

Title: Intestinal Ischemic preconditioning ameliorates hepatic ischemia reperfusion injury in rats: Role of heme oxygenase-1 in the second-window of protection

Authors: Shoichi Kageyama, M.D.^{1,2}, Koichiro Hata, M.D., Ph.D.¹, Hirokazu Tanaka, M.D.¹, Hirofumi Hirao, M.D.¹, Toyonari Kubota, M.D.¹, Yusuke Okamura, M.D.¹, Keiko Iwaisako, M.D., Ph.D.¹, Yasutsugu Takada, M.D., Ph.D.², and Shinji Uemoto, M.D., Ph.D.¹

Institutional affiliations:

¹ Department of Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

² Department of Hepato-Biliary-Pancreatic and Brest Surgery, Ehime University Graduate School of Medicine, Ehime, Japan

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1. Abbreviations:

ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; HO-1, heme oxygenase-1; IL-6, interleukin-6; IL-10, interleukin-10; IPC, Ischemic preconditioning; IRI, Ischemia reperfusion injury; LDH, lactate dehydrogenase; RIPC, remote ischemic preconditioning; SEM, standard error of mean; SMA, superior mesenteric artery; SWOP, 2nd window of protection; THI, total hepatic ischemia; TNF- α , tumor necrosis factor- α ; ZnPP, zinc protoporphyrin

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4. Corresponding author contact information, and reprint request to:

Koichiro Hata, M.D., Ph.D.,

Department of Surgery, Division of Hepato-Pancreato-Biliary Surgery and Transplantation, Graduate School of Medicine, Kyoto University. 54 Kawaharacho, Shogoin, Sakyo-ku, Kyoto, 608-8507, Japan.

Telephone: +81-75-751-4323; FAX: +81-75-751-4348

e-mail: khata@kuhp.kyoto-u.ac.jp

Abstract

Introduction: Preconditioning by brief ischemia protects not only the concerned organ, but also other distant organs against subsequent lethal damage, called remote ischemic preconditioning (RIPC). This study was designed to investigate the impact of intestinal RIPC against hepatic ischemia/reperfusion injury (IRI), with special interests in heme oxygenase-1 (HO-1) induction in the 2nd-window of protection (SWOP).

Materials & Methods: Male Wistar rats were randomly assigned into 2 groups; Group-RIPC or Group-Sham. Prior to hepatic IRI, either intestinal RIPC, consisting of 2 cycles of 4-minute SMA clamping separated by 11-minute declamping (Group-RIPC), or sham procedure (Group-Sham), was performed. After 48-hour recovery, the rats were exposed to 30-minute total hepatic IRI. Transaminase releases and proinflammatory cytokines were determined at several time-points after reperfusion. Histopathological analysis and animal survival were also investigated.

Results: Intestinal RIPC significantly lowered transaminase release (ALT at 2 hours, Group-RIPC vs. Group-Sham; 873.3 ± 176.4 vs. 3378.7 ± 871.1 IU/L; $p < 0.001$), as well as proinflammatory cytokines production (TNF- α at 2 hours, 930 ± 42 vs. 387 ± 17 pg/ μ L; $p < 0.001$). Morphological integrity of the liver and the ileum were significantly better maintained by intestinal RIPC, reaching to statistical significance not only in Suzuki's liver injury score (3.5 ± 0.2 vs. 0.7 ± 0.5 ; $p = 0.0074$) but in Park's score for intestinal damages (4.0 ± 0.4 vs. 2.0 ± 0.2 ; $p = 0.0074$). Animal survival was also markedly improved (83.1 vs. 15.4%, $p < 0.001$). As a mechanism underlying the protection, HO-1 was substantially induced in liver tissue, especially into hepatocytes, with remarkable up-regulation of bradykinin in the portal blood, while HO-1 protein induction in enterocytes was not significant.

Conclusion: Intestinal RIPC remarkably attenuates hepatic IRI in the SWOP, presumably by HO-1 induction in hepatocytes.

Introduction

Ischemia reperfusion injury (IRI) is a pathophysiologic process where hypoxic organ damage is accentuated by following restoration of blood flow and oxygen delivery to the ischemically-damaged tissue. Then the liver is exposed not only to direct cellular damage from ischemic insult but also to delayed dysfunction and tissue injuries resulting from activation of inflammatory cascades after reperfusion (1). Hepatic IRI is often encountered in various clinical situations including liver transplantation, trauma, shock state followed by resuscitation, and elective liver resections with inflow occlusion that is frequently used to minimize blood loss.

Ischemic preconditioning (IPC) refers to a strategy in which prior transient ischemia induces a state of protection against subsequent prolonged damage (2). In hepatobiliary surgery, total hepatic inflow occlusion, termed as Pringle's maneuver (3), represents an effective strategy to reduce blood loss and transfusion requirements (4). However, noble Pringle's maneuver easily results in adverse effects, especially on marginal livers such as cirrhosis (5) and steatosis (6), because a safe ischemic period for normal liver might be conversely crucial for marginal livers to be yielded to liver failure (7). Same difficulties are in ischemic insults for preconditioning. To date, there has been several randomized, controlled clinical trials evaluating the efficacy of IPC in liver resections and transplantations, however, most of which failed to support clinical benefits of IPC despite various protective results from numerous experimental settings (8, 9). One of the major obstacles is thought to be in the difficulty to determine the ideal protocol of IPC in humans. In clinical practice, patients' characteristics including systemic as well as hepatic conditions are quite different in each case. Ten minutes IPC seems to be beneficial for normal or less-damaged livers, whereas it might be harmful for cirrhotic patients. In other words, we could neither know the best IPC protocol for liver resection with 25% microvesicular steatosis, nor predict the suitable regimen for

“Blue liver” with liver metastasis from colorectal cancer. Alternatively, much attention has recently been paid to a novel method, remote organ preconditioning, in which a preceding stress to remote organ/tissue confers protection to the concerned organs (10-12). Actually, remote ischemic preconditioning (RIPC) to the hind-limb has been shown to exert substantial protection to hepatic IRI in several experimental studies (11, 12). Because RIPC is a recently-explored phenomenon, there has been little evidence about RIPC to the liver: Could splanchnic organs instead of hind-limb be the target for RIPC to the liver? ; Does RIPC also exhibit delayed-phase effect/ 2nd window of protection (SWOP) like IPC?

The beneficial effects of IPC and RIPC are, at least in part, associated with the induction of heat shock proteins, especially in the SWOP. Among them, heme oxygenase-1 (HO-1) is induced in a variety of organs during diverse stress-related conditions, and exerts a cytoprotective function in hepatic IRI and liver transplantation (13, 14). However, less is known about the involvement and the impact of HO-1 induction to the liver in RIPC.

This study was thus designed to investigate whether intestinal IPC, as a RIPC to the liver, could provide protective effects against hepatic IRI, with special interests in the role of HO-1 induction both in liver and in intestinal tissues. In addition, we tried to provide possible mechanisms underlying distant HO-1 induction in liver tissue by RIPC, as well as the advantage of intestinal RIPC on signal transduction to the liver, in comparison with the other organ preconditioning.

Experimental Procedures

Animals

All experimental protocols were approved by the Animal Research Committee of Kyoto University, and all animals received humane care according to Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No 86-23, revised, 1985). Male Wistar rats weighing 180-220 g were obtained from SLC (Shizuoka, Japan). The rats were kept in a temperature-controlled environment with a 12-hour light-dark cycle and allowed tap water and standard chow pellets ad libitum. During the last 12 hours before the experiments, the animals had no access to solid food but free access to water.

1. Intestinal RIPC followed by THI

1-1. Surgical Procedures

After an acclimatization period of 7 days, rats were randomly assigned into 2 groups ($n = 18$ each), Group-RIPC or Group-Sham. The rats in the both groups were anesthetized with Isoflurane (Escaïn®, Mylan, Osaka, Japan) via small animal anesthetizer (MK-A110, Muromachi Kikai Co., Ltd., Tokyo, Japan). The animals' body temperature was maintained at $36.5 \pm 0.5^{\circ}\text{C}$ with a heating pad (Midori shokai, Hiroshima, Japan).

First, laparotomy was performed through a midline incision, and superior mesenteric artery (SMA) was exposed. Free peritoneum was covered with a plastic wrap (Saran Wrap®, Asahi Kasei, Tokyo, Japan) to minimize evaporative heat and fluid loss. In Group-RIPC, intestinal RIPC, consisting of 2 cycles of 4-minute SMA clamping with an atraumatic vessel clip (Sugita clip, Mizuho Co., Ltd, Tokyo, Japan) separated by 11-minute reperfusion, was performed, while the rats in Group-Sham SMA received sham procedure without any clamping maneuver. Abdominal wall was then closed.

After 48-hour recovery, the rats in both groups were exposed to total hepatic ischemia (THI). Prior to relaparotomy, 24G Teflon catheter (Surflow®, Terumo Co., Ltd, Tokyo,

Japan) was inserted into the penile vein, enabling continuous infusion of 3 ml/kg/hr of Ringer's solution. After laparotomy, the portal vein and the proper hepatic artery were both occluded by the aforementioned clip. After 30 minutes of THI, the clip was removed and the liver was reperfused. Abdominal wall was closed again to allow transient recovery from the operation. After 2, 6, and 24 hours from reperfusion, the rats were re-anesthetized, and both portal venous and peripheral blood, liver and ileal tissue samples were collected (Fig. 1A). A half of tissue samples was immediately frozen in liquid nitrogen and stored at -80°C until later analysis, and the other half was embedded in 4% paraformaldehyde for histopathological evaluation.

1-2. Measurement of Liver Enzymes

Serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) were measured by a standard spectrophotometric method with an automated clinical analyzer (JCA-BM9030, JEOL Ltd., Tokyo, Japan).

1-3. Evaluation of cytokines production

Serum concentration of interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), and IL-10 were determined by using BD Cytometric Bead Array Rat Flex Set (Cat; 558308, 558309, and 558306, respectively, Becton, Dickinson and Company Japan, Tokyo, Japan) according to the manufacture's protocol. Flow cytometry analysis was carried out in a FACS Calibur flow cytometer (Becton Dickinson and Company Japan, Tokyo, Japan).

1-4. Histopathological Assessment

Ileal and hepatic tissues were fixed with 4% paraformaldehyde, embedded in paraffin wax and sectioned at 4 μ m thickness. The slides were then stained with hematoxylin and eosin. Three pathologists assessed and scored ileal and hepatic tissue damage in a blind fashion, according to Suzuki's classification for hepatic injury (15) and to Park's score for intestinal damages (16), respectively.

1-5. Scanning Electron Microscopy

With respect to liver samples for electron microscopy, we first perfused rats with saline through the aorta and then with a fixative containing 2% glutaraldehyde and 4% paraformaldehyde. The livers were cut into larger pieces (5 mm³) and postfixed in 2% glutaraldehyde and 4% paraformaldehyde at 4°C for 2 hours. Each sample was ion-sputter-coated and observed with a S-4700 scanning electron microscope (Hitachi, Ltd., Tokyo, Japan).

1-6. Survival Study

Animal survival was also investigated as an end-point parameter for the efficacy of the treatment. For this purpose, additional 13 rats in each group were subjected to 35-minute THI by Pringle's maneuver that is thought to be almost lethal for rats. After the operation, all rats were carefully observed up to 10 days.

2. *Intestinal RIPC without hepatic IRI*

To investigate the effect of RIPC with a special interest in HO-1 induction to the liver and the gut, additional rats ($n = 30$ in each group) were prepared, and subjected to either intestinal RIPC or sham procedure, without hepatic IRI. Blood and tissue samples from liver and ileum were obtained at 2, 6, 24, 72 and 168 hours after RIPC/sham procedure (Fig. 1B).

2-1. Liver Damage after intestinal RIPC

To evaluate the influence of intestinal RIPC to the liver, serum AST and ALT were measured after intestinal RIPC in the absence of liver IRI by the same manner.

2-2. Reverse-Transcriptase Polymerase Chain Reaction

To analyze the chronological alteration of the gene expression in the liver and the intestinal tissue, total RNA of the both tissues was extracted with TRIzol® (Life Technologies Japan Ltd., Tokyo, Japan) according to the manufacture's protocol. The equal

amounts of RNA were adjusted using NanoDrop2000® (NanoDrop Technologies, Wilmington, Delaware, USA) and complementary DNA was reverse-transcribed by incubation with Omniscript RT Kit (Qiagen, Tokyo, Japan) according to the manufacture's protocol. Real-time PCR was performed using TaqMan® technology and the real-time PCR system (StepOnePlus®, Life Technologies Japan, Tokyo, Japan). TaqMan® probe and primers for TNF- α (assay ID Rn99999017_m1), IL-6 (assay ID Rn01410330_m1), and HO-1 (assay ID Rn01536933_m1), and β -actin (assay ID Rn00667869_m1) were obtained from TaqMan® gene expression assays (Applied Biosystems, Life Technologies Japan, Tokyo, Japan).

2-3. Western Blot Analysis

Tissue samples were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 6.8), 10% glycerol, and 2% sodium dodecylsulfate. Protein quantification of samples was performed with bicinchoninic acid protein assay. Detergent-soluble protein lysates were separated by SDS-polyacrylamide gel electrophoresis on a 12.5% acrylamide gel, and proteins were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) and incubated with a primary antibody recognizing HO-1 (SPA-896; Stressgene, Victoria, Canada), or β -actin (KO0305, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:1000 dilution overnight at 4°C. After washing, membranes were reacted with horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Chemiluminescence was detected with immobilon western horseradish peroxidase substrate (Millipore, Billerica, MA.) and visualized with a charge-coupled device camera (Ez-capture, Atto Corporation, Tokyo, Japan). The intensity of the bands was quantified with imaging analysis software (CS Analyzer, Atto Corporation, Tokyo, Japan).

