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Luminal injection of hydrogen-rich solution attenuates intestinal ischemia-reperfusion injury in rats

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Abbreviations:

8-OHdG: 8-hydroxydeoxyguanosine; GS: glucose saline; HMGB1: high mobility group box chromosomal protein 1; HRGS: hydrogen-rich glucose saline; ICAM-1: intercellular adhesion molecule-1; IL: Interleukin; iNOS: inducible nitric oxide synthase; IRI: ischemia-reperfusion injury; MDA: malondialdehyde; TNF: tumor necrosis factor; TUNEL: terminal deoxynucleotudyl transferase-mediated dUTP nick end-labeling; VCAM-1: vascular cell adhesion molecule-1

Abstract

Background: Luminal preservation of the intestine is an attractive method to locally mitigate preservation injury and ischemic-reperfusion injury (IRI) in small bowel transplantation (SBT), because this method has a potential to maintain the intestinal graft integrity. Hydrogen is noted as an antioxidant material by reducing hydroxyl radicals. We hypothesized that hydrogen-containing solution can be an optimum material for luminal preservation method in SBT.

Methods: Ischemic reperfusion was induced in Lewis rats by occlusion of the supramesenteric artery and vein for 90 minutes. Experimental protocols were divided into 4 groups: sham operation group, no luminal injection (control) group, luminal injection of 5% glucose saline solution (GS) group, and luminal injection of hydrogen-rich GS (HRGS) group. Two ml of experimental solution was locally injected into the lumen of the intestine before declamping of vessels. Oxidative stress markers, proinflammatory cytokines, apoptosis in the crypt cells, and morphological changes of the intestine were assessed.

Results: The production of malondialdehyde and 8-hydroxydeoxyguanosine, as oxidative stress markers, were markedly suppressed in HRGS group. The level of proinflammatory cytokines, such as inducible nitric oxide synthase and interleukin-6,

were significantly inhibited in HRGS group. Crypt apoptosis was also significantly suppressed in HRGS group. Histopathologically, integrity of villus in intestine was maintained in HRGS group in comparison to the other groups.

Conclusions: Luminal injection of hydrogen-rich solution can reduce oxidative stress and consequently ameliorate IRI. Hydrogen-containing solution can be a novel and promising luminal preservation material in SBT.

Introduction

Small bowel transplantation (SBT) is the only treatment for end stage intestinal failure. The patient and graft survival are still dismal, because of the lack of adequate immunosuppression and ischemia-reperfusion injury (IRI) (**1**). IRI is one of the obstacles to success in SBT. The destruction of intestinal barrier function induced by IRI may easily stimulate bacterial translocation, which can trigger systemic inflammatory response syndrome with high mortality **(2, 3)**.

Two preservation methods exist in SBT: intravascular and luminal preservation. Intravascular preservation cannot solely preserve mucosal damage caused by intestinal IRI for an extended period of storage time **(4, 5)**. Luminal preservation showed better graft integrity in comparison to intravascular preservation **(6-8)**.

Hydroxyl radicals and peroxinitrites released by IRI have strong cytotoxic effect by producing lipid peroxidation, DNA oxidation, and mitochondrial depolarization. It has been reported hydrogen gas had protective effect against IRI by decreasing hydroxyl radicals and peroxinitrites **(9)**. However, hydrogen gas is highly flammable in the air over the concentration of 4.6%. On the other hand, hydrogen-rich solution resolves this problem since it is portable, easily administered and a safe means of delivering hydrogen **(10)**.

Rodent models of intestinal IRI via intravenous administration and SBT via inhalation and using hydrogen-rich solution as a preservation solution are reported **(11-13)**. It is hypothesized that luminal preservation by using hydrogen-rich solution can be a novel and unique method to attenuate intestinal IRI in the clinical setting of SBT. Our purpose of this study was to evaluate the efficacy of luminal injection with hydrogen-rich solution against intestinal IRI in rat.

Results

Hydrogen concentration of small intestine by various administration methods

Hydrogen concentration of small intestine was significantly higher by oral administration, compared to other administration at 5 and 15 min after injection (**Fig. 1A)**. Hydrogen concentration at 30 and 60 min after injection was no significant difference between the groups.

Time dependent change of hydrogen concentration and hydrogen consumption in normal group and 90 min ischemic group by luminal injection

Time dependent variation of hydrogen concentration by luminal injection in small intestine was shown in **Figure 1B**. The mean hydrogen concentration in 90 min

ischemic group was significantly lower than that in normal group at 1 and 5 min after injection (1,265 vs. 2,150 ppm; $p<0.05$, 232.2 vs. 1395.2 ppm; $p<0.01$, respectively).

Effect of hydrogen-rich glucose saline on oxidative stress

The tissue value of malondialdehyde (MDA) was significantly decreased in Hydrogen-rich glucose saline (HRGS) group at 1 hour after reperfusion **(Fig. 2A)**. Moreover, 8-hydroxydeoxyguanosine (8-OHdG) positive cells in the intestinal tissues significantly increased in control and glucose saline (GS) group, and suppressed in HRGS group at 1 hour after reperfusion **(Fig. 2B)**. The distribution of 8-OHdG positive cells was significantly suppressed in HRGS group at 1hours after reperfusion **(Fig. 2C)**.

Messenger RNA expression of small intestine

Interleukin (IL) -6 and inducible nitric oxide synthase (iNOS) mRNA levels in the intestinal tissues were significantly suppressed in HRGS group at 1 hour after reperfusion **(Fig. 3)**. Tumor necrosis factor (TNF)-α and IL-1β mRNA levels in the intestinal tissues tended to decrease in HRGS group at 1 hour after reperfusion with no significant difference. The mRNA levels of intercellular adhesion molecule-1

(ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and high mobility group box chromosomal protein 1 (HMGB1) had a tendency to be lower in the HRGS group at 1 hour after reperfusion, although no significant differences were found for the conditions examined.

Hydrogen-rich glucose saline attenuates apoptosis of crypt cells after intestinal ischemia-reperfusion injury

Crypt apoptosis was stimulated at 3 hours after reperfusion in control and GS group. On the other hand, crypt apoptosis was significantly inhibited in HRGS group **(Fig. 4A)**. The distribution of crypt apoptosis at 3 hours after reperfusion was significantly suppressed in HRGS group **(Fig. 4B)**.

Hydrogen-rich solution histologically reduced ischemia-reperfusion injury

The damage of the intestinal mucosa in control and GS groups was severer than that in HRGS group at 1 and 3 hours after reperfusion **(Fig. 5A)**. Crypt cells revealed to be necrotic in control group. The construction of villi was preserved in HRGS group in spite of the presence of shortened villi and edema. The grade of Park/Chiu classification was significantly lower in HRGS group at 1 and 3 hour after reperfusion

(Fig. 5B, C).

Discussion

Our results indicate that luminal injection can reduce oxidative stress, the production of inflammatory cytokines, and apoptotic change in crypt cells in the intestinal tissues, which are compatible with those from the other reports of intestinal IRI **(11-14)**. Significant suppression of MDA and 8-OHdG in the intestinal tissue may indicate that luminal injection of hydrogen-rich solution neutralizes hydroxyl radicals. Marked suppression of hydroxyl radicals result in reduction of proinflammatory cytokines. Especially, iNOS expression was significantly suppressed in HRGS group. Nitric oxide generated by iNOS reacts with superoxide radicals to form peroxynitrite, which has strong oxidative power. Therefore, hydrogen might reduce not only hydroxyl radicals but also peroxynitrites. Suppression of IL-1β, TNFα and IL-6 indicates are compatible with inhibition of macrophage infiltration because these cytokines are produced by macrophages. Moreover, it is known the Janus kinase/signal transducers and activators of transcription pathway, which plays an essential role in intestinal apoptosis, is activated by IL-6 **(15, 16)**. Consequently, apoptosis and intestinal tissue injury after intestinal ischemia reperfusion are suppressed by hydrogen-rich solution. The expression of HMGB1, which is known to be a crucial mediator of shock and

inflammation, is promoted by IL-1β and TNFα, and the protein is passively released by necrotic cells (17). Stimulation of endothelial cells with HMGB1 causes increased expression of ICAM-1 and VCAM-1, which mediate the adhesion and emigration of activated leukocytes in postcapillary venules (2). The expression of HMGB1, ICAM-1 and VCAM-1 may be later than that of IL-1β and TNFα. Thus, in our study, the decreases in the HMGB1, ICAM-1 and VCAM-1 gene expression in the HRGS group is compatible with the reduction of proinflammatory cytokines resulting from the suppression of hydroxyl radicals.

We showed the variation of hydrogen concentration by various administration methods. Superoxide anion, generated in the mitochondria, stimulates the production of hydrogen peroxide, followed by hydroxyl radicals via the Fenton reaction, which has strong oxidative power **(18)**. In addition, hydroxyl radicals are mainly released at early phase after reperfusion **(19)**. Ohsawa et al. reported that molecular hydrogen had antioxidant effect against IRI by selective neutralization of hydroxyl radicals **(9)**. Chuai Y et al. showed the reduction rate of hydroxyl radicals had strong correlation with hydrogen concentration **(20)**. Zao L et al. reported the protective effect of hydrogen-rich solution on the abdominal skin flap after ischemia reperfusion by intraperitoneal administration. They demonstrated that survival rate of skin flap was

improved hydrogen-dose-dependently **(21)**. Therefore, it is important to maintain high hydrogen concentration within target organ to reduce hydroxyl radicals, especially at early phase after reperfusion.

