Luminal Preloading with Hydrogen-Rich Saline Ameliorates Ischemia Reperfusion Injury Following Intestinal Transplantation in Rats

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Abbreviations

COX-2: cyclooxygenase-2

HBSS: Hank's Balanced Salt Solution

HO-1: heme oxygenase-1

iNOS: inducible nitric oxide synthase

IR: ischemia reperfusion

IL: interleukin

LEW: Lewis

LDU: Laser Doppler Units

MDA: malondialdehyde

mRNA: messenger ribonucleic acid

ppm: parts per million

RIPA: radioimmunoprecipitation assay

RNA: ribonucleic acid

PCR: polymerase chain reaction

SDS: sodium dodecyl sulfate

UW: University of Wisconsin

ZO-1: zonula occludens-1

AUTHORS' CONTRIBUTION

Hirotsugu Yamamoto,Toshiyuki Aokage, Takahiro Hirayama, Mizuki Seya, Tsuyoshi Nojima collected and analyzed the data; Takuro Igawa performed histological analysis; Atsunori Nakao conceived the concept/design, drafted the manuscript and performed animal surgery. Michiko Ishikawa-Aoyama and Hiromichi Naito conceived the concept, design and drafted the manuscript. All authors approved the article.

ABSTRACT (249 WORDS)

Background: Prolonged intestinal cold storage causes considerable mucosal breakdown, which could bolster bacterial translocation and cause life-threatening infection for the transplant recipient. The intestine has an intraluminal compartment, which could be a target for intervention, but has not yet been fully investigated. Hydrogen gas exerts organ protection and has used been recently in several clinical and basic research studies on topics including intestinal transplantation. In this study, we aimed to investigate the cytoprotective efficacy of intraluminally-administered hydrogen-rich saline on cold ischemia reperfusion (IR) injury in intestinal transplantation.

Methods: Isogeneic intestinal transplantation with six hours of cold ischemia was performed on Lewis rats. Hydrogen-rich saline (H_2 concentration at 5 ppm) or normal saline was intraluminally introduced immediately before preservation. Graft intestine was excised three hours after reperfusion and analyzed.

Results: Histopathological analysis of control grafts revealed blunting of the villi and erosion. These mucosal changes were notably attenuated by intraluminal hydrogen. Intestinal mucosa damage caused by IR injury led to considerable deterioration of gut barrier function 3h post-reperfusion. However, this decline in permeability was critically prevented by hydrogen treatment. IR-induced upregulation of proinflammatory cytokine mRNAs such as IL-6 were mitigated by hydrogen treatment. Western blot revealed that hydrogen treatment regulated loss of the transmembrane protein zonula occludens-1.

Conclusions: Hydrogen-rich saline intraluminally administered in the graft intestine modulated IR injury to transplanted intestine in rats. Successful abrogation of intestinal

IR injury with a novel strategy using intraluminal hydrogen may be easily clinically applicable and will compellingly improve patient care after transplantation.

INTRODUCTION

Intestinal grafts are exceptionally susceptible to ischemia reperfusion (IR) injury compared to other organs. Intestinal IR injury, obligatory to the consequences of cold preservation and transplantation of the intestinal grafts, can result in early graft failure and life-threatening events for recipients.[1-3] Advanced IR injury and subsequent mucosal barrier damage can cause considerable bacterial translocation and systemic inflammation, as well as fluid and electrolyte shifts.[4]

Hydrogen gas, a stable molecule that may have oxidant or reductive characteristics depending upon the environment in which it is placed, has been applied in several experimental models in the organ transplantation field.[5-7] Graft protection with inhaled hydrogen gas has been reported in lung IR injury[8] and liver transplantation.[9] In addition, hydrogen delivered in a water-soluble form is a more practical and controllable method in biological systems. The beneficial effects of oral administration of hydrogen-rich solutions for chronic graft changes[10-12], as well as supplementation of hydrogen in the preservation solution[13, 14], have been proven in various experimental transplant models. Thus, there is no doubt that hydrogen molecules provide beneficial effects for organ transplantation. Researchers must proceed in reducing the risk of any environmental/systemic toxicity and promoting hydrogen use in the clinical organ transplantation setting.

As demonstrated in other organs, hydrogen's efficient graft protective effects have been shown in small intestinal transplant rodent models, showing ameliorated graft contractility and mucosal barrier function, as well as improvement of remote organ inflammation.[15, 16] However, managing patients with hydrogen for prolonged tine periods would pose some

operational problems for most hospitals due to hydrogen's flammability risks, reducing the appeal of this therapeutic approach.

The intestine has an intraluminal compartment, which could be a target for intervention, but it has not yet been fully investigated. Administration of hydrogen through the luminal membrane, the site for the uptake of nutrients, water, and electrolytes, can directly deliver hydrogen molecules to enterocytes. Therefore, in this study, we hypothesized that luminal loading of hydrogen-rich solution during cold preservation can reduce intestinal mucosal injury and consequently ameliorate IR injury of the intestinal graft. Luminal introduction of hydrogen during cold storage may be an attractive approach due to its technical simplicity and safety.

MATERIALS AND METHODS

Animals

Inbred male 200-250-gram LEW ($RT.1^{l}$) rats supplied from Japan SLC (Hamamatsu, Shizuoka, Japan) were housed in a laminar flow animal facility at Okayama University and fed a standard diet and were given water *ad libitum*. All procedures were performed in compliance with Okayama University institutional animal care and use committee guidelines.

Preparation of Hydrogen-rich Saline

We prepared hydrogen-rich solution using a hydrogen-generating agent (10 WATER; MiZ Co Ltd, Kanagawa, Japan).[17] The agent containing calcium hydroxide and metal

aluminum grains was placed in a nonwoven bag, dipped in pure water to start the chemical reaction, and placed inside a small bottle; the bottle was closed using a cap with a check valve. We placed the small bottle into a 500mL bottle with normal saline solution at room temperature. The bottle was tightly closed to dissolve the hydrogen produced in the normal saline solution. Hydrogen-rich saline was generated within five minutes, hydrogen gas accumulated in the bottle, and the bottle expanded. Finally, the bottle was shaken for 30 seconds to sufficiently dissolve the hydrogen.

