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

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elastase inhibitor; sivelestat; adherent platelets.

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-1 β 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]. Neutrophils 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, polyethylen 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 μ L/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 (2×10^{-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 mm²). (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 hematoxylin (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 \pm 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 zones 1 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-1 β , 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 suppress 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-1 β , 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.

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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 1×10^8 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 zones 1 and 2 at 120 min after reperfusion. * $P < 0.001$; *versus* zones 1 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 recognized (HE stain x 400).

Figure.5: Serum liver enzymes.

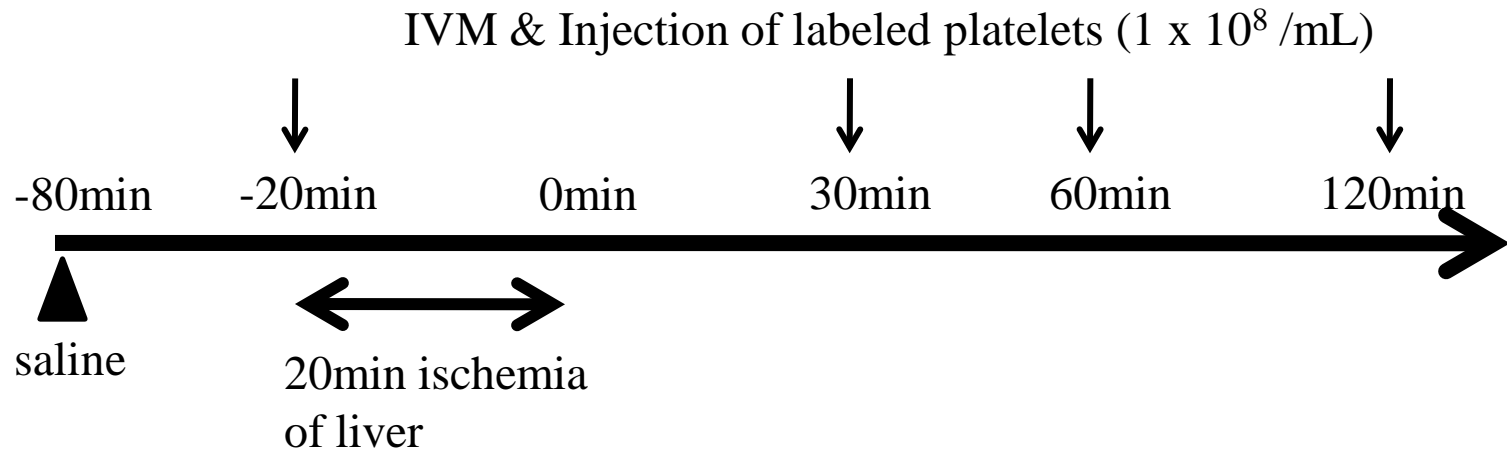
Serum ALT and LDH, reflecting hepatic parenchymal injury, increased after reperfusion in the control group. *P<0.05; *versus* before induction of ischemia in the control group.

