Int Surg 2018;**103:**191–198
DOI: 10.9738/INTSURG-D-17-00033.1

A Protective Effect of Sivelestat From Ischemia/Reperfusion Injury in a Porcine Hepatectomy Model

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Summary of background data: Sivelestat sodium hydrate (Sive), a neutrophil elastase inhibitor, has been approved as a worldwide therapeutic drug for acute lung injury associated with systemic inflammatory response syndrome. Yet how Sive influences hepatic ischemic reperfusion (I/R) injury and liver regeneration has not been clarified.

Objective: We investigated the effect of Sive against hepatic I/R injury and liver regeneration using porcine hepatectomy model, and found that Sive contributes significantly in increasing the liver volume.

Methods: We induced 1-hour ischemia by occluding the vessels and the bile duct of the right and median lobes. About 40% left hepatectomy was performed after reperfusion. A total of 6 animals received Sive (10 mg/kg/h) intravenously and 6 control animals received physiologic saline (10 mg/kg/h) from commencement of laparotomy. Remnant liver volume, hemodynamics, and liver function test were compared between the groups. Expressions of TRL4 mRNA in hepatic tissues were examined using RT-PCR. Apoptosis and cell proliferation were demonstrated by TUNEL staining.

Results: AST, LDH, and LA levels at 5 minutes after reperfusion were significantly lower in Sive group than in the control group. Sive significantly increased the liver volume, yet did not have any effect for liver regeneration.

Conclusion: Sive is considered to reduce hepatic injury in the early phase of I/R injury.

Key words: Liver resection – Ischemia-reperfusion injury – Sivelestat – Liver volume – TRL4

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Sivelestat sodium hydrate (Sive), a neutrophil elastase inhibitor, has been approved as a therapeutic drug for acute lung injury associated with systemic inflammatory response syndrome, which may occur after infection, surgical invasion, and traumatic and burn injury.[1] Some reports have also shown a protective effect of Sive in the liver.[2–4] However, the effect for liver regeneration remains unknown.

Furthermore, Kupffer cells play a prominent role in I/R injury in liver. I/R injury in liver induces release of HMGB1 from damaged liver cells, which then stimulates nonparenchymal cell, such as Kupffer cells, through toll-like receptor 4 (TLR4).[5] TLR4 triggers the secretion of tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6 from Kupffer cells, resulting in liver regeneration.[5]

Clinical experiments using animal models are essential to explore the effect of Sive against I/R injury in liver, and to expand the application of Sive clinically. We have conducted this trial to define the effects of Sive after I/R injury, and its contribution to liver regeneration in the porcine liver resection model.

In this study, we investigated the effect of Sive against hepatic I/R injury and effect on liver regeneration, and found that Sive contributes significantly in increasing the liver volume.

Material and Methods

Experimental groups

The study was performed using male pigs, weighing 23 to 29 kg (SEASCO, Saitama, Japan), in accordance with the Guidelines for the Care and Use of Laboratory Animals. This animal study was approved by the research and ethics committee at the Dokkyo Medical University (Number: 00-082). We prepared 2 groups of animals: a Sive group (n=6) in which Sive (supplied by Ono Pharmaceutical Co., Ltd., Osaka, Japan) was administered intravenously at a dose of 10 mg/kg/h, just from commencement of reperfusion to completion of the liver resection, and a control group (n = 6) in which physiologic saline was administered intravenously for the similar period. The Sive dose was the same as treatment for a human brain infarction dose[6] (Fig. 1).

Surgical procedure

A chevron incision was made under general anesthesia, and each branch of the portal vein, hepatic artery and bile duct were carefully isolated and taped. Hemi-hepatic (approximately 60%) liver ischemia was induced by clamping right and middle hepatic vessels and bile duct using vessels tapes, and maintained for 60 minutes (Fig. 2a); the left portal branch was kept patent to avoid bowel congestion, and the left lobe played the role of bypass. After declamping, the left portal vein and artery was ligated, and a left hemi-hepatectomy was performed (approximately 40%, Fig. 2b). Liver transection was achieved by the crush-clamping method. During liver transection, the exposed Glisson's vessels were ligated and cut. The hepatic vein was closed by continuous sutures using a 4-0 monofilament. The influence of I/R injury was assumed to be on the right lobe only. During this procedure, hemodynamic parameters (systolic and diastolic arterial pressure) were monitored by femoral arterial line. All pigs received Ringer solution during the procedure. After operation, pigs were taken care in the animal center and oral intake was free after the first postoperative day. No antibiotics were administered either orally or intravenously after surgery.