Transplant Procedure

We performed isolated orthotopic isogeneic small bowel transplantation with caval venous drainage as previously described.[18] We isolated the entire donor small intestine from the ligament of Treitz to the ileocecal valve on a vascular pedicle comprising the superior mesenteric and artery portal vein in continuity with an aortic segment. The graft was perfused via the aortic segment with 5 ml chilled saline, and 20 ml of cold saline solution was used to irrigate the intestinal lumen to remove intraluminal contents. Then, approximately 15 milliliters 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's lactate solution for six hours at 4°C.

Experimental Groups

Recipient animals were either followed for 14 days to assess animal survival or sacrificed at three hours after transplantation to determine the early protective effects of intraluminal hydrogen. Three groups of animals were studied: (i) sham animals; (ii) recipients grafted with intestine containing intraluminal saline without hydrogen; (iii) recipients grafted with

intestine containing intraluminal saline with hydrogen. Animals in the sham group were only anesthetized (naïve animals).

Histopathological Analysis

Three hours after reperfusion, we fixed whole intestinal segments in 10% buffered formalin, embedded them in paraffin, and stained 4 μm thick sections with hematoxylin and eosin. A pathologist (IT) evaluated the degree of mucosal injury microscopically in a blinded fashion based on a 0-9 summary score: grade 0 indicated healthy mucosa, while submucosal edema, erosions, and hemorrhages were each rated by grades 1 to 3. All slides, at least eight sections per animal, were reviewed.

Intestinal Blood Flow Measurement

We monitored intestinal microvascular blood flow at three hours after reperfusion using a laser Doppler flowmeter (BLF 21D; Transonic Systems, Ithaca, NY) on the serosal surface of the graft jejunum and ileum adjacent to the mesenteric border. An individual without knowledge of the experimental groups repeated this measurement three times each for the proximal, middle, and distal portions of the intestinal grafts (nine measurements per animal). Superior mesenteric artery and marginal artery blood flows were also analyzed.

SYBR Green Two-Step Real-Time Reverse Transcriptase Polymerase Chain Reaction (PCR)

Messenger RNA (mRNA) levels for interleukin (IL)-6, IL-1β, IL-10, cycloxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), heme oxygenase-1 (HO-1), and β-actin

were assessed using SYBR Green 2-step real-time reverse transcription PCR.[19-21] In brief, 30mg of powdered frozen graft tissue was used for RNA extraction with Nucleospin® RNA (Takara Bio Inc., Kusatsu, Japan) according to the manufacturer's recommendations. One µg of the extracted total RNA was used for reverse transcription with ReverTra Ace® qPCR RT Master Mix (TOYOBO INC., Osaka, Japan). SYBR green PCR mixture was prepared with THUNDERBIRD SYBR qPCR Mix(R) (TOYOBO CO. LTD., Osaka Japan) with primers (Table 1). The thermal cycling protocol consisted of 10 minutes at 95℃ to activate the polymerase, followed by 40 cycles at 95℃ for 15 seconds and at 60℃ for one minute on StepOnePlu™ Realtime PCR (Thermo Fisher Scientific, Waltham, Massachusetts).

Graft Permeability

As an indicator of barrier integrity, we estimated intestinal permeability to fluorescein isothiocyanate-labeled dextran with a molecular weight of 4kDa (FD-4) in the everted intestine of rats as previously described.[22] The intestinal segments (4.0 cm) were everted with a blunt plastic rod and bound with 3-0 nylon at one end. The other end was bound after injecting 0.8mL Hank's Balanced Salt Solution [HBSS, 137 NaCl, 4.17 $NaHCO_3$, 0.34 Na_2HPO_4, 5.37 KCl, 0.44 KH_2PO_4, 1.66 $CaCl_2$, 0.81 $MgSO_4$, and 5.56 D-glucose (mmol/L)]. The everted intestine was incubated in the HBSS, which was dissolved with 25 µmol/L FD-4 for 30 minutes at 37°C in a water bath. After incubation, the content solution was retrieved by pricking with a 18G needle. The fluorescent intensity of the content solution was measured using a fluorescence plate reader (Flexstation 3,

Molecular Devices). The intestinal permeability to FD-4 was calculated as pmol/cm intestine/min.

Measurement of Tissue Malondialdehyde (MDA)

We harvested and homogenized flushed jejunal full thickness segments from each experimental group three hours after reperfusion. Tissue MDA concentration, a marker of lipid peroxidation, was measured using the manufacturer's kit directions (Kit MDA-586; Oxidresearch, Portland, OR*).* We expressed the results as micrometer per liter of protein for the serum and micrometer per microgram of protein for the tissue levels.

Western Blot

Protein was extracted using radioimmunoprecipitation assay (RIPA) buffer, which consists of 50mM Tris-HCl (pH 8.0), 150mM NaCl, 1% Igepal® CA-630 (Merck, Darmstadt, Germany), 0.5% sodium deoxychoate, 0.1% sodium dodecyl sulfate (SDS), and 1mM EDTA with cOmplete™ Mini Protease Inhibitor Cocktail (Merck, Darmstadt, Germany). Thirty milligrams of powdered frozen graft tissue were mixed with RIPA buffer, homogenized, and centrifuged. The supernatant where the protein was dissolved was used for protein assay. Intestinal cytosolic proteins (10 µg) were separated using electrophoresis on 10% acrylamide SDS gels and transferred to Immobilon®-P polyvinylidene difluoride membrane (0.45 µm) (Merck, Darmstadt, Germany). After blocking the nonspecific binding with 5% skim milk, the membrane was incubated at 4°C overnight with primary polyclonal rabbit antibody against zonula occludens-1 (ZO-1) 1:1,000 dilution, #61-7300, ThermoFisher Scientific, Waltham, MA (1:1,000 dilution, #61-7300, ThermoFisher Scientific, Waltham, MA), polyclonal rabbit antibody against Claudin