Previous studies described about the effectiveness of hydrogen in intestinal IRI model and SBT in rats by various administration methods **(11-13)**. Zheng et al. and Chen et al. respectively reported that the intravenous administration of hydrogen-rich saline 10 min and 30 min prior to reperfusion attenuated IRI in rats **(11, 12)**. Buchholz BM et al. demonstrated the effect of hydrogen in SBT model in rats, 2% of hydrogen gas inhalation during donor and recipient operation **(13)**. These papers did not demonstrate tissue hydrogen concentration after reperfusion. Our study indicated these administration methods could not maintain hydrogen concentration in small intestine at early phase of reperfusion. Hydrogen effect of reducing hydroxyl radicals presented with existence of hydrogen in the target organ, because hydroxyl radicals have the highest reactivity of all reactive oxygen spices **(9, 10)**. If tissue hydrogen concentration was low at early phase after reperfusion in these studies, we should consider new mechanism of hydrogen cytoprotective effect. Recent paper have shown that hydrogen increased the levels of antiapoptotic proteins, such as B-cell lymphoma-2 and B-cell lymphoma-extra large, as a signaling molecules in both the extrinsic and intrinsic

apoptotic pathway **(22)** However, the details of the biologic mechanisms associated with hydrogen were unclear.

In this study, we focused on luminal preservation in SBT. The most important feature of intestinal lumen provides route for absorption of water and nutrients. Luminal preservation reduces mucosal damage caused by intestinal IRI compared to intravascular preservation **(6-8)**. Luminal injection of hydrogen-rich solution has 2 beneficial effects. First, it is possible to inject tailored hydrogen-rich solution because hydrogen can be easily added to the preservation solution. For example, addition of amino acid or polyethylene glycol to preservation solution by luminal injection has better effect to attenuate intestinal IRI and preserve tight junction **(6, 8)**. Second, luminal injection of hydrogen-rich solution shows the highest hydrogen concentration of small intestine compared to other administration methods. As previously indicated, luminal injection of hydrogen-rich solution can suppress oxidative stress effectively. Thus, luminal injection of hydrogen-rich solution has enormous potential for reducing intestinal IRI. Moreover, it is possible to apply luminal injection of hydrogen-rich solution in the clinical setting, such as abdominal aortic aneurysm surgery, supramesenteric artery occlusion and SBT. This method ameliorates local injury effectively, followed by bacterial translocation and systemic inflammatory response

syndrome, eventually improve mortality rate for intestinal IRI. In case of SBT, hydrogen is expected to prolong preservation time of graft as reported in rodent model of kidney and heart transplantation **(23, 24)**. Large animal study should be necessary to evaluate the effectiveness of hydrogen-rich solution for the clinical application.

There might be a limitation in this study, warm ischemia is caused during ischemic period, thus, this may not apply equally to clinical SBT. Further investigation should be required to examine the efficacy of luminal injection of hydrogen-rich solution in SBT model.

In conclusion, we demonstrated the effectiveness of hydrogen-rich solution by luminal injection, which luminal injection was specific method for small intestine. We suggest luminal injection of tailored hydrogen-rich solution can be a breakthrough of intestinal graft preservation method for SBT.

Materials and Methods

Animals

Inbred male Lewis rats, weighing 170- 200 g, were purchased from SLC Japan (Hamamatsu, Japan). The animals were maintained in an environment with controlled temperature and light and allowed free access to a standard diet and water throughout

the experimental period. All studies were performed in accordance with the principles of the Guidelines for Animal Experimentation at the National Research Institute for Child Health and Development.

Preparation of hydrogen-rich glucose saline and the measurement of hydrogen concentration in small intestine

High concentration of hydrogen-rich solution (5ppm) was employed, which was described by Ishibashi et al **(25)**. Briefly, 450 ml of saline and 50 ml of 50 % glucose were mixed into 530 ml plastic bottle (5 % GS solution) to provide a carbonated drink. Hydrogen-rich glucose saline (HRGS) was prepared using hydrogen generating agent (MiZ Co., Ltd., Kanagawa, Japan) including metal alminium grains and calcium hydroxide; 0.5 g of the materials was enclosed and heat-sealed within a non-woven fabric. Hydrogen generating agent was inserted into small bottle, and then added 0.8 ml of pure water, a cap with a check valve was tightly closed. Small bottle was inserted into 530 ml bottle filled with 5 % GS.

Approximately 1 g of tissue sample was obtained and put into a small container (24 ml) for the measurement of hydrogen concentration. The hydrogen concentration was measured using a sensor gas chromatograph, SGHA-FA (FIS Inc., Hyogo, Japan) after

pulverizing the tissue using gentleMACS Octo Dissociator (Miltenyi biotec GmbH, Bergisch Gladbach, Germany). Hydrogen concentration of tissue samples was measured after cleaning tissue samples with saline to avoid mixture of hydrogen rich solution into a small container. Hydrogen concentration in 24 ml container was converted into concentration per 1 ml.

Distribution of hydrogen in small intestine by oral, intraperitoneal, intravenous and inhalation administration

Hydrogen concentration in small intestine was measured by various administration methods, such as oral, intraperitoneal and intravenous administration of HRGS, and 4 % of hydrogen gas inhalation. Two ml of HRGS was injected via the oral route, abdominal puncture, and dorsal vein of penis. Hydrogen gas was given until sacrificed. Rats were sacrificed to obtain tissue samples (oral and intraperitoneal administration: before injection, 5, 15, 30 and 60 min after injection, intravenous administration; before injection and 5 min after injection, inhalation; before injection, 5, 30 and 60 min after injection, n=3, respectively).

Experimental protocols

Experimental protocols in this study were divided into 4 groups; sham group, no treatment group (control), GS and HRGS treatment group. Ischemic reperfusion was induced by occlusion of the supramesenteric artery and vein for 90 min by a micro vascular clamp. Two points of small intestine were ligated at the points of 5 cm and 10 cm from treitz ligament to make injection space. Two ml of GS or HRGS was injected into injection space using 24G catheter (SURFLO®, TERUMO CORPORATION, Tokyo, Japan), and then vascular clamp was removed. Tissue samples were obtained at 1 and 3 hours after reperfusion.

The measurement of hydrogen consumption after 90 min ischemia-reperfusion injury

Hydrogen concentration of small intestine was measured before injection, at 1, 5, 15, 30 and 60 min after luminal injection of HRGS in 90 min ischemic group (n=3, respectively) and no ischemic group (normal group, n=3 respectively). Hydrogen consumption was defined as difference between mean hydrogen concentration of normal group and 90 min ischemic group.

Oxidative damage measurement

Tissue MDA concentration and 8-OHdG immunostaining were performed for the

analysis of oxidative stress. As for MDA measurement, tissue samples were frozen immediately in liquid nitrogen, then, stored at -80ºC. Tissue samples were thawed on ice and divided 50μg of samples each after all samples collected. Divided samples were homogenized in RIPA buffer (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to prevent sample oxidation. All samples were centrifuged (10000×g, 10 min, 4 ºC) to collect supernatant. Tissue MDA levels were assessed using an OxiSelectTM TBARS Assay kit (Cell Biolabs, Inc., San Diego, CA) according to manufacturing protocol. The absorbance at 532 nm was measured using a NanoDrop (Thermo Fisher Scientific, Yokohama, Japan).

Subsequently, in 8-OHdG immunostaining, tissue samples were soaked in Bouin solution for 3- 5 days, and then embedded in paraffin. The specimens were cut into 3-μm tissue sections, deparaffinized with xylene and alcohol. The sections were treated with autoclave for 10 min, 120 ℃ in citric buffer for antigen retrieval. The primary antibody (anti-8-Hydroxy-2'-deoxyguanosine monoclonal antibody N45.1, Japan institute for the Control of Aging, Nikken Seil Co., Ltd., Shizuoka, Japan) was applied overnight at 4℃ after incubation with 8% skimmed milk for 30 min to block non-specific reaction. They were then incubated using LSABTM2 kits/HRP (Dako, Tokyo, Japan) and Streptavidin Biotin Complex (Dako) according to manufacturing

protocol as the secondary antibody, and dyed with 3,3'-Diaminobenzidine,tetrahydrochloride. As for 8-OHdG immunostaining, 10 viewing fields randomly selected on each slide section were examined at ×400 magnification and the rate of 8-OHdG positive cells was calculated in the crypt.

RNA isolation and quantitative real-time reverse-transcriptase polymerase chain reaction

Total RNA was extracted from the small intestine using a sepasol RNA 1 super G (Nacalai tesque, Kyoto, Japan) and DNA-freeTM kit (Life technologies, Carlsbad, CA) according to manufacturing protocol. Aliquots of 800 ng of RNA were reverse transcripted to cDNA using PrimeScript RT reagent kit (Perfect Real Time) (Takara Bio Inc., Shiga, Japan) according to manufacturing protocol. Quantitative real-time reverse-transcriptase polymerase chain reaction was performed using TaqMan on an Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Primers amplifying the rat mRNA regions and a specific TaqMan probe were designed using the Primer Express software package (Applied Biosystems). Data are expressed as the comparative cycle threshold. The normalized cycle threshold value of each gene was obtained by subtracting the cycle threshold value of 18S rRNA.

Analysis of crypt apoptosis

Terminal deoxynucleotudyl transferase-mediated dUTP nick end-labeling (TUNEL) staining was performed using a Peroxidase in situ Apoptosis Detection Kit S7100 (CHEMICON International, Inc. CA), according to the manufacturer's instructions. Briefly, the sections were deparaffinized and treated with proteinase K (20 μg/ml) for 15 min at room temperature. The slides were incubated with 3 % hydrogen peroxide in PBS for 10 min to block endogenous peroxidase activity, followed by incubation in Working Strength TdT enzyme solution for 60 min at 37 ºC. The reaction was terminated by incubation in a Working Strength Stop/Wash Buffer for 10 min. The slides were incubated with Anti-digoxigenin peroxidase for 30 min at room temperature. Chromogenic color was developed with 3,3'-diaminobenzidine and nuclei were counterstained with hematoxylin. Tissue samples for TUNEL staining were obtained at 3 hours after reperfusion. The rate of TUNEL positive cells in the crypt was examined at ×400 magnification in the 10 viewing fields randomly selected on each slide.