2-4. Immunohistochemistry

For immunohistochemistry of HO-1, paraffin sections were pretreated with 0.3% H₂O₂ in methanol and then subjected to antigen retrieval in citrate buffer (10 mM, pH 6.0) in a pressure cooker. After blocking with 3% bovine serum albumin-10% normal serum for 1 hour, the sections were incubated with the above-mentioned anti-HO-1 antibody at 1:200 dilution overnight at 4°C. Subsequently, the sections were incubated with Labeled Polymer in an Envision + System HRP Kit (Dako, Tokyo, Japan) at room temperature for 1 hour. The sections were then examined with a Liquid DAB Substrate Chromogen System (Dako, Tokyo, Japan).

2-5. Bradykinin Measurement after intestinal RIPC, compared with hindlimb RIPC

In order to investigate what signals are transduced from the preconditioned intestine into the liver, we measured serum concentration of bradykinin, one of the central humoral mediators in RIPC [ref.1], both in the portal and in systemic blood, according to the manufacturer's protocol (#ADI-900-206, Enzo Life Sciences AG, Switzerland). To assess the advantage of intestinal RIPC compared with hindlimb RIPC that was the only method ever reported to be effective against hepatic IRI, additional rats were exposed to either intestinal or hindlimb RIPC ($n=6$ each). The regimens used here were both 2 cycles of 4-minute clamping and 11-minute relief of either SMA or right femoral artery, respectively.

Statistical Analysis

All results are expressed as means $\pm$ standard error of mean (SEM), unless otherwise indicated. Two-way repeated-measurement analysis of variance (ANOVA) followed by Bonferroni's post-test was used to assess the time-dependent parameters between the groups. Animal survival was assessed by the Kaplan-Meier method followed by a log-rank test. The other comparisons were performed by two-tailed Student's *t*-tests/Mann-Whitney test between the 2 groups, as appropriate. All calculations were performed with Prism Software

Version 5.0c (GraphPad Software Inc., CA, USA). *P*-values less than 0.05 were considered statistically significant.

Results

Serum transaminase release after THI

To determine whether intestinal RIPC could attenuate hepatic IRI, we measured serum transaminase releases at 2, 6, and 24 hours after reperfusion. As shown in Figure-2A, AST was significantly lower in Group-RIPC ($p=0.0005$ between the groups by 2-way repeated measurement ANOVA: time-point assessment, Group-Sham vs. Group-RIPC; 5393.3 ± 1517.7 vs. 1130.7 ± 216.6 IU/L; $p<0.001$ by posttest at 2 hours). Intestinal RIPC significantly reduced ALT (Fig. 2B: $p<0.0001$ between the groups, Posttest at 2 hours; Group-Sham vs.. Group-RIPC; 3378.7 ± 871.1 vs. 873.3 ± 176.4 IU/L; $p<0.001$, Posttest at 6 hours; Group-Sham vs. Group-RIPC; 2245.8 ± 321.0 vs. 426.8 ± 67.9 IU/L; $p<0.01$). Similar data were obtained also in LDH release (Fig. 2C: $p=0.0102$ by 2-way ANOVA, Posttest at 2 hours; Group-Sham vs. Group-RIPC; 29990.2 ± 9838.7 vs. 7981.7 ± 1733.2 IU/L; $p<0.01$).

Serum cytokine release after THI

Serum inflammatory cytokines release of IL-6 (Fig. 3A) and TNF- α (Fig. 3B), and anti-inflammatory mediator, IL-10 (Fig. 3C) were measured at 2, 6 and 24 hours after reperfusion. Intestinal RIPC significantly reduced both IL-6 ($p=0.0347$ by 2-way ANOVA, Posttest at 2 hours; Group-Sham vs. Group-RIPC; 1998 ± 775 vs. 562 ± 117 pg/ μ L; $p<0.05$) and TNF- α ($p<0.0001$, Posttest at 2 hours, Group-Sham vs. Group-RIPC; 930 ± 42 vs. 387 ± 17 pg/ μ L; $p<0.001$). Of note, anti-inflammatory IL-10 elevation was also attenuated by intestinal RIPC compared with Group-Sham ($p<0.0065$, Posttest at 2 hours, Group-Sham vs. Group-RIPC; 11942 ± 3448 vs. 2248 ± 404 pg/ μ L; $p<0.001$)

Tissue cytokine expression after THI

In order to reveal when and where inflammatory cytokines burst was attenuated by intestinal RIPC, we then quantified relative IL-6 (Fig. 4A, 4C) and TNF- α (Fig. 4B, 4D) mRNA expression both in hepatic and in ileal tissues at 2, 6, and 24 hours after reperfusion.

Intestinal RIPC significantly lowered hepatic TNF- α release ($p < 0.0019$, Posttest at 2 hours, Group-Sham vs. Group-RIPC; 7.90 ± 0.67 vs. 1.98 ± 0.64 relative value; $p < 0.001$). Although the standard deviation is too large to reach statistical significance ($p = 0.14$), IL-6 in liver tissue also showed same tendency. In contrast, intestinal RIPC had no influence on both IL-6 ($p = 0.75$) and TNF- α expression ($p = 0.35$) in intestinal tissues. These results indicate that intestinal RIPC significantly alleviates proinflammatory cytokines production in the liver, but failed to attenuate inflammatory response from the gut.

Histopathological Analysis and Tissue Damage Scores

Tissue sections of the liver and the ileum at 2 hours after reperfusion were evaluated histopathologically, and their tissue damages were quantified by using the scoring systems. The ileum in Group-Sham showed mucosal bleeding, denuded villi and loss of villus tissue (Fig. 5A), whereas in Group-RIPC, intestinal mucosa could maintain subepithelial space at villi, and displayed almost normal mucosal construction (Fig. 5B). Thus, Park's injury score in Group-RIPC (Fig. 5C), 2.0 ± 0.2 , was significantly lower than 4.0 ± 0.4 in Group-Sham ($p = 0.0074$). Liver tissues in Group-Sham exhibited cytoplasmic vacuolization, sinusoidal congestion, and massive cellular infiltration (Fig. 5D), while in Group-RIPC, these damages were substantially alleviated (Fig. 5E), reaching to statistical significance in Suzuki's criteria, as summarized in Fig. 5F (3.5 ± 0.2 vs. 0.7 ± 0.5 ; $p = 0.0074$).

Ultrastructural Analysis using Electron Microscopy

Hepatic microstructure at 2 hours after reperfusion was evaluated by means of scanning electron microscopy. In Group-Sham, destroyed and enlarged sinusoidal pores were observed (Fig. 5G), whereas sinusoidal wall structure was well preserved in Group-RIPC (Fig. 5H).

Animal Survival

There were neither postoperative complications nor mortality until sacrifice. None of the rats suffered any problems with digestion during this interval.

In lethal THI for 35 minutes, the 10-day survival in Group-Sham was just 15.4%, and all deaths occurred within 12 hours after reperfusion. In contrast, animal survival was remarkably improved by intestinal RIPC up to 83.1% ($p<0.001$, Fig. 6).

Serum transaminase release after intestinal RIPC

As documented in the supplemental figure, our regimen of intestinal RIPC did not promote liver damage. The highest value of serum ALT was just 70 IU/L at 6 hours of reperfusion, which is thought to be much lower than by direct IPC to the liver.

Chronological analysis of HO-1 induction by intestinal RIPC

The time course of HO-1 mRNA and protein expression in the both tissues was summarized in Fig.-7 and -8, respectively. As shown in Fig. 7A, intestinal RIPC significantly up-regulated HO-1 mRNA in liver tissue, up to 7-folds increase compared with Group-sham, at just 2 hours after the preconditioning. Thereafter, the expression gradually decreased, and returned to the level of the controls by 24 hours. In good agreement with the time-course of mRNA expression, HO-1 protein induction in liver tissue peaked at 6 to 48 hours after RIPC, then returning to the control level by 7 days (Fig.8A). In contrast to the liver, HO-1 mRNA expression in intestinal tissue was relatively low. Although mRNA expression reached statistical significance because of very small standard deviations (Fig.7B), it was not enough to drive significant induction of HO-1 protein to the intestine, as shown in Fig.8B.

The site of HO-1 induction evaluated by immunohistochemistry

To detail the localization of HO-1 induction, we performed immunohistochemical staining of HO-1 of the both tissues at 48 hours after intestinal RIPC. In accordance with the Western-blot, no significant difference in HO-1 staining could be observed in ileal mucosa between the groups (Fig. 9A, 9B), affirming that our regimen of intestinal preconditioning failed to induce HO-1 substantially to intestinal epithelium. Meanwhile in the liver, Kupffer cells were stained in the both groups (Fig. 9C, 9D), reflecting physiological and constitutive

expression of HO-1 therein. However, Kupffer cells' staining in Group-RIPC was more intense than in Group-C. Moreover, it is noteworthy that HO-1-stained granules in hepatocytes spread all over the lobule in Group-RIPC (Fig. 9D), indicating that intestinal RIPC could promote significant induction of HO-1 into hepatocytes.

Signal transduction in intestinal RIPC, compared with hindlimb preconditioning

As shown in Fig.10, intestinal RIPC significantly up-regulated bradykinin release into the portal blood than by hindlimb RIPC ($p<0.05$). In systemic blood, however, no difference of bradykinin concentration was observed between the 2 methods. Taken together, these results suggest that intestinal RIPC promotes the direct inflow of bradykinin signals into the liver through portal blood stream.

Discussion

This is the first report demonstrating that splanchnic organs, such as intestine, could be the target of RIPC to hepatic IRI, as well as that RIPC also exhibits SWOP like IPC, by means of stress proteins induction. In this study, we demonstrated the significant protection of both liver and intestine against total hepatic IRI, as evidenced by a) less hepatocellular damage verified by reduced transaminase release; b) attenuation of proinflammatory cytokines releases of TNF- α , IL-6, and IL-10 after THI; c) better-preserved tissue integrity both of the liver and of the intestine; and d) markedly improved animal survival in a lethal model of THI.

Clinically, hepatic IRI has been shown to correlate with poor outcomes after major hepatectomy and liver transplantation (17). Numerous and various attempts have been made so far, aiming at attenuating liver injury following prolonged periods of vascular occlusion, or of cold storage of liver grafts. Number of studies have demonstrated that HO-1 plays a protective role on liver IRI (18), also in the protection by IPC (19) and RIPC (12, 20). Under the physiological condition, HO-1 in the liver tissue was observed only in the non-parenchymal Kupffer cells (21), and various stressors have been shown to induce HO-1 in hepatocytes (22). Recently HO-1 was reported to ameliorate hepatic IRI by suppressing proinflammatory cytokines release via inactivation of Kupffer cells (23). In the early phase of reperfusion, Kupffer cell activation is thought to occur first, giving rise to endothelial activation and dysfunction. Such responses by Kupffer cells to various stimuli seem to be biphasic, initiated by secretion of pro-inflammatory cytokines such as TNF- α , IFN- β , IL-6, and IL-1, followed by subsequent release of anti-inflammatory mediators, such as IL-10 (24). In the present study, TNF- α and IL-6 were both significantly down-regulated in Group-RIPC, and subsequent up-regulation of IL-10 was also attenuated, representing the inactivation of Kupffer cells by intestinal RIPC. Moreover, intestinal RIPC induced substantial HO-1 into

hepatocytes, which certainly contributed to attenuate parenchymal damage on liver IRI. Taken together, HO-1 up-regulation in Kupffer cells and massive induction of HO-1 in hepatocytes cooperate with each other, thus leading to alleviation of liver IRI.

As a consequence of intestinal ischemic preconditioning, intestinal damages after THI were remarkably attenuated, despite HO-1 protein induction in intestinal tissue was not significant. In the meanwhile, there have been several reports demonstrating that intestinal IPC induces HO-1 in intestinal tissue and mucosa (20, 25). This contradiction of HO-1 induction may be due to the difference of the protocols used for preconditioning. Most of previous studies using SMA clamping adopted the protocol of 15-minute ischemia followed by 5 to 30 minutes of reperfusion (26), which was longer ischemic insult compared with the current study. Liver anatomically locates just the down-stream of intestine in portal circulation. In THI, portal triad clamping results in congestion of the splanchnic organs, which, in turn, damages up-stream organs including the gut. As documented in Fig.5, livers in Group-Sham were more damaged by IRI with a loss of sinusoidal endothelial cell integrity and subsequent disruption of the microcirculatory blood flow (27). Regarding the fact that impaired ileal mucosa after THI results from the disturbance of portal circulation, maintenance of the tissue integrity of hepatic microstructures in Group-RIPC certainly contributes to attenuate intestinal damages.

As for the regimen of intestinal RIPC, we adopted 2 cycles of 4-minute SMA clamping separated by 11-minute reperfusion, because of the following 3 reasons:

1. Repeated ischemia has been proven to induce stronger protective effect than single ischemia (28). As a preliminary study, we tested 3 patterns of intestinal RIPC against liver IRI: 2 cycles of 2-, 4-, and 6-minute SMA clamping. Liver injury tended to be more alleviated by 4-minute clamping regimen than by the others (data not shown).

2. We would like to avoid the direct ischemic insult to the liver occurred secondary to the reduction in portal perfusion by longer SMA clamping. As a preliminary observation, we measured the alteration of hepatic and intestinal perfusion during intestinal RIPC, by using laser Doppler flowmetry. By SMA clamping, intestinal (ileal) perfusion immediately fell down to 10-15% of pre-clamping value, however, total hepatic perfusion was maintained over 75-80% of pre-clamping value (not significant reduction). This phenomenon seems to be attributed to a well-known endogenous response, hepatic arterial buffer response (HABR), which immediately functions to maintain total hepatic blood flow by increasing arterial flow when portal perfusion decreases.

