Platelet adhesion in the sinusoid caused hepatic injury by neutrophils after hepatic ischemia reperfusion

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Abstract

Liver ischemia-reperfusion (I/R) injury is one of the most serious complications of hepatic surgery. In I/R, activated Kupffer cells cause platelet adhesion to sinusoidal endothelium as well as neutrophils and cause liver dysfunction. The aim of this study was to evaluate platelet dynamics in the hepatic microcirculation after I/R by intravital microscopy (IVM) and to clarify the relationship between platelet adhesion and neutrophil activation. Male Sprague-Dawley (SD) rats were divided into two groups: the control (administration of saline) group and the sivelestat group in which neutrophil activation was suppressed by sivelestat before I/R. The number of adherent platelets in sinusoid was observed up to 120 minutes after I/R by IVM. Samples of liver tissue and blood were taken for examination of histological findings, liver enzymes and inflammatory cytokines. The number of adherent platelets was significantly increased after I/R in both groups. Compared with the control group, the number of adherent platelets significantly decreased after hepatic I/R in the sivelestat group. Moreover, sivelestat improved changes of histological findings and elevation of liver enzymes. However, there was no significant difference in inflammatory cytokines of TNF-α, IL-1β and IL-6. Platelet adhesion in the sinusoid is associated with liver dysfunction after I/R as well as neutrophils. Activated neutrophils induce the platelet adhesion in the sinusoid of the liver.

Introduction

Liver injury caused by hepatic ischemia-reperfusion (I/R) is a major complication of liver resection and liver transplantation. The disturbance of hepatic microcirculation is considered the primary cause of hepatic I/R injury[1]. An excessive inflammatory response is a critical component of hepatic I/R injury. Previous studies have reported that there are two distinct phases of liver injury after warm hepatic I/R[2,3]. The early phase is characterized by activation of Kupffer cells (KCs) which generate reactive oxygen species and aggravate the early injury occurring within 120 minutes[4,5]. Neutrohphils accumulate in the liver and contribute to hepatocyte injury in the late phase, which appears more than 6 hours after reperfusion[2,3].

A neutrophil elastase inhibitor, sivelestat, has been approved as a therapeutic drug for acute lung injury associated with systemic inflammatory response syndrome[6]. In the previous report we demonstrated that after the induction of liver ischemia, sivelestat attenuated the number of accumulated neutrophils and suppressed I/R injury[7].

Recently some studies have focused on the role of platelets in hepatic I/R. Activated platelets produce proinflammatory mediators, such as chemokines and cytokines[8,9]. Sindram et al. reported that without platelets and leukocytes, the function of KCs was decreased and I/R injury was suppressed[10]. However, the relationship between platelets and neutrophils is still unclear. The aim of this study was to investigate platelet adhesion on I/R of the livers *in vivo* using IVM and the relationship between platelet adhesion and neutrophil activation.

Materials and Methods

Animals

Male Sprague-Dawley (SD) rats, weighing 250 to 300 g, were obtained from CLEA Japan (Tokyo, Japan). Animal experiments were carried out in a humane manner after receiving approval from the Institutional University Experiment Committee of the University of Tsukuba, and in accordance with the Regulation for Animal Experiments in the university and Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology.

Experimental groups

Total warm hepatic ischemia was induced for 20 minutes by clamping the portal triad. Animals were divided into two groups as follows: (A) a control group, in which saline was continuously infused via the jugular vein for 60 minutes 10 ml/kg/h before hepatic ischemia (n=6), and (B) the sivelestat group, in which 10 mg/kg/h sivelestat (Ono Pharmaceutical Co., Osaka, Japan) was continuously infused via the jugular vein 60 minutes before hepatic ischemia (n=6). The dose of sivelestat described above was determined by previous reports[7,11]. Sivelestat, ONO-5046; N- [2-(4-[2,2-dimethylpropionyloxy] phenylsulphonyl-amino)benzoyl] was dissolved in saline and the pH was modulated to 7.8 using Na2 CO3. All animals also received a continuous infusion of saline via the jugular vein to prevent dehydration (10 ml/kg/h).

Administration of sivelestat and saline was started 60 minutes before hepatic ischemia

to achieve an adequate blood concentration and continued up to 120 minutes after

reperfusion[12]. Platelet dynamics and hepatic microcirculation were observed just

before ischemia and at 30, 60, and 120 minutes after reperfusion (Fig.1).

Surgical Procedure

Under anesthesia with intraperitoneal sodium pentobarbital (50 mg/kg), the animals

were tracheotomized. To reduce spontaneous breathing, the animals were ventilated

mechanically (KN-55; Natsume Co. Ltd., Tokyo, Japan). When spontaneous breathing

interfered with mechanical ventilation, additional pentobarbital (10 mg/kg) was

administered intravenously. The animals were placed in a supine position on a heated

pad to maintain a rectal temperature of 37°C. To monitor arterial blood pressure and

allow continuous infusion of sivelestat and saline, polyehylen catheters (PE-50,

0.58/0.96 mm internal/external diameter; Becton Dickinson, Sparks, MD) were inserted

into the left carotid artery and left jugular vein. After laparotomy had been performed by

a transversal incision, the ligaments around the liver were dissected to mobilize the left

lobe. At the same time, the hepatoduodenal ligament was taped in preparation of

clamping later. The left lobe was exteriorized on a plate specially designed to minimize

movement caused by respiration and covered with a cover glass. After 60 minutes of

continuous sivelestat or physiological saline infusion, intravital fluorescence

microscopy (IVM) was performed as a baseline study. Hepatic ischemia was then

induced by portal triad clamping, i.e., hepatic artery, portal vein, and bile duct, by

means of a microclip (Aesculap, Tuttlingen, Germany) for 20 minutes. IVM was

performed at 30, 60, and 120 minutes after reperfusion. Blood samples were taken for the analysis of enzyme activities in serum at the same time as IVM. At the end of the experiments, liver tissue was taken for histological examination.

Platelet Preparation

Platelets were isolated from whole blood of syngeneic rats and labeled with rhodamine-6G (50 μL/ml whole blood: R-4127; Sigma, St. Louis, MO), as described by Massberg et al[13]. Briefly, the collected blood was diluted with buffer after the addition of prostaglandin E1 and rhodamine-6G. After 2-cycle centrifugation, fluorescent platelets were resuspended in PBS. In this study, a total amount of 1×10^8 fluorescence-labeled platelets, approximately 1% of all circulating platelets in the recipient rat, were injected through the left carotid artery at 5 minutes before IVM.