(CLDN) 1 (1:1,000 dilution, catalog no. ab15098, Abcam, Cambridge, UK), or monoclonal mouse antibody against β-actin (1:10,000 dilution, catalog no. A5441 Sigma-Aldrich, St. Louis, MO). Antibodies against ZO-1 and CLDN-1 were diluted in Can Get Signal immunoreaction enhancer solution 1 (Toyobo, Osaka, Japan). After incubating at room temperature for one hour in the peroxidase-conjugated secondary antibody against rabbit IgG (111-035-144. Jackson ImmunoResearch Inc., West Grove, PA) (1:10000) or mouse IgG (115-036-020. Jackson ImmunoResearch Inc., West Grove, PA) (1:10000), the membrane was developed with ECL Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, Illinois) and the signal was detected with WSE-6100 LuminoGraph Ⅰ (ATTO Corporation, Japan). Band intensity was measured using the software Image J.

Data Analysis

The results are expressed as means plus or minus the standard error of the mean. Statistical analysis was performed using the unpaired Student's t-test or analysis of variance 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

Hydrogen Treatment Mitigated Histopathological Changes and Barrier Function Damage in the Intestinal Grafts

IR-induced intestinal damage is characterized by massive intestinal epithelial loss, resulting in mucosal erosions, villous congestions, and hemorrhagic ulcerations.

Histopathological analysis demonstrated a significant attenuation of these mucosal changes in the grafts treated with intraluminal administration of hydrogen-containing saline (Figure 1A). The histopathological score obtained from blinded evaluation of the control grafts was 5.6 ± 1.1, while the score of the grafts treated with hydrogen was 3.1 ± 1.8. Correlating with histopathological changes associated with IR injury, gut barrier function determined by permeability in everted gut sac method at three hours post-reperfusion deteriorated considerably to 4.94 ± 2.21 pmoL/cm·min in the grafts containing saline without hydrogen, while the permeability of naïve intestine was 1.08 ± 0.55 pmoL/cm·min. However, this decline in permeability was critically prevented by luminal injection with hydrogen-containing saline with permeability of 1.91 ± 0.95 pmoL/cm·min (Figure 1C).

Intraluminal Hydrogen-containing Saline Reduced Upregulation of mRNA for Inflammatory Mediators

Realtime RT-PCR demonstrated marked upregulations of proinflammatory mediator mRNAs including IL-6, IL-1β, iNOS, and COX-2 compared with the intestines of sham groups three hours after reperfusion (Figure 2A, B, C, D). These upregulations were significantly mitigated by intraluminal hydrogen treatment. Levels of IL-10 mRNA, known as anti-inflammatory cytokine, were significantly increased three hours after reperfusion, which was inhibited by intraluminal hydrogen (Figure 2E). HO-1 mRNA was also upregulated three hours after reperfusion compared to controls. Hydrogen treatment significantly attenuated HO-1 mRNA upregulation to approximately 30% of the value of untreated grafts (Figure 2F).

Intraluminal Hydrogen-Rich Solution Ameliorated Oxidative Injury in the Intestinal Grafts

IR resulted in tissue lipid peroxidation of the tissue and graft failure. MDA, an oxidative stress marker, was 0.089 ± 0.01 nmol/ml tissue in the naïve intestine. MDA levels increased to 0.30 ± 0.04 nmol/ml tissue three hours after reperfusion. Intraluminal hydrogen significantly reduced intestinal tissue oxidative injury to 0.18 ± 0.03 nmol/ml tissue of MDA (Figure 3A).

Intraluminal Hydrogen-rich Solution Improved Deteriorated Intestinal Serosal Microcirculation

Blood flow of the intestinal marginal arteries did not differ in normal intestine of sham groups and transplanted intestine regardless of intraluminal hydrogen content ranging between 118 and 132 Laser Doppler Units (LDU) (Figure 3B). Blood flow on the serosal side of the naïve intestine detected by Doppler flowmeter was 38.7 ± 4.2 LDU. Intestinal graft microcirculation on the serosa was significantly reduced to 15.4 ± 4.5 LDU three hours after reperfusion compared to sham animals. Serosal microcirculation was significantly improved to 22.4 ± 3.2 LDU in the grafts preserved with luminal hydrogen administration (Figure 3C).

Intraluminal Hydrogen-Containing Saline Maintained Tight Junction Barrier-Related Proteins

ZO-1 and CLDN-1 are the primary transmembrane proteins connecting the junctional complex with cytoskeletal proteins. Western blot revealed that the expressions of these

tight junction barrier-related proteins were reduced in the graft intestine three hours after reperfusion (Figure 4A). Intraluminal hydrogen did not alter ZO-1 expression (Figure 4B). However, hydrogen treatment regulated loss of the transmembrane protein CLDN-1 (Figure 4C).

Recipient Survival

Because animal survival depended entirely on the function of transplanted intestinal grafts, animal survival was considered identical to graft survival in this model. Prolonged cold preservation for six hours in Ringer's lactate solution and following transplantation caused severe graft injury. Only four of 13 (30.8%) recipients survived for 14 days after transplantation. In contrast, survival of recipients with intestinal grafts treated by intraluminal hydrogen was improved and was eight of 12 (66.7%). In addition, survival of animals with hydrogen-treated grafts was good and the animals gained weight throughout the follow-up period, indicating the safety and feasibility of this novel therapeutic strategy using hydrogen-rich saline.