3. We think RIPC to the liver *per se* should not be harmful to the liver. As shown in the supplemental data, our regimen of intestinal RIPC did not promote liver damage. The highest value of serum ALT after the preconditioning was just 70 IU/L at 6 hours of reperfusion, which is thought to be much lower than by direct IPC to the liver.

There have been a few reports describing RIPC against liver IRI, but all of which adopt the hindlimb ischemia as the site for RIPC (11, 12). Advantage of our regimen is the site exposed to preceding ischemia and shorter ischemic stress. Although the precise mechanisms have not been fully elucidated, by which the stimuli are transmitted from the preconditioned tissue to the target organs, both neural and humoral pathways are thought to be involved (29). Though ischemic periods shorter than 5 minutes or longer than 15 minutes are reported to fail to promote protection (30-32), our protocol strongly protects the liver as well as the intestine from THI-induced tissue damage. As a reason for this, we have revealed that our regimen of intestinal RIPC significantly amplified the bradykinin signal directly into the hepatic inflow, one of the central humoral mediators in RIPC [ref.1]. This, in turn, undoubtedly promotes endothelial NOS (nitric oxide synthase) activation, provoking NO burst into the liver, thus leading to the protein kinase-C activation and subsequent expression

of stress response. It is also noteworthy that the bradykinin signal in systemic blood was not different between these 2 different methods. In view of the fact that the gut is anatomically situated in immediate up-stream to the liver in portal circulation, even a little signal/stimulus certainly affect to just the down-stream organ, liver.

Our results indicate that the protective effects by intestinal RIPC are presumably related to HO-1 induction. Numerous animal studies describing HO-1 administer zinc protoporphyrin-9 (ZnPP), to inhibit HO-1 activity and to prove the contribution of HO-1(20). However, ZnPP abolish not only induced HO-1 but also constitutively expressed HO-2 in hepatocytes. Hepatocellular HO-2 plays a pivotal role to lower vascular resistance of hepatic parenchyma, and to maintain sinusoidal circulation physiologically (21, 33, 34). Because ZnPP is just a competitive inhibitor to all isoforms of HO, we assume the administration of ZnPP might disrupt the physiological condition of the sinusoidal, portal circulation (33, 34).

In conclusion, RIPC produced by repeated brief intestinal ischemia exerts substantial protection not only against liver IRI but also to the intestinal damage due to THI. The RIPC protocol of 2 cycles of 4-minute intestinal ischemia separated by 11-minute reperfusion induces substantial amount of HO-1 to the liver, thereby confers the significant protection as SWOP in RIPC. Although methods to identify and modify these protective pathways are areas of intense investigation, further studies extrapolating the mechanisms of RIPC between the liver and the intestine will be required.

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Legends for Figures:

Figure 1. (A) Experimental protocol for studying the effect of intestinal remote ischemic preconditioning (RIPC) against total hepatic ischemia (THI). Rats were subjected either to sham operation or to intestinal RIPC, consisting of 2 cycles of 4 minutes of superior mesenteric arterial clamping separated by 11 minutes of declamping/reperfusion.

(B) Experimental protocol for evaluating heme oxygenase-1 (HO-1) induction by intestinal RIPC. Rats were subjected either to sham operation or to intestinal RIPC without hepatic ischemia.

In the both protocols, blood, liver and ileal tissue samples were obtained at indicated time-points.

Figure 2. Serum (A) aspartate aminotransferase (AST), (B) alanine aminotransferase (ALT), and (C) lactate dehydrogenase (LDH) release after THI in sham operated rats (Group-Sham, $n = 6$) and in intestinal RIPC rats (Group-RIPC, $n = 6$). All data are presented as mean $\pm$ standard error of the mean (SEM). Two-way analysis of variance (2-way ANOVA) was used to assess intergroup difference, followed by Bonferroni's post-test, if appropriate, to analyze the difference at each time-point. *: $p < 0.001$, †: $p < 0.01$, and ‡: $p < 0.05$ vs. Group-Sham in post-test, respectively.

Figure 3. Serum (A) interleukin-6 (IL-6), (B) tumor necrosis factor-alpha (TNF- α), and (C) interleukin-10 (IL-10) release after total hepatic ischemia in Group-Sham, and in Group-RIPC, ($n = 6$ each). All data represent as mean $\pm$ SEM. Two-way ANOVA was used to assess intergroup difference, followed by Bonferroni's post-test, if appropriate, to analyze the difference at each time-point. *: $p < 0.001$, †: $p < 0.01$, and ‡: $p < 0.05$ vs. Group-Sham in post-test, respectively.

Figure 4. Chronological analysis of relative interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) mRNA expression in liver tissues (Fig-4A and -4B, respectively), and in ileal tissues (Fig-4C and - 4D, respectively) after THI in Group-Sham and in Group-RIPC ($n = 6$ each). All data are presented as mean $\pm$ SEM, analyzed by 2-way ANOVA, followed by Bonferroni's post-test. *: $p < 0.001$, †: $p < 0.01$, and ‡: $p < 0.05$ vs. Group-Sham in post-test, respectively.

Figure 5. Representative tissue sections of the ileum (A and B) and the hepatic acini (D and E) after 2 hours of reperfusion, stained with hematoxylin and eosin. Intestinal and hepatic damage after THI were quantified by using Park's (Fig 5C) and Suzuki's (Fig 5F) scoring system, respectively ($n = 6$ each), represented by box-and-whiskers plot. The boxes represent the 25% to 75%, the whiskers represent the data range, and the lines in the box are given as the median values of the distribution. †: $p < 0.01$ vs. Group-Sham. Black bars indicate 100 μm .

Photomicrographs of the representative tissue sections by scanning electron microscopy, displaying the sinusoidal wall structures in Group-Sham (Fig. 5G) and Group-RIPC (Fig. 5H). The original magnification was $\times 8,000$.

Figure 6. Animal survival in the Group-Sham (gray line) and Group-RIPC (black line) in the prolonged ischemic time of 35-minute THI ($n = 13$ each). The probabilities of survival in Group-RIPC and Group-Sham were 83.1% and 15.4%, respectively. *: $p < 0.001$ vs. Group-Sham.

Figure 7. Chronological analysis of heme oxygenase-1 (HO-1) gene expression in liver tissues (Fig. 7A) and in ileal tissues (Fig. 7B) after intestinal ischemic preconditioning (Group-RIPC) or sham operation (Group-Sham) by Real-Time Polymerase Chain Reaction ($n = 6$ each). β -actin was used as internal control. All data are presented as mean $\pm$ SEM, analyzed by 2-way ANOVA, followed by Bonferroni's posttest. *: $p < 0.001$, †: $p < 0.01$, and ‡: $p < 0.05$ vs. Group-Sham in posttests, respectively.

Figure 8. Chronological analysis of heme oxygenase-1 (HO-1) protein induction in liver tissues (Fig. 8A) and in ileal tissues (Fig. 8B) after intestinal ischemic preconditioning (Group-RIPC) and sham operation (Group-Sham) by Western Bolts ($n = 6$ each). β -actin was used as internal control. All data represent mean $\pm$ SEM, analyzed by 2-way ANOVA, followed by Bonferroni's posttest. *: $p < 0.001$, †: $p < 0.01$, and ‡: $p < 0.05$ vs. Group-Sham in posttests, respectively.

Figure 9. Immunohistochemistry of HO-1 in ileal (A, B) and liver (C, D) tissues in Group-Sham and Group-RIPC. Arrowheads (C, D) show HO-1 stained Kupffer cells and arrows (D) point HO-1 stained granules in hepatocytes. Black bars show length of 25 μ m.

Figure 10. Serum concentration of bradykinin both in portal and in systemic blood after either intestinal or hindlimb RIPC ($n=6$ each). The preconditioning regimens used here were both 2 cycles of 4-minute clamping, separated by 11-minute relief of either SMA or right femoral artery, respectively. ‡: $p < 0.05$ vs. Systemic in intestinal RIPC, and Portal in hindlimb RIPC by 1-way ANOVA, respectively.