Measurements and sampling protocol levels

Blood samples were obtained from the arterial line immediately after laparotomy, 5 and 180 minutes after reperfusion, and after 1 month. The level of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and lactic acid (LA) were evaluated. Hepatic tissues were obtained from the right lobe at laparotomy, after reperfusion, and after 1 month, and were subjected to TUNEL staining, and TLR4 mRNA. Serum AST, ALT, LDH, and LA were measured using standard clinical methods for automated analysis (Model 7170, Hitachi, Inc, Tokyo, Japan).

Total liver weight was calculated on the basis of previous experimental data from 30 pigs (data not shown). We calculated the average percentage of total liver weight to body weight (2.64%), estimated the total liver weight from preoperative body weight, and calculated the estimated remnant liver weight 1 month after operation by subtracting the resected liver weight at the operation from estimated total liver weight. Liver weight increasing rate (%) was calculated as follows; liver weight at 1 month − liver weight at resection / liver weight at resection × 100.

Quantitative real-time PCR

On laparotomy, 30 mg of surgical tissue sample was stored in liquid nitrogen and kept at –80°C until

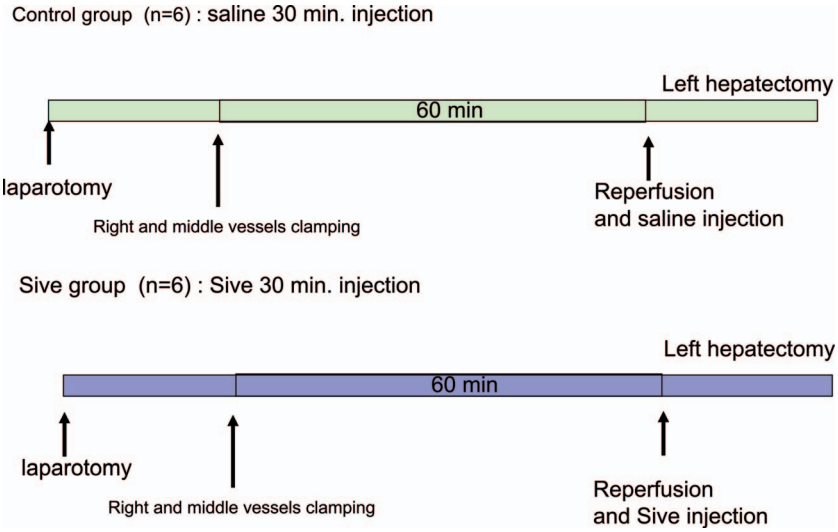

Fig. 1 Study protocol. Two groups of animals were prepared: both groups in which Sive and saline were administered intravenously at a dose of 10 mg/kg/h, just from commencement of reperfusion to completion of the liver resection.

extraction of total RNA using a commercial kit (Nucleospin II, Macherey-Nagel, Düren, Germany). Reverse transcription reactions were performed using a commercial system for t-PCR (SuperScript III First-strand Synthesis System, Invitrogen, Carlsbad, California). Briefly, 1 μg of total RNA, oligo dT primer, and dNTPs were incubated at 65°C for 5 minutes, and then 10 μL of cDNA synthesis mixture was added and incubated at 50°C for 50 minutes. The reaction was terminated by adding 1 μL of RNase H and incubated at 37°C for 20 minutes. Real-time PCR was performed with a sequence detector (ABI Prism 7700, Applied Biosystems, Warrington, UK). The PCR reaction was carried out in a final volume of 1 μL cDNA, 2 μL 10X SYBR Green (Applied Biosystems), using 40 cycles at 95°C for 30 seconds and at 60°C for 30 seconds. The specific primers were designed using Primer 3 software (http://frodo.wi.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized by Sigma Genosys (Hokkaido, Japan). The sequences of each primer were as follows: GAPDH: sense 5′-CCA CCC AGA AGA CTG TGG AT-3′, anti-sense 5′-TTC AGC TCA GGG ATG ACC TT-3′; TLR4: sense 5′-CCC CTG TCC ATC CCT TTA TT-3′, anti-sense 5′-AAG CCC CAG TTC CAA TTC TT-3′. For each PCR run, a standard curve was constructed from serial dilutions of cDNA from the PANCI cell line. The level of expression of TLR4 was calculated using the formula: relative expression (t) = (copy number of TLR4 number / copy number of GAPDH) × 1000. For non-template reactions and standard cDNA dilutions from PANCI cells, liver samples were assayed in triplicate. The average and standard deviation were calculated and the t value was determined from the averages.