Intravital Fluorescence Microscopy (IVM)

IVM was performed using a modified microscope (BX30 FLA-SP; Olympus, Tokyo, Japan) with a 100 W mercury lamp attached to a filter block. The hepatic microcirculation was recorded by means of a CCD camera (C5810; Hamamatsu Photonics, Hamamatsu, Japan) and a digital video recorder (GV-D1000 NTSC; Sony, Tokyo, Japan) for off-line analysis. Using objective lenses (10 x 0.3 to 20 x 0.7; Olympus, Tokyo, Japan), a final magnification of 325x to 650x was achieved on the video screen. To assess sinusoidal perfusion, sodium fluorescein (2 x 10-3 M/kg, F-6377; Sigma, St. Louis, MO) was injected via the jugular catheter. Rhodamine-6G

labeling platelets were infused intra-arterially just before ischemia and at 30, 60, and 120 minutes after reperfusion, and 7 to 10 randomly chosen acini were visualized.

Quantitative assessment of microcirculatory parameters was performed offline using WinRoof image software (version 5.0; Mitani Shoji, Tokyo, Japan).

Microcirculatory Analysis

The following two parameters were analyzed. (1) Number of adherent platelets; platelets firmly attached to the endothelium within the sinusoid for longer than 20 seconds. The number of adherent platelets was counted in scanned acini. The results were expressed as the number of adherent platelets per field (1 field = approximately 0.2 mm^2). (2) Zonal distribution of platelets (zone 1 + zone 2 and zone 3).

Histological Analysis

After 120 minutes of reperfusion, liver tissues were obtained from each group, fixed with 10% formaldehyde, and embedded in paraffin. Thin sections (4μm) were prepared and stained with hematozylin (HE). Tissue damage was evaluated in five randomly selected high-power fields (x 400).

Biochemical Assays

As a marker of liver deterioration, the activities of alanine transaminase (ALT) and lactate dehydrogenase (LDH) were measured in serum using a Drychem 7000V auto

analyzer (Fuji Film, Tokyo, Japan).

Serum was stored at -80°C until use for cytokine determination. Levels of tumor necrosis factor (TNF) α, interleukin (IL) 1β, and IL-6 were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN).

Statistical Analysis

All data are expressed as mean ± standard deviation (SD). The Mann-Whitney test and analysis of variance (ANOVA) were used, followed by Scheffe's test. *P* values <0.05 were considered statistically significant.

Results

Number and Zonal Distribution of Adherent Platelets in Acini

In the control group, the number of adherent platelets was increased after reperfusion (P<0.001). This continued in proportion to the duration of reperfusion up to 120 minutes (Fig.2A and B). In the sivelestat group, platelet adhesion was significantly prevented compared with the control group (Fig. 2A and B). In the control group approximately 67% of adherent platelets were located in zones 1 and 2 at 120 minutes after reperfusion. In the sivelestat group, the number of adherent platelets in zones1 and 2 was significantly decreased compared with the control group (Fig. 3).

Histological Findings

In the control group vacuolation of hepatocyte and sinusoidal narrowing were demonstrated histologically after 120 minutes of reperfusion (Fig. 4). These findings were not recognized in the sivelestat group.

Serum Liver Enzymes and Cytokines Levels

The number of white blood cell (WBC) and platelet (PLT) in the blood was not significantly different between the control and sivelestat groups within 120 minutes after I/R (Data are not shown). However, serum ALT and LDH reflecting parenchymal injury significantly increased after I/R in the control group. In the sivelestat group, ALT

and LDH after I/R were significantly lower than in the control group (Fig.5). In addition,

the concentration of serum TNF-α, IL-1β, and IL-6 was not significantly different

between the two groups (Table.1).

Discussion

The role of neutrophils in hepatic I/R has been investigated in many studies[1,2,5]. Recently, some studies have focused on the role of platelets in the stress condition of the liver[14-16]. Platelets have a pivotal function of biological improvement or deterioration in the liver stress[17]. Some reports demonstrated that platelets contribute toward the prevention of liver fibrosis and promote liver regeneration after hepatectomy[16-18]. Conversely, several reports have demonstrated the contribution of platelets on hepatic I/R injury[8,9,14]. In this study, we evaluated the dynamics of platelets in liver microcirculation and tried to clarify the mechanism of platelet adhesion after I/R. As a result, adherent platelets were significantly increased after I/R and platelet adhesion was induced by neutrophil activation.

Hepatic I/R stimulated KCs and neutrophils and the interaction between these cells also reactivated themselves[9,19]. Activated neutrophils released neutrophil elastase and adhered to sinusoidal endothelial cells and migrated to parenchymal cells[1,3,5]. In addition, platelets also have a correlation with hepatic I/R[14]. Platelet adhesion was increased after hepatic I/R[14,21]. Neutrophils and platelets synergistically exacerbate sinusoidal endothelial cell apoptosis in hepatic I/R and KCs alone did not cause the hepatic I/R injury[10]. In our previous study we demonstrated that the number of adherent platelets was suppressed by the elimination of KCs[14]. The present study revealed that zonal distribution of adherent platelets was mostly located in zones 1 and 2. The distribution of platelets was similar to that of KCs[14]. It is likely that there is a correlation between adherent platelets and KCs. In the control group, vacuolation of hepatocyte and sinusoidal narrowing were demonstrated after I/R on histological

examination, but in the sivelestat group these findings were not detected. Sivelestat suppressed the number of adherent platelets and elevation of liver enzymes. Adherent platelets are likely to be related to neutrophil activation. Therefore, platelets also induced hepatic microvascular dysfunction as well as neutrophils and KCs[1,3,5].

Activated neutrophils cause conversion of xanthine dehydrogenase to xanthine oxidase in endothelial cells, and the mechanism is associated with the cytotoxic effect of activated neutrophils[22]. Sivelestat is a highly selective neutrophil elastase inhibitor[23]. Some studies reported that sivelestat was implicated in the conversion from xanthine dehydrogenase to xanthine oxidase during ischemia[11,22]. Kusner et al. reported that neutrophil elastase promoted neutrophil activation[24]. Administration of sivelestat before induction of ischemia protects against hypoxia-induced neutrophil activation. This so-called "neutrophil priming" has been considered to be an indispensable step in reperfusion injury[25,26]. Our previous study demonstrated that administration of sivelestat suppressed the number of adherent neutrophils[7]. Therefore, the suppression of neutrophil elastase is likely to attenuate neutrophil priming by suppressing xanthine oxidase release after reperfusion. On the other hand, Yamaguchi et al. indicated that suppression of inflammatory cytokines was a possible mechanism of the neutrophil elastase suppression[27]. However in this study we demonstrated that sivelestat did not suppress the inflammatory cytokines but did suppressed the number of adherent platelets after I/R. From these findings, it is highly possible that suppression of neutrophil activation by sivelestat attenuated the number of adherent platelets in sinusoid.

In this study, sivelestat did not suppress the levels of serum cytokines such as TNF-α, IL-1β, and IL-6. Activated KCs released a large amount of both proinflammatory and

anti-inflammatory mediators after I/R, i.e., TNF-α, IL-1, IL-6, PG, IL-10 and IL-13[9]. In general, following the activation of KCs, platelets react and immediately accumulate in the hepatic sinusoids[3]. The present study demonstrated that sivelestat did not suppress the levels of inflammatory cytokines. It is likely that there was no correlation between KCs and administration of sivelestat. Our results indicated that platelet adhesion was induced by neutrophil activation without KCs.

To reduce hepatic I/R injury, various agents and methods have been reported[11,20]. However, they are not suitable for clinical use due to toxicity or other side effects. Sivelestat actually suppressed the number of adherent neutrophils[7] and platelets and sivelestat prevented apoptosis of sinusoidal endothelial cells after hepatic I/R. Therefore, pre-administration of sivelestat would be effective in the prevention against liver injury.

Conclusion

Adhesion of platelets was involved in hepatic I/R injury. The activation of neutrophils was strongly associated with adherent platelets. Sivelestat appears to be useful for hepatic I/R in a clinical setting.

Acknowledgments

Figure legend

Figure.1: Experimental groups.

Total warm hepatic ischemia was performed for 20 min by clamping the portal triad in each group. Animals were divided into two groups: control group, and sivelestat group. A total of 1 x 10^8 fluorescence-labeled platelets, approximately 1% of all circulating platelets in the recipient rat, were injected through the left carotid artery before 5 min of IVM.

Figure.2: Platelet dynamics in sinusoid.

(A) In the sivelestat group, the number of adherent platelets was significantly decreased compared with the control group before induction of ischemia and at 30 min after reperfusion and with duration of reperfusion. *P<0.001; *versus* the control group (n=6).
(B) Video images of adherent platelets in acini 120 min after reperfusion. Many platelets adhered in the sinusoid in the control group. However, in the sivelestat group only a few platelets were recognized.

Figure.3: Zonal distribution of adherent platelets.

In the control group, approximately 67% of adherent platelets were located in zones1 and 2 at 120 min after reperfusion. *P<0.001; *versus* zones1 and 2 in the control group; **P<0.005; *versus* zones 1 and 2 in the control group; ***P<0.005; *versus* zone 3 in the control group (n=6).

Figure.4: Histological findings.

In the control group, vacuolation of hepatocyte and sinusoidal narrowing (arrow head) were demonstrated after 120 min of reperfusion on histological examination. In the sivelestat group, these findings were not recoginized (HE stain x 400).
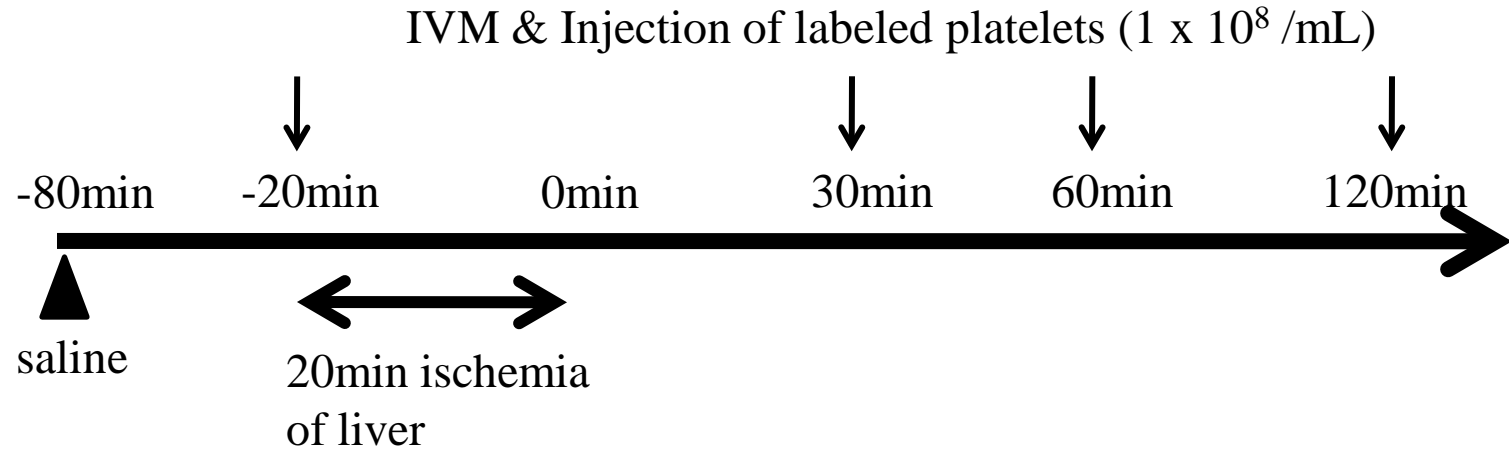
Figure.5: Serum liver enzymes.

Serum ALT and LDH, reflecting hepatic parenchymal injury, increased after reperfusion in the control group. *P<0.05; *versus* before induction of ischemia in the control group. In the sivelestat group serum ALT and LDH significantly decreased after reperfusion. **P<0.05; *versus* the control group (n=6).