DISCUSSION

In this study, we demonstrated that intraluminal administration of hydrogen-rich saline significantly mitigated IR-induced mucosal injury associated with inhibition of inflammation. Previously, our hypothesis was tested using a warm intestinal IR injury model created by clamping mesenteric vessels for 90 minutes. In this previous experiment, 2 mL of 5% glucose saline containing hydrogen (5 ppm of concentration) was injected into ischemic intestinal lumen.[23] However, such a warm ischemia model was not

applicable for clinical organ transplantation, as the graft intestine was not perfused or preserved in cold preservation solution. Our study is novel because we used a more clinically relevant experimental model of cold preservation and transplantation. In order to determine the applicability of hydrogen to clinical transplantation, *in vivo* animal experiments using organ transplantation after cold preservation are required.

Hydrogen represents an effective and non-toxic molecule with wide potential for treating various diseases associated with oxidative injury, including transplantation-related organ damage.[24, 25] Protective mechanisms have not been fully elucidated. Previous literature has advocated for the role of hydrogen as a scavenger of reactive oxygen species. In our study, hydrogen's scavenging properties may not play a major role in improvement of intestinal graft IR injury, but we do not exclude the possibility. The possible beneficial mechanisms of molecular hydrogen include the indirect effect of inducing the expression of protective genes such as HO-1 or IL-10.[26, 27] However, our data did not show an involvement of these protective genes for protection conferred by luminal hydrogen. Currently, we do not have a clear explanation for how intraluminal administration was able to mitigate mucosal injury in the intestinal grafts, which was consistent with previous reports showing hydrogen's organ protective properties.

The intestinal barrier comprises epithelial cells and the apical junctional complex, which enables the establishment of an immunological environment permissive to colonization by commensal bacteria.[28] The tight junction is the most luminal component of the barrier apparatus and forms an effective barrier against the influx of microbes, antigens, digestive enzymes, microbial toxins, and other noxious substances from the gastrointestinal lumen to the internal milieu. While mucosal breakdown occurs during preservation, intestinal

mucosal injury was further promoted, leading to impaired mucosal barrier function and bacterial translocation on perfusion. Thus, the intestinal mucosa is very susceptible to cold storage injury. Furthermore, the release of proinflammatory mediators from the intestinal graft results in systemic inflammatory responses in distant organs.[15] Advanced IR injury may stimulate adaptive alloimmunity and promote acute/chronic rejection.

Previously, basic/clinical researchers had aimed to develop ideal intravascular solutions for organ perfusion and preservation. The intestinal lumen, the site for uptake of nutrients, water, and electrolytes, is an alternative route of access and should be a therapeutic target for prevention of IR injury. Intraluminally-administered preservation solution and its ingredients may directly reach the intestinal mucosa, which should be a reasonable target to prevent intestinal graft injury. Current intestinal graft preservation practice consists of an *in situ* vascular flush with iced University of Wisconsin (UW) solution or histidine-tryptophan-ketoglutarate solution followed by cold storage at 4°C without luminal interventions.

Several studies have attempted to demonstrate the benefits of transluminal interventions for mitigating intestinal IR injury. Luminal contact between the mucosa of intestinal grafts and UW solution improved the quality of human small bowel preservation.[29] Likewise, luminal administration of Celsior solution could mitigate rat intestinal IR injury compared with UW solution.[30] Thus, supplemental luminal interventions during cold preservation may contribute to extending the safe cold ischemic time, reduce reperfusion injury, and improve graft viability. As our study has shown, intraluminal administration of hydrogen-rich saline before/during cold storage could be easily incorporated into current transplant clinical practice due to its technical simplicity.

One limitation of this study was its lack of clinical relevance. While we are aware that UW solution or histidine-tryptophan-ketoglutarate solution is the current gold standard[4], saline or Ringer's solution was used for organ storage in this study. These preservation solutions contain several ingredients, including antioxidants or metabolically inert substances, all of which are considered beneficial for preventing organ injury during cold ischemia. These additives may mask the effects of intraluminal hydrogen. Also, we used Lactated Ringer's Solution in our previous study investigating the effect of inhaled hydrogen in rodent small bowel transplantation[15, 16] and chose to keep the experimental protocol consistent among the three studies.

We also used an isograft model, which is an artificial experimental model that does not reflect what happens in the clinical transplantation setting (except for in the rare case of identical twins). However, the isograft model is considered an ideal experimental model to study IR injury by allowing isolation of factors related to IR injury from other factors involved in alloimmune reactions. It is widely used as a tool to study the pathophysiology of IR injury and evaluate the effect of therapeutic manipulations.

Finally, in this study we analyzed intestinal graft samples at only a short-term time point, which may not detain the study's quality. Previous studies have demonstrated that the severity of intestinal graft injury closely correlates with long-term outcome.[15,16,20,22] Previous studies have also shown the benefits of some macromolecules (polyethylene, glycol, lactobionate, dextrane) to retain water in the lumen.[4,31,32] Future research may focus on more clinically relevant interventions using molecular hydrogen.

CONCLUSION

Luminal administration of hydrogen-rich saline prevents intestinal mucosal damage and breakdown of the mucosal barrier associated with graft inflammation. Intraluminal treatment using hydrogen-rich saline can potentially be a novel, promising, and clinically applicable therapeutic strategy for the prevention of intestinal IR injury. Ideal preservation of the intestine may incorporate a combination of several approaches. Since we have already shown the beneficial effects of hydrogen administration via inhalation, vascular flush, and preservation, our intraluminal approach is a meaningful additional therapeutic strategy.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest

AUTHORS' CONTRIBUTIONS

HY and TA participated in the research design, research performance, data acquisition, data interpretation, and data analysis and wrote the manuscript. TI participated in performing the histopathological data analysis. TH, MS, and TN participated in performing the research. AN provided the working hypothesis, participated in the research design, performance of the research, and data acquisition, and wrote the manuscript. HN provided the working hypothesis, contributed to the study design, and was involved in revising the article for intellectual content. All authors reviewed and approved the final manuscript.