Histologic examination

Tissue samples were obtained from; at the time of laparotomy, the remnant liver just after hepatectomy, 3 hours after declamp of right and middle hepatic arteries and portal vein, and after 1 month, which then were fixed with 10% formalin for 24 hours and embedded in paraffin. Sections of 3-μm thickness were stained by the *in situ* terminal TUNEL method using an apoptosis *in situ* detection kit (Wako Pure Chemical, Inc, Osaka, Japan) according to the manufacturer's instructions. The mean numbers of apoptotic cells per 10 high-power fields randomly were calculated and compared between the 2 groups.

Statistical analysis

Statistical analyses were performed with a statistical software package (SPSS, version 13.0; SPSS Inc, Chicago, Illinois). At first, associations between different categoric variables were assessed using Fisher exact test or Mann-Whitney U-test. Continuous variables were compared between the 2 groups using Welch t test (F-test: $P < 0.05$) or Student's t-test (F-test: $P > 0.05$). All values are expressed as median (min-max) or mean ± SD. Differences between the 2 groups were evaluated using analysis of variance with $P < 0.05$ considered to be significant.

Results

There were no significant differences in weight, amount of intraoperative hemorrhage, liver resection time, and weight of resected liver (Table 1). There were

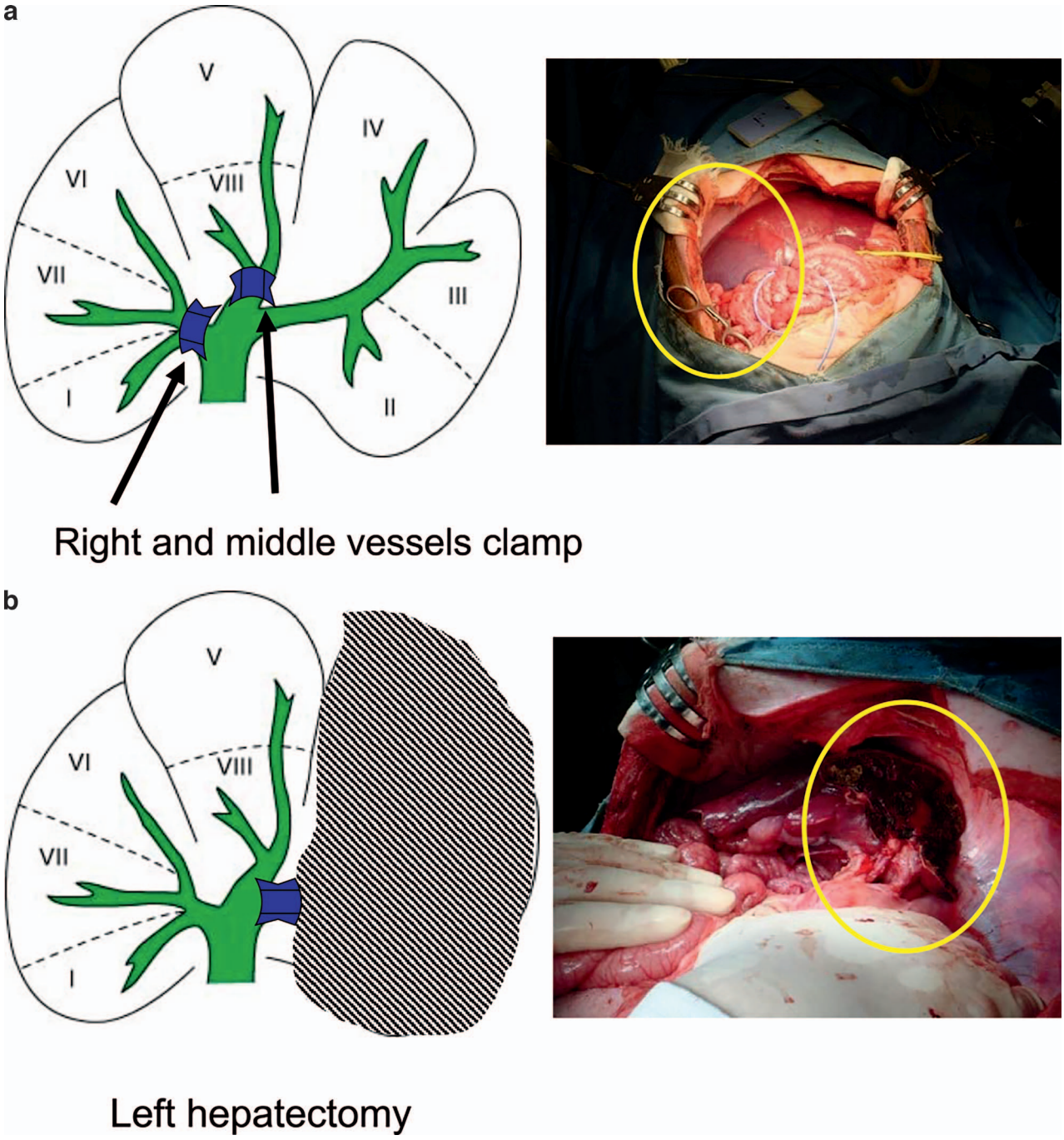

Fig. 2 Surgical procedure. (a) The hepatic pedicle was isolated, and the hepatic pedicle, containing the all branch of the portal veins and hepatic arteries, were exposed. Partial hepatic ischemia was created by clamping of the right and middle hepatic pedicle (yellow circle). Clamping was maintained for 60 minutes. (b) Almost 40% of the left section was resected (yellow circle), after 60 minutes of clamping.

no significant differences in systolic and diastolic blood pressures between the 2 groups (Fig. 3a and 3b)

