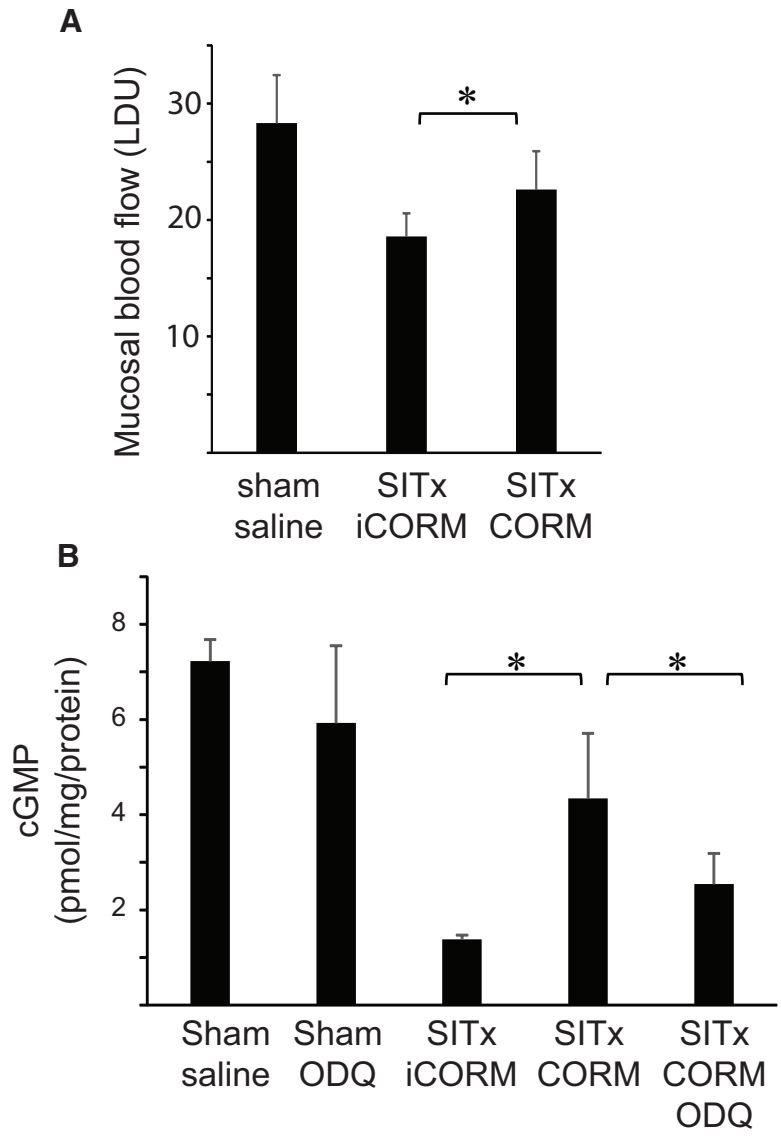

FIGURE 4. Luminal administration of carbon monoxide–releasing molecule 3 (CORM-3) increases mucosal blood flow and intestinal cGMP levels. A, Graft blood flows on the mucosal surface were measured using a laser Doppler flowmeter. Ischemia/reperfusion injury induced by transplantation (small intestinal transplantation [SITx]) reduced mucosal blood flow 3 h after reperfusion. Intraluminal CORM-3 treatment during cold preservation in Ringer solution significantly increased mucosal blood flow compared with those treated with inactive counterpart of CORM-3 (iCORM-3) (n = 5 from each group, $^*P < 0.05$). B, The levels of tissue cGMP in sham-operated intestinal grafts decreased at 3 h after reperfusion compared with basal levels. There was a significant increase in cGMP levels in grafts treated with CORM-3 (n = 4, $^*P < 0.05$). LDU, laser doppler unit; ODQ, 1H- [1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one.

assessed the levels of cGMP in the groups studied.[11] Tissue concentrations of cGMP in the sham-operated intestine in the presence or absence of ODQ were 5.93 ± 1.62 pmol/mg protein and 7.23 ± 0.46 pmol/mg protein, respectively. Following intestinal transplantation, grafts previously treated with iCORM-3 displayed a marked decrease in tissue cGMP (1.38 ± 0.09 pmol/mg protein), whereas cGMP levels in CORM-3–treated intestinal grafts were much better preserved (4.34 ± 1.37 pmol/mg protein). Inhibition of sGC by ODQ in the CORM-3 grafts reduced tissue cGMP to 2.55 ± 0.64 pmol/mg protein (Figure 4B).