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



sivelestat group

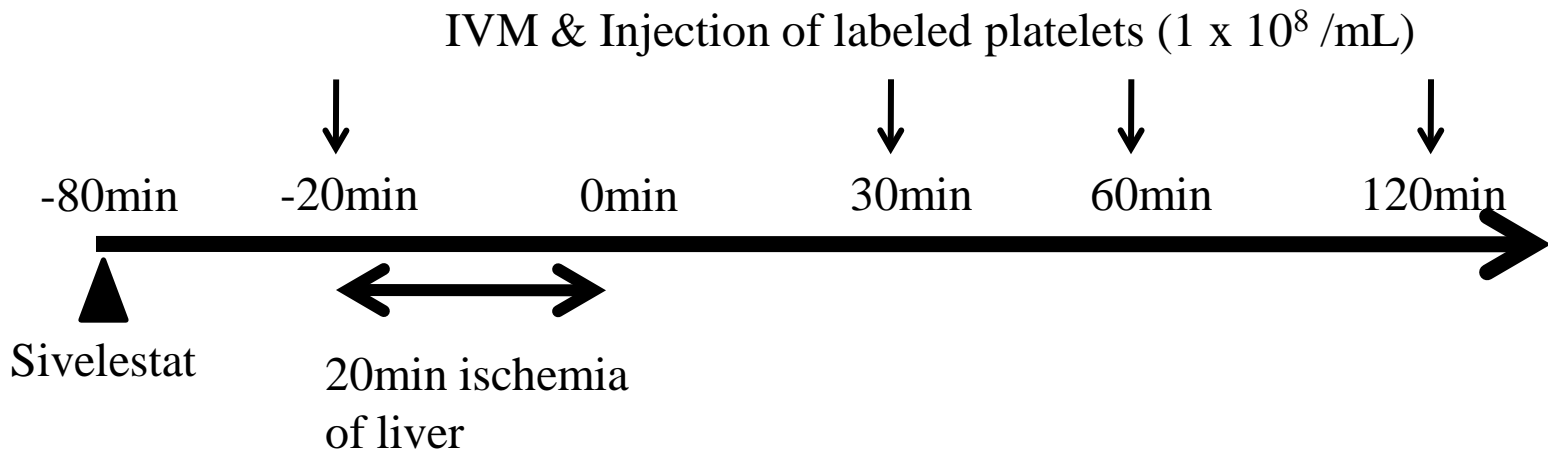


