Interaction between Kupffer cells and platelets in the early period of hepatic ischemia-reperfusion injury—an in vivo study

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Running title: Kupffer cells and platelet dynamics in the postischemic liver

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Abstract

**Background.** Hepatic ischemia-reperfusion (I/R) leads to activation of Kupffer cells (KCs). The activated KCs cause platelet and leukocyte adhesion to the sinusoidal endothelium. Previously, we reported that platelet-endothelium interactions occur earlier than leukocyte responses. The aim of this study was to evaluate the interaction between platelets and KCs in the hepatic microcirculation after I/R.

**Materials and Methods.** Sprague-Dawley rats were divided into 3 groups: the no-ischemia group (control group; n=6); the 20-minute ischemia group (I/R group; n=6); and the 20-minute ischemia + anti-rat platelet serum group (APS group; n=6). KCs were labeled using the liposome entrapment method. The number of adherent platelets was observed for up to 120 minutes after reperfusion by intravital microscopy. To investigate the effects of platelets on I/R injury, rats were injected intravenously with rabbit APS for platelet depletion.

**Results.** In the I/R group, the number of adherent platelets increased significantly after I/R. More than 50% of the adherent platelets were adhered to KCs. Electron microscopy indicated that the platelets attached to the KCs after hepatic ischemