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Title:

Oral administration of polyamines ameliorates liver ischemia-reperfusion injury and promotes liver regeneration in rats.

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FOOTNOTES

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Abbreviations:

ALT, alanine aminotransferase; ATP, adenosine triphosphate, AST, aspartate aminotransferase; CXCL, chemokine(C-X-C motif) ligand; DNA, deoxyribonucleic acid; IL, interleukin; INF, interferon; IRI, ischemia-reperfusion injury; IVC, inferior vena cava, LDLT, living donor liver transplantation; LT, liver transplantation; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; PCNA, proliferating cell nuclear antigen; PFA, paraformaldehyde; POD, postoperative day; PV, portal vein; RANTES, regulated on activation normal T cell expressed and secreted; Rb, retinoblastoma protein; RECA, rat endothelial cell antigen; RNA, ribonucleic acid; SD, standard deviation; SEM, scanning electron microscopy; SPD, spermidine; SPM, spermine; TEM, transmission electron microscopy; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling; VEGF, vascular endothelial growth factor

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Key words: Polyamine; Oral administration; Ischemia-reperfusion injury; Liver regeneration; Liver transplantation

ABSTRACT

Introduction: Polyamines are essential for cell growth and differentiation. They play important roles in protection from liver damage and promotion of liver regeneration. However, little is known about the effect of oral exogenous polyamine administration on liver damage and regeneration. This study investigated the impact of polyamines (spermidine and spermine) on ischemia-reperfusion injury (IRI) and liver regeneration.

Material and Methods: We used a rat model in which 70% hepatectomy after 40 minutes of ischemia was performed to mimic the clinical condition of living donor partial liver transplantation. Male Lewis rats were separated into two groups: a polyamine group given polyamines before and after operation as treatment, and a vehicle group given distilled water as placebo.

Results: The levels of serum aspartate aminotransferase and alanine aminotransferase at 6 h, 24 h and 48 h after reperfusion were significantly lower in the polyamine group compared with those in the vehicle group. Polyamine treatment reduced the expression of several proinflammatory cytokines and chemokines at 6 h after reperfusion. Histological analysis showed significantly less necrosis and apoptosis in the polyamine group at 6 h after reperfusion. Sinusoidal endothelial cells were also well preserved in the polyamine group. In addition, the regeneration of the remnant liver at 24 h, 48 h, and 168 h after reperfusion was significantly accelerated, and the Ki-67 labeling index and the expressions of PCNA and phosphorylated Rb at 24 h after reperfusion were significantly higher in the polyamine group compared with those in the vehicle group.

Conclusion: Perioperative oral polyamine administration attenuates liver IRI and promotes liver regeneration. It might be a new therapeutic option to improve the outcomes of partial liver transplantation.

(273 words)

INTRODUCTION

Liver transplantation (LT) is one of the most effective treatments for patients with end-stage liver disease and acute liver failure, and has been widely accepted (1) (2). However, the shortage of donor organs is still a major problem for LT because of a limited pool of donors. Due to the donor organ shortage, living donor LT (LDLT) is a possible alternative, and shows the most promising outcomes (3) (4) (5). In LT, the liver graft is damaged during cold and warm ischemia time and blood reperfusion thereafter; this damage is called ischemia-reperfusion injury (IRI). Moreover, in LDLT, the first priority is donor safety, and in the clinical setting, the minimum-sized liver grafts as small as possible are selected (6). In adult-to-adult LDLT, size mismatch between graft and recipient sometimes leads to primary graft non-function or dysfunction, a critical problem called ‘small-for-size syndrome’, which occurs as a result of liver graft IRI and secondary liver tissue regeneration impairment (7) (8). Therefore, the effective treatments to reduce hepatic IRI and accelerate liver regeneration are crucial and offer major benefits to improve the outcomes after LDLT.

Polyamines [putrescine, spermidine (SPD), and spermine (SPM)] are aliphatic cations derived from ornithine (9) and essential for cell growth. These molecules play important roles in the stabilization of deoxyribonucleic acid (DNA) structure, regulation of gene expression, translation, protein synthesis, and signal transduction, as well as modulation of cell growth and cell differentiation (9-11). Several previous studies have demonstrated that the polyamines suppressed inflammatory mediators, such as proinflammatory cytokines and chemokines (12) (13). Polyamine depletion was found to be related to the suppression of liver regeneration after hepatectomy (14), and also to the enhanced liver damage induced by drugs, such as carbon tetrachloride (15) and ethanol (16). These results suggest that polyamines play important roles in attenuating liver damage and accelerating liver regeneration; however, many of these studies were performed using models under the condition of endogenous polyamine depletion, or were experiments in vitro. Few studies have investigated the effect of the exogenous

polyamine administration as treatment in the ischemia-injured partial liver model.

The present study examined the effect of oral administration of polyamines (SPD and SPM) using a rat model of 70% liver resection with IRI, and investigated the impact of polyamines on liver IRI and liver regeneration.

MATERIALS AND METHODS:

Animals and treatment:

Male Lewis rats (9-10 weeks old, weighing 270-330 g, Charles River, Kanagawa, Japan) were used in this study. All animals were housed in a specific pathogen-free animal facility at Kyoto University under the following conditions: 50% \pm 10% relative humidity, 12 h/12 h light–dark cycle, and 24°C \pm 2°C. Rats were fed a standard diet (F-2; Oriental Bio Service, Kyoto, Japan) and tap water ad libitum. Animal handling and care met the institutional guidelines for animal welfare. The institutional ethics committee of Kyoto University approved the experimental protocol (Medkyo-13612), which met the ethical guidelines of the Declaration of Helsinki.

The experiment was conducted in two groups of rats: a polyamine treated group and a vehicle group (**Fig.1A**). In the polyamine-treated group, the rats were administered polyamines (250 μ mol/kg) by gavage once a day from 3 days to 2 hours before operation; after the operation, this treatment was continued from postoperative day (POD) 1 to POD 6. In the vehicle group, the rats were given an equivalent volume of distilled water with the same procedure. The polyamines were a mixture of spermidine trihydrochloride (200 μ mol/kg) and spermine tetrahydrochloride (50 μ mol/kg), diluted with distilled water. Their relative proportion was determined to be approximate with that of soybeans (17).

Surgical procedure:

We used a rat model of 70% hepatectomy with 40 minutes of partial warm hepatic ischemia,

mimicking the clinical situation of LDLT with 30% partial liver graft and 40 minutes of warm ischemia (18). All procedures were performed under inhalation anesthesia using 1.5% isoflurane with endotracheal intubation (endotracheal catheter: 16G SURFLO I.V. catheter; Terumo, Tokyo, Japan) and artificial respirator (SN-480-7; Shinano, Tokyo, Japan). The abdomen was opened through a midline incision. An atraumatic micro vessel clip (Sugita Titanium 2 Aneurysm Clip, Mizuho Medical, Tokyo, Japan) was used to interrupt the arterial and portal venous blood supply to the left and middle hepatic lobes. This procedure avoided intestinal congestion. After 40 minutes of partial hepatic ischemia, the vessel clip was removed to initiate hepatic reperfusion. Immediately after the onset of reperfusion, the left lateral lobe, left portion of the medial lobe, right inferior and superior lobes, and caudate lobes were resected with ligation using 4-0 silk, leaving only the ischemic right portion of the medial lobe behind (**Fig. 1B**). The abdomen was closed with 4-0 nylon sutures, and the rats were allowed to awaken. After the operation, the rats were given free access to food and water. The animals were sacrificed at 6 h, 24 h, 48 h, and 168 h after the reperfusion. Blood samples were taken from the inferior vena cava (IVC), and liver was harvested and weighed.

Assessment of polyamine concentration in the blood:

To assess the pharmacokinetics of the polyamines after oral administration, the concentration of polyamines in the whole blood was analyzed in an additional experiment. The rats were administered polyamines in the same way as mentioned above for 3 days; on the fourth day, the rats were sacrificed before administration (0 minute; the trough level of polyamines), at 30 minutes, 1 h, 2 h, and 12 h after administration (n = 3 at each time point) of the polyamines. Blood samples were collected from portal vein (PV) and IVC, and were stored in ethylenediaminetetraacetic acid-coated tubes at -80°C. To measure the concentrations of the polyamines, each whole blood sample (1 mL) was transferred to a new microfuge tube containing 10% trichloroacetic acid (1 mL). After centrifugation at 2000 g for 15

minutes, each of the resulting supernatant was collected, and was double-diluted with 0.1 N hydrochloric acid. Then the concentrations of SPD and SPM were measured by high performance liquid chromatography (19).

Assessment of hepatocellular function and proinflammatory cytokines:

The blood samples were centrifuged for 10 min at 3000 rpm, and serum was collected. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured as standard indicators of hepatocellular injury by a standard spectrophotometric method with an automated analyzer (JCA-BM9030, JEOL Ltd., Tokyo, Japan).

To assess the serum proinflammatory cytokines and chemokines, the Bio-Plex multiplex system (Bio-Rad Laboratories, Hercules, CA, USA) was used in conjunction with the Bio-Plex 200 (Bio-Rad Laboratories) to measure inflammatory mediators, according to the manufacturer's directions (n = 5-7 in each group). Data were analyzed using Bio-Plex Manager 6.1 (Bio-Rad Laboratories).

Histological analysis of liver IRI:

Formalin-fixed, paraffin-embedded sections (4 μ m thickness) were stained with hematoxylin and eosin. Tissue sections were all examined with a blinded fashion by two independent investigators. The severity of IRI (sinusoidal congestion, vacuolization and necrosis) was scored with modified Suzuki's criteria on a scale from 0 - 4 (20). The scores were evaluated in 10 random fields (magnification, \times 200) per slide and averaged for each slide.

TUNEL analysis:

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) analysis was performed on 4 μ m sections using the FD Apop Kit (FD NeuroTechnologies,

Columbia, MD, USA) according to the manufacturer's instructions. The number of TUNEL-positive cells / 10^3 hepatocytes was calculated from 10 randomly selected high-power field (magnification, $\times 200$) per slides ($n = 5$ / group).

Assessment of injury to sinusoidal endothelial cells:

Assessment of the damage to sinusoidal endothelial cells (SECs) was performed by immunohistochemical staining for rat endothelial cell antigen (RECA-1; MCA-970R; AbD Serotec, Oxford, UK). Ten high-power fields ($\times 200$) were randomly selected for calculating the staining area of the SECs. The staining area was morphometrically quantified using ImageJ (National Institutes of Health) ($n = 5-7$ / group).

Electron microscopy:

The rat livers were first perfused with phosphate buffered saline through the abdominal aorta and then perfused with a fixative containing 2% glutaraldehyde and 4% paraformaldehyde (PFA). The livers were cut into small pieces (approximately 1 mm^3) for transmission electron microscopy (TEM) and into larger pieces (approximately 5 mm^3) for scanning electron microscopy (SEM). The samples were fixed in 2% glutaraldehyde and 4% PFA at 4°C for overnight. Thin sections were stained with saturated uranyl acetate and lead citrate and were observed with a Hitachi H-7650 electron microscope (Hitachi, Tokyo, Japan) for TEM. For SEM, the samples were ion-sputter-coated and observed with a Hitachi S-4700 scanning electron microscope.

Liver regeneration rate:

The liver regeneration rate was calculated as remnant liver weight / estimated whole liver weight (18). The estimated whole liver weight was calculated as the resected liver weight / 0.67, based on the

results of our pilot study in which the resected liver weight in our model was proven to be equal to 67.0% of the original whole liver weight ($67.0 \pm 2.6\%$, $n = 4$). The liver regeneration rates were calculated at the time points of 6 h, 24 h, 48 h, and 168 h after reperfusion.

Ki-67 analysis:

Deparaffinized hepatic sections (4 μm thickness) were stained for Ki-67 using the Ki-67 antibody (monoclonal mouse anti-rat Ki-67 antigen; Dako, Tokyo, Japan) and the EnVision+ System HRP Kit (Dako, Tokyo, Japan). Immunoreactivity was detected with a diaminobenzidine substrate kit (liquid 3,3'-diaminobenzidine Substrate-Chromogen System; Dako), and the sections were counterstained with hematoxylin. Ten high-power fields ($\times 200$) were randomly selected, and the Ki-67 labeling index (%) was calculated in accordance with the percentage of positive cells ($n = 6 / \text{group}$).

Western blot assay:

Western blot analyses were conducted, using primary antibodies recognizing cleaved caspase-3 [Asp175; #9664; Cell Signaling Technology (CST), Danvers, MA, USA], proliferating cell nuclear antigen (PCNA) (#13110; CST), p27^{Kip1} (#3686; CST), phosphorylated retinoblastoma protein (Phospho-Rb) (Ser608) (#2181; CST), Phospho-Rb (Ser807/811) (#8516; CST), and β -actin (#4970; CST) at a 1:1000 dilution. Anti-rabbit immunoglobulin G horseradish peroxidase (NA934V, GE Healthcare UK Ltd, Buckinghamshire, UK) was used as a secondary antibody at a 1:2000 dilution. The bands were detected using ECL reagent (GE Healthcare UK Ltd,) and the Ez-Capture II camera (ATTO, Tokyo, Japan). Results were analyzed using Image J.

Statistical analysis:

All data are expressed as the mean \pm standard deviation (SD). Student's t-test was used for comparison

between groups. *P* values of < 0.05 were considered statistically significant. All statistical analyses were performed using JMP 11 (SAS Institute, Cary, NC, USA) and Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Concentrations of polyamines in whole blood of PV and IVC

The concentrations of SPD and SPM in the blood after polyamine administration are shown in **Fig. 1C**. The values at 0 h indicate the trough levels after 3 days of administration. The concentration levels of SPD and SPM in the whole blood of the PV and IVC were both elevated after oral administration, peaked at 1 h after oral administration, and gradually decreased afterward.

Polyamine administration ameliorated liver IRI

All rats survived until sacrifice. The serum AST and ALT levels of the polyamine group at the points of 6 h, 24 h and 48 h after reperfusion were significantly lower compared with those of the vehicle group ($P < 0.05$, $n = 5-6$ / group) (**Fig. 2A**).

Several serum levels of proinflammatory cytokines and chemokines at 6 h after reperfusion are shown in **Fig. 2B**. The analyzed mediators shown are interleukin (IL)-1 β , IL-6, IL-10, tumor necrosis factor- α (TNF- α), interferon- γ (INF- γ), regulated on activation normal T cell expressed and secreted (RANTES), chemokine(C-X-C motif) ligand-1 (CXCL-1), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-3a (MIP-3a), and vascular endothelial growth factor (VEGF). The levels of serum IL-1 β , IL-6, TNF- α , INF- γ , RANTES, CXCL-1, MCP-1, and MIP-3a of the polyamine group were significantly lower compared with the vehicle group ($P < 0.05$, $n = 5-7$ / group). On the other hand, the level of serum IL-10, which is one of the anti-inflammatory cytokines, was slightly higher in the

polyamine group, but the difference was not statistically significant ($n = 7 / \text{group}$). Moreover, there was no difference in the level of serum VEGF between the two groups ($n = 6 / \text{group}$).

In the histological findings, as shown in **Fig. 3**, the livers in the vehicle group at 6 h after reperfusion revealed partial necrosis, congestion and histological damage (**Fig. 3A**). In contrast, the livers in the polyamine group at 6 h after reperfusion showed less necrotic area, good preservation of architecture and histological detail (**Fig. 3B**). Indeed, the Suzuki score significantly improved at 6 h after reperfusion in the polyamine group compared with the vehicle group ($P = 0.01$, $n = 7 / \text{group}$) (**Fig. 3C**).

Polyamine administration suppressed IR-induced apoptosis of hepatocytes

We examined how polyamine pretreatment affected the apoptosis of hepatocytes induced by IRI (**Fig. 4**). A representative liver section stained by TUNEL in the vehicle group at 6 h after reperfusion is shown in **Fig. 4A** and that of the polyamine group at 6 h after reperfusion is shown in **Fig. 4B**. The numbers of the cells in apoptosis (TUNEL positive cells / 10^3 hepatocytes) induced by IR in the polyamine group were significantly fewer compared with the vehicle group ($P < 0.01$, $n = 5 / \text{group}$) (**Fig. 4C**).

In addition, pretreatment with polyamines inhibited the expression of the cleaved caspase-3 level at 6 h after reperfusion ($P < 0.05$, $n = 4 / \text{group}$) (**Fig. 4D, 4E**).

Polyamine administration suppressed IR-induced endothelial cell injury

To quantitatively assess the impact of polyamine on SEC injury, anti-endothelial cell antibody (RECA-1) immunohistochemistry was performed, and areas with relative RECA-1 positivity were examined (**Fig. 5**). Representative liver sections stained by RECA-1 at 6 h and 24 h after reperfusion are shown in **Fig. 5A**. RECA-1 expression gradually decreased after reperfusion in both groups; however, areas with relative RECA-1 positivity in the vehicle group significantly decreased with time from 6 h to 24 h after reperfusion. In contrast, areas with RECA-1 positivity in the polyamine group were

significantly larger than those in the vehicle group both at 6 h and 24 h after reperfusion ($P < 0.05$, $n = 5$ / group) (**Fig. 5B**). These results suggest that polyamine attenuated endothelial cell injury.

Next, we morphometrically examined endothelial cell injury with SEM and TEM. Representative SEM images in the vehicle group and the polyamine group 6 h after reperfusion are shown in **Fig. 6A** and **6B**, and representative TEM images in each group at the same time point are shown in **Fig. 6C** and **6D**. At 6 h after reperfusion, SEM demonstrated that the architecture of the sieve plate arrangement in the polyamine group was well preserved in comparison with that in the vehicle group (**Fig. 6A, B**). TEM demonstrated that SECs in the polyamine group were well preserved in comparison with those in the vehicle group (**Fig. 6C, D**). These results suggest that the polyamine treatment attenuated the damage of endothelial cells induced by IR.

Polyamine administration promoted liver regeneration

The liver regeneration rates at 24 h, 48 h, and 168 h after reperfusion are shown in **Fig. 7A**. The liver regeneration rates in the polyamine group were significantly higher at 24 h, 48 h, and 168 h compared with the rates in the vehicle group ($P < 0.01$, $n = 5-7$ at each time point / group) (**Fig. 7A**). The liver regeneration rates at 168 h after reperfusion with dose escalation of polyamines are shown in **Fig. 7B**. The liver regeneration rates increased with the dose escalation of polyamines up to 250 $\mu\text{mol/kg}$, but the dose over 250 $\mu\text{mol/kg}$ had no positive impact on liver regeneration ($n = 5-7$ at each dose).

To elucidate cell proliferation, we investigated the Ki-67 labeling index, which indicates DNA synthesis in the cell cycle. A representative liver section stained by Ki-67 in the vehicle group at 24 h after reperfusion is shown in **Fig. 7C**, and that of the polyamine group at 24 h after reperfusion is shown in **Fig. 7D**. The Ki-67 labeling index was significantly higher in the polyamine group ($58.5 \pm 9.2\%$) compared with the vehicle group ($18.5 \pm 6.6\%$) ($P < 0.01$, $n = 6$ / group) (**Fig. 7E**). Next, the expression of PCNA, which is expressed in the nuclei of cells especially in the G1 and S phase of the cell cycle, was

measured by Western blot analysis. The expression level of PCNA in the liver tissue in the polyamine group was significantly higher at 24 h after reperfusion compared with the vehicle group ($P < 0.05$, $n = 5 / \text{group}$) (**Fig. 7F, 7G**). These results suggest that postoperative cell proliferation, DNA synthesis, and liver regeneration were promoted in the polyamine treated group.

To investigate the detailed mechanisms of accelerated regeneration of the remnant liver, the expressions of p27^{Kip1} and Phospho-Rb were measured by Western blot analyses. The expression level of p27^{Kip1} in the liver tissue in the polyamine group was lower at 24 h after reperfusion compared with that in the vehicle group, although the difference was not statistically significant ($P = 0.08$, $n = 5 / \text{group}$) (**Fig. 8A, 8B**). On the other hand, the expression levels of Phospho-Rb proteins [Phospho-Rb (Ser608) and Phospho-Rb (Ser807/811)] in the polyamine group were significantly higher at 24 h after reperfusion compared with those in the vehicle group ($P < 0.05$, $n = 5-6 / \text{group}$) (**Fig. 8C, 8D, 8E**).

DISCUSSION

The results of the present study revealed that oral polyamine administration attenuated liver IRI and promoted liver regeneration after 70 % hepatectomy with IRI in the rat model. To the best of our knowledge, this is the first study to investigate the impact of oral polyamine administration on both liver IRI and liver regeneration.

Polyamines are derived from ornithine, including three kinds of molecules: putrescine, SPD, and SPM. They are present in all higher eukaryotic cells and essential for cell growth and differentiation (9, 10). Although the effects of intraperitoneal injection of polyamines have been investigated (15, 16), few studies have investigated the effects of oral administration of polyamines. In the clinical settings, simple route of administration is preferable, and oral administration would be acceptable. When orally administered, putrescine is mostly converted to other non-polyamine metabolites in the intestine by the enzyme ‘diamine oxidase’, while neither SPD nor SPM are enzymatically degraded in the alimentary

tract (21). Most of these two polyamines can be quickly absorbed from the intestinal lumen and distributed to the liver through the PV (22). In the present study, the concentration levels of SPD and SPM in the blood of the PV and IVC were both elevated and peaked at 1 h after oral administration, which indicated that they were absorbed quickly from the intestinal lumen. The concentration levels of polyamines in the PV were higher than those in IVC, which suggested that these polyamines were accumulated in the liver. Based on this result, the timing of the operation was decided at 2 h after oral administration of polyamines. The optimal dose of polyamines for liver regeneration was determined to be 250 $\mu\text{mol/kg/day}$ in this study with the analysis of dose escalation.

The present study revealed the protective effect of polyamines against liver IRI. IRI is still one of the major causes of morbidity and mortality after LT. Ischemic conditions result in adenosine triphosphate depletion and accumulation of toxic metabolites, whereas reperfusion results in the production of reactive oxygen intermediates, which can lead to oxidative stress (23). The initial phase of IRI is a Kupffer cell-mediated response, in which the oxidant stress results in the release of a number of proinflammatory cytokines, and the second phase of IRI is characterized by the recruitment of activated neutrophils into the liver parenchyma (24) (25). The present study showed that the levels of TNF- α , IL-1 β , and CXCL-1, as well as transaminases, other proinflammatory cytokines and chemokines were significantly lower in the polyamine group. Moreover, histological analysis showed less necrosis in the polyamine group. These data strongly support the idea that polyamines have protective effects against IRI by suppressing these inflammatory cascades. Polyamines are reported to play important roles in protection from oxidative stress as free radical scavengers (26, 27), and reduce lipid peroxidation (28). In addition, the role of SPD in inducing autophagy has also been emphasized as an anti-inflammatory mechanism (9, 29).