Figure.2A

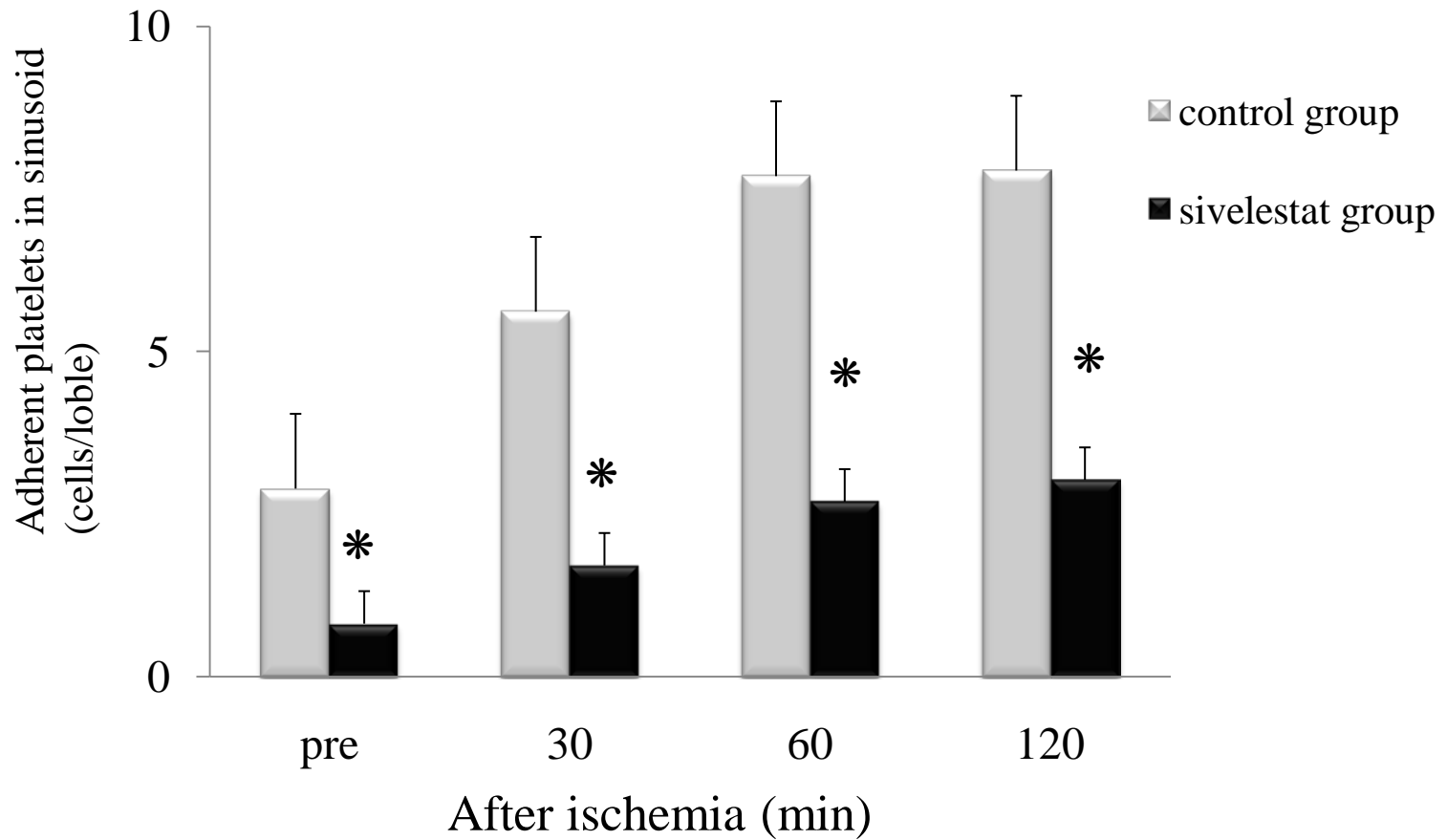
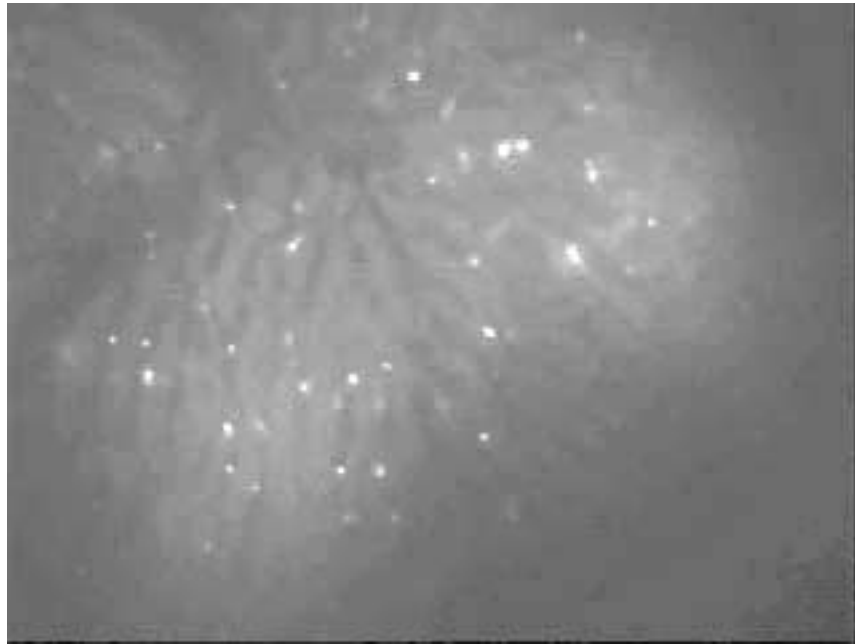