Figure 1

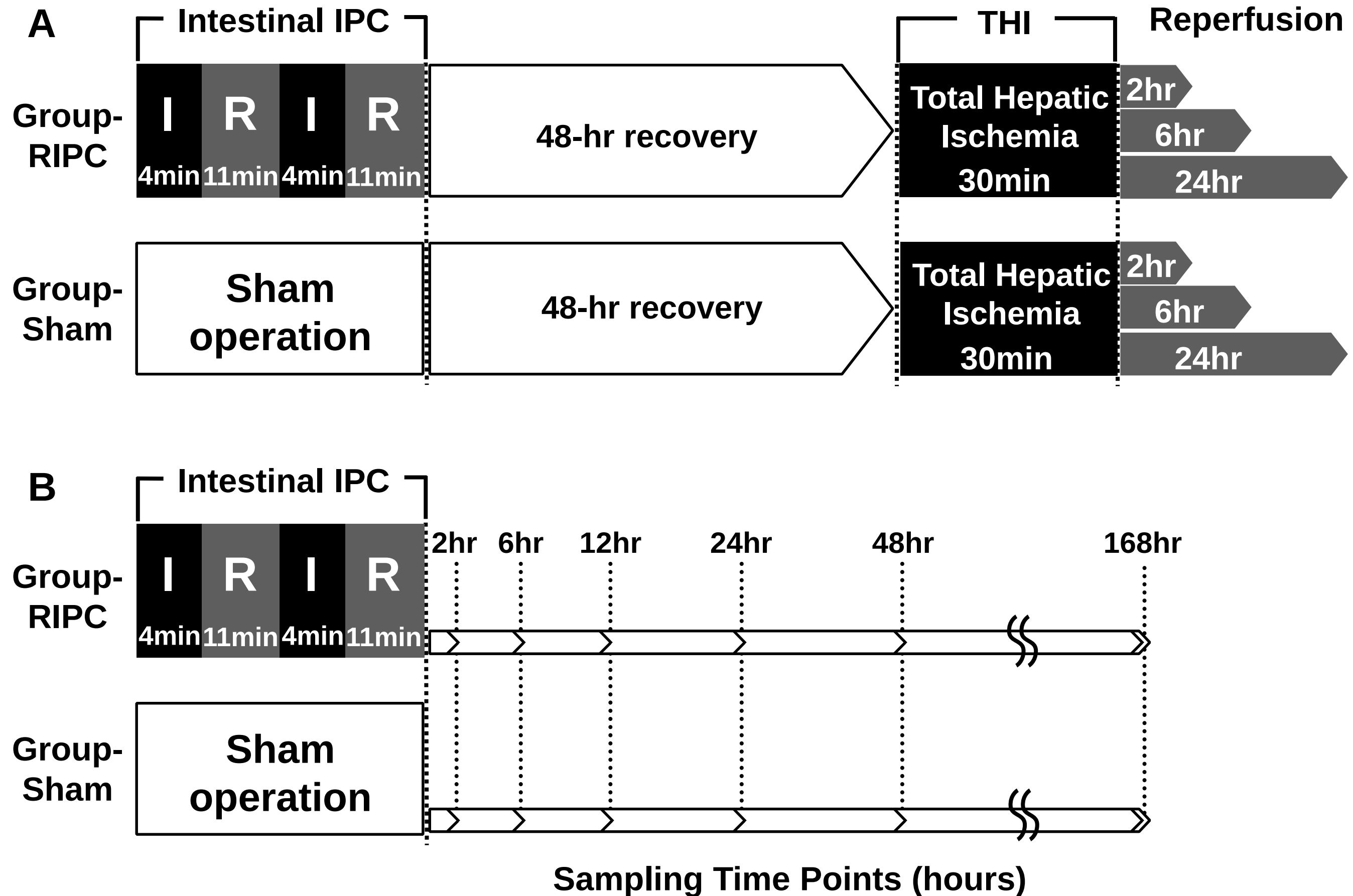


Figure 2

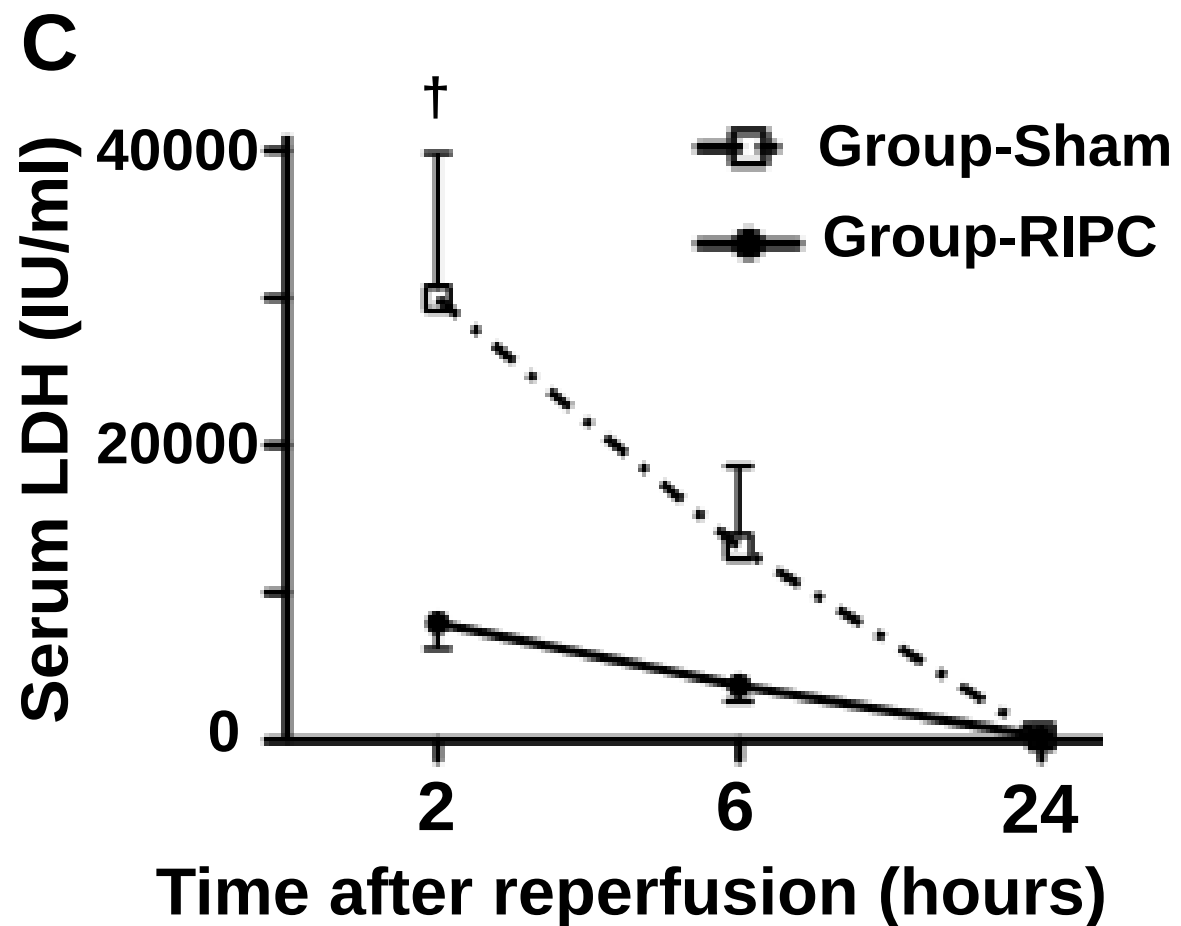
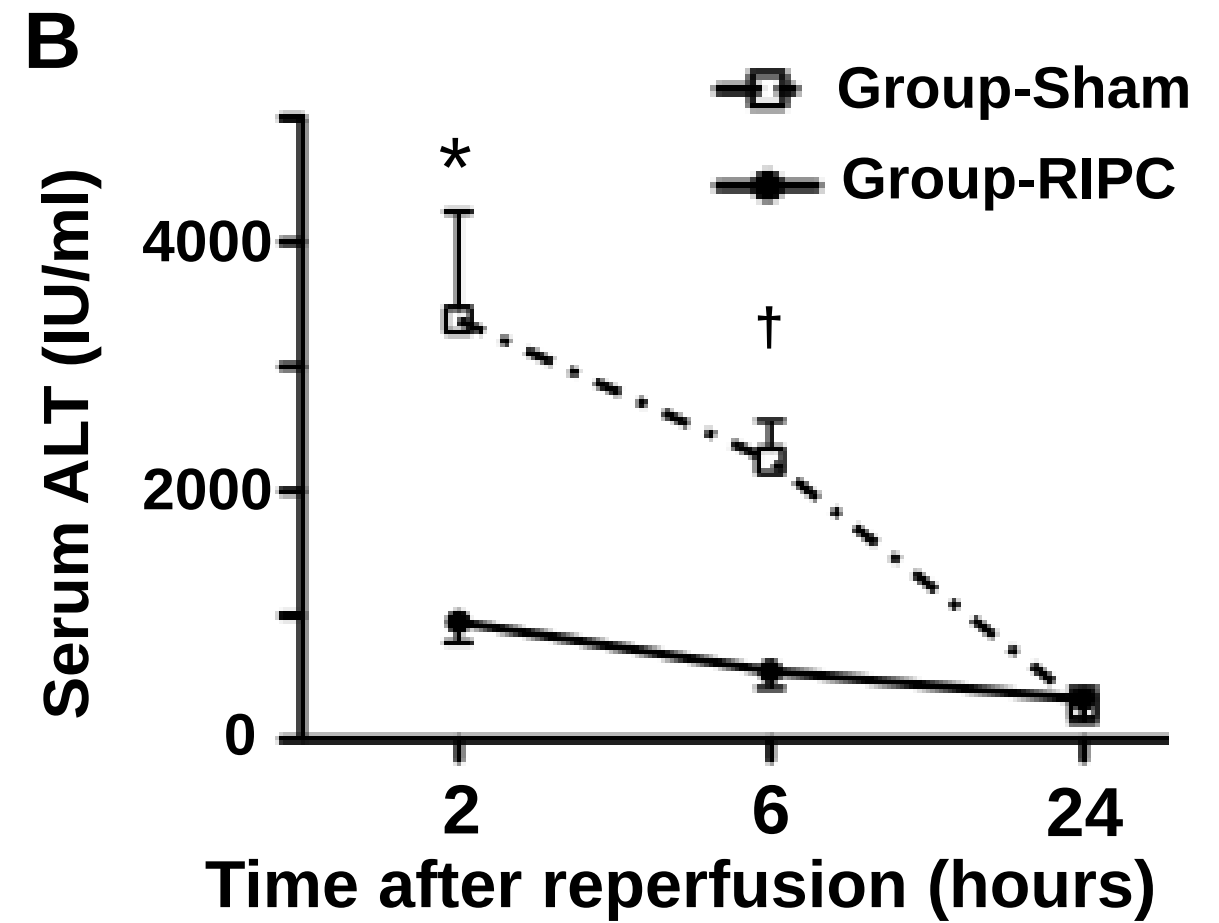
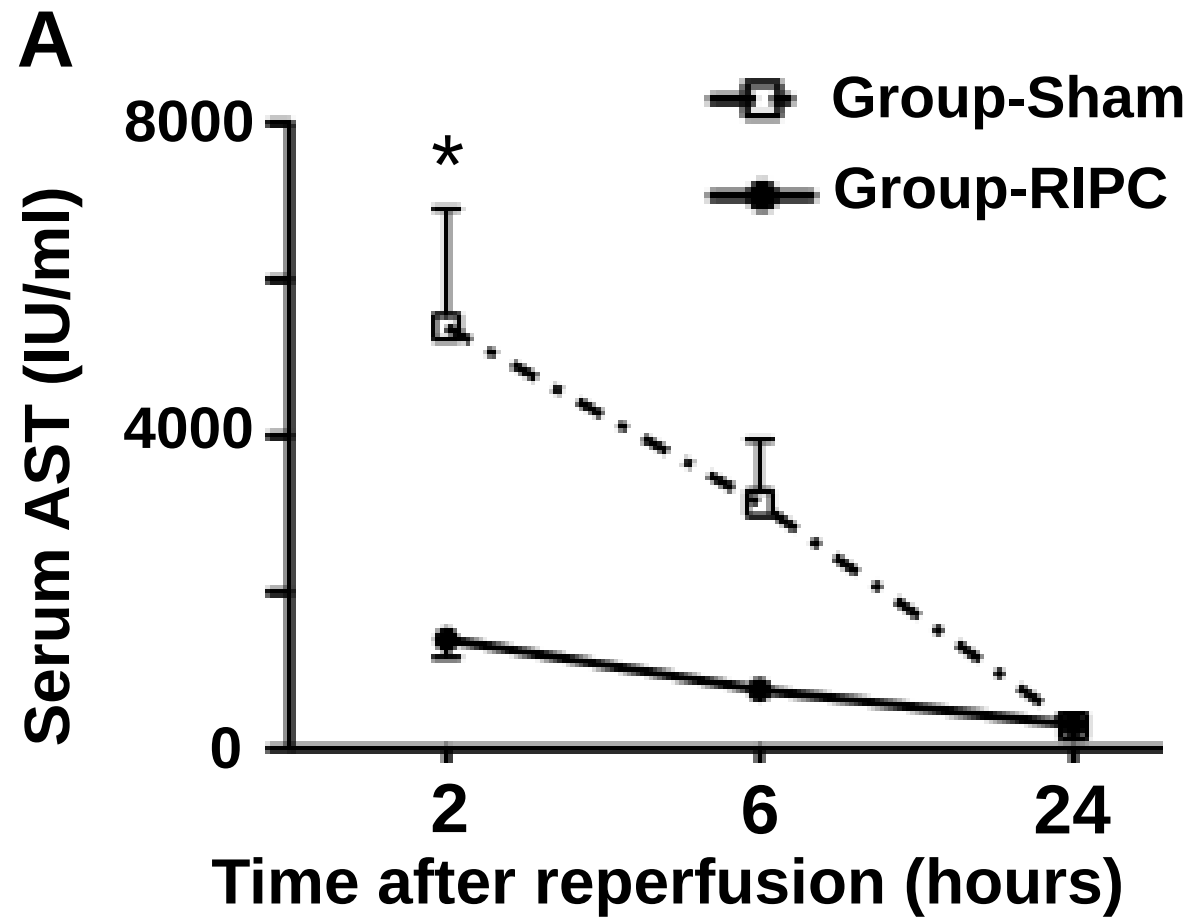
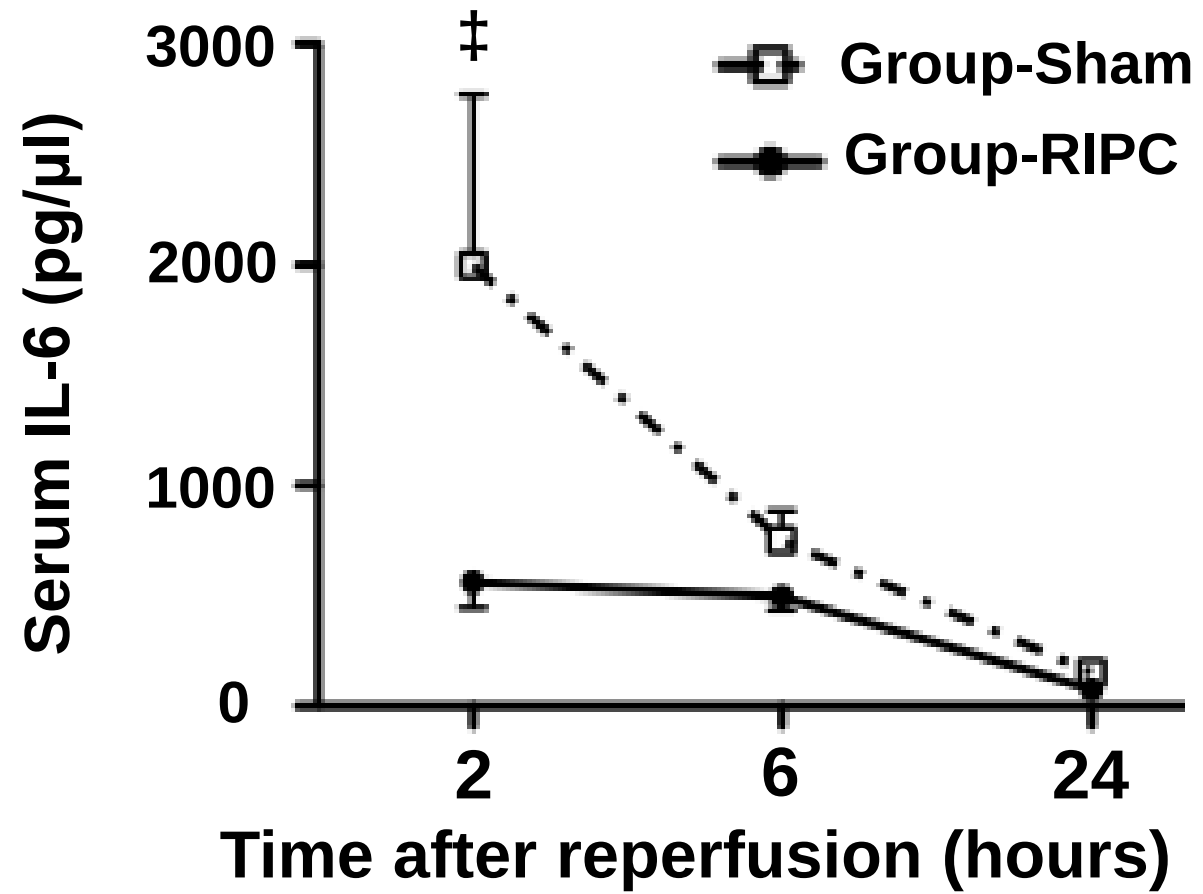
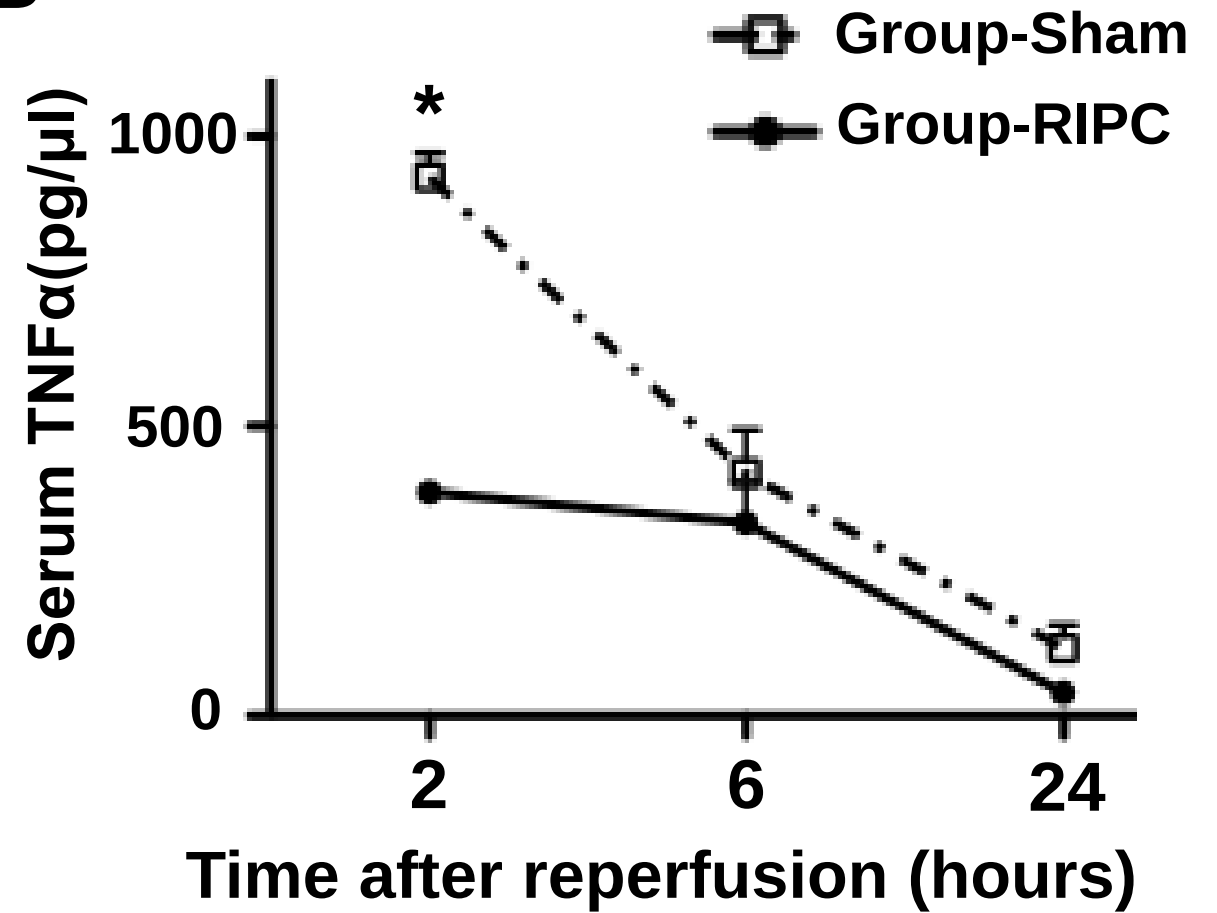