Intraluminal Administration of CORM-3 Protected Intestinal Grafts From IR Injury After Cold Preservation in Belzer-UW Solution

The experiments described above were performed using saline for intraluminal injection and a Ringer lactate solution for cold preservation to be consistent with our previous works. The use of these solutions was also important to assess the effects of CORM-3 without any interference of additional ingredients supplemented into other preservation solutions commonly used in the clinic. Therefore, we wanted to verify whether intraluminal administration of CORM-3 before cold preservation had similar effects when grafts were stored in a clinically feasible Belzer-UW solution. We found that histopathological analysis of the intestinal tissue revealed massive epithelial loss and bleeding in iCORM-3–treated grafts 3 h after reperfusion. These severe changes in mucosa morphology were substantially mitigated by intraluminal treatment with CORM-3 (Figure 5A–C). The tissue pathology score of the graft intestines stored with intraluminal iCORM-3 was 6.67 ± 0.58, whereas the score of tissues treated with CORM-3 was 3.67 ± 0.58 (Figure 5D). Similarly, at 3 h after reperfusion,

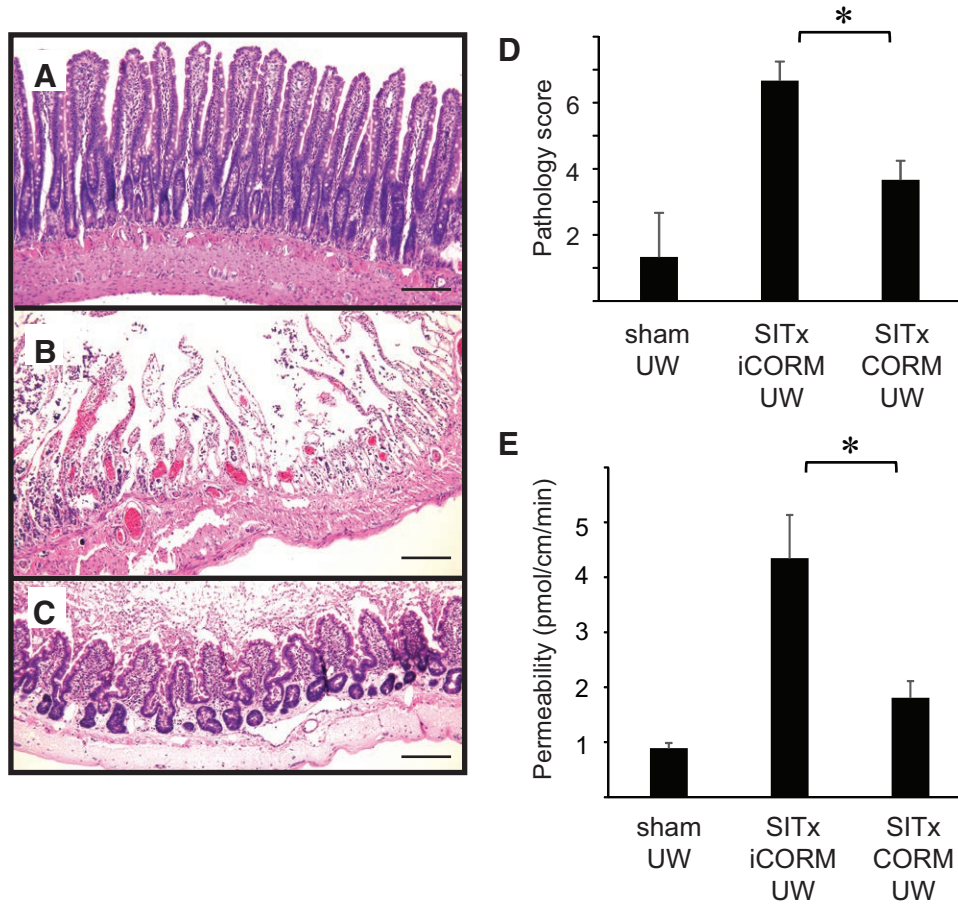

FIGURE 5. Intraluminal administration of carbon monoxide–releasing molecule 3 (CORM-3) protects intestinal grafts from ischemia/reperfusion (IR) injury after cold preservation in University of Wisconsin (UW) solution. Representative histological images of the intestinal grafts stored in UW solution for 6 h from sham-treated tissue (A, n = 6), graft intestine stored with inactive counterpart of CORM-3 (iCORM-3) following transplantation (B, n = 6), or graft intestine stored with CORM-3 following transplantation (C, n = 7). D, Histological score indicating that IR-induced alterations in mucosal morphological changes were higher in the intestine treated with iCORM-3 (n = 6) than those treated with intraluminal injection of CORM-3 (n = 7). E, Gut permeability increased 3 h after reperfusion, suggesting loss of mucosal barrier function in iCORM-3 treated intestines stored in UW solution (n = 6). Intraluminal CORM-3 significantly prevented the increase in intestinal permeability, averting mucosal barrier breakdown (n = 6) (*$P < 0.05$). SITx, small intestinal transplantation.

the permeability of the grafts stored with iCORM in Belzer-UW solution for 6 h was 4.35 ± 0.79 pmoL/cm·min, whereas an intraluminal injection of CORM-3 significantly reversed the impaired barrier function as indicated by a permeability of 1.81 ± 0.3 pmoL/cm·min (Figure 5E).

DISCUSSION

The results presented here demonstrate that an intraluminal injection of CORM-3, a water-soluble compound that liberates CO, before cold storage of intestinal grafts effectively mitigates mucosal injury induced by IR after transplantation. These data are in line with previous work from our group showing that CO gas is extremely effective in preventing organ dysfunction and inflammation and provide important information on novel technical approaches to exploit CO as an adjuvant in transplantation procedures. In particular, the current study using an intraluminal injection of CORM-3 in the excised grafts ex vivo during cold preservation of the intestine before transplantation represents an easy, safe, and practical procedure to preserve organ function and viability in the clinical transplant setting.

CORMs were initially designed in 2002 to carry and deliver controlled amounts of CO in vitro and in vivo for therapeutic applications.[21] The development of CORMs provided a concrete opportunity to optimize the delivery of CO in a more practical, controllable, and accurate way to the target tissues in comparison to the use of CO gas by inhalation.[18,32] A wide range of CORMs have been substantially characterized from a biochemical and biological viewpoint and confirmed to be therapeutically active by different laboratories.

Substantial published data have now corroborated the ability of CORMs to exert therapeutic effects through the delivery of CO in a variety of animal disease models ranging from tissue ischemia to inflammation, infection, and metabolic disorders.[32,33] In the context of pathologies specifically affecting the intestinal tract, several studies have shown the efficacies of CORMs on inflammatory bowel disease, including ulcerative colitis and Crohn disease.[34,35] In addition, it has been reported that an intravenous injection of CORM-2 confers anti-inflammatory effects by interfering with nuclear factor (NF)-kappaB activation and subsequent upregulation of vascular proadhesive phenotype after warm ischemia in the small intestine.[36] Similarly, an intraperitoneal administration of either CORM-2 or CORM-3 significantly inhibited inflammatory response in the gut after induction of colitis in mice by inhibition of cytokine production and suppression of the inflammatory

response.[37,38] Zhang et al[39] reported that administration of CORM-3 by intestinal injection exerts protective effects against systemic inflammation following exposure to traumatic brain injury with subsequent hemorrhagic shock and resuscitation in rats, indicating that CORMs certainly can be absorbed from the intestinal mucosa and possess therapeutic effects against bowel inflammation. Both water-soluble CORM-3 and CORM-A1 have also been reported to markedly alleviate postoperative ileus by restoring contractility and transit of the small intestine, effects that were associated with reduced leukocyte infiltration and a decrease in both oxidative stress and inflammation.[40]

IR injury triggers innate immunity and thereby may enhance the alloimmune response toward the graft and promote rejection.[41,42] Also, impairment of the barrier function of intestinal grafts is accompanied by bacterial translocation, resulting in stimulation of danger signals and alloreactivity.[43] Amelioration of IR injury by CORMs may also inhibit impairment of both acute and long-term graft function and thereby reduce organ rejection. CORM-3 can also inhibit dendritic cell maturation and may be able to reduce organ rejection episodes.[44] Previous studies have shown that CO gas inhalation attenuates organ graft rejection via inhibition of proinflammatory mediators, such as IL-2 and interferon γ, and suppressing adhesion molecules in rat heart or kidney allografts.[45,46] Martin et al[47] reported that treatment with CO reduced the migration of donor-derived dendritic cells, CD4+ T cells, and alloreactive T cells following the engraftment of organs. Thus, CORMs may confer beneficial effects on immune cells in allogeneic transplantation settings.