Figure.1

control group

IVM & Injection of labeled platelets (1×10^8 /mL)

-80min   -20min   0min   30min   60min   120min

saline

20min ischemia
of liver

sivelestat group

IVM & Injection of labeled platelets (1×10^8 /mL)

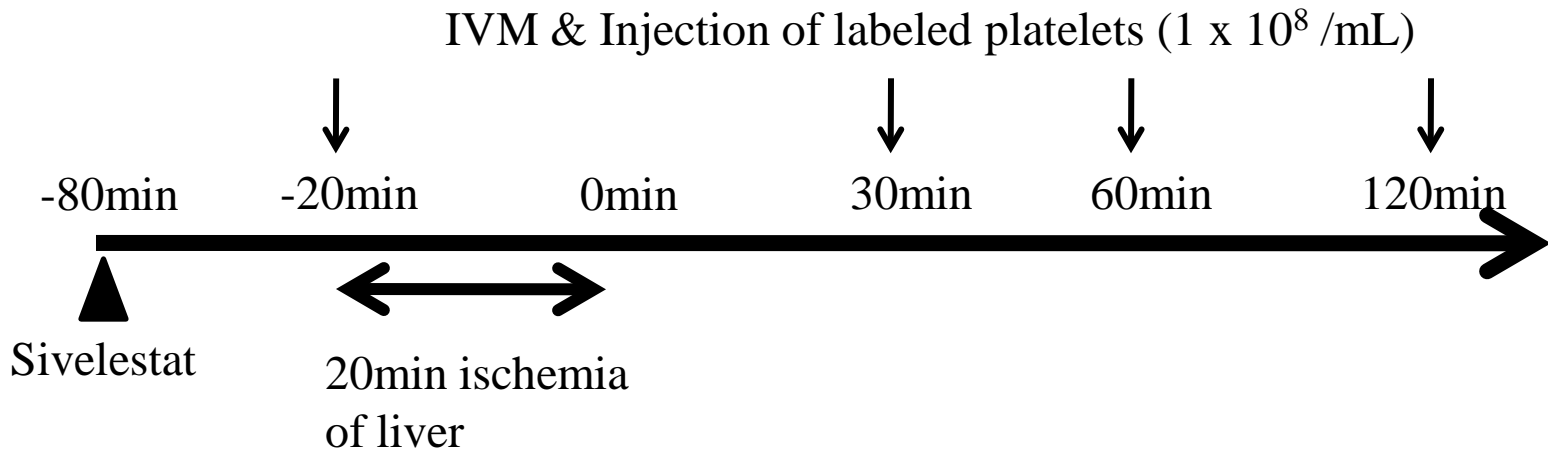-80min   -20min   0min   30min   60min   120min
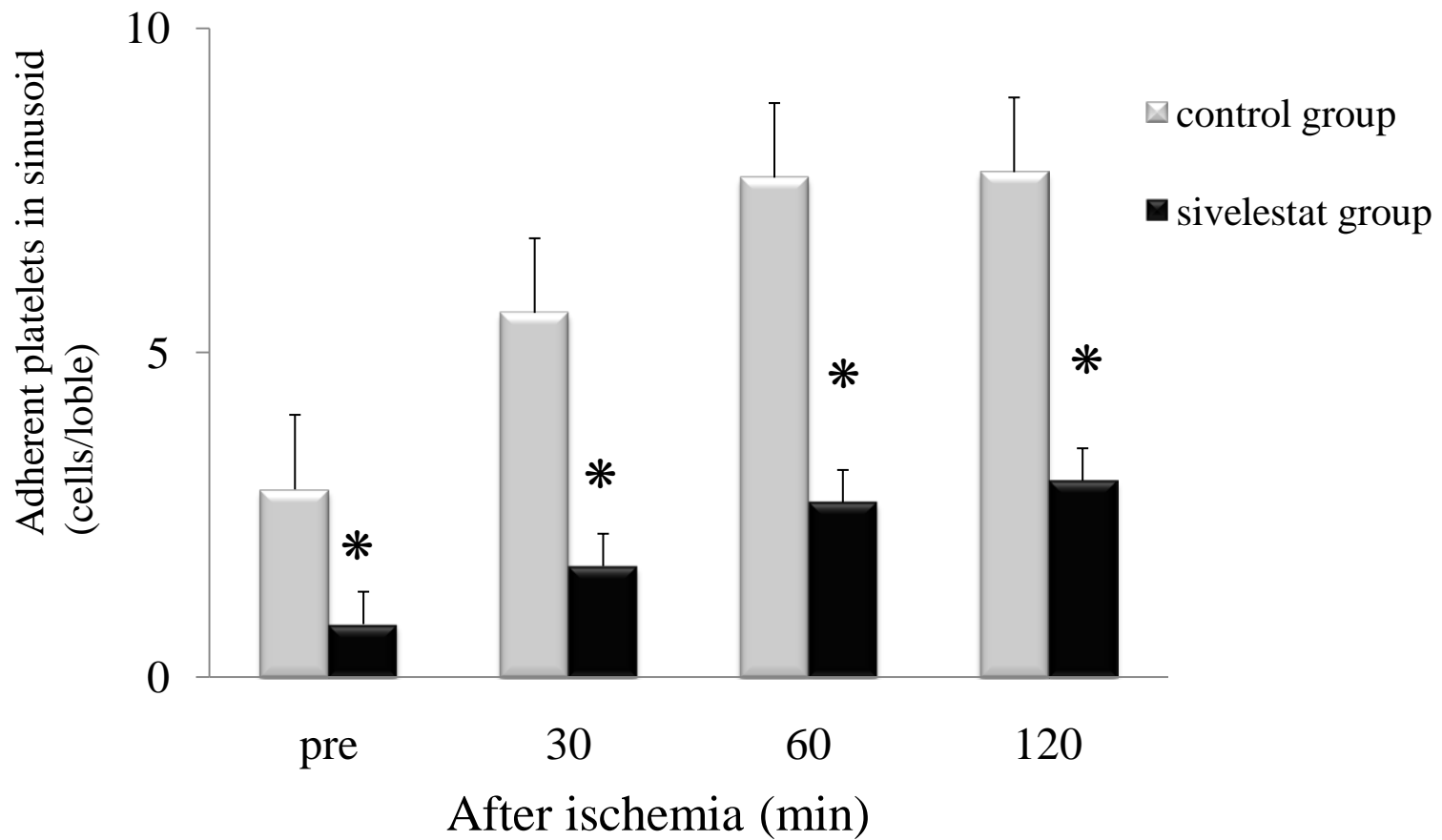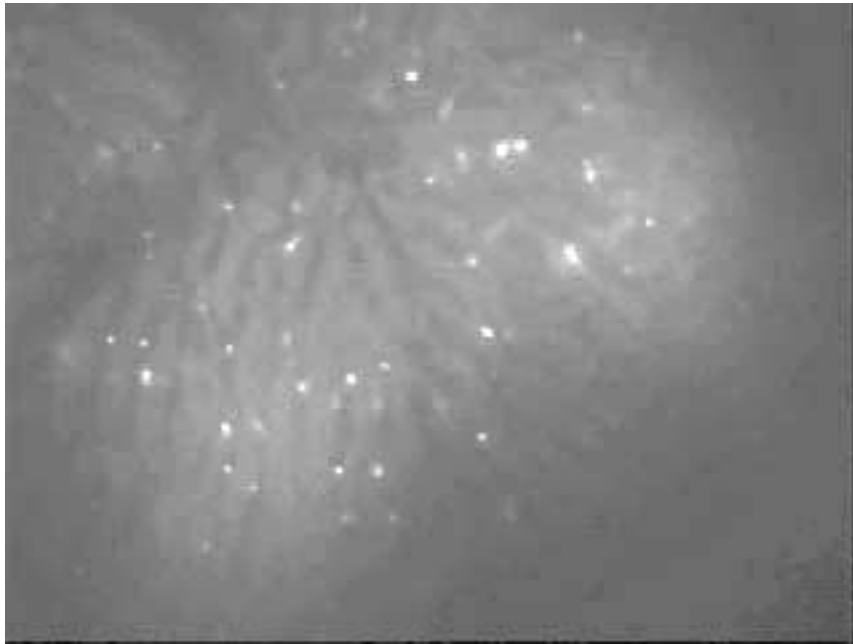
Sivelestat