Figure.2B

control group



sivelestat group



Figure.3

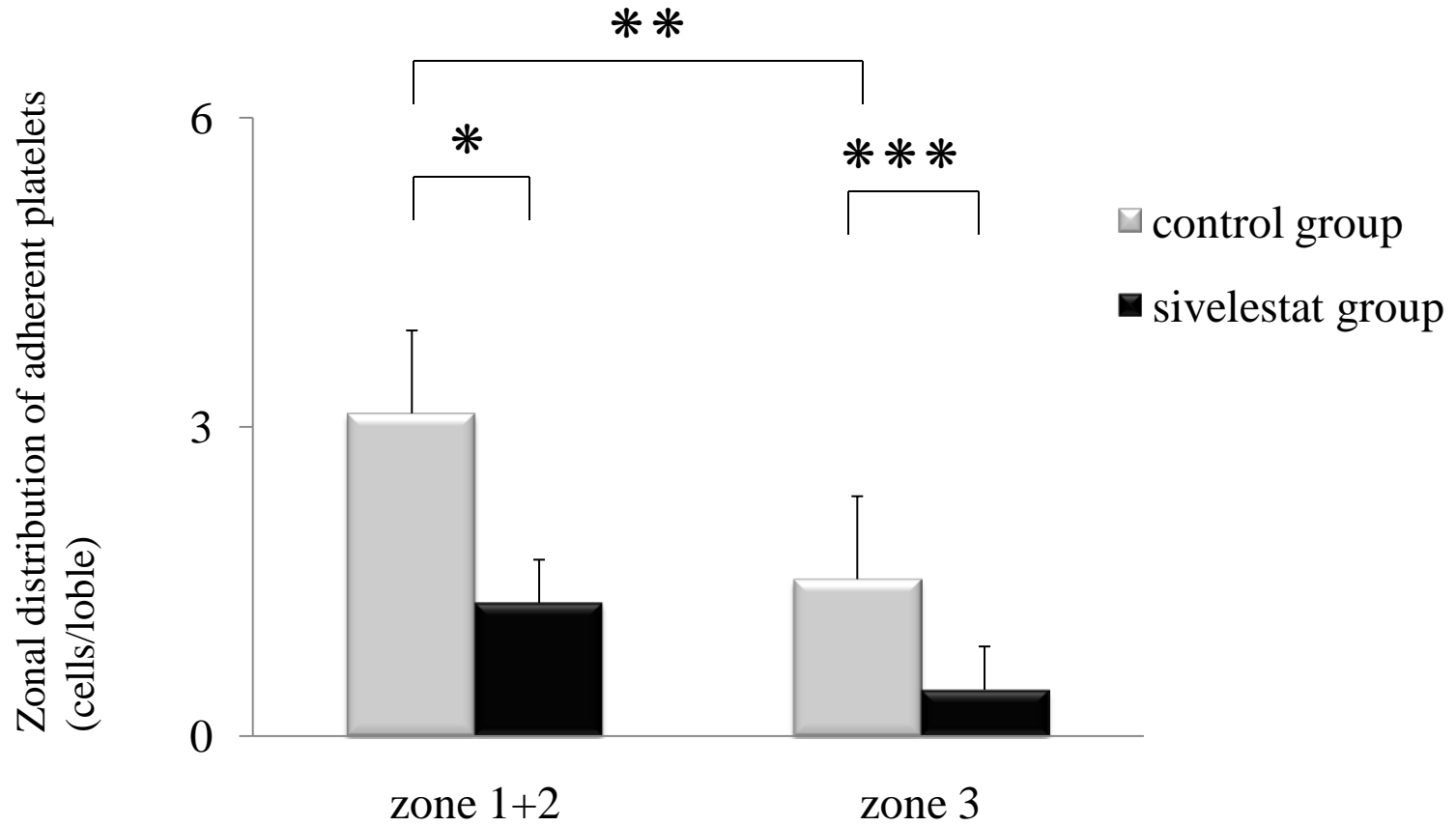