FIGURE LEGENDS

Figure 1.

A. Ischemia reperfusion (IR)-induced alterations in graft mucosal morphological changes are shown (representative images from eight individual animals from each group). B. The degree of graft mucosal damage is presented as a mean histopathological score of each animal. Histopathological analysis of control grafts revealed massive epithelial loss. Hydrogen treatment significantly ameliorated histopathological injuries three hours after reperfusion ($n = 8$ for each group, $*p < 0.05$). C. Gut permeability increased three hours after reperfusion, suggesting loss of mucosal barrier function. Hydrogen significantly prevented an increase in intestinal permeability, averting mucosal barrier breakdown ($n = 5$ for each group, $*p < 0.05$).

Figure 2.

Tissue inflammation-related mRNA expression of IL-6(A), IL-1β (B), iNOS (C), COX-2 (D), IL-10 (E), and HO-1 (F) were remarkably upregulated at 3 h after reperfusion in the control grafts. Hydrogen treatment significantly downregulated mRNA expressions for IL-6, IL-1β, iNOS, and COX-2 ($n=8$ for each group, $*p< 0.05$). Anti-inflammatory cytokine IL-10 and antioxidant HO-1 mRNAs were also significantly reduced by intraluminal hydrogen treatment ($n=8$ for each group, $*p< 0.05$).

Figure 3.

A. Malondialdehyde (MDA) levels indicating that lipid peroxidation increased three hours after reperfusion in the transplanted intestine. Luminal loading of hydrogen during cold preservation significantly reduced tissue MDA levels (n=5-6 for each group, *p< 0.05).

Graft blood flows in the marginal artery (B) and intestinal serosal wall (C) were measured using a Laser Doppler flowmeter. B: Intestinal marginal artery blood flows were similar in all groups (B). IR injury reduced serosal blood flow three hours after reperfusion. Hydrogen treatment significantly increased serosal blood flow compared to those without hydrogen (n=5-6 for each group, *p< 0.05).

Figure 4.

A. Western blot for the tight junction barrier-related proteins ZO-1 and CLDN-1 was performed using intestinal grafts taken three hours after reperfusion (representative data from six independent experiments). Band density was quantified and expressed as the percentage of ZO-1 to β-actin (B, n = 5–6 for each group) and CLDN-1 to β-actin (C, n = 5–6 for each group). Protein expression of ZO-1 was significantly reduced three hours after reperfusion, regardless of the presence of hydrogen. However, hydrogen inhibited the loss of CLDN-1 in the transplanted intestine three hours after reperfusion.

Figure 5.

Recipient survival. Cold preservation of intestinal grafts for 6 h in control UW solution resulted in intestinal graft dysfunction, and the overall survival rate at 14 days was 30.8%

(4/13). In contrast, recipient survival was significantly enhanced to 66.7% (8/12) with intraluminal hydrogen treatment.