20min ischemia
of liver

Figure.2A

Figure.2B

control   group

sivelestat group

Figure.3
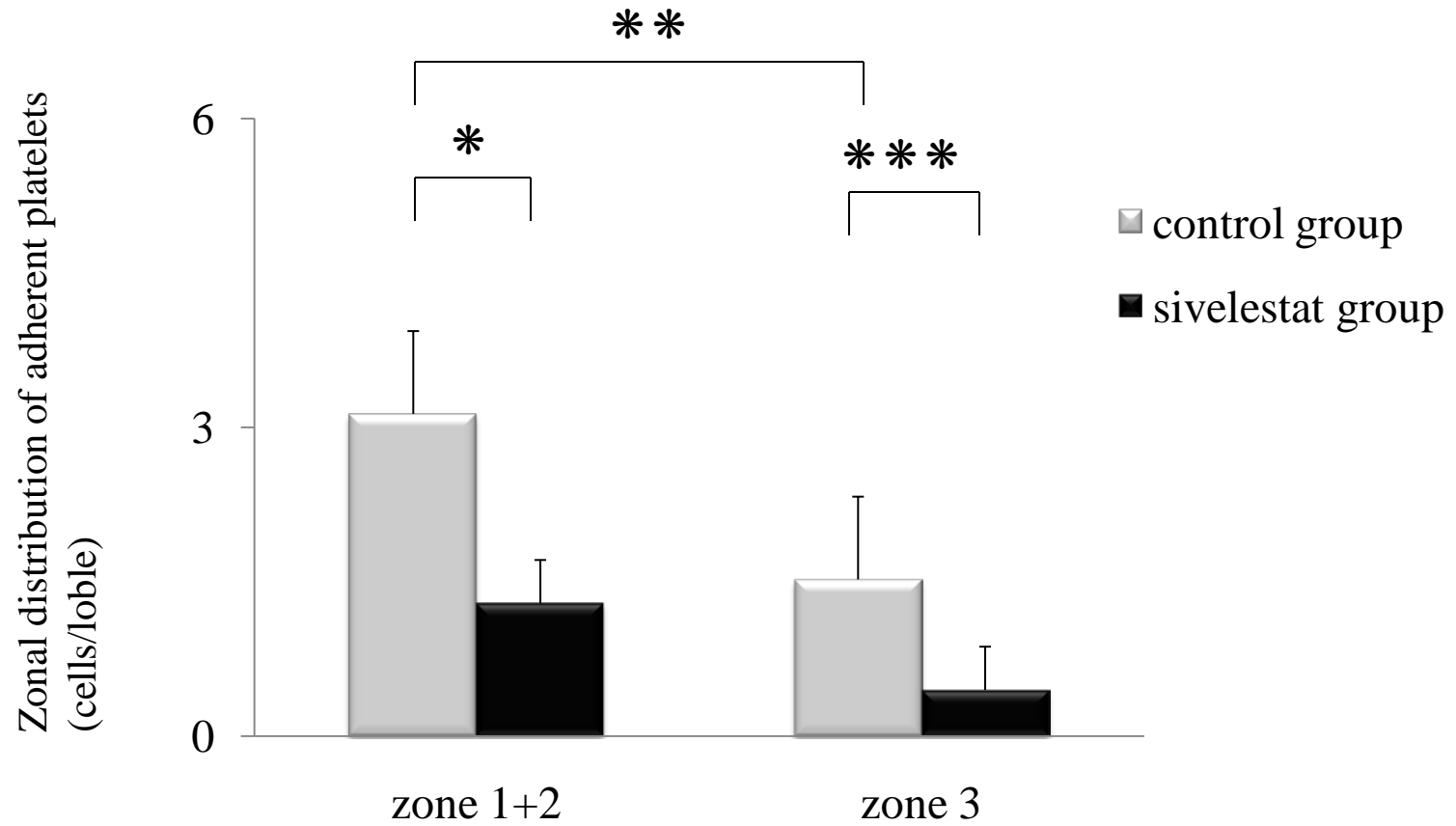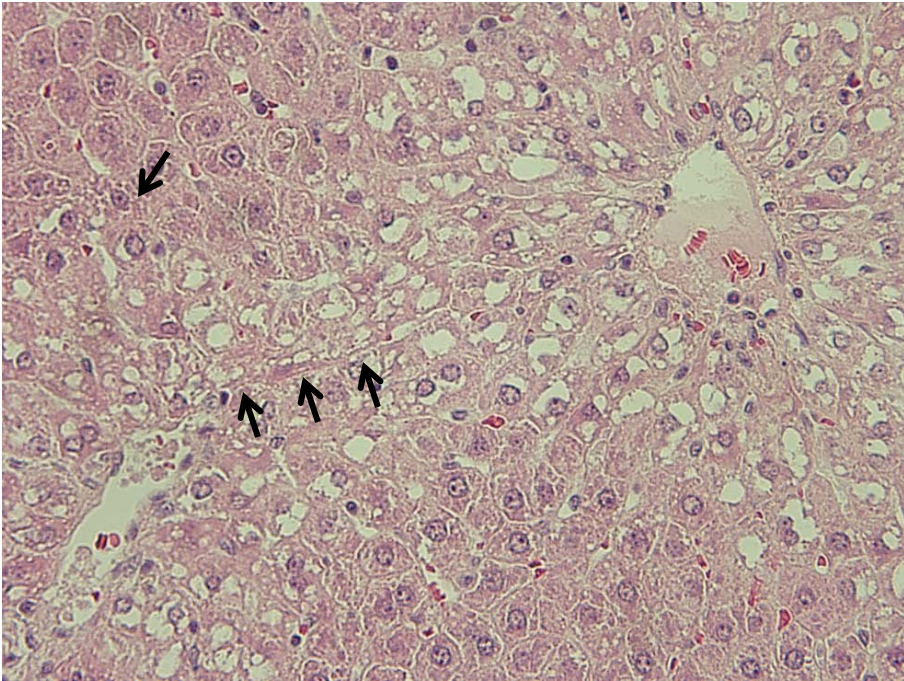
Figure.4
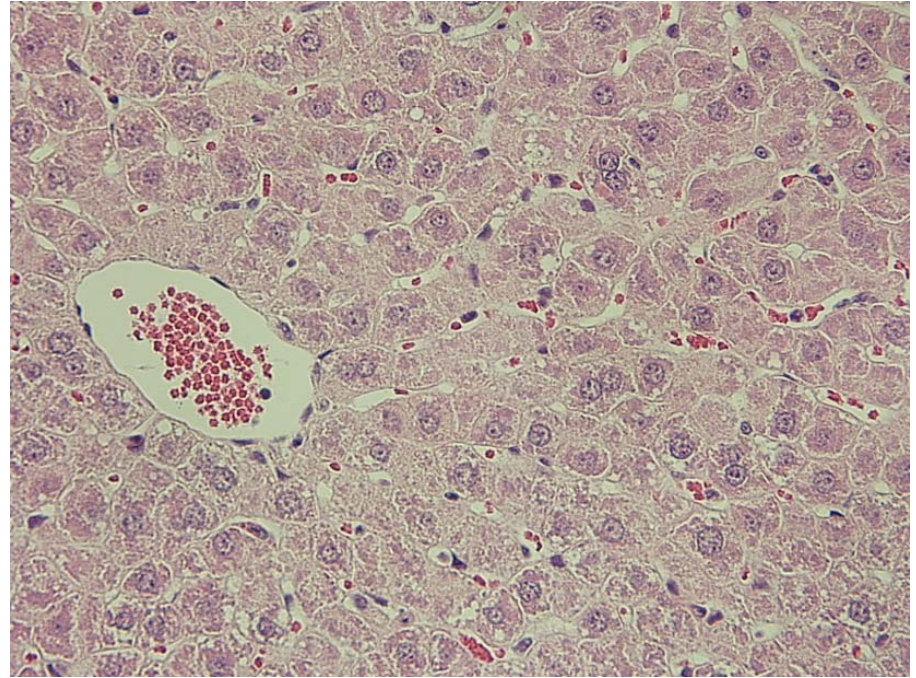
control group x400   sivelestat group x400