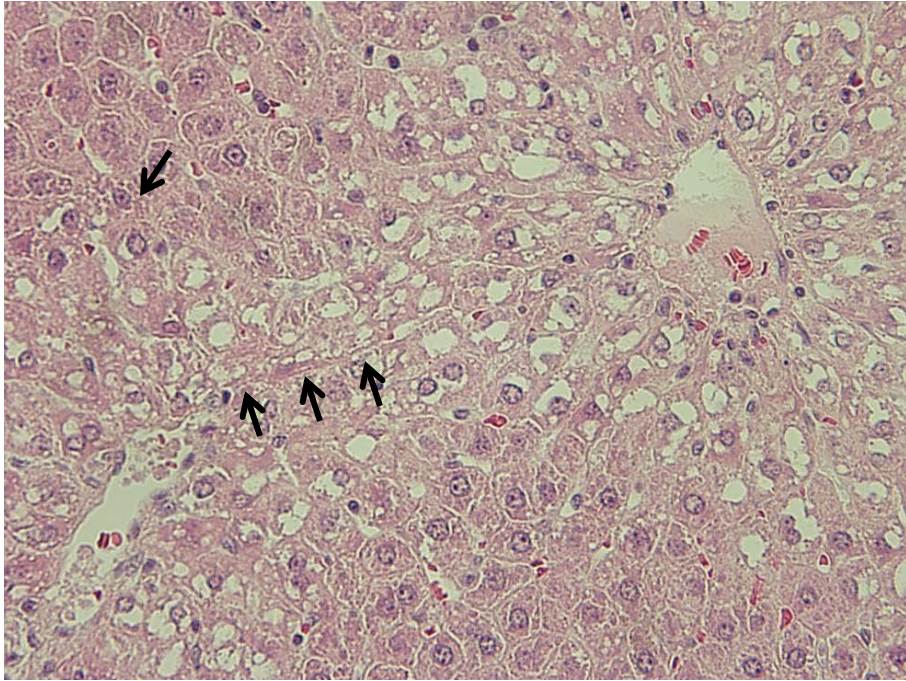
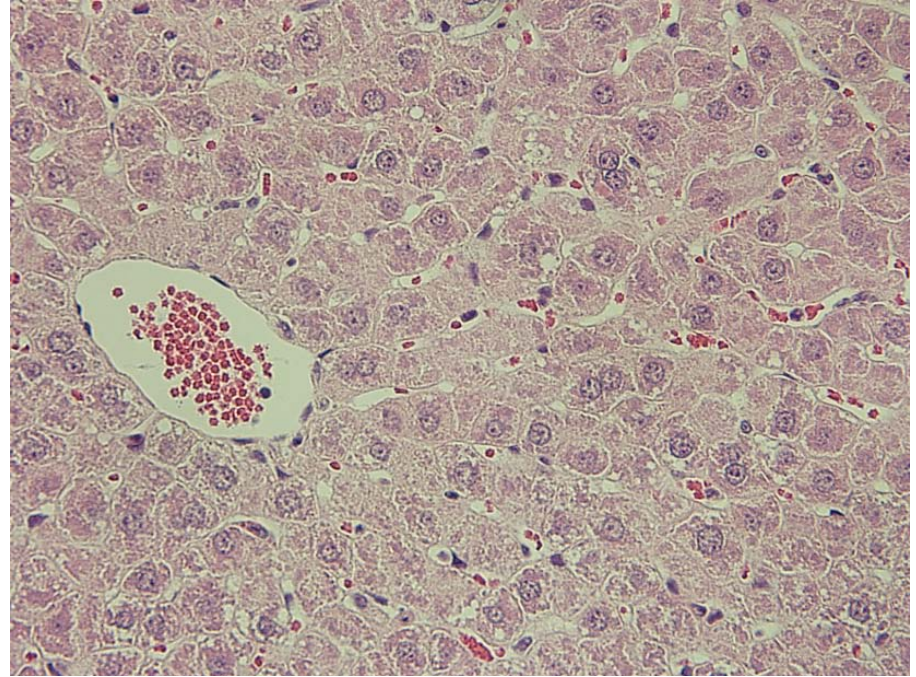


Figure.4



control group x400



sivelestat group x400

Figure.5

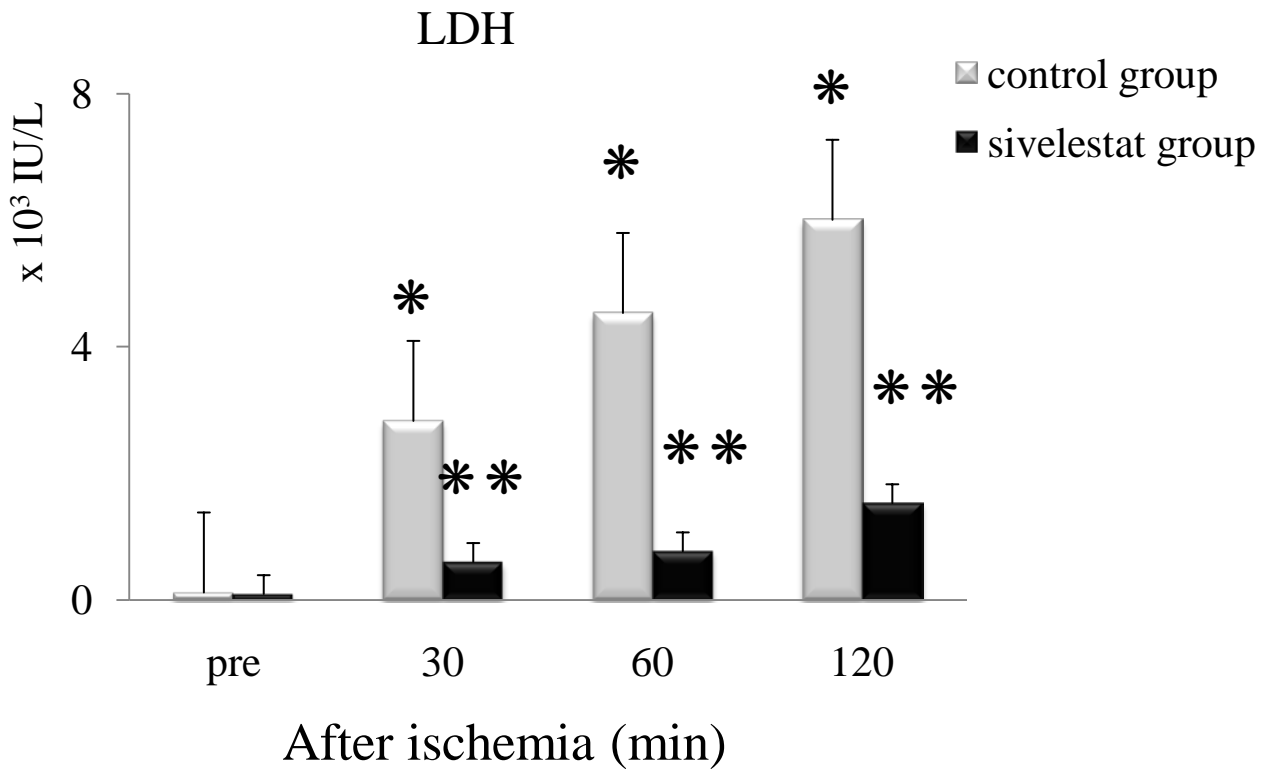
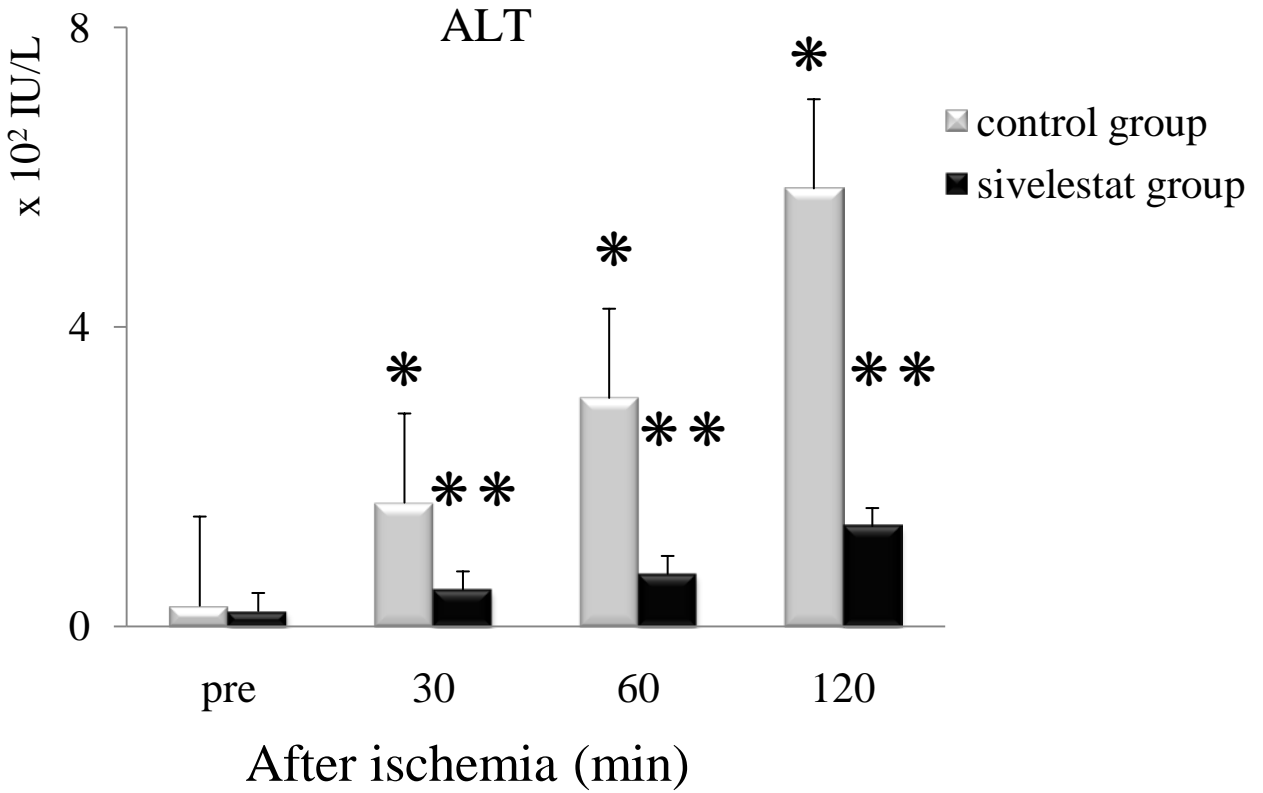


Table.1: Serum cytokines levels at before ischemia and after reperfusion in both groups.

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

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