In serum chemistry, AST, LDH, and LA levels at 5 minutes after reperfusion were significantly lower in the Sive group than in the control group (190.3 ± 61.3 versus 163.0 ± 22.3 IU/L: $P = 0.009$, 904.0 ± 154.3 versus 697.7 ± 107.0 IU/L: $P = 0.020$ and 29.2 ± 8.3 versus 17.1 ± 4.9 mg/dL: $P = 0.012$; Fig. 4a, 4b, and 4c). Fig. 5 shows expression of TLR4-mRNA in liver tissues; there was no significant difference

Table 1 Operative findings in control group and Sive treatment group

	Control group	Sive group	*P* value
Body weight at operation, kg (range)	25.0 (24.0–25.0)	24.0 (23.0–26.0)	0.21
Body weight (after 1 month), kg (range)	29.0 (26.5–33.0)	28.0 (24.0–29.0)	0.18
Bleeding, g(range)	80.0 (50.0–200.0)	40.0 (0–120.0)	0.14
Resection time, min(range)	21.5 (13.0–32.0)	23.5 (17.0–28.0)	0.84
Resected liver weight (RxLw), g (range)	295.0 (248.0–334.0)	273.0 (220.0–318.0)	0.23
Liver weight (after 1 month), g (range)	552.5 (420.0–625.0)	575.5 (510.0–660.0)	0.21
Liver weight (after 1 month)-RxLw/ RxLw, % (range)	78.4 (30.0–142.0)	119.7 (84.0–146.0)	0.18

between both groups. Also, there were no significant differences between the 2 groups regarding the expression of TUNEL staining 1 month after operation.

Discussion

In 1908, Pringle[7] first reported a hepatectomy procedure for traumatic liver damage, with control of hemorrhage by blockage of blood circulation into the liver. Since then, The Pringle method has usually been performed for the reduction of the amount of hemorrhage during hepatectomy.[7] However, this method inevitably causes I/R injury, and has a risk of inducing abnormally high hepatic enzyme levels, icterus, hyperammonemia, lactacidemia, and intractable accumulation of pleural and peritoneal effusion postoperatively. In addition, we experienced a quite severe decrease in the blood pressure at the time of I/R. I/R injury associated with Pringle maneuver may affect outcome when the ischemic time is long, or in hepatectomy for a diseased liver, such as in cases of hepatic cirrhosis and fatty liver. Despite the reduction in the intraoperative hemorrhage achieved by the Pringle method,[8] recently,