Figure 3

A



B



C

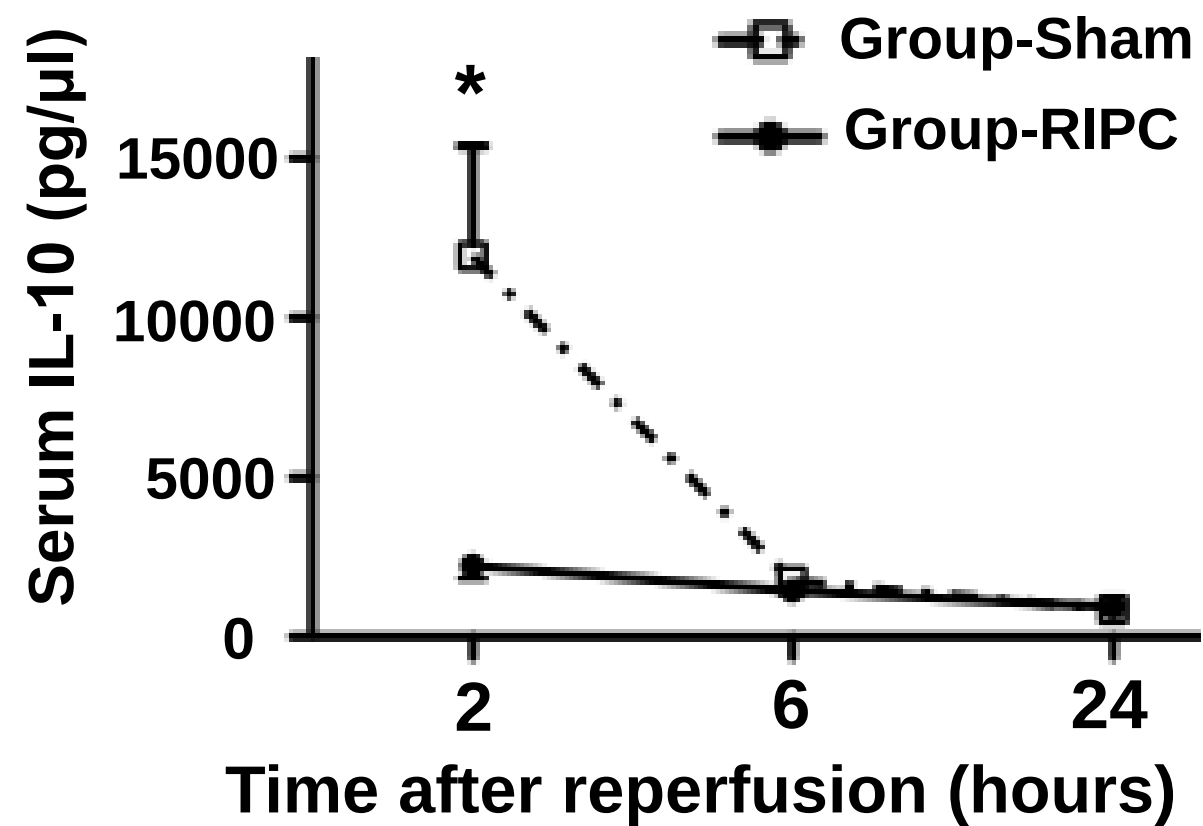


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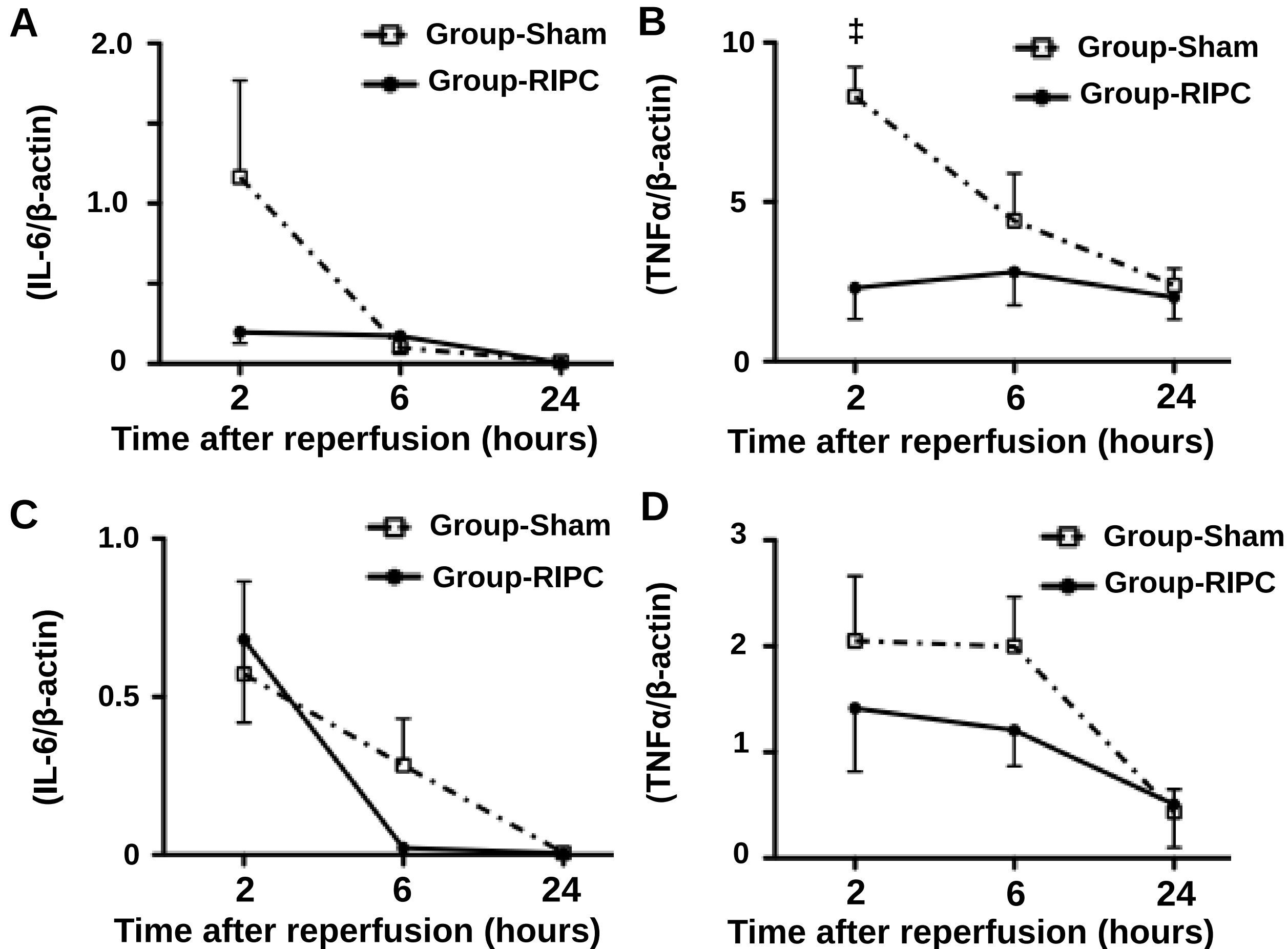
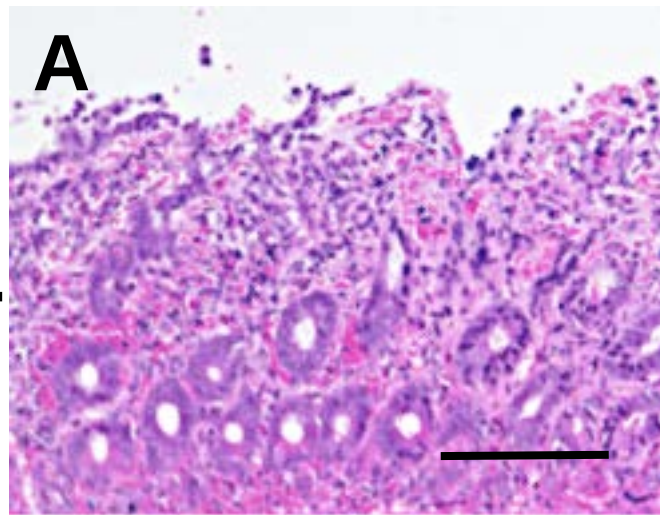


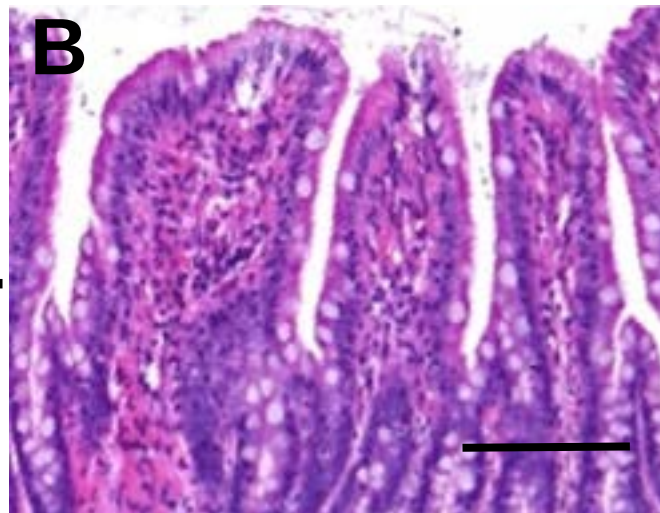
Figure 5

Ileum

Group-Sham

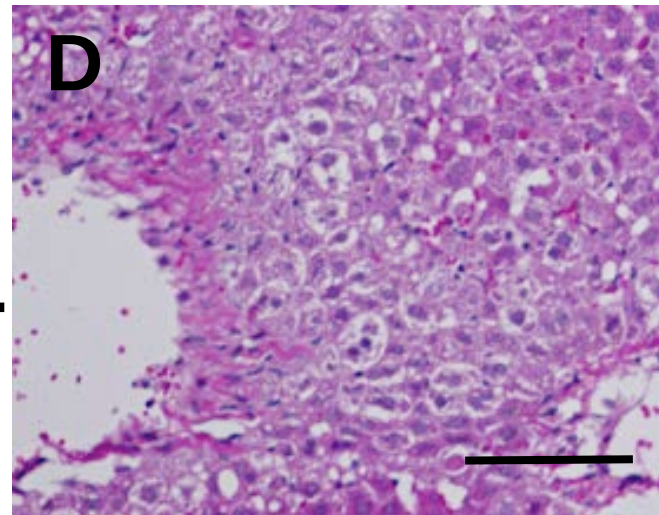


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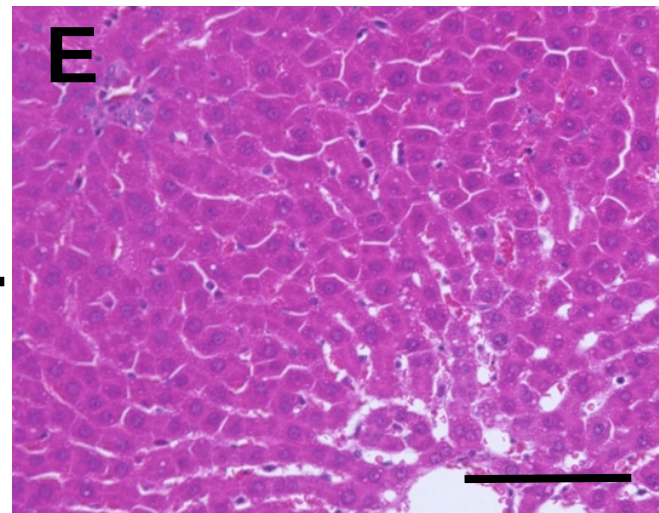