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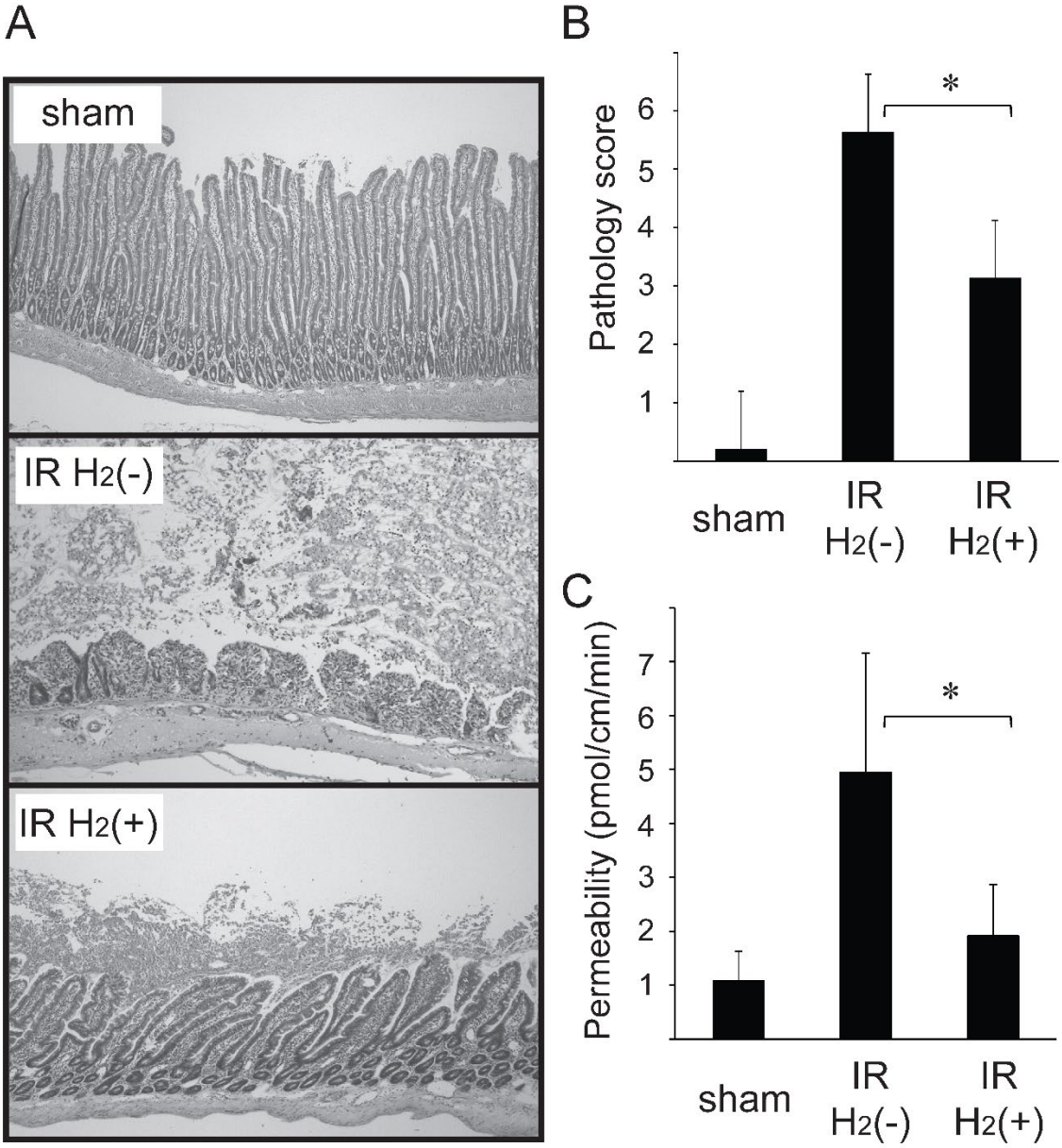
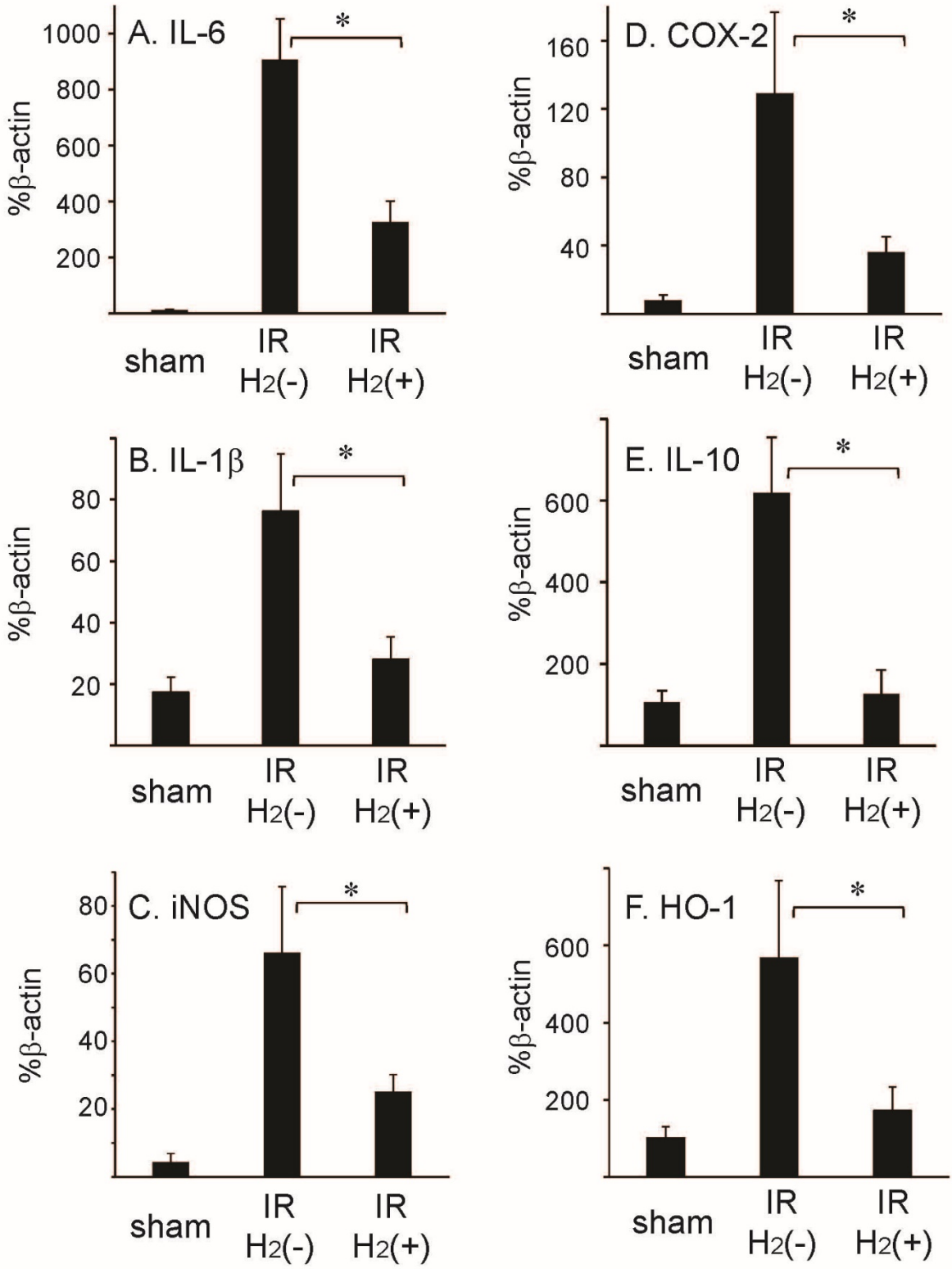