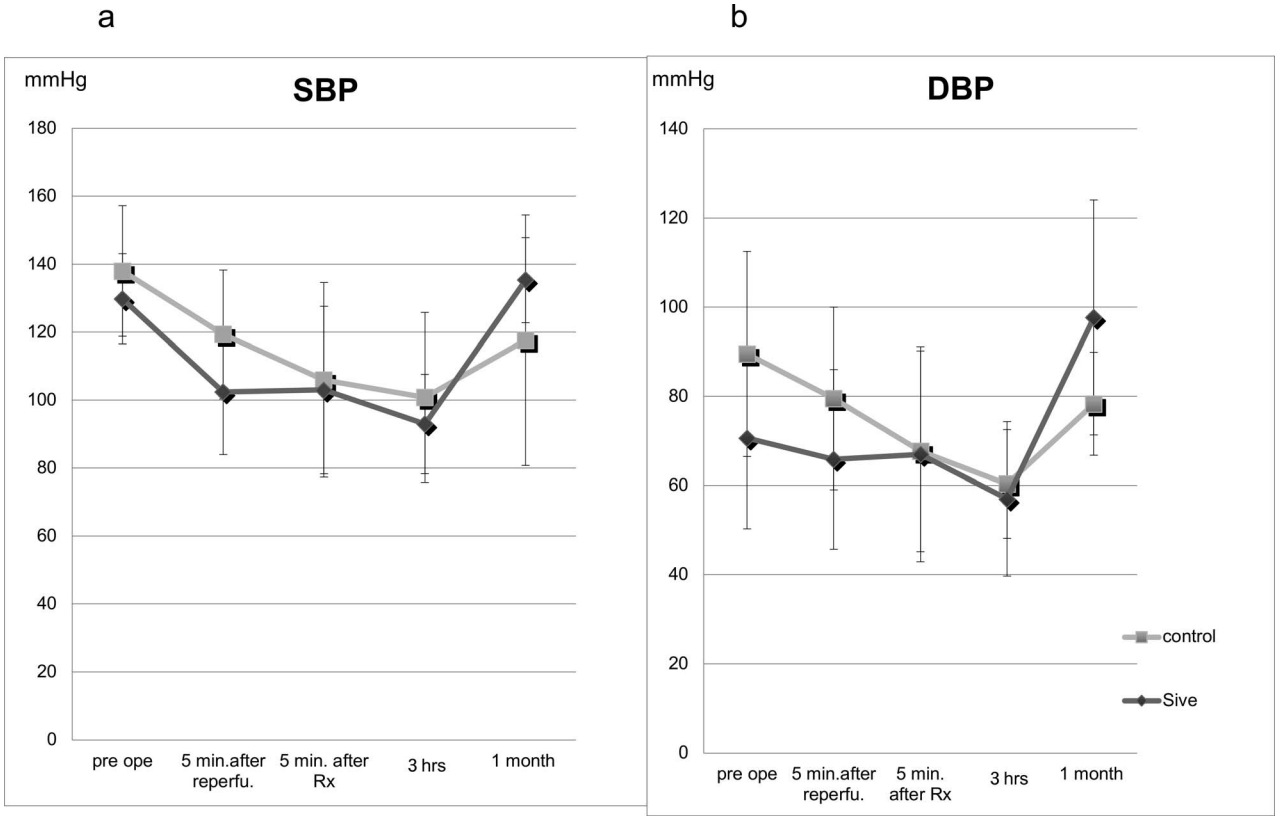

Fig. 3 Circulation dynamics. Systolic blood pressure [SBP (a)] and diastolic blood pressure [DBP (b)] had no differences between the 2 parameters. All values are expressed as mean ± SD. Parameters were evaluated using Student's *t*-test.