Morphological analysis

Tissue samples were fixed in 10 % formalin, embedded in paraffin. Three-μm tissue sections were mounted on slides, deparaffinized with xylene and alcohol, and stained with hematoxylin-eosin. Histological damage was assessed using Park/Chiu classification **(26, 27)**. Park/Chiu classification was graded by a skillful pathologist.

Statistical analysis

The SPSS software program was used for the statistical analysis (SPSS version 18.0, SPSS Inc. Chicago, IL). All results are given as the means ± standard deviation. Statistical analyses were performed using the Tukey test for parametric multiple comparisons. P-values < 0.05 were considered to be significant.

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Figure legends

Figure 1: Changes and distribution of hydrogen concentration by various administration methods in small intestine.

(A) Hydrogen concentration of small intestine by various administration methods. The intestinal hydrogen concentration by oral administration showed significantly higher compared with other administration methods at 5 and 15 min after injection (n=3 per groups, *p<0.05, **p<0.01). (B) Comparison of intestinal hydrogen concentration between normal and 90 min ischemic model by luminal injection. Intestinal hydrogen concentrations in 90 min ischemia group at 1, 5 and 15 min after injection were significantly reduced in comparison to normal group (n=3 per groups, *p<0.05, **p<0.01).

Figure 2: The analysis of malondialdehyde (MDA) and 8-hydroxydeoxyguanosine (8-OHdG) in the intestinal tissues.

HRGS significantly suppressed MDA and 8-OHdG in the intestinal tissues. (A) Tissue MDA level (n=5 per group) and **(B)** 8-OHdG immunostaining at 1h after reperfusion were measured as oxidative stress markers. 8-OHdG positive cells were counted in the crypt at ×400 magnification in 10 viewing of fields. **(C)** The distribution rate of

8-OHdG positive cells in the crypt was significantly reduced in HRGS group, compared with control and GS group (n=3 per groups, $p<0.05$).

Figure 3: The levels of proinflammatory cytokines after intestinal ischemia-reperfusion injury.

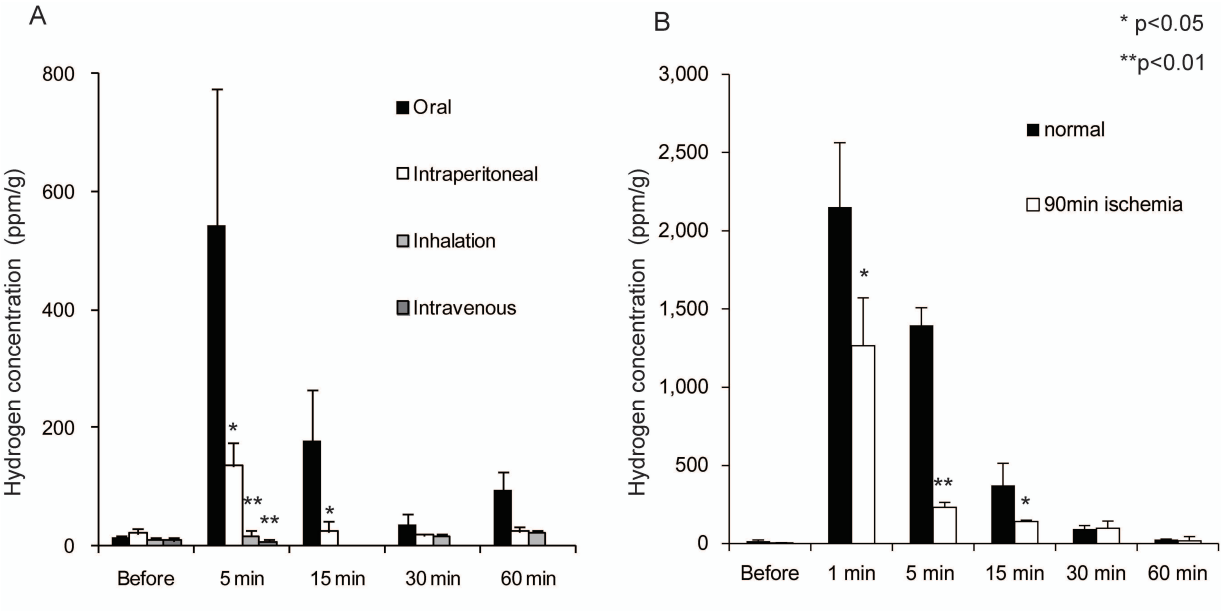
HRGS significantly suppressed proinflammatory cytokine mRNA expression. Tissue inflammatory mRNA expression of interleukin (IL) -1β, IL-6, inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)-α, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and high mobility group box chromosomal protein 1 (HMGB1) at 1h after reperfusion (n=3- 4 per groups). The levels of IL-6 and iNOS were significantly suppressed in HRGS group, compared with control at 1h after reperfusion (IL-6; $p<0.05$, iNOS; $p<0.01$). The levels of IL-1β, TNF-α, ICAM-1, VCAM-1 and HMGB1 tended to suppress in HRGS, compared with control.