One of the key events after liver IRI is apoptosis of hepatocytes (30). The role of polyamines in the regulation of apoptosis has been investigated in several studies. These previous studies have suggested

that decreased polyamines were responsible for apoptosis (31, 32). Although few studies have investigated the role of polyamines in regulating apoptosis of the liver cells, the present study revealed that exogenous polyamine administration before liver IRI reduced apoptosis of hepatocytes. Moreover, we also assessed the extent of SEC injury by RECA-1 immunohistochemistry and electron microscopy. Although little is known about the relationship between polyamines and liver SEC injury, our study revealed that polyamines attenuated SEC injury as well as hepatocyte injury.

The present study also revealed the promoting effect of polyamines on liver regeneration. Most polyamines in the liver exist as a polyamine- ribonucleic acid (RNA) complex, and play important roles in transcription and translation in the cell cycle through a structural change of RNA (10), especially in the transition from the G1 phase to the S phase and the G2 phase to the M phase (33). In the present study, Ki-67 labeling index and the expression of PCNA were significantly higher in the polyamine group, which suggested that polyamine treatment promoted the cell cycle and cell proliferation. Moreover, the expression level of p27^{Kip1}, which is the inhibitor of cyclin-dependent-kinase-2 and one of the inhibitory factors of cell cycle progression, was lower, and the expression level of phospho-Rb, which plays an important role to accelerate cell cycle progression from G1 to S phase, was higher in the polyamine group compared with those in the vehicle group. Previous studies reported that the level of p27^{Kip1} was increased by polyamine deficiency, which resulted in the arrest of the cell cycle (33) (34). The analysis at the molecular level revealed that the synthesis of p27^{Kip1} was inhibited by polyamines at the level of translation (33). The results of the present study also support the notion that polyamines downregulated the synthesis of p27^{Kip1} and promoted the phosphorylation of Rb protein, which resulted in the progression of the cell cycle from G1 to S phase. Further investigations are necessary to reveal the detailed mechanisms of polyamines and liver regeneration.

Small-for-size syndrome is a critical problem in LDLT. In adult-to-adult LDLT, to maintain the safety of the living donors, the size of the grafts has been reduced as much as possible, and small grafts such as

left lobe grafts or posterior lobe grafts are often used instead of right lobe grafts (6). However, size mismatch between graft and recipient sometimes leads to primary graft non-function or dysfunction, as a result of liver graft IRI and secondary liver tissue regeneration impairment (7, 8). If supportive treatment fails, re-transplantation is eventually required to rescue the recipient. Perioperative polyamine treatment could be a new therapeutic option to reduce the risk of small-for-size syndrome and improve the outcome of LDLT by reducing liver IRI and promoting liver regeneration.

When considering clinical applications, oral administration of polyamines would be easily accepted. Polyamines can be safely extracted from soybeans, which contain the highest amounts of polyamines among various natural foods (17). Polyamines can be administered to living donors as well as recipients. The treatment could be effective in promoting the regeneration of the remnant livers of the donors as well as that of the liver grafts transplanted to the recipients. Moreover, polyamines could be administered even to deceased donors using feeding tubes during the evaluation time. Although this study was performed with once daily administration regimen, divided dosage might be more effective, as the half-life period of polyamine concentration in the blood after oral administration was relatively short. Further assessments will be needed to evaluate the safety, optimal dose and timing of administration.

In conclusion, the data of the present study revealed that perioperative oral polyamine administration attenuates liver IRI and promotes liver regeneration. Polyamines work as protective factors against liver damage and play important roles in liver regeneration. Perioperative exogenous polyamine treatment will be a new therapeutic option to improve the outcomes of LT.

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FIGURE LEGENDS

Figure 1. Experimental protocol and the concentrations of polyamines in the whole blood.

(A) In the polyamine treated group, the rats were administered polyamines (250 $\mu\text{mol/kg}$) by gavage once a day from 3 days to 2 hours before operation; after the operation, this treatment was continued from POD 1 to POD 6. In the vehicle group, the rats were given an equivalent volume of distilled water with the same procedure. The polyamines were a mixture of spermidine trihydrochloride (SPD) (200 $\mu\text{mol/kg}$) and spermine tetrahydrochloride (SPM) (50 $\mu\text{mol/kg}$) (POD: postoperative day).

(B) Our rat model of 70% hepatectomy with IRI (40 minutes of warm ischemia). Arrows indicate the microvascular clip used to clamp the inflow. * indicates the remnant right portion of the median lobe.

(C) The concentrations of polyamines (SPD and SPM) in the whole blood (PV and IVC) after the administration of polyamines. The data are expressed as means \pm standard deviations ($n = 3$ in each time point).

Figure 2. Serum concentrations of AST, ALT, and several cytokines and chemokines.

(A) Serum concentrations of AST and ALT at the time points of 6 h, 24 h, 48 h, and 168 h after reperfusion.

The serum levels of AST and ALT at 6 h, 24 h, and 48 h after reperfusion were significantly lower in the polyamine group compared with those in the vehicle group.

(B) Serum concentrations of several cytokines and chemokines (IL-1 β , IL-6, IL-10, TNF- α , INF- γ , RANTES, CXCL-1, MCP-1, MIP-3a, VEGF) at 6 h after reperfusion.

The data are expressed as means \pm standard deviations (* $P < 0.05$, ** $P < 0.01$, $n = 5-7$ / group).

Figure 3. Histological analyses of liver IRI.

(A) (B) Representative liver histology in the vehicle group (A) and in the polyamine group (B) at 6 h after reperfusion. The livers in the vehicle group at 6 h after reperfusion revealed partial necrosis, congestion and histological damage. The livers in the polyamine group at 6 h after reperfusion showed

less necrotic area, good preservation of architecture and histological detail (HE staining, Original magnification: $\times 200$, Scale bars represent $100\ \mu\text{m}$, CV: central vein, PV: portal vein).

(C) Quantification of modified Suzuki's criteria of the hepatocellular damage at 6 h after reperfusion. Suzuki score was significantly lower at 6 h after reperfusion in the polyamine group compared with that in the vehicle group (vehicle group: 2.45 ± 0.77 vs. polyamine group: 1.33 ± 0.66). The data are expressed as means \pm standard deviations ($*P < 0.05$, $n = 7$ / group).

Figure 4. Assessments of apoptosis.

(A) (B) Representative liver sections stained by TUNEL in the vehicle group (A) and in the polyamine group (B) at 6 h after reperfusion. Arrows indicate TUNEL positive nuclei of hepatocytes (Original magnification: $\times 200$, Scale bars represent $100\ \mu\text{m}$).

(C) Quantification of TUNEL positive hepatocytes in both groups. The numbers of the hepatocytes in apoptosis (TUNEL positive hepatocytes / 10^3 hepatocytes) in the polyamine group were significantly fewer compared with those in the vehicle group. The data are expressed as means \pm standard deviations ($***P < 0.001$, $n = 5$ / group).

(D) The expression of cleaved caspase-3 in the liver tissue at 6 h after reperfusion measured by Western blot analysis.

(E) Quantification of the relative ratio of cleaved caspase-3 / β -actin by western blot analysis. The expression level of cleaved caspase-3 of the liver tissue in the polyamine group was significantly lower compared with that in the vehicle group. The data are expressed as means \pm standard deviations ($*P < 0.05$, $n = 5$ / group).