Liver

Group-Sham

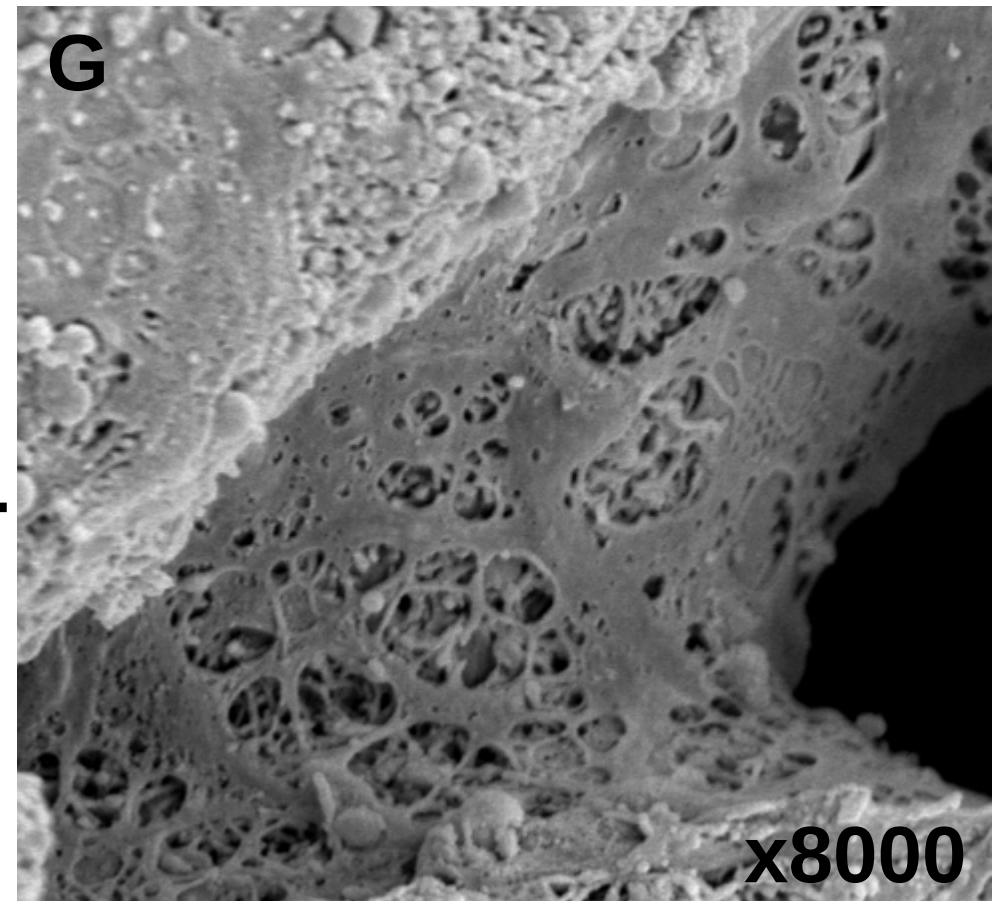


Group-RIPC



SEM

Group-Sham



Group-RIPC

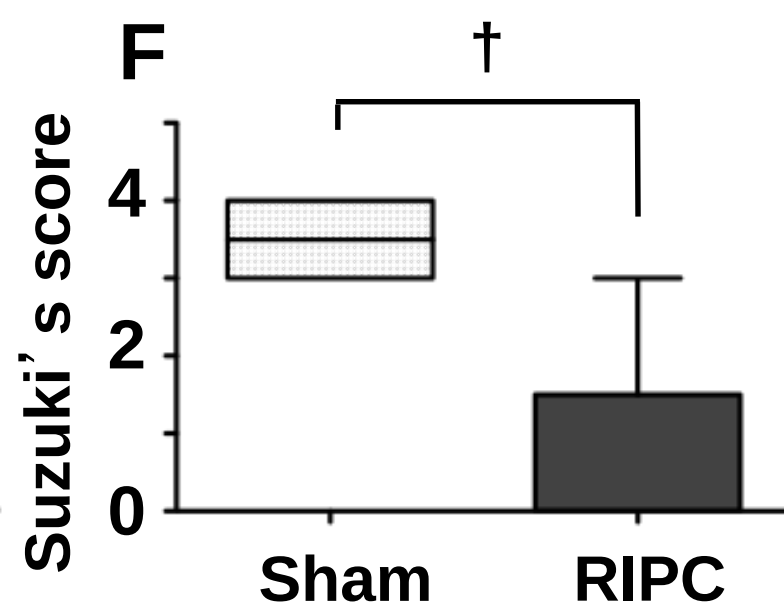
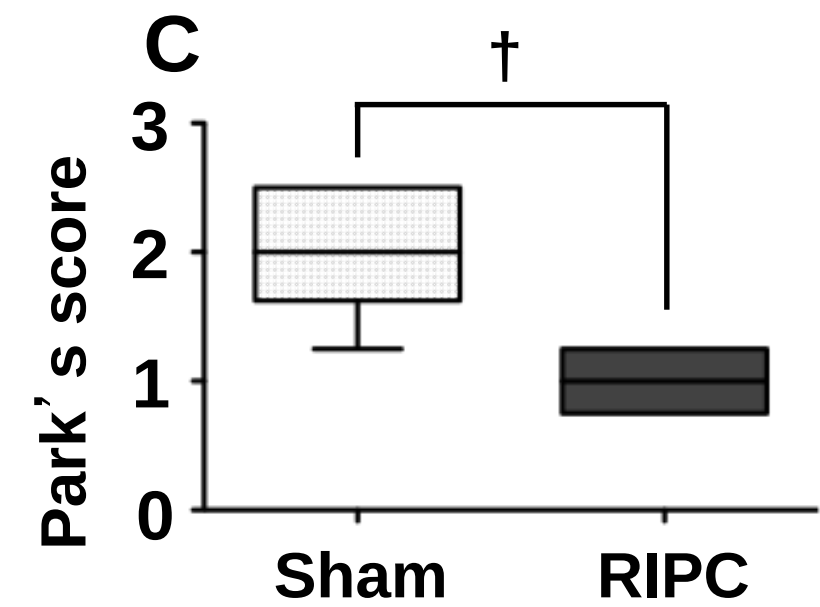
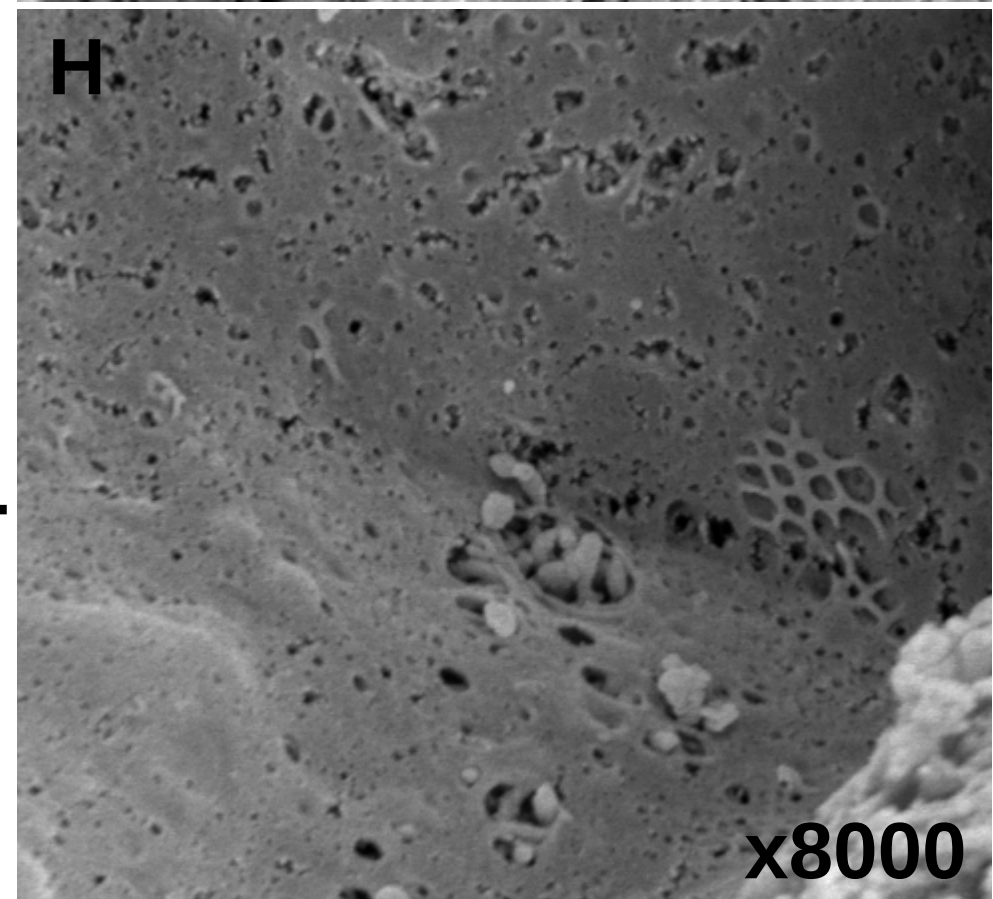