Figure 4: The analysis of apoptosis in the crypt cells after intestinal ischemia-reperfusion injury.

HRGS significantly suppressed intestinal crypt apoptosis. (A) Terminal deoxynucleotudyl transferase-mediated dUTP nick end-labeling (TUNEL) staining for the analysis of crypt apoptosis at 3h after reperfusion. TUNEL positive cells were counted in the crypt at ×400 magnification in 10 viewing of fields. **(B)** The distribution of TUNEL positive cells was significantly suppressed in HRGS group compared with control and GS group (n=3 per groups, $p<0.01$).

Figure 5: Histopathological assessment by using Park/Chiu classification after intestinal ischemia-reperfusion injury.

(A) Histopathological findings at 1h and 3h after reperfusion (×200 magnification). HRGS significantly decreased intestinal tissue injury. Histological score by Park/Chiu classification at 1h (B) and 3h (C) after reperfusion (n=12 per groups). Histological score was significantly decreased in HRGS group compared with control and GS group ($p<0.01$ at 1h after reperfusion, $p<0.05$ at 3h after reperfusion).



Changes in and distribution of the hydrogen concentrations in the small intestine following various administration methods

(A) Hydrogen concentrations in the small intestine following various administration methods. The intestinal hydrogen concentrations after oral administration were significantly higher than those observed following the other administration methods at five and 15 minutes after injection (n=3 per group, *p<0.05, **p<0.01). (B) Comparison of the intestinal hydrogen concentrations between the normal control and 90 minutes of ischemia groups following luminal injection. The intestinal hydrogen concentrations in the 90 minutes of ischemia group at one, five and 15 minutes after injection were significantly reduced in comparison to those observed in the normal control group (n=3 per group, *p<0.05, **p<0.01).

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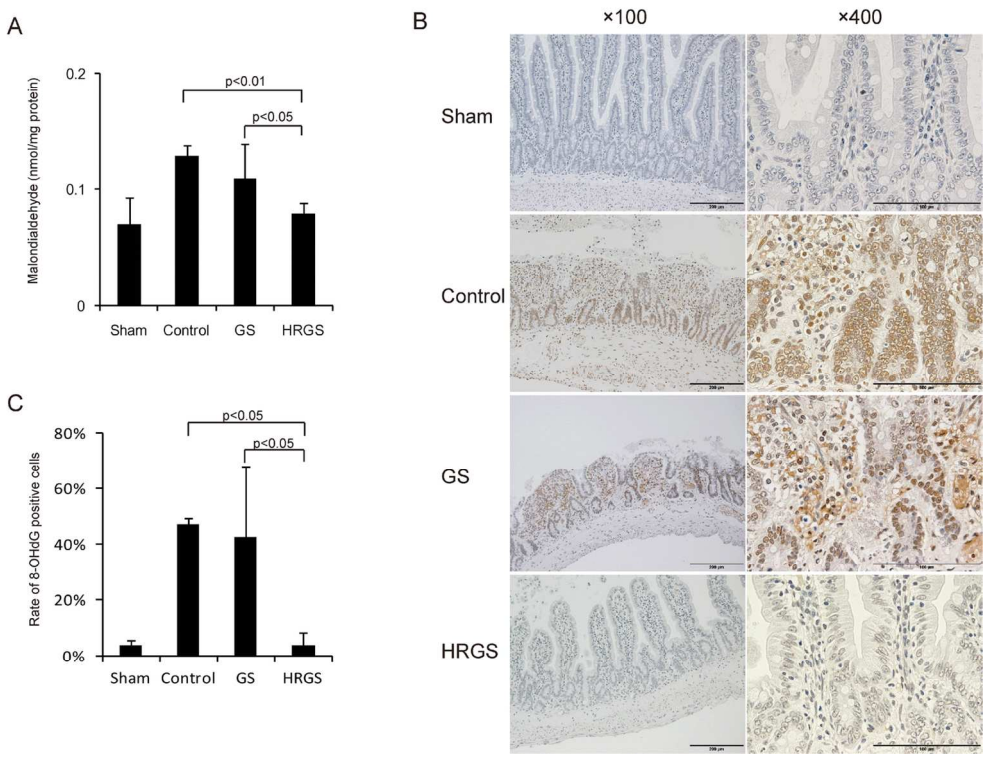
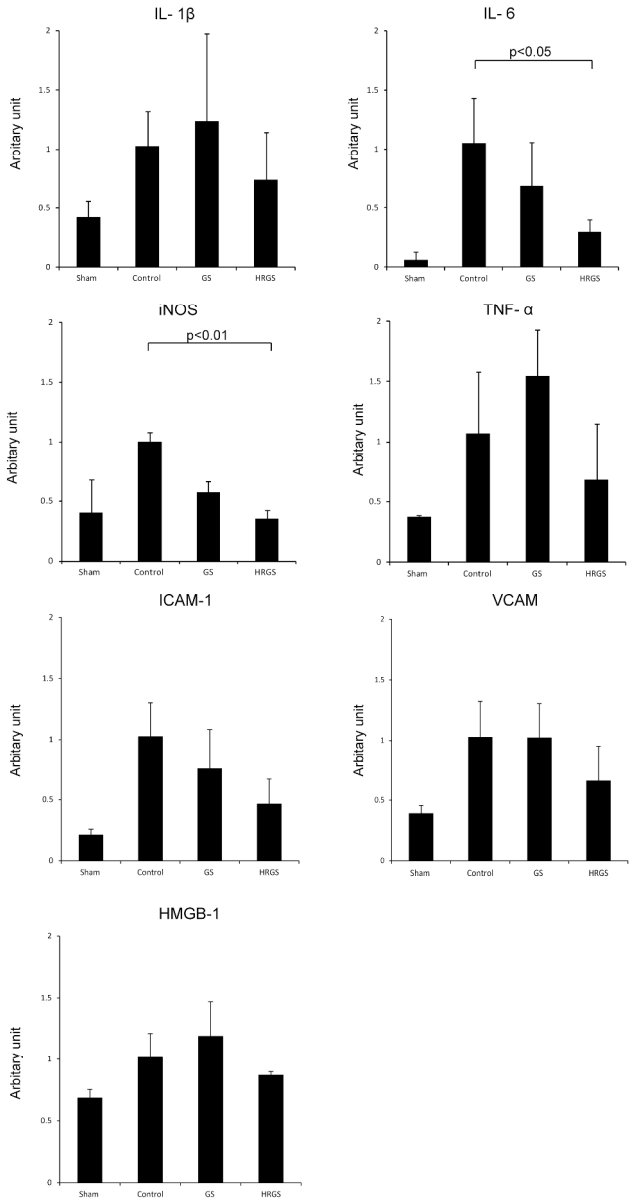