Figure.5

ALT

x 10² IU/L

* control group
■ sivelestat group

pre   30   60   120

After ischemia (min)
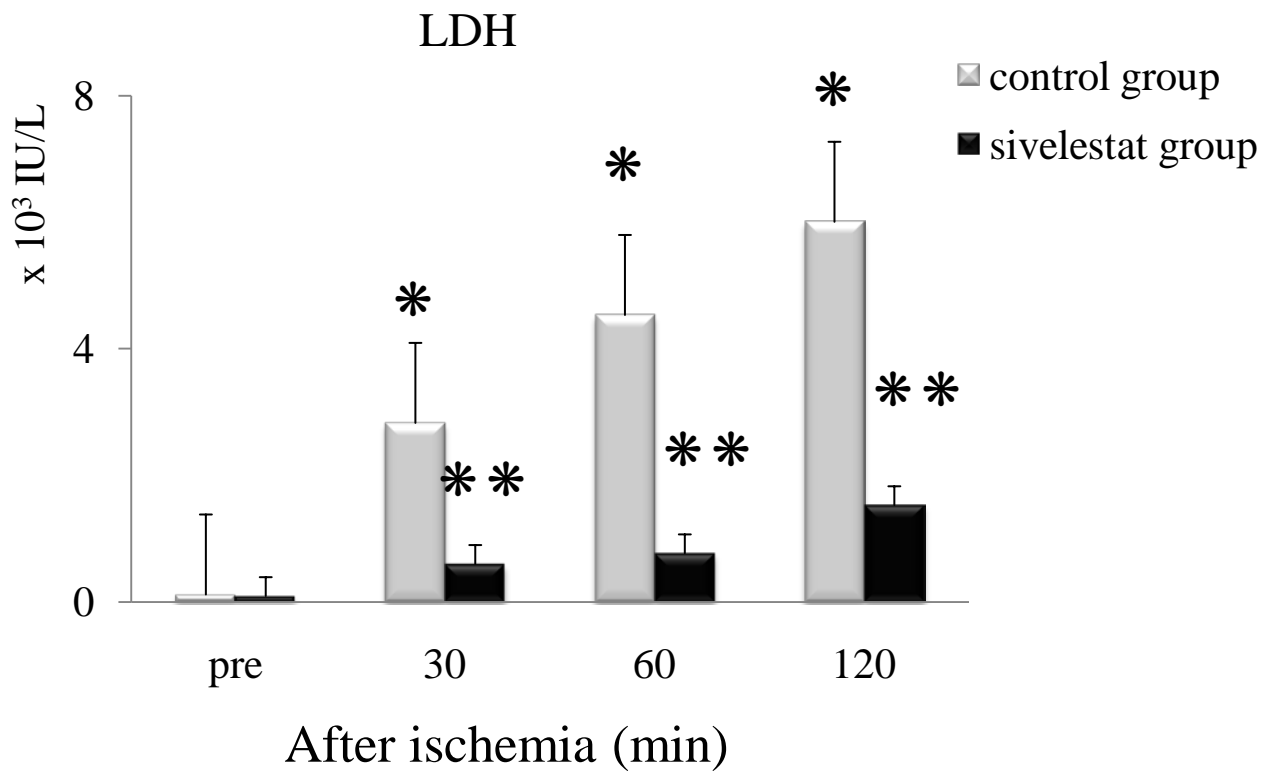
LDH

x 10³ IU/L

* control group
■ sivelestat group

pre   30   60   120
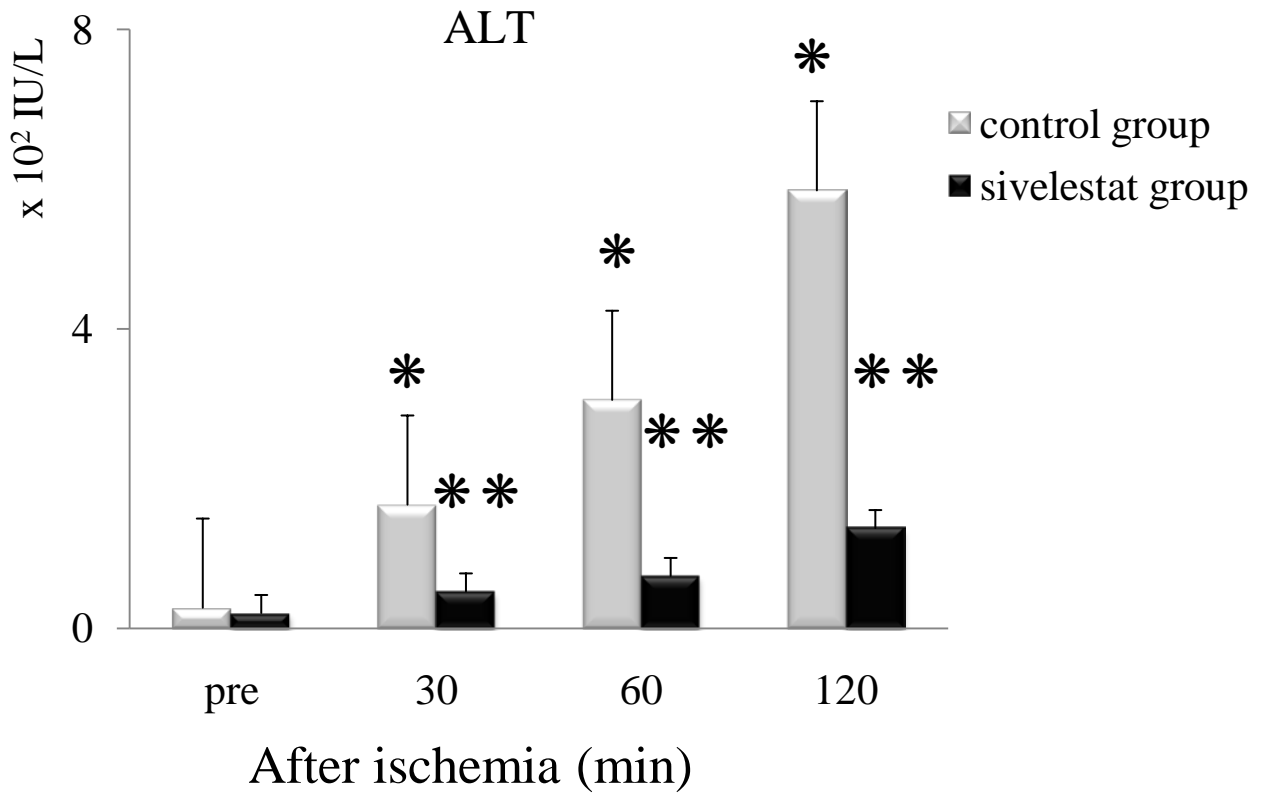
After ischemia (min)

Table.1: Serum cytokines levels at before ischemia and after reperfusion in both groups.

After ischemia		pre	30 min	60 min	120 min
TNF-α	control group	-	34.3±15.7	113.0±73.3	194.3±142.6
	sivelestat group	-	54.5±43.1	142.6±84.3	237.2±100.2
	P-value	0.34	0.11	0.23	0.26
IL-1β	control group	26.6±28.6	38.8±34.9	71.2±50.6	149.7±71.2
	sivelestat group	28.5±13.2	23.9±16.9	58.3±24.20	225.7±151.6
	P-value	0.43	0.15	0.26	0.11
IL-6	control group	67.8±34.5	220.91±179.8	1419.7±1575.2	4000.5±2406.5
	sivelestat group	74.0±33.8	519.1±617.3	2551.7±2213.9	4401.1±2250.5
	P-value	0.36	0.11	0.13	0.37

No significant differences were found between control and sivelestat groups.
pre, before induction of ischemia

References

1. H Jaeschke. Mechanisms of reperfusion injury after warm ischemia of the liver. J Hepatobiliary Pancreat Surg 1998;5:402-408.

2. H Jaeschke, Farhood A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. Am J Physiol 1991;260:G355-362.

3. Arii S, Teramoto K, Kawamura T. Current progress in the understanding of and therapeutic strategies for ischemia and reperfusion injury of the liver. J Hepatobiliary Pancreat Surg 2003;10:189-194.

4. Bilzer M, Roggel F, Gerbes AL. Role of Kupffer cells in host defense and liver disease. Liver Int 2006;26:1175−1186.

5. H Jaeschke. Mechanism of Liver Injury.II Mechanism of neutrophil-induced liver cell injury during hepatic ischemia-reperfusion and other acute inflammatory conditions. Am J Physiol Gastrointest Liver Physiol 2006;290:G1083-1088.