Figure 6

Animal Survival

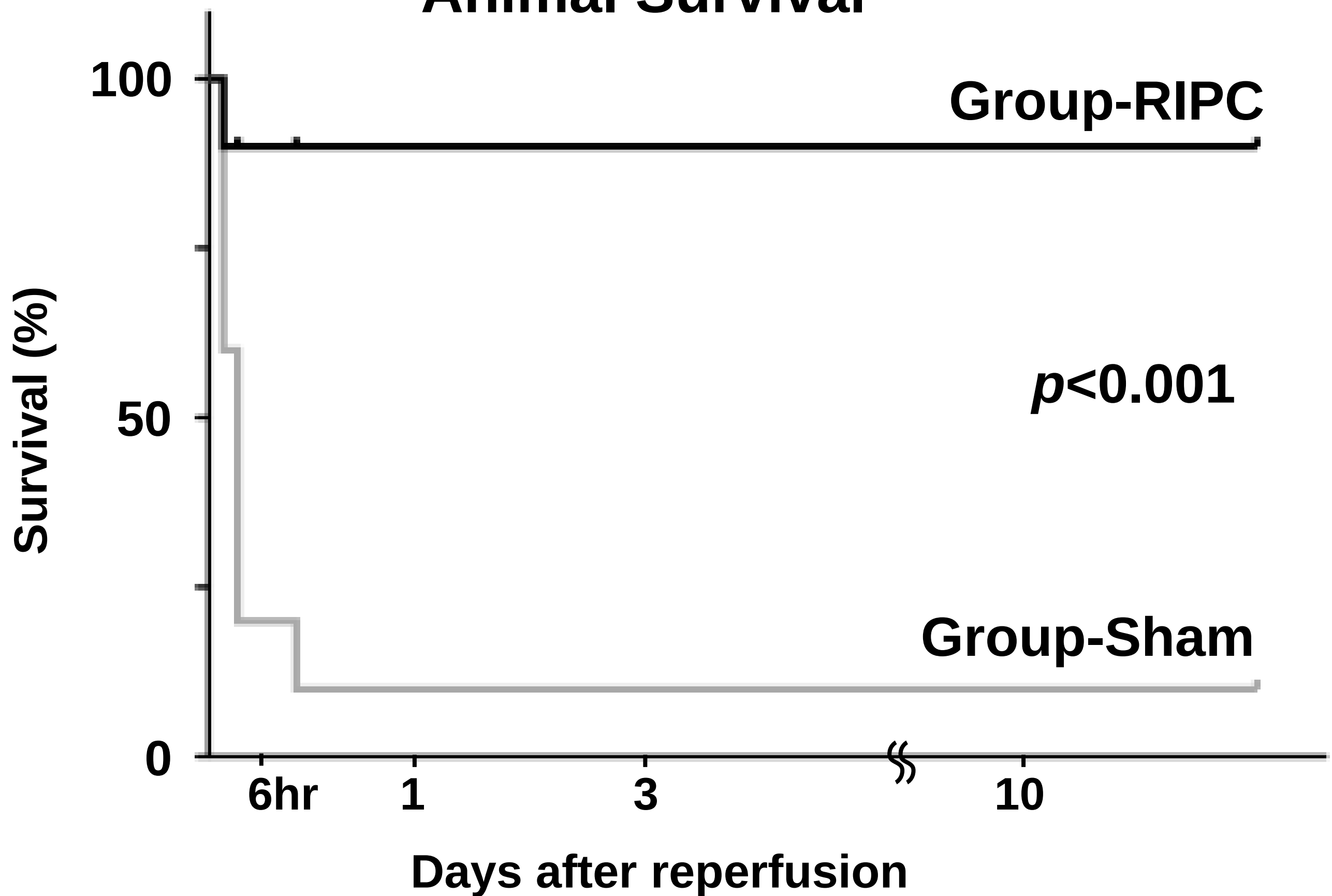


Figure 7

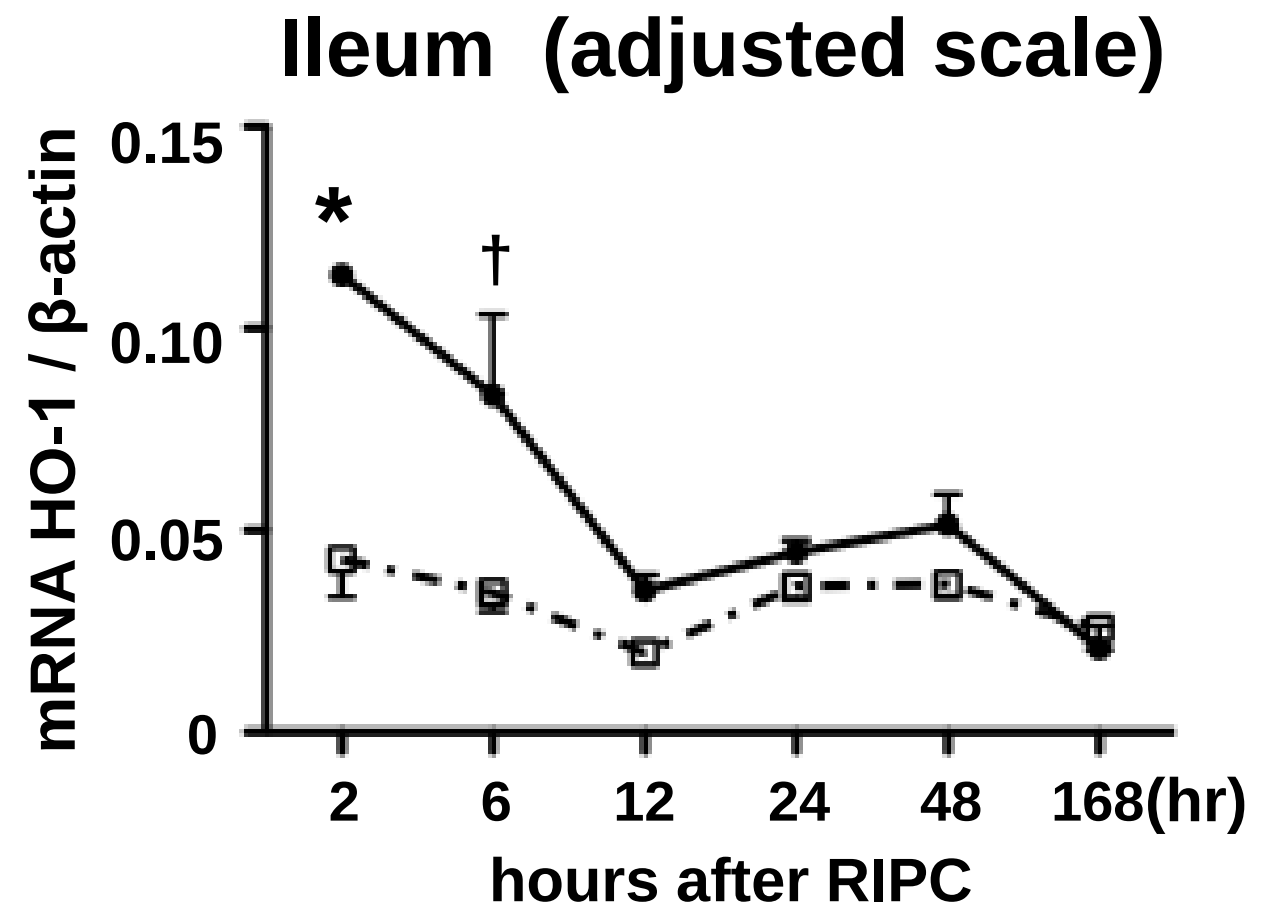
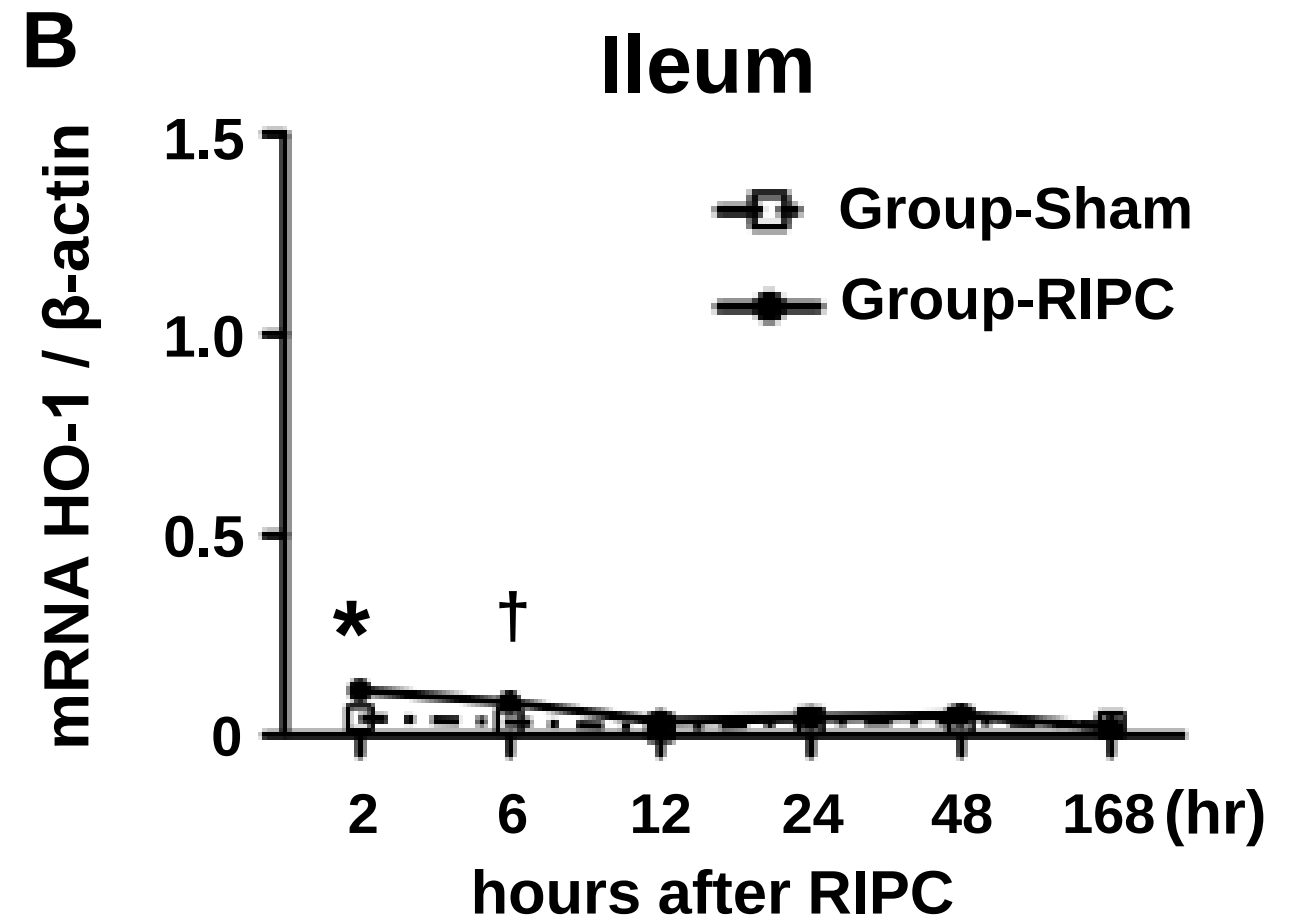
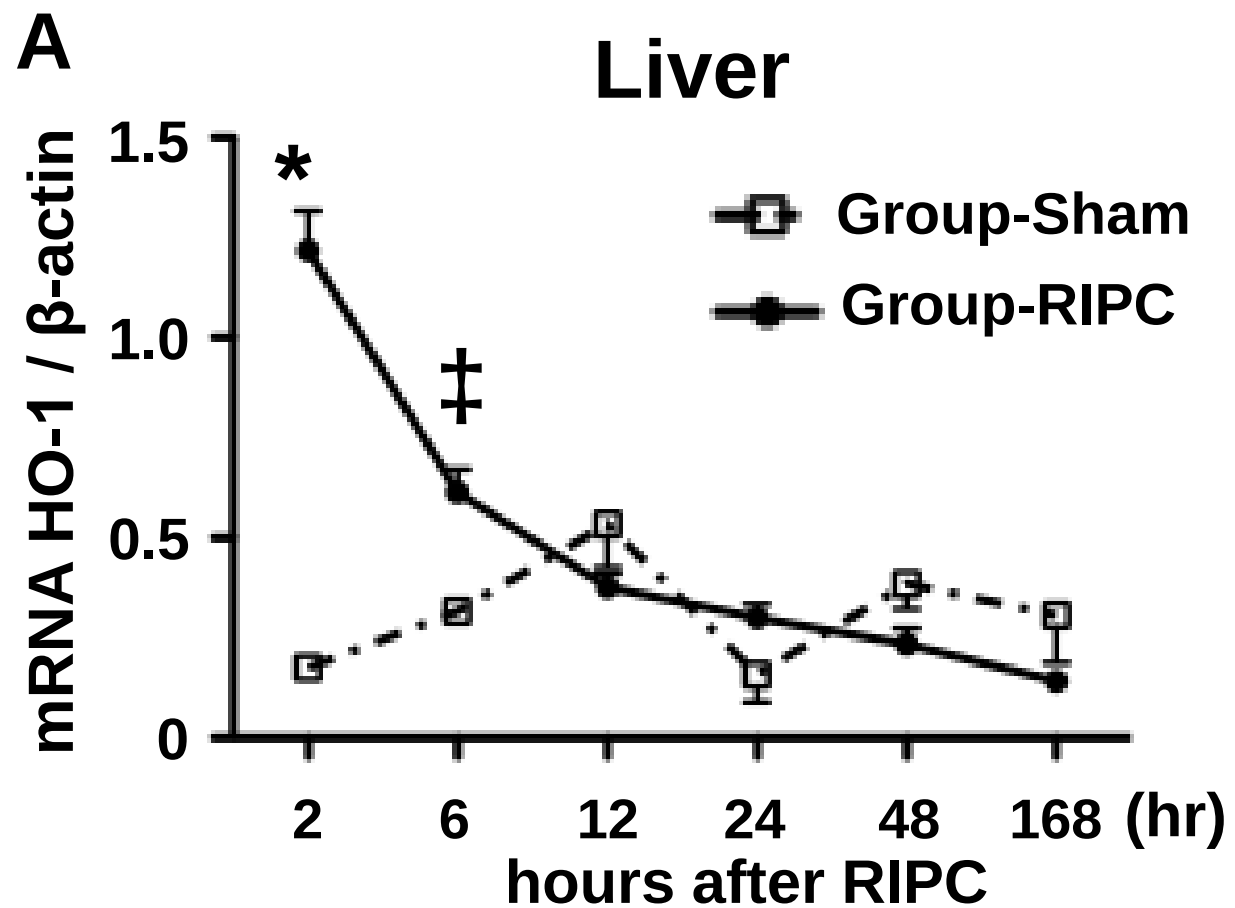