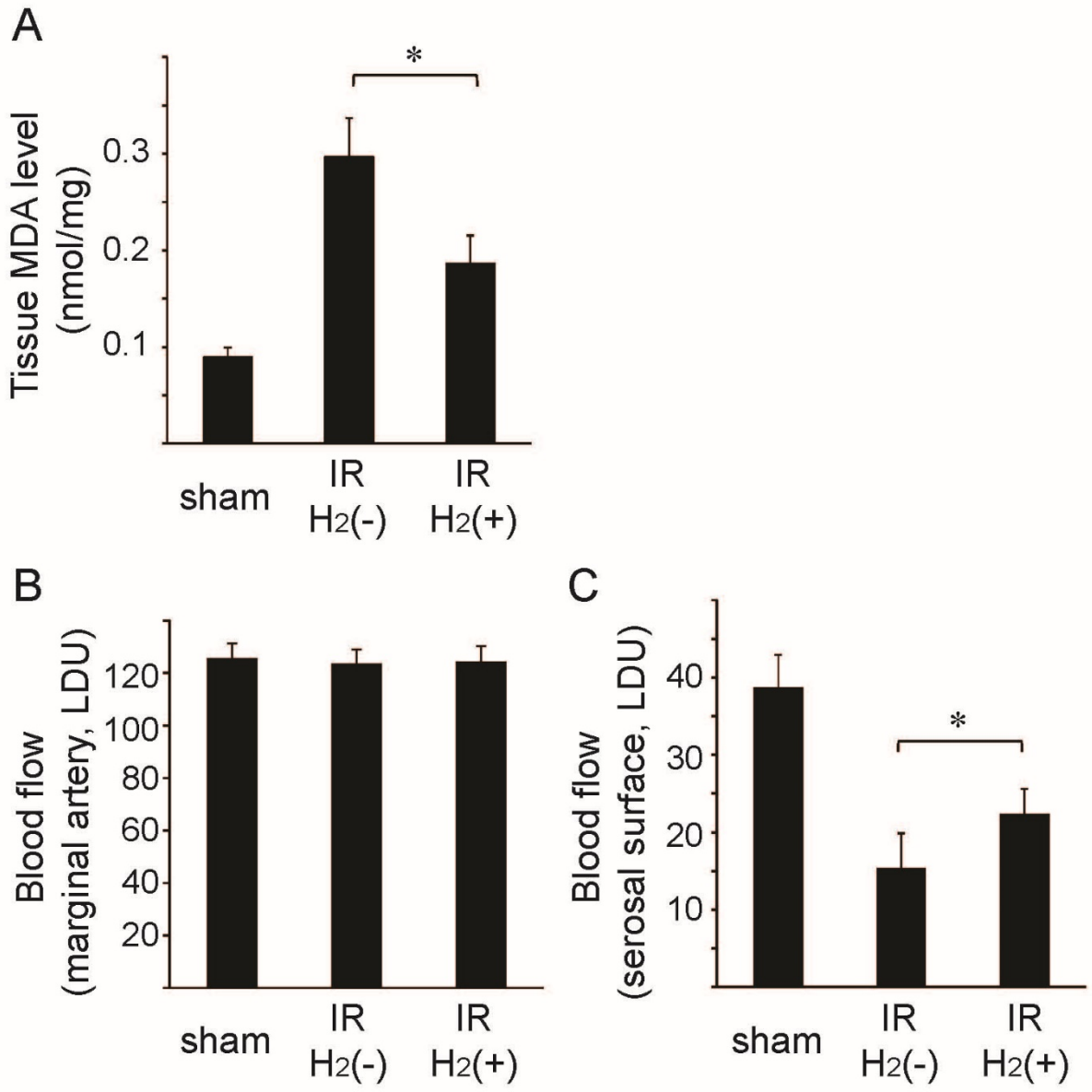
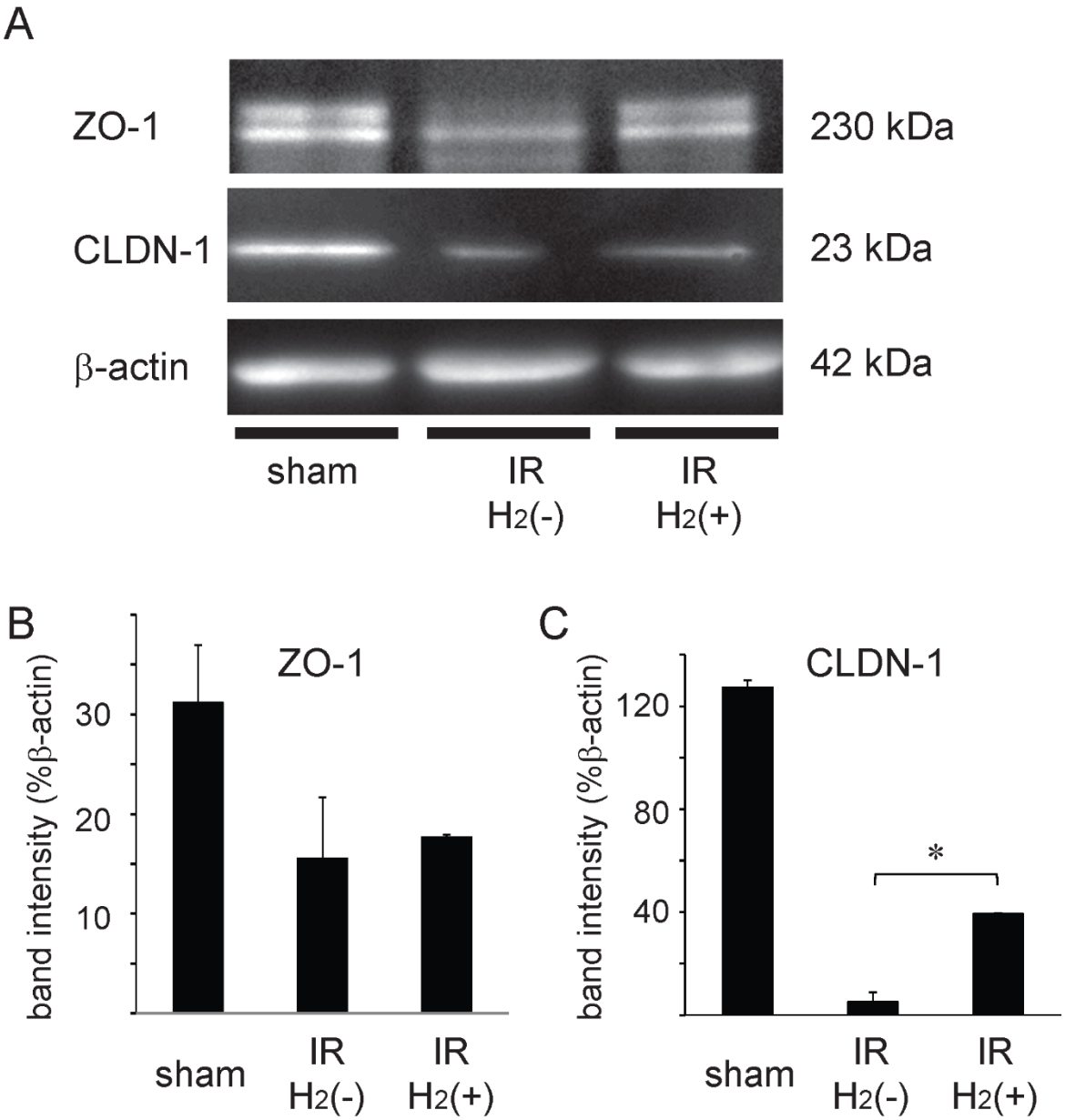
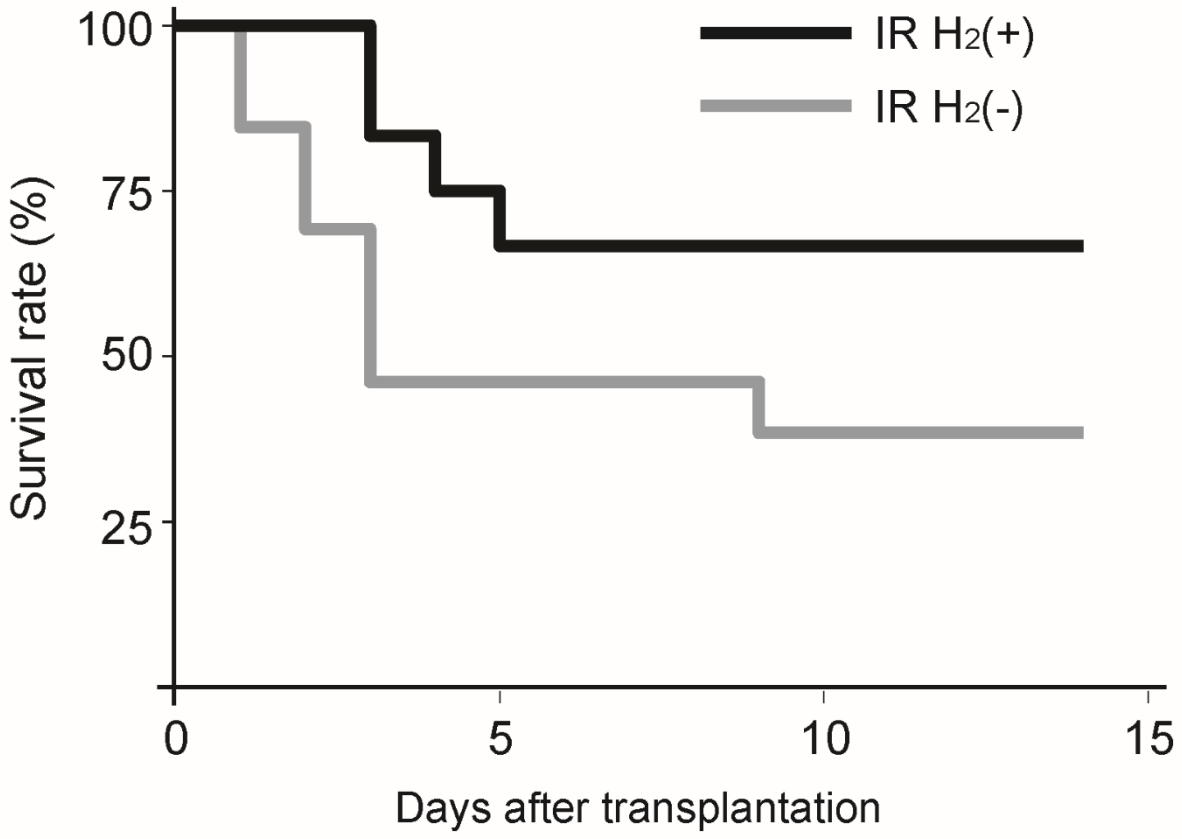