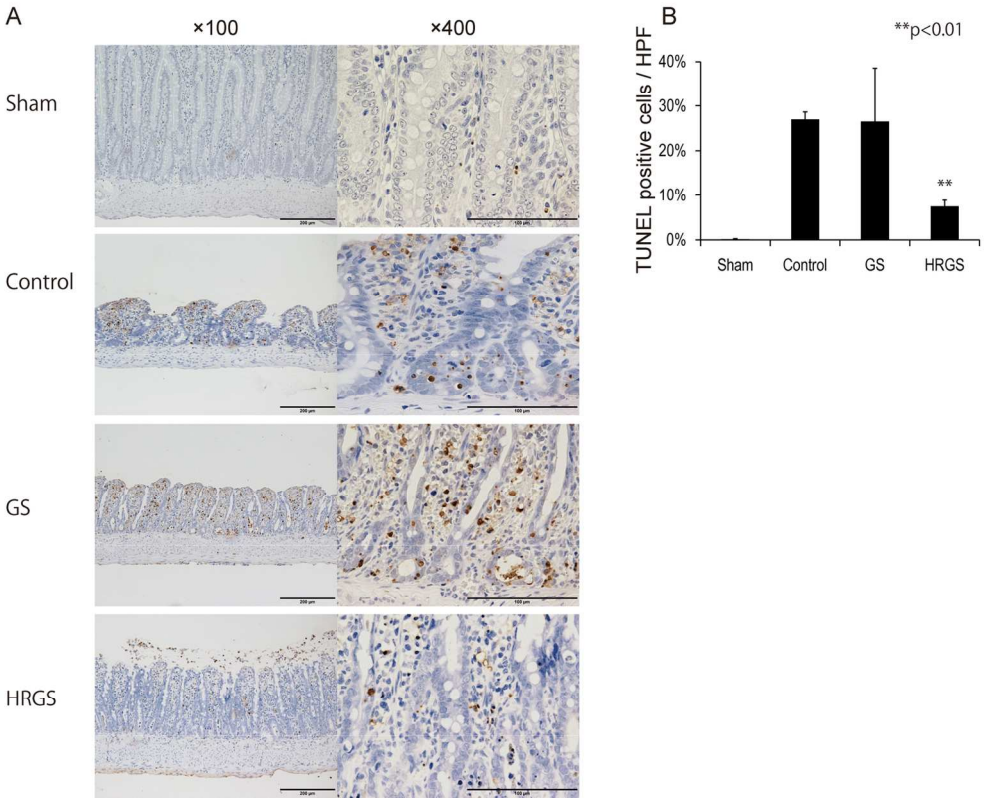
Analysis of the malondialdehyde (MDA) and 8-hydroxydeoxyguanosine (8-OHdG) levels in the intestinal tissues

HRGS significantly suppressed the MDA and 8-OHdG levels in the intestinal tissues. (A) The tissue MDA levels (n=5 per group) and (B) 8-OHdG immunostaining results at one hour after reperfusion were assessed as markers of oxidative stress. The number of 8-OHdG-positive cells among the crypt cells was counted at ×400 magnification in 10 fields of view. (C) The proportion of 8-OHdG-positive cells among the crypt cells was significantly reduced in the HRGS group compared with that observed in the control and GS groups (n=3 per group, $p<0.05$).