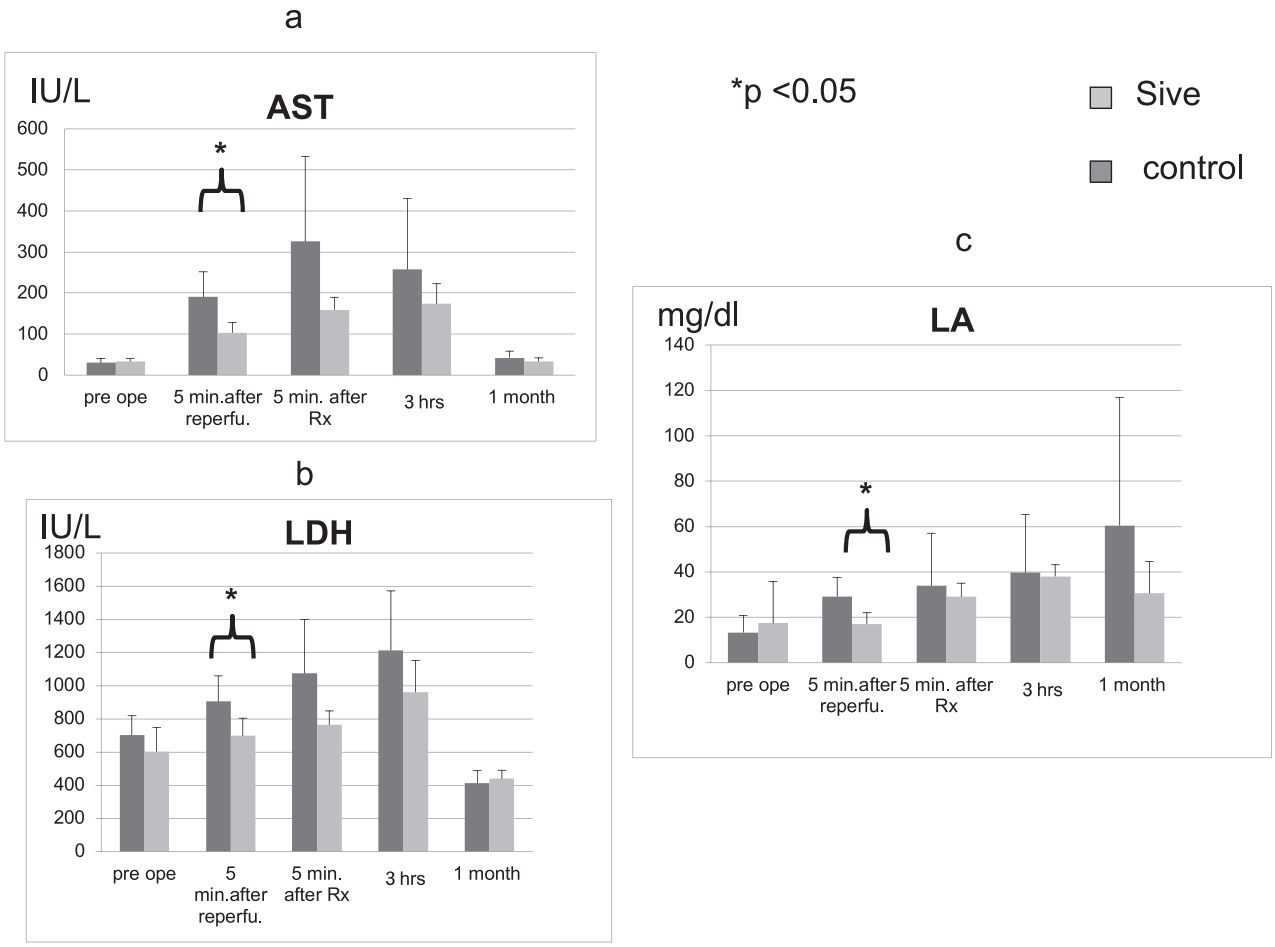

Fig. 4 Serum biochemical parameters. The AST LDH and LA were significantly lower 5 minutes after reperfusion in the Sive group compared to the control group. All values are expressed as means ± S.D.. Parameters were evaluated using Student's *t*-test.

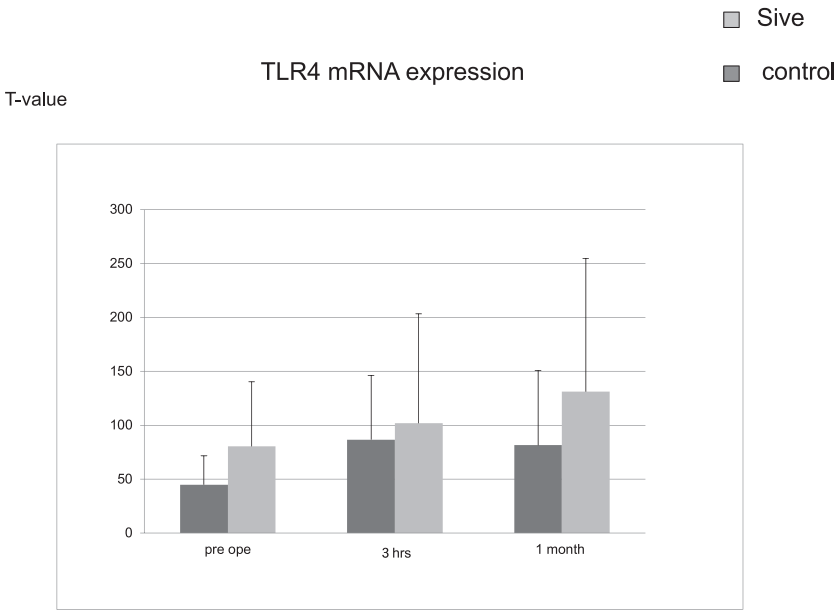

Fig. 5 TLR4 mRNA expression. TLR4 mRNA level was no significant difference in both groups. All values are expressed as mean ± SD. Parameters were evaluated using Mann-Whitney *U* test.

some reports described that prolonging Pringle time or the Pringle method itself were independent risk factors for tumor recurrence in the liver malignancy.[9–11]

I/R injury may be a causal factor of the above symptoms, and various countermeasures have been proposed. These include modified surgical methods, such as an intermittent Pringle method, partial hepatic pedicle clamping, and ischemic preconditioning, as well as drug therapy with steroids, prostaglandin E1, edarabone, or erythropoietin.[12–18] However, assessment of the effectiveness of these approaches has been limited. We have sought that such assessments can be clarified through studies in animals of similar size to humans. In general, the process of I/R injury consists of multiple steps. Hypoxia due to cessation of blood supply impairs oxidative phosphorylation in the mitochondria, leading to profound cellular damage.[19] Reperfusion further exacerbates cellular damage by producing reactive oxygen species, activating anti-inflammatory cytokines such as IL-10 and TNF-α,[20] and upregulating cell adhesion molecules such as P-, E-, and L-selectins,[21,22] resulting in tissue devastation.