6. Tamakuma S, Ogawa M, Aikawa N, Kubota T, Hirasawa H, Ishizuka A, Taenaka N, Hamada C, Matsuoka S, Abiru T. Relationship between neutrophil elastase and acute lung injury in humans. Pulm Pharmacol Ther 2004;17:271-279.

7. Nakano Y, Kondo T, Matsuo R, Murata S, Fukunaga K, Ohkohchi N. Prevention of leukocyte activation by the neutrophil elastase inhibitor, sivelestat, in the hepatic microcirculation after ischemia-reperfusion. J Surg Res 2009;155:311-317.

8. Khandoga A, Biberthaler P, Messmer K, Krombach F. Platelet-endothelial cell interactions during hepatic ischemia-reperfusion in vivo: a systematic analysis. Microvasc Res 2003;65:71-77.

9. Montalvo-Jave EE, Escalante-Tattersfield T, Ortega-Salgado JA, Pina E, Geller DA. Factors in the pathophysiology of the liver ischemia-reperfusion injury. J Surg Res

2008;147: 153-159.

10. Sindram D, Porte RJ, Hoffman MR, Bentley RC, Clavien PA. Synergism between platelets and leukocytes in inducing endothelial cell apoptosis in the cold ischemic rat liver: a Kupffer cell-mediated injury. FASEB J 2001;15:1230-1232.

11. Kotake Y, Yamamoto M, Matsumoto M, Morisaki H, Takeda J. Sivelestat, a neutrophil elastase inhibitor, attenuates neutrophil priming after hepatoenteric ischemia in rabbits. Shock 2005;23:156-160.

12. Fujimoto H, Shintani T, Takamoto M, Yamamoto R, Hiraku S. Studies on the metabolic fate of neutrophil elastase inhibitor ONO-5046Na (5): Plasma concentration-profile after intravenous bolus administration or intravenous infusion to rats and dogs. Yakubutudoutai 1998;13:28-35.

13. Massberg S, Eisenmenger S, Enders G, Krombach F, Messmer K. Quantitative analysis of small intestinal microcirculation in the mouse. Res Exp Med (Berl) 1998;198:23-35.

14. Nakano Y, Kondo T, Matsuo R, Hashimoto I, Kawasaki T, Kohno K, Myronovych A, Tadano S, Hisakura K, Ikeda O, Watanabe M, Murata S, Fukunaga K, Ohkohchi N. Platelet dynamics in the early phase of postischemic liver in vivo. J Surg Res 2008;149:192-198.

15. Myronovych A, Murata S, Chiba M, Matsuo R, Ikeda O, Watanabe M, Hisakura K, Nakano Y, Kohno K, Kawasaki T, Hashimoto I, Shibasaki Y, Yasue H, Ohkohchi N. Role of platelets on liver regeneration after 90% hepatectomy in mice. J Hepatol 2008;49:363-372.

16. Watanabe M, Murata S, Hashimoto I, Nakano Y, Ikeda O, Aoyagi Y, Matsuo R, Fukunaga K, Yasue H, Ohkohchi N. Platelets contribute to the reduction of liver

2

fibrosis in mice. J Gastroenterol Hepatol 2009;24:78-89.

17. Pereboom IT, Lisman T, Porte RJ. Platelets in liver transplantation: friend or foe? Liver Transpl 2008;14:923-931.

18. Murata S, Matsuo R, Ikeda O, Myronovych A, Watanabe M, Hisakura K, Nakano Y, Hashimoto I, Ohkohchi N. Platelets promote liver regeneration under conditions of Kupffer cell depletion after hepatectomy in mice. World J Surg 2008;32:1088-1096.

19. Kobayashi A, Imamura H, Isobe M, Matsuyama Y, Soeda J, Matsunaga K, Kawasaki S. Mac-1 (CD11b/CD18) and intercellular adhesion molecule-1 in ischemia-reperfusion injury of rat liver. Am J Physiol Gastrointest Liver Physiol 2001;281:G577-585.

20. Khandoga A, Hanschen M, Kessler JS, Krombach F. CD4+ T cells contribute to postischemic liver injury in mice by interacting with sinusoidal endothelium and platelets. Hepatology 2006;43:306-315.

21. Fondevila C, Busuttil RW, Kupiec-Weglinski JW. Hepatic ischemia/reperfusion injury-a fresh look. Exp Mol Pathol 2003;74:86-93. [PubMed: 12710939]

22. Phan SH, Gannon DE, Ward PA, Karmiol S. Mechanism of neutrophil-induced xanthine dehydrogenase to xanthine oxidase conversion in endothelial cells: evidence of a role for elastase. Am J Respir Cell Biol 1992;6:270-278.

23. Kawabata K, Suzuki M, Sugitani M, Imaki K, Toda M, Miyamoto T. ONO-5046, a novel inhibitor of human neutrophil elastase. Biochem Biophys Res Commun 1991;177:814-820.

24. Kusner DJ, Aucott JN, Franceschi D, Sarasua MM, Spangnuolo PJ, King CH. Protease priming of neutrophil superoxide production. Effects on membrane lipid order and lateral mobility. J Biol Chem 1991;266:16465-16471.

25. Jaeschke H, Farhood A, Bautista AP, Spolarics Z, Spitzer JJ, Smith CW. Functional inactivation of neutrophils with a Mac-1 (CD11b/CD18) monoclonal antibody protects against ischemia-reperfusion injury in rat liver. Hepatology. 1993;17:915-923.

26. Yamada K, Horikawa N, Matsui K, Yamamoto S, Kawamura N, Ohashi N. Inhibitory effect of SM-20550, an Na/H exchange inhibitor, on accumulation of leukocytes in ischemia and reperfusion. Naunyn Schmiedebergs Arch Pharmacol 2001;363:597-603.

27. Yamaguchi Y, Akizuki E, Ichiguchi O, Matsumura F, Goto M, Miyanari N, Mori K, Yamada S, Ogawa M. Neutrophil elastase inhibitor reduces neutrophil chemoattractant production after ischemia-reperfusion in rat liver. Gastroenterology. 1997;112:551-560.