Figure 8

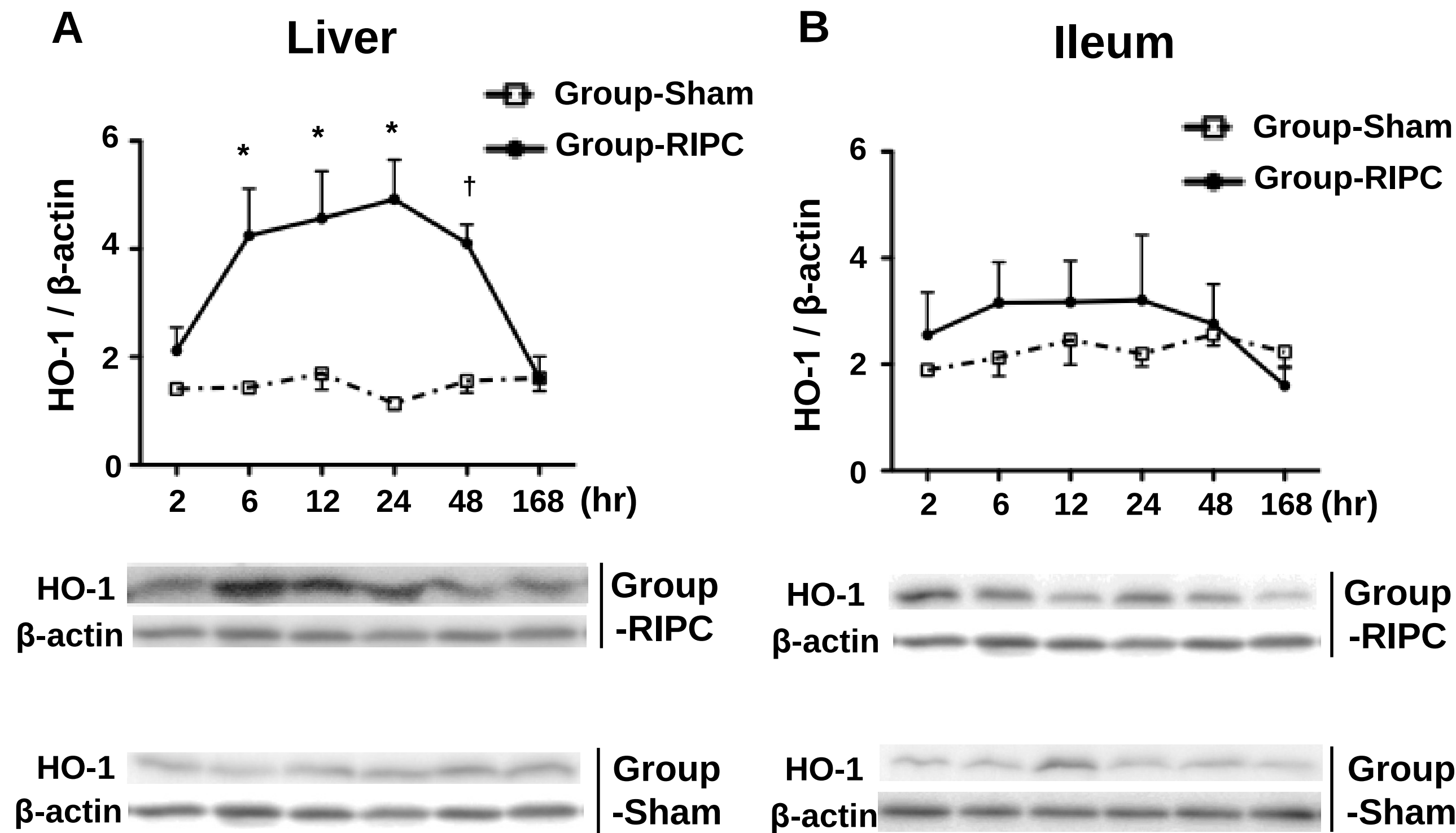
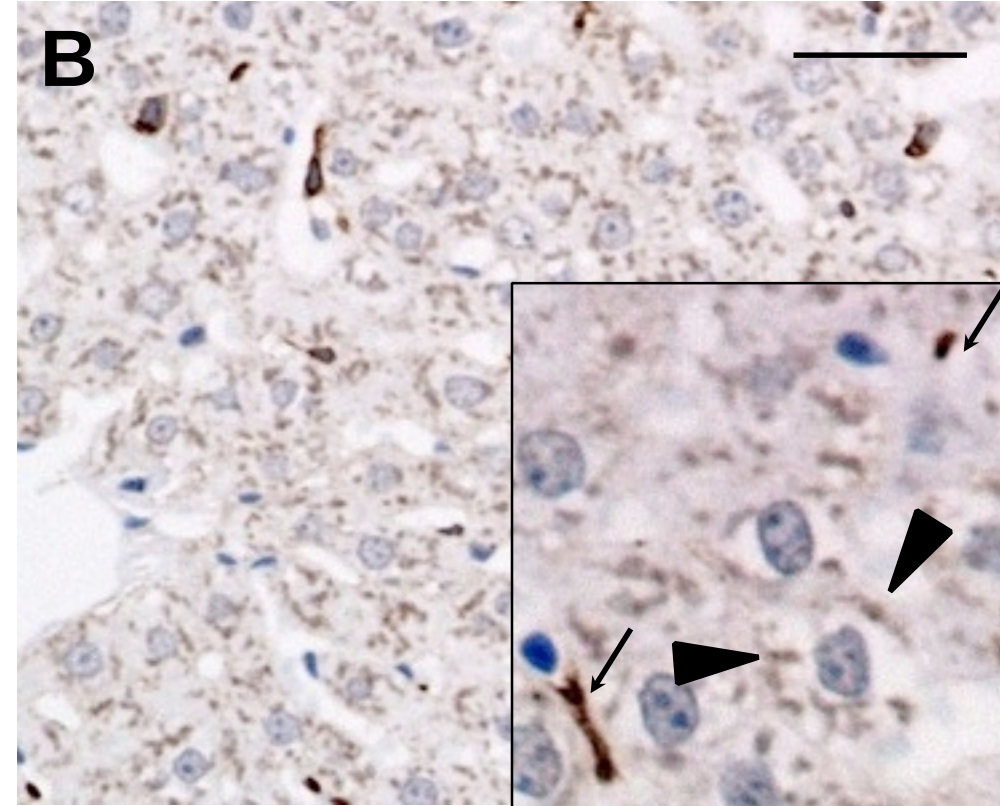
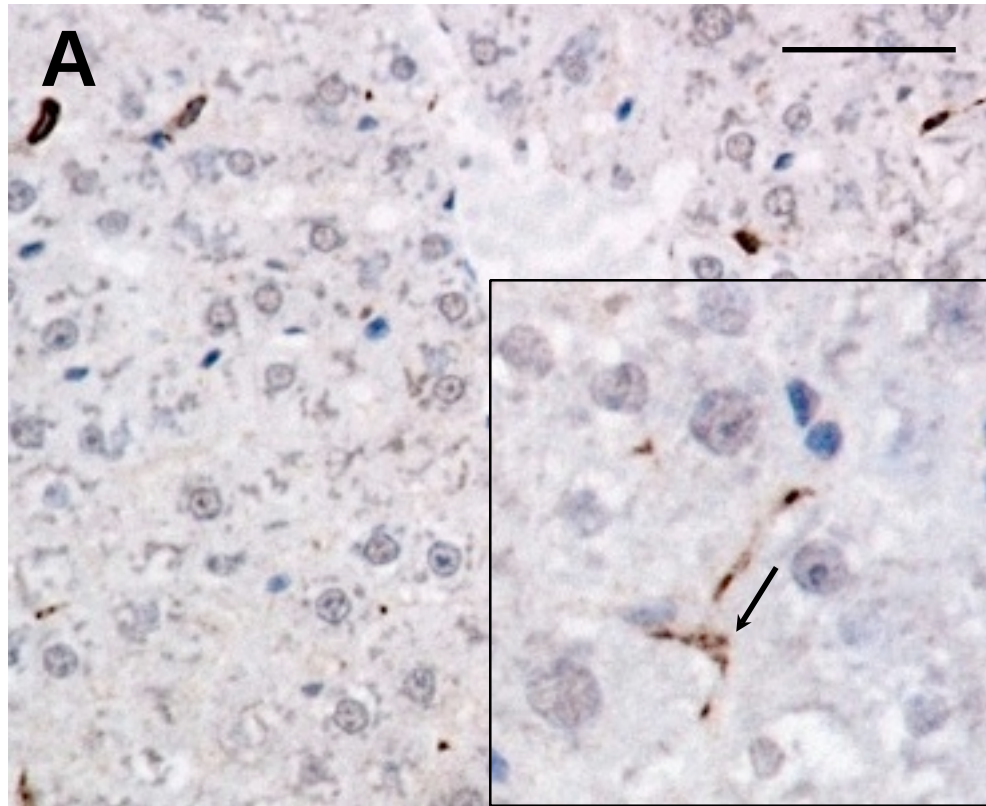


Figure 9

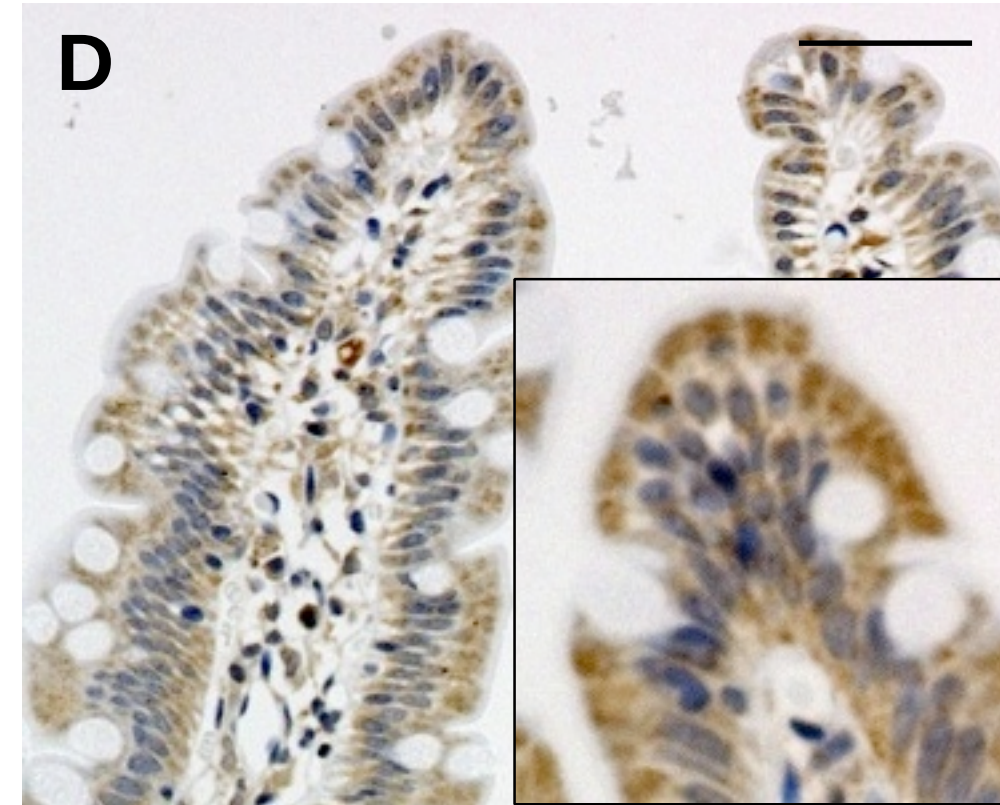
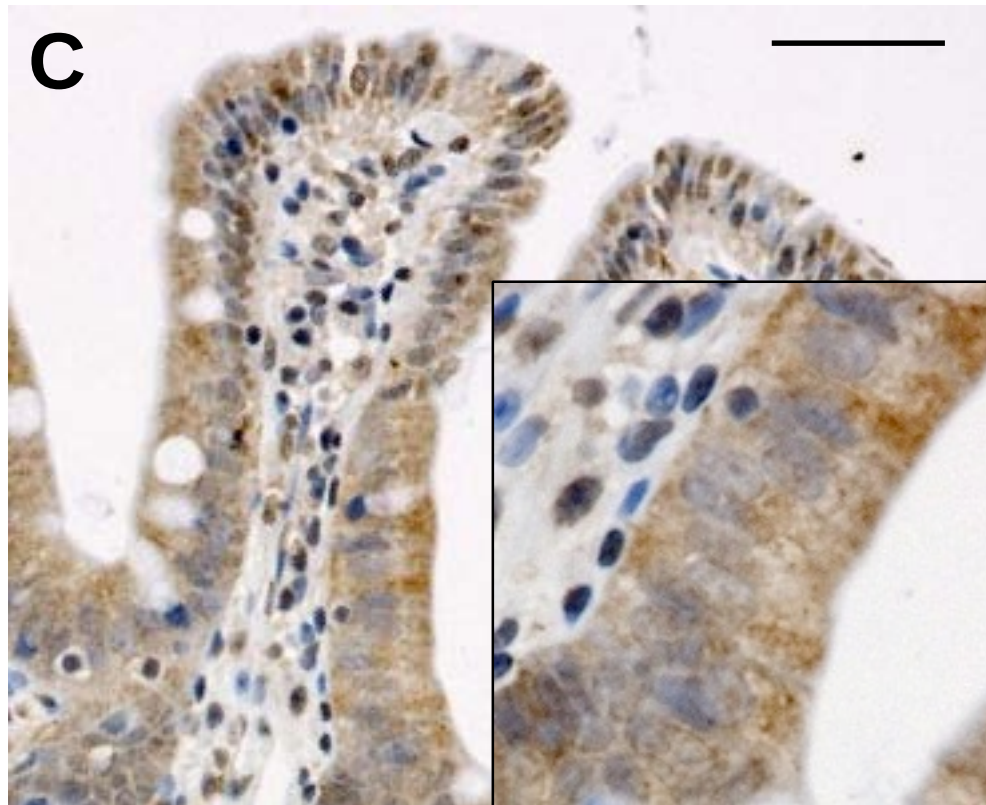
Group-Sham

Group-RIPC

Liver

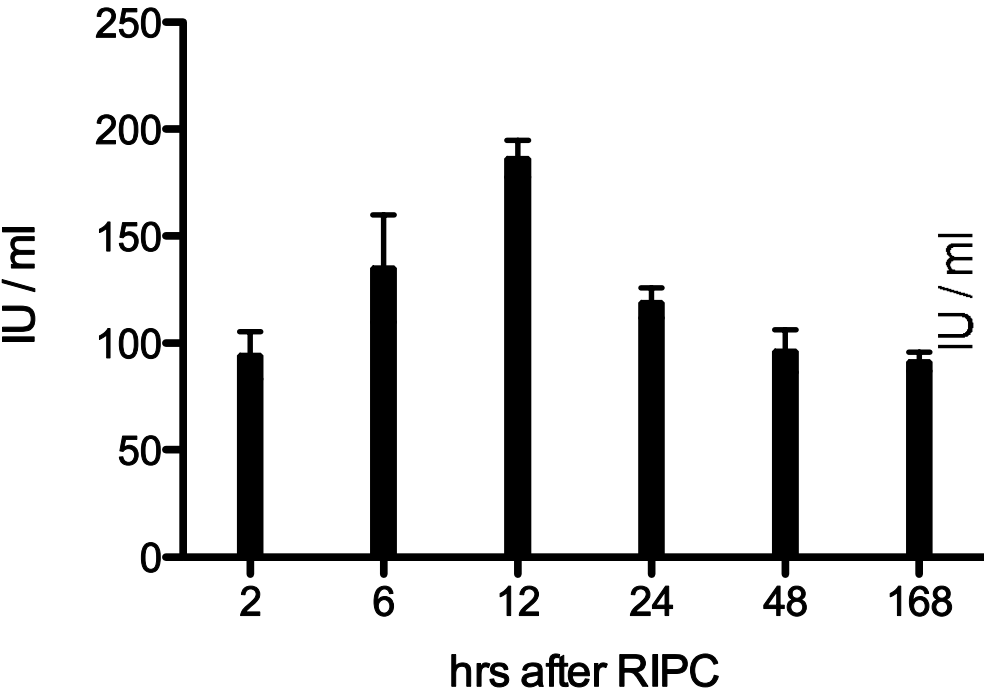


Ileum



Supporting Information

AST



ALT