Neutrophils play an important role in biophylaxis. These cells include high concentrations of lysozymes and proteases, including neutrophil elastase, a lysosomal serine protease with a molecular weight of approximately 30 KDa. The natural roles of neutrophil elastase include sterilization and decomposition of foreign proteins in biophylaxis. But neutrophil elastase also degrades proteins such as elastin, collagen, and proteoglycan, which occurs when the enzyme is released in an activated form.[23] In serious inflammation or reperfusion injury, a large amount of neutrophil elastase is released, and this generates reactive oxygen species, cytokines, and tumor necrosis factors, which may lead to organ failure.

Sive is a specific neutrophil elastase inhibitor that prevents organ failure by inhibiting an increase in vascular permeability, as well as reducing production of cytokines and reactive oxygen species. The clinical efficacy of Sive for acute lung injury has been established.[1–3,24] The action of Sive in inhibiting neutrophil elastase activity may control expression of reactive oxygen species, suppress activation of Kupffer cells in the liver, and indirectly inhibit generation of TNF-α and NO, thus exerting a protective effect on the liver.[4] In this study, we observed Sive successfully reducing liver dysfunction at 5 minutes after reperfusion. However, we did not observe anti-apoptotic reaction and liver regeneration at 1 month after operation. Thus, the results suggest that Sive has a protective effect against I/R injury in very early phase after the injury, which was reflected in the decrease of markers.

TLRs consist of 13 mammalian members containing a conserved TIR Toll/IL-1 receptor domain in their intracellular domain and an individual leucine rich repeat domain in their extracellular domain. TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface and TLR3, TLR7, TLR8, and TLR9 are expressed on the endosome-lysosome membrane. TLR4 and TLR5 are the receptors for the Gram-negative bacterial cell wall components, lipopolysaccharide, and bacterial flagellin, respectively.[25] In the liver, TL4 is one of the important key factors for liver fibrosis, I/R injury, and liver regeneration. Upon I/R injury, TLR4 ligand HMGB1 is released from damaged hepatocytes and subsequently stimulates Kupffer cells through TLR4.[26] Suppression of the TLR4 at I/R injury leads to the control of liver regeneration by controlling liver fibrosis. Unfortunately, in this study, Sive never decreased TLR4 levels before and after liver resection. This result suggested that Sive did not have any effect on liver fibrosis and regeneration in cellular level.

In conclusion, our results demonstrated that Sive has a protective effect against early phase I/R injury in the liver. Further studies are needed to determine the dose, timing, and duration of Sive. Studies of Sive should be conducted under clinical setting as this study is conducted using small number of animal model, and had some limitations of sample sampling. However, we have shown, for the first time in a porcine model, that Sive would be a promising agent for ameliorating I/R injury during hepatectomy.

Acknowledgments

Conflicts of Interests: The authors declare no conflicts of interest. The authors do not have any grant and drug supports.

References

1. Tamakuma S, Ogawa M, Aikawa N, Kubota T, Hirasawa H, Ishizaka A *et al*. Relationship between neutrophil elastase and acute lung injury in humans. *Pulm Pharmacol Ther* 2004;**17**(5): 271–279