Figure 5. Assessments of sinusoidal endothelial cell injury.

(A) Representative liver sections stained by RECA-1 in the vehicle group and in the polyamine group at 6 h and 24 h after reperfusion.

(B) Relative RECA-1 positive area was morphologically quantified. Areas with RECA-1 positivity in

the polyamine group were significantly larger than those in the vehicle group both at 6 h and 24 h after reperfusion. The data are expressed as means \pm standard deviations ($*P < 0.05$, $n = 5-7$ / group).

Figure 6. Electron microscopy findings.

(A) (B) Scanning electron microscopy in the vehicle group (A) and in the polyamine group (B) at 6 h after reperfusion. The architecture of the sieve plate arrangement in the polyamine group was well preserved in comparison with that in the vehicle group (Original magnification: $\times 6000$).

(C) (D) Transmission electron microscopy in the vehicle group (C) and in the polyamine group (D) at 6 h after reperfusion. Sinusoidal endothelial cells in the polyamine group were well preserved in comparison with those in the vehicle group (Original magnification: $\times 2000$, Scale bars indicate 2 μm , HC: hepatocyte, SEC: sinusoidal endothelial cell).

Figure 7. Assessments of liver regeneration.

(A) Liver regeneration rates (%) at 6 h, 24 h, 48 h, and 168 h after reperfusion. The liver regeneration rates in the polyamine group (250 $\mu\text{mol/kg}$) were significantly higher at 24 h, 48 h, and 168 h compared with those in the vehicle group. The data are expressed as means \pm standard deviations ($***P < 0.01$, $n = 5-7$ / group).

(B) Liver regeneration rates (%) at 168h after reperfusion with several doses of polyamines. The liver regeneration rates increased with the dose escalation of polyamines up to 250 $\mu\text{mol/kg}$, but the dose over 250 $\mu\text{mol/kg}$ had no positive impact on liver regeneration ($***P < 0.01$, n.s.: no significant difference, $n = 5-7$ / group).

(C) (D) Representative liver sections stained by Ki-67 in the vehicle group (C) and in the polyamine group (D) at 24 h after reperfusion (Original magnification: $\times 200$, Scale bars represent 100 μm).

(E) Quantification of Ki-67 labeling index in both groups. Ki-67 labeling index was significantly higher in the polyamine group compared with that in the vehicle group ($58.5 \pm 9.2\%$ vs. $18.5 \pm 6.6\%$).

The data are expressed as means \pm standard deviations ($***P < 0.001$, $n = 6$ / group).

(F) The expression of PCNA in the liver tissue measured by Western blot analysis.

(G) Quantification of the relative ratio of PCNA / β -actin by western blot analysis. The expression level of PCNA in the liver tissue in the polyamine group was significantly higher at 24 h after reperfusion compared with that in the vehicle group. The data are expressed as means \pm standard deviations (* $P < 0.05$, $n = 5$ / group).

Figure 8. Western blot analysis of p27^{Kip1} and phospho-Rb.

(A) The expression of p27^{Kip1} in the liver tissue measured by Western blot analysis.

(B) Quantification of the relative ratio of p27^{Kip1} / β -actin by western blot analysis. The expression level of p27^{Kip1} in the liver tissue in the polyamine group was lower at 24 h after reperfusion compared with that in the vehicle group. The data are expressed as means \pm standard deviations ($P = 0.08$, $n = 5$ / group).

(C) The expression of phospho-Rb in the liver tissue measured by Western blot analysis.

(D) (E) Quantification of the relative ratio of phospho-Rb / β -actin by western blot analysis. The expression levels of phospho-Rb (Ser608) (D) and phospho-Rb (Ser807/811) (E) in the liver tissue in the polyamine group were significantly higher at 24 h after reperfusion compared with those in the vehicle group. The data are expressed as means \pm standard deviations (* $P < 0.05$, $n = 5-6$ / group).