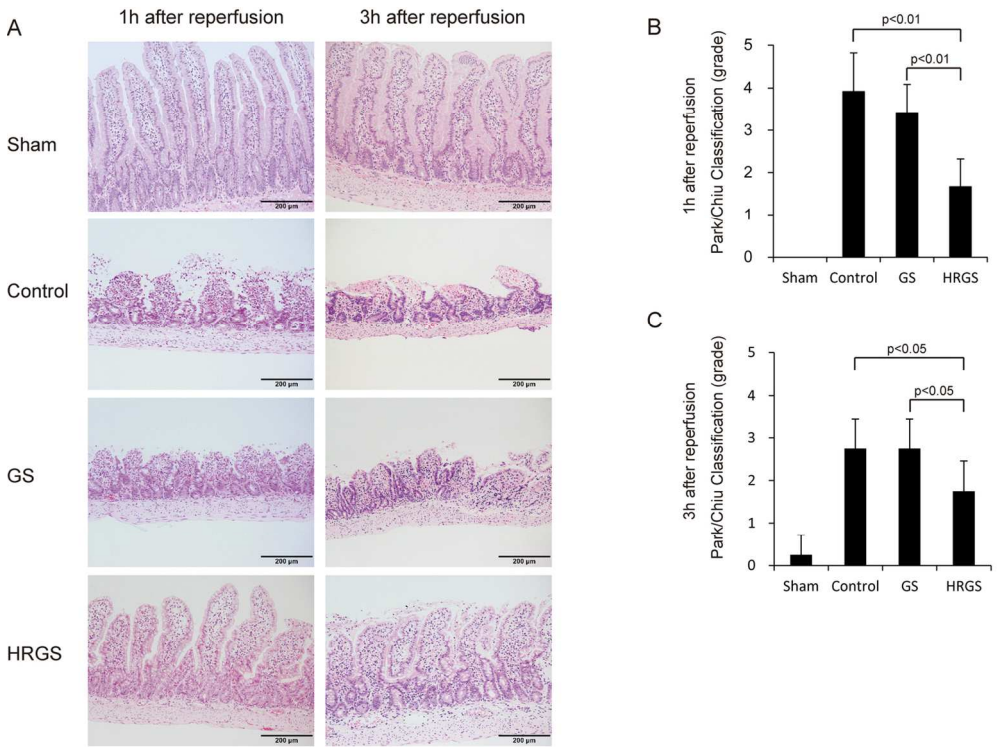
136x103mm (300 x 300 DPI)



The levels of proinflammatory cytokines after intestinal ischemia-reperfusion injury.
HRGS significantly suppressed proinflammatory cytokine mRNA expression. Tissue inflammatory mRNA expression of interleukin (IL) -1β, IL-6, inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)-α, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and high mobility group box chromosomal protein 1 (HMGB1) at 1h after reperfusion (n=3- 4 per groups). The levels of IL-6 and iNOS were significantly suppressed in HRGS group, compared with control at 1h after reperfusion (IL-6; $p<0.05$, iNOS; $p<0.01$). The levels of IL-1β, TNF-α, ICAM-1, VCAM-1 and HMGB1 tended to suppress in HRGS, compared with control.
265x502mm (300 x 300 DPI)



Analysis of apoptosis in the crypt cells after intestinal ischemia-reperfusion injury.
HRGS significantly suppressed intestinal crypt apoptosis. (A) Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining for the analysis of crypt apoptosis at three hours after reperfusion. The number of TUNEL-positive cells among the crypt cells was counted at ×400 magnification in 10 fields of view. (B) The proportion of TUNEL-positive cells was significantly suppressed in the HRGS group compared with that observed in the control and GS groups (n=3 per group, $p<0.01$).
144x116mm (300 x 300 DPI)



Histopathological assessment using the Park/Chiu classification after intestinal ischemia-reperfusion injury and survival analysis.
(A) Histopathological findings at one and three hours after reperfusion (×200 magnification). HRGS significantly decreased intestinal tissue injury. Histological scores according to the Park/Chiu classification at one (B) and three (C) hours after reperfusion (n=12 per group). The histological scores were significantly decreased in the HRGS group compared with those observed in the control and GS groups ($p<0.01$ at one hour after reperfusion, $p<0.05$ at three hours after reperfusion).
132x98mm (300 x 300 DPI)