In the context of organ preservation and transplantation, our previous data provide interesting evidence that CO gas can act as an ex vivo protective adjuvant when bubbled into cold preservation solutions in which intestine grafts are stored before transplantation.[16] This study was inspired by a previous work showing that also the addition of CORM-A1 and CORM-3 to UW solutions during cold preservation of kidney results in a marked increase in glomerular filtration rate, vascular activity, and renal function at reperfusion.[48] Likewise, using an isolated ex vivo organ perfusion system in a porcine model of controlled nonheart-beating donor kidney transplantation, supplementation with CORM-3 significantly ameliorated renal hemodynamics and function following reperfusion.[4] Most recently, similar results were obtained with the manganese-containing CORM-401, providing additional strong evidence that CO liberated by CORMs exerts renal protection following reperfusion with normothermic isogeneic porcine blood through oxygenated pulsatile perfusion for 10 h.[49] This suggests that cold preservation solutions supplemented with CORMs could truly materialize in a novel, clinically relevant strategy for ex vivo organ preservation including ex vivo machine perfusion, which has already seen great success in the context of liver transplantation.[50] Despite the fact that no human studies utilizing CORMs have been conducted so far, we believe that their translation to the clinic as therapeutic agents in the context of transplantation is highly promising and probably applicable in the near future. In particular, ex vivo administration of CORMs as adjuvants to cold-preserved organ grafts represents a very feasible and valuable opportunity to deliver CO in a targeted and safe manner, thus avoiding the long process required for a drug approval when used systemically in vivo.

Our present study reveals for the first time that preservation of intestinal morphology and function after transplantation can be achieved by treating intestinal grafts with CORM-3 during the cold storage phase. This was evident by the beneficial effects mediated by CORM-3 and assessed 3 h after graft transplantation, which included (1) reduced epithelial loss, villous congestion, and tissue erosion; (2) better preserved intestinal permeability and mucosa blood flow associated with an increased activation of tissue cGMP production; (3) improved survival rate of mice receiving the intestine grafts treated with CORM-3; (4) decreased production of proinflammatory markers, such as IL-6, iNOS, and CCL2. The fact that the inactive CORM-3, which does not liberate CO, did not provide any improvement of the intestine function after IR clearly indicates that the CO delivered by the transition carbonyl complex is responsible for the observed protective effects. It is known that CO gas as well as CORMs relax blood vessels and exert anti-thrombotic effects by inhibiting platelet aggregation and derepressing fibrinolysis.[32] CO preferably targets heme-containing proteins, such as hemoglobin, complexes in the mitochondria respiratory chain, and sGC, which is a well-established target that regulates vessel relaxation and blood flow.[2,33] In fact, our data indicate that increased levels of the second messenger cGMP in the intestine correlate with the cytoprotective actions by CORM-3 and that an sGC/cGMP-dependent mechanism is at least partially involved since a specific inhibitor of sGC (ODQ) abrogates the beneficial effects mediated by CORM-3. CO has also been reported to inhibit apoptosis in endothelial and epithelial cells and to reduce proliferation of smooth muscle cells, fibroblasts, and T lymphocytes.[51,52] Thus, there is accumulating evidence to support the notion that CO treatment of transplant donors, organs, or recipients can prevent graft dysfunction due to rejection or IR injury.[9,53] Our data on the anti-inflammatory effects of CORM-3 in the intestine following transplantation are in line with previous data showing that both CO gas and CO liberated by CORMs differentially affect cytokine production by decreasing the expression of proinflammatory interleukins and increasing the expression of anti-inflammatory interleukins.[2,54,55] Upregulation of HO-1, which degrades heme to CO, is also known to be associated with an anti-inflammatory profile and be protective in transplanted organs.[56,57]