A
sham
IR H2(-)
IR H2(+)
B
Pathology score
6
5
4
3
2
1
*
sham
IR H2(-)
IR H2(+)
C
Permeability (pmol/cm/min)
7
6
5
4
3
2
1
*
sham
IR H2(-)
IR H2(+)


A. IL-6
%β-actin
1000
800
600
400
200
*
sham
IR H2(-)
IR H2(+)
D. COX-2
%β-actin
160
120
80
40
*
sham
IR H2(-)
IR H2(+)
B. IL-1β
%β-actin
80
60
40
20
*
sham
IR H2(-)
IR H2(+)
E. IL-10
%β-actin
600
400
200
*
sham
IR H2(-)
IR H2(+)
C. iNOS
%β-actin
80
60
40
20
*
sham
IR H2(-)
IR H2(+)
F. HO-1
%β-actin
600
400
200
*
sham
IR H2(-)
IR H2(+)


A
Tissue MDA level
(nmol/mg)
0.3
0.2
0.1
*
sham
IR
H2(-)
IR
H2(+)
B
Blood flow
(marginal artery, LDU)
120
100
80
60
40
20
sham
IR
H2(-)
IR
H2(+)
C
Blood flow
(serosal surface, LDU)
40
30
20
10
*
sham
IR
H2(-)
IR
H2(+)


A
ZO-1
230 kDa
CLDN-1
23 kDa
β-actin
42 kDa
sham
IR
H2(-)
IR
H2(+)
B
ZO-1
band intensity (%β-actin)
30
20
10
sham
IR
H2(-)
IR
H2(+)
C
CLDN-1
band intensity (%β-actin)
120
80
40
*
sham
IR
H2(-)
IR
H2(+)


100
75
50
25
Survival rate (%)
0
5
10
15
Days after transplantation
IR H2(+)
IR H2(-)