Figure. 1

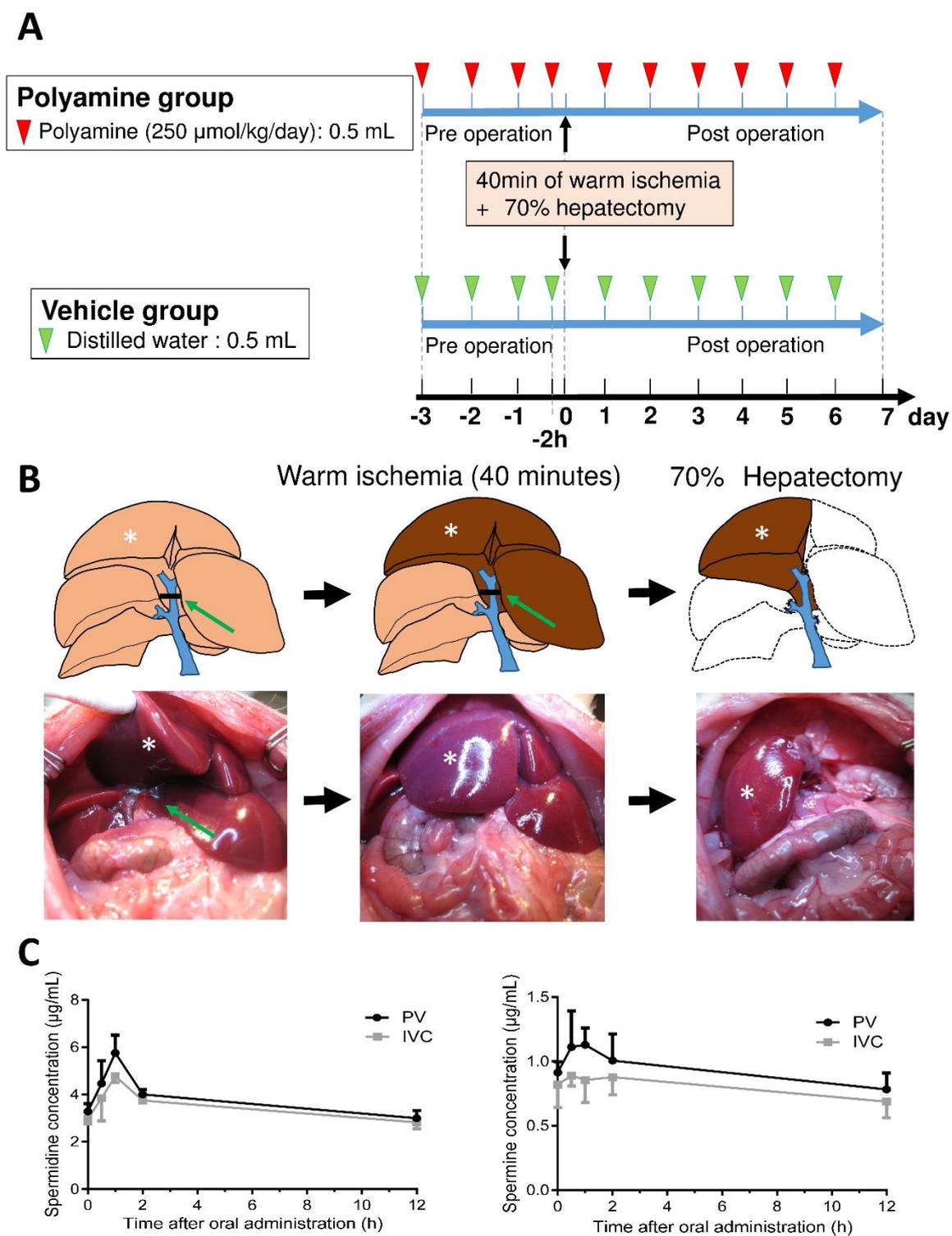


Figure. 2

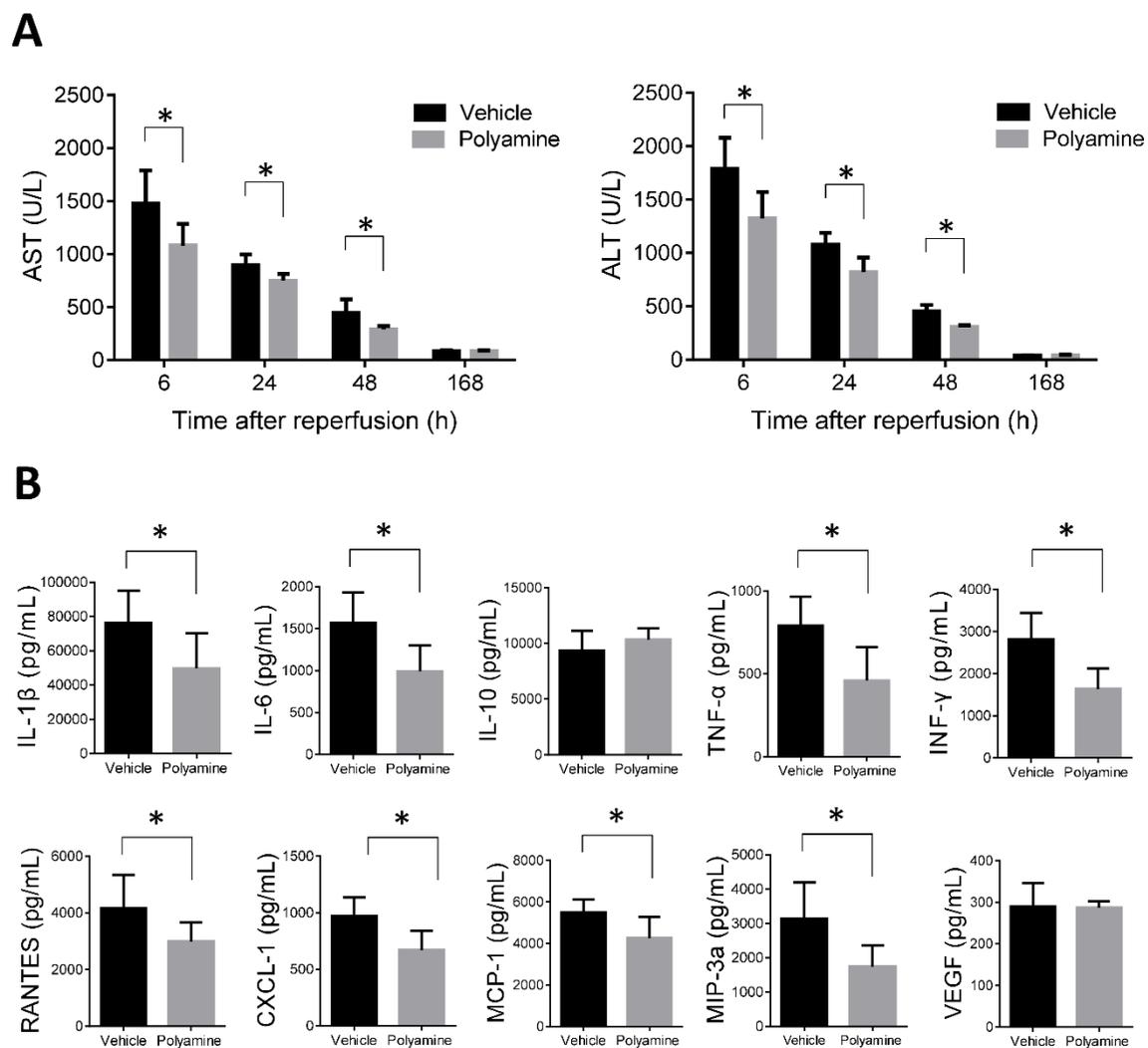


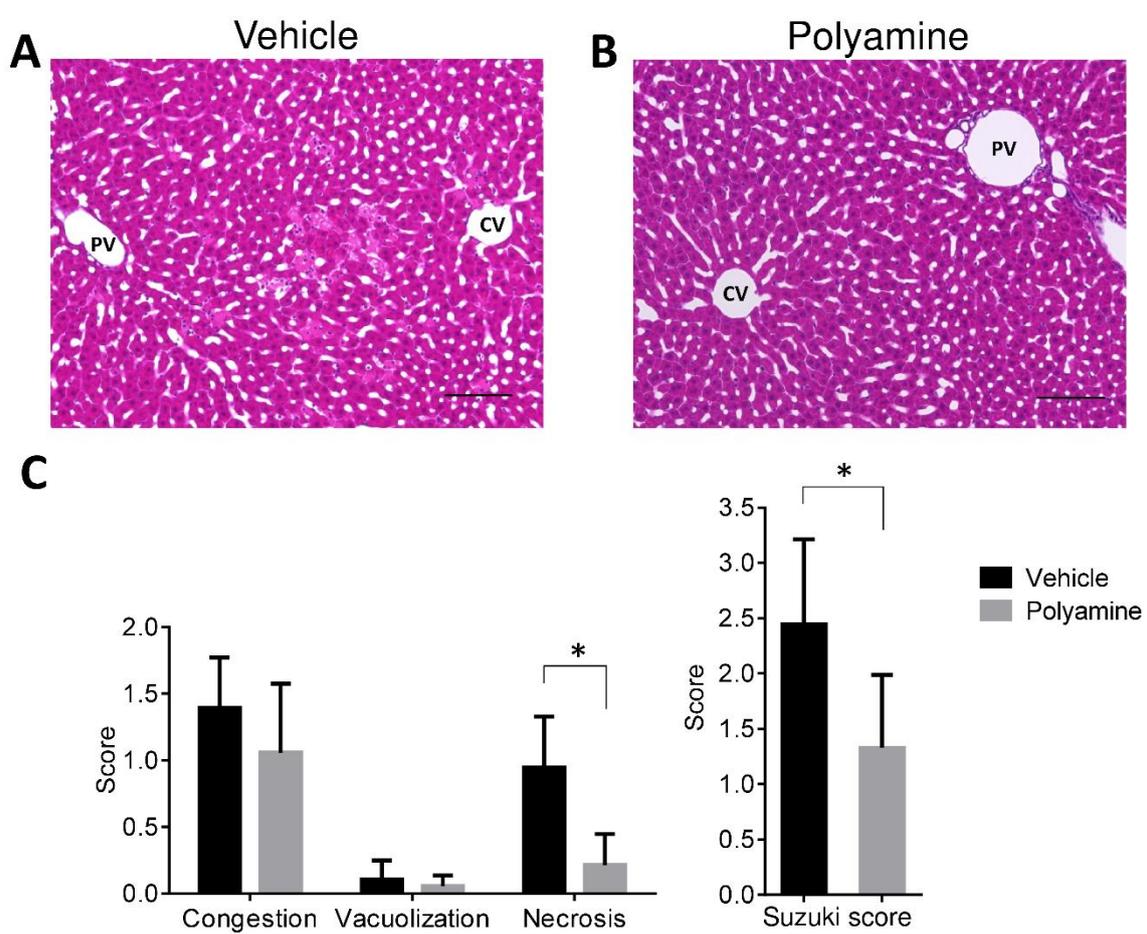
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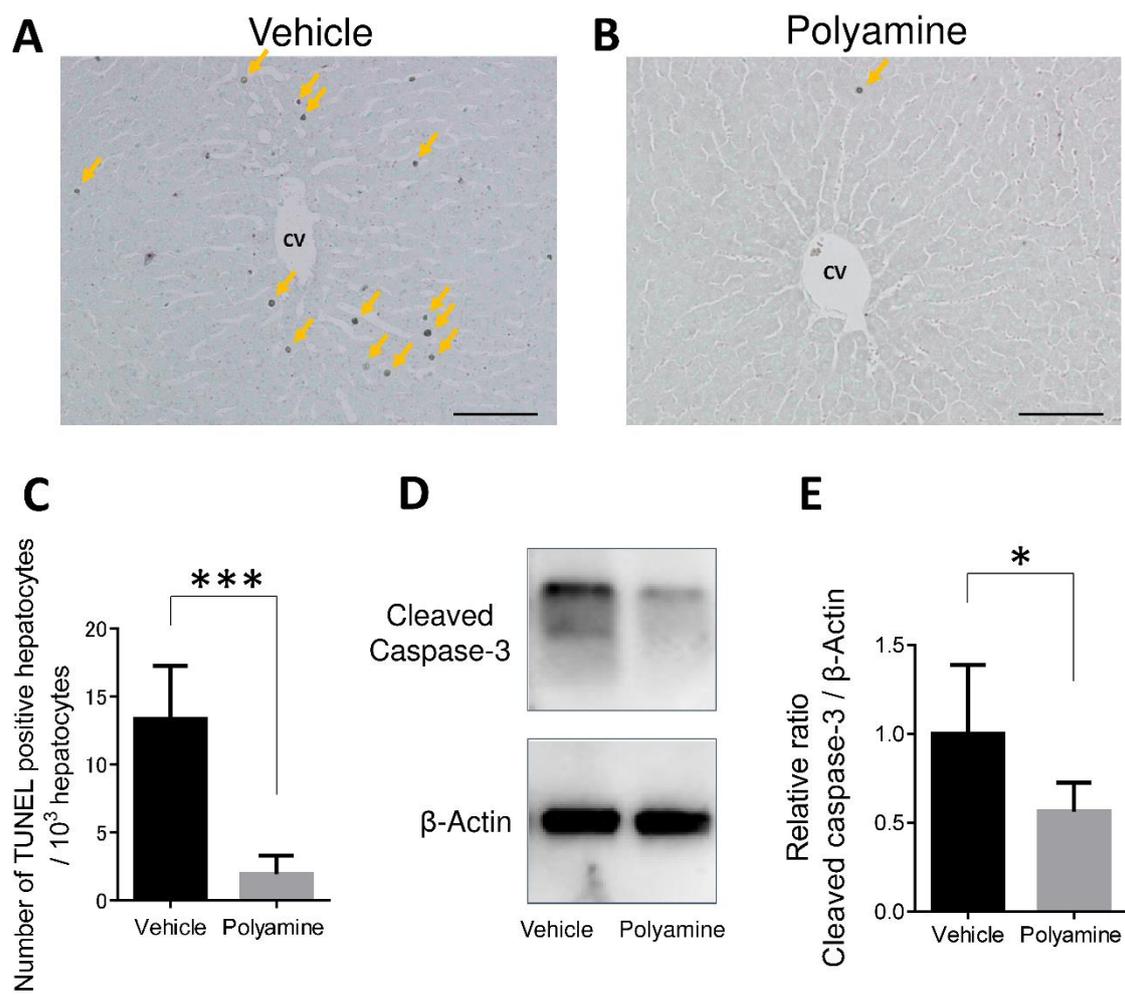
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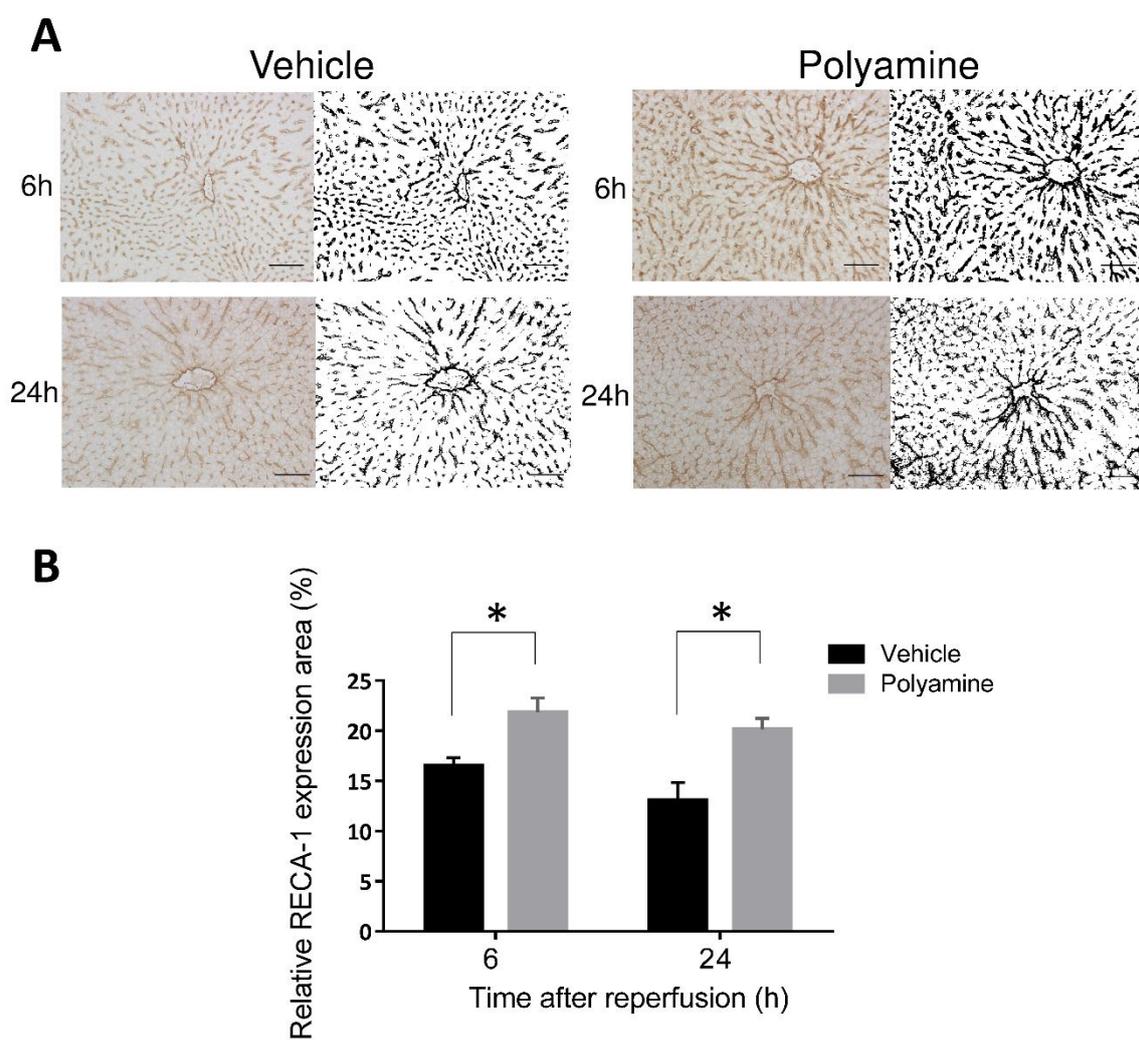
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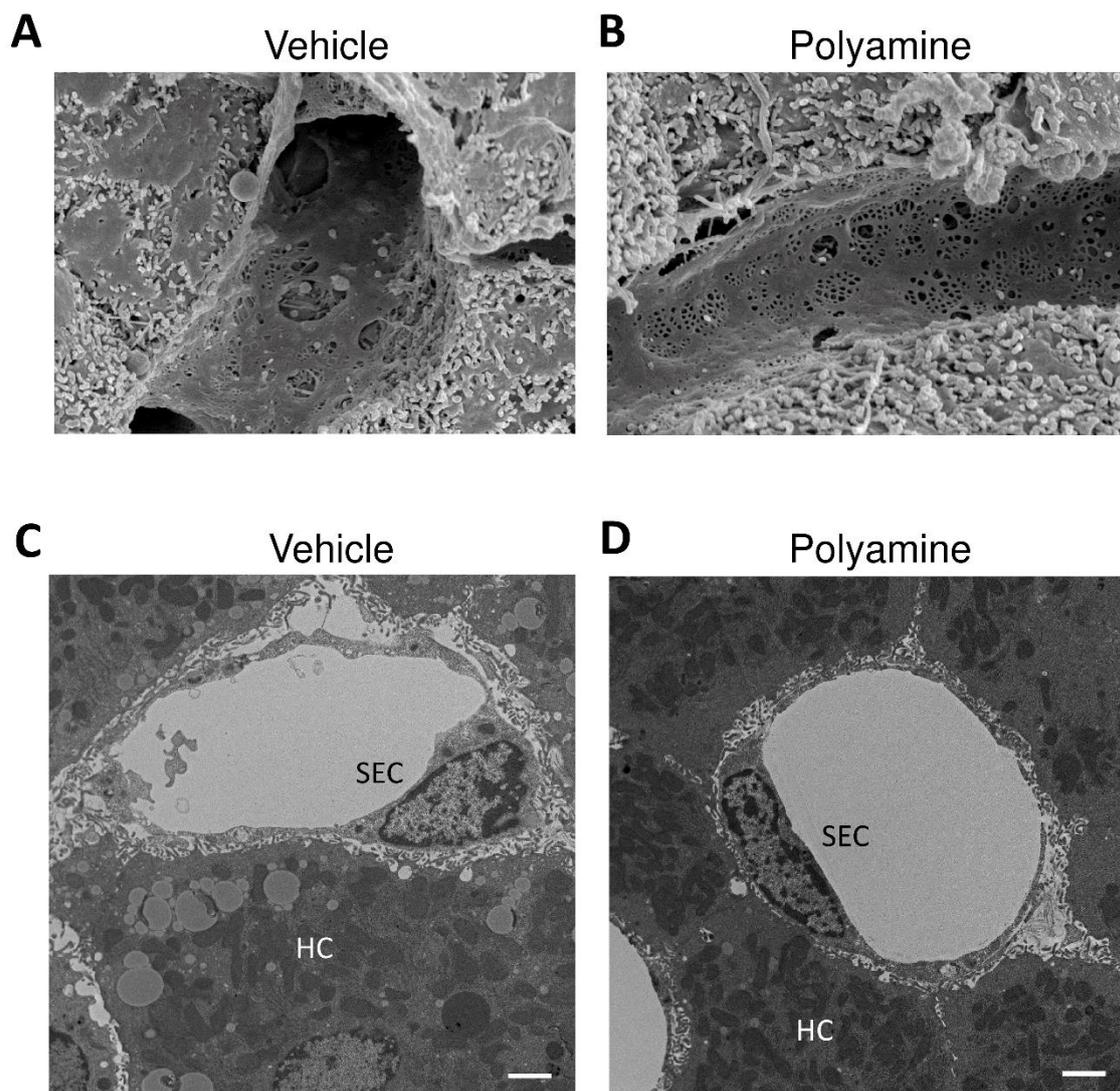
Figure. 6