It is also important to highlight that the beneficial effects exerted by CORM-3 administered in the lumen of the intestinal grafts preserved in Ringer lactate were recapitulated in Belzer-UW solution, suggesting that addition of CORMs as adjuvants to cold preservation solutions can be easily translated into the clinic for improving the viability of organs during transplantation procedures. The reason we initially explored the effect of CORM-3 in a simple crystalloid solution such as Ringer lactate was specifically to avoid a possible interference of the several additives, including antioxidants or metabolically inert substances, that are commonly present in the preservation solutions. These additives may have masked the effects of intraluminal CORM-3 treatment that we observed. Another important reason to use Ringer lactate solution was to be consistent with the experimental protocol used in previous

studies because we used this preservation solution in our previous study investigating the effect of various agents in rodent small bowel transplantation.[27,31,58,59]

The concentration of CORM-3 used in our current study (100 μM) was comparable to previously published works; however, we must emphasize that our approach consisted of treating the intestinal grafts ex vivo, not in vivo, thus providing the important information that metal carbonyls could be used to treat tissues and organs at much higher concentrations than previously thought. As the appropriate concentration is still unknown, future experiments to determine the minimal and most effective doses of CORM-3 in this setting will be required. A potential limitation of our study is that an isograft transplant is very rare to occur in the clinical setting of transplantation except in the case of identical twins; however, syngeneic transplantation, consistent with our previous series of experiments, is considered an ideal experimental model to study IR injury because it allows the isolation of factors related to the ischemic insult from factors involved in alloimmune reactions.[11,12,16,27]

Finally, the potential toxicity of CORMs is an important issue to consider for moving toward clinical applications. There is a general unjustified perception or bias that CORMs, such as CORM-3, which contains ruthenium, are toxic per se primarily because of the presence of a transition metal. First of all, we would like to point out that as for any substance or drug, toxicity is primarily dictated by the dose or concentration tested in a given experimental setting, and, thus, it is fundamentally wrong to merely define a compound toxic without reporting the amount(s) used. Second, considering that in the present study, CORM-3 (100 μmol/L in the flushing solution) was used ex vivo, thus avoiding the systemic circulation, we believe that the issue of potential toxicity of this compound becomes even less relevant. Last, and most importantly, there is no clear, substantial, or robust experimental evidence that, at the doses of metal-containing CORMs reported in the literature so far, these compounds are toxic. If anything, the contrary is true since, apart from plenty of studies that clearly indicate a beneficial rather than a toxic effect of CORMs ex vivo or in vivo,[25,32,33,40] there are only 2 articles to the best of our knowledge that have evaluated the toxicity of these compounds. Specifically, it was found that following acute oral administration in mice, the LD50 (the dose causing a 50% mortality) for CORM-3 was 800 to 1000 mg/kg and for other CORMs containing either ruthenium or molybdenum was 100 to 450 mg/kg.[60,61] We should point out that CORM-3 and molybdenum-based CORMs have been shown to exert pharmacological effects in vivo at doses ranging from 3 up to 60 mg/kg. Recently developed CORMs containing manganese and administered orally at 30 to 40 mg/kg in mice for several days or months revealed potent therapeutic effects in models of obesity, skin wound, multiple sclerosis, and systemic inflammation.[62-64] Thus, although more studies are required to fully assess the toxicity profile of metal-based CORMs, these important observations suggest that their therapeutic window is significantly below the doses causing toxic effects. There is also the possibility that the metal within the CORM structure will ultimately facilitate the delivery of CO to tissues, thus being a key element for the efficacy of CO-mediated therapeutic effects in vivo. This possibility has never been investigated but may prove to be an important aspect in the assessment of toxicity/efficacy of metal CORMs in mammals.

CONCLUSIONS

We provide here the first evidence that intraluminal administration of CORM-3 prevents IR injury following cold preservation and transplantation of the intestine. This effect was associated with preserved intestinal permeability, increased mucosa blood flow, and mitigation of the proinflammatory responses. Thus, CORMs may offer a feasible strategy to safely deliver controlled amounts of CO to organ grafts in the clinical setting of transplantation.

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