2. Soejima Y, Yanaga K, Nishizaki T, Yoshizumi T, Uchiyama H, Sugimachi K. Effect of specific neutrophil elastase inhibitor on ischemia/reperfusion injury in rat liver transplantation. J Surg Res 1999;**86**(1):150–154
3. Tomizawa N, Ohwada S, Ohya T, Takeyoshi I, Ogawa T, Kawashima Y *et al*. The effects of a neutrophil elastase inhibitor (ONO-5046.Na) and neutrophil depletion using a granulotrap (G-1) column on lung reperfusion injury in dogs. *J Heart Lung Transplant* 1999;**18**(7):637–645
4. Shimoda M, Iwasaki Y, Okada T, Sawada T, Kubota K. Protective effect of Sivelestat in a porcine hepatectomy model prepared using an intermittent Pringle method. *Eur J Pharmacol* 2008;**587**(1–3):248–252
5. Seki E, Brenner DA. Toll-like receptors and adaptor molecules in liver disease: update. *Hepatology* 2008;**48**(1):322–335
6. Watanabe T, Yuki S, Egawa M, Nishi H. Protective effects of MCI-186 on cerebral ischemia: possible involvement of free radical scavenging and antioxidant actions. *J Pharmacol Exp Ther* 1994;**268**(3):1597–1604
7. Pringle JH. V. Notes on the arrest of hepatic hemorrhage due to trauma. *Ann Surg* 1908;**48**(4):541–549
8. Belghiti J, Noun R, Malafosse R, Jagot P, Sauvanet A, Pierangeli F *et al*. Continuous versus intermittent portal triad clamping for liver resection: a controlled study. *Ann Surg* 1999; **229**(3):369–375
9. Liu S, Li X, Li H, Guo L, Zhang B, Gong Z *et al*. Longer duration of the Pringle maneuver is associated with hepatocellular carcinoma recurrence following curative resection. *J Surg Oncol* 2016;**114**(1):112–118
10. Yang Y, Fu SY, Lau WY, Lai E, Li AJ, Pan ZY *et al*. Selective main portal vein clamping to minimize the risk of recurrence after curative liver resection for hepatocellular carcinoma. *Hepatogastroenterology* 2012;**59**(117):1560–1565
11. Huang J, Tang W, Hernandez-Alejandro R, Bertens KA, Wu H, Liao M *et al*. Intermittent hepatic inflow occlusion during partial hepatectomy for hepatocellular carcinoma does not shorten overall survival or increase the likelihood of tumor recurrence. *Medicine (Baltimore)* 2014;**93**(28):e288
12. Shimoda M, Iwasaki Y, Sawada T, Kubota K. Protective effect of ischemic preconditioning against liver injury after major hepatectomy using the intermittent pringle maneuver in swine. *Pathobiology.* 2007;**74**(1):42–49
13. Clavien PA, Yadav S, Sindram D, Bentley RC. Protective effects of ischemic preconditioning for liver resection performed under inflow occlusion in humans. *Ann Surg* 2000;**232**(2):155–162
14. Makuuchi M, Mori T, Gunven P, Yamazaki S, Hasegawa H. Safety of hemihepatic vascular occlusion during resection of the liver. *Surg Gynecol Obstet* 1987;**164**(2):155–158
15. Hanazaki K, Kajikawa S, Fujimori Y, Nakata S, Shimozawa N, Koide N *et al*. Effects of prostaglandin E1 administration during hepatectomy for cirrhotic hepatocellular carcinoma. *Hepatogastroenterology* 2000;47(32):461–464
16. Shimoda M, Iwasaki Y, Okada T, Kubota K. Edaravone inhibits apoptosis caused by ischemia/reperfusion injury in a porcine hepatectomy model. *World J Gastroenterol* 2012;**18**(27):3520–3526
17. Kato M, Sawada T, Kita J, Shimoda M, Kubota K. Erythropoietin ameliorates early ischemia-reperfusion injury following the Pringle maneuver. *World J Gastroenterol* 2010;**16**(38):4838–4845
18. Shimoda M, Sawada T, Iwasaki Y, Mori S, Kijima H, Okada T *et al*. Erythropoietin strongly protects the liver from ischemia-reperfusion injury in a pig model. *Hepatogastroenterology* 2009; **56**(90):470–475
19. Grace PA. Ischaemia-reperfusion injury. *Br J Surg* 1994;**81**(5): 637–647
20. Lopez-Neblina F, Toledo-Pereyra LH. Anti-ischemic effect of selectin blocker through modulation of tumor necrosis factor-alpha and interleukin-10. *J Surg Res* 2007;**138**(2):275–283
21. Clavien PA, Harvey PR, Sanabria JR, Cywes R, Levy GA, Strasberg SM. Lymphocyte adherence in the reperfused rat liver: mechanisms and effects. *Hepatology* 1993;**17**(1):131–142
22. Tsuchihashi S, Fondevila C, Shaw GD, Lorenz M, Marquette K, Benard S *et al*. Molecular characterization of rat leukocyte P-selectin glycoprotein ligand-1 and effect of its blockade: protection from ischemia-reperfusion injury in liver transplantation. *J Immunol* 2006;**176**(1):616–624
23. Janoff A. Elastases and emphysema. Current assessment of the protease-antiprotease hypothesis. *Am Rev Respir Dis* 1985; **132**(2):417–433
24. Yamaguchi Y, Matsumura F, Liang J, Okabe K, Ohshiro H, Ishihara K *et al*. Neutrophil elastase and oxygen radicals enhance monocyte chemoattractant protein- expression after ischemia/reperfusion in rat liver. *Transplantation* 1999;**68**(10): 1459–1468
25. Akira S. Pathogen recognition by innate immunity and its signaling. *Proc Jpn Acad Ser B Phys Biol Sci* 2009;**85**(4):143–156
26. Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT *et al*. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. *J Exp Med* 2005;**201**(7): 1135–1143