Figure. 7

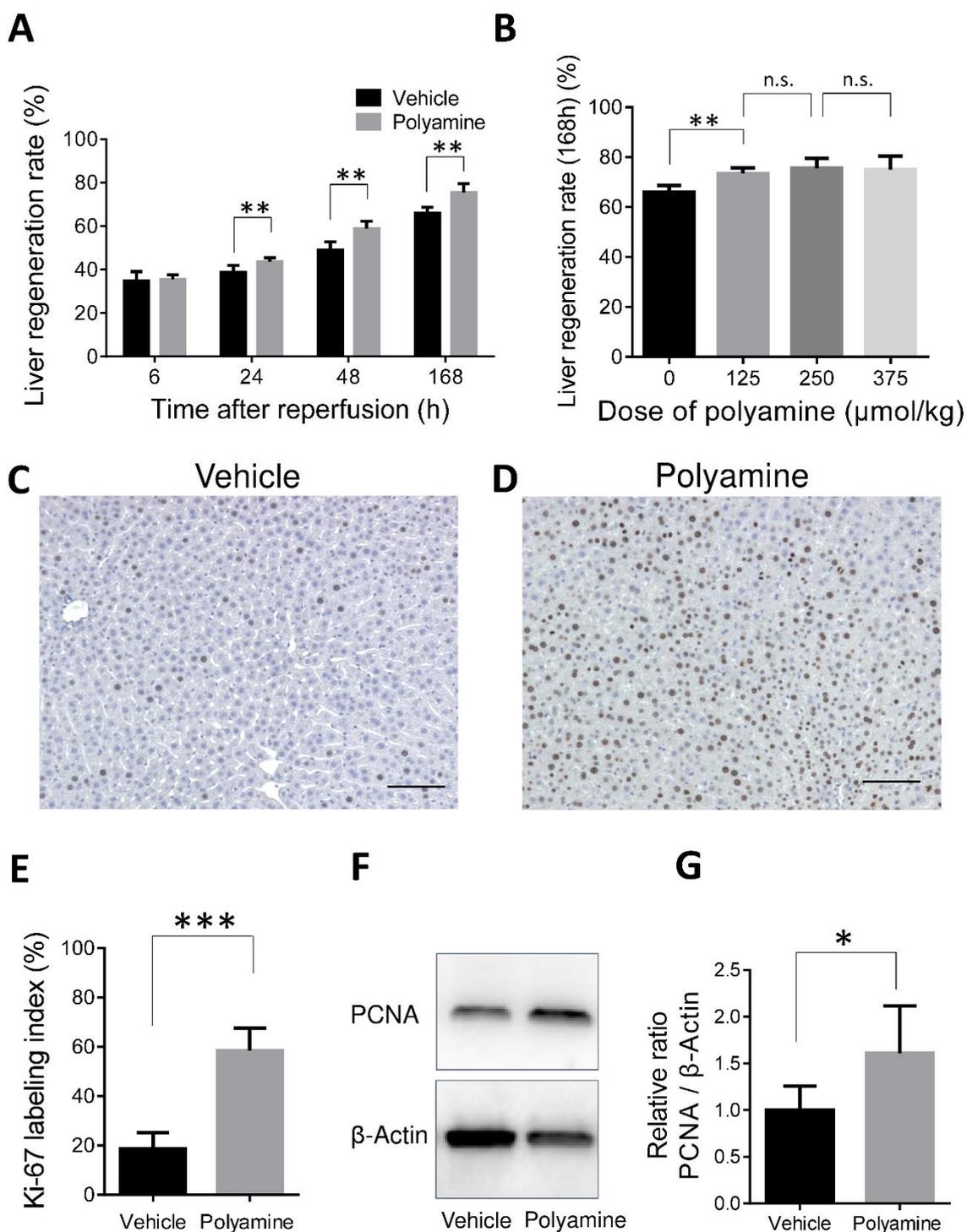


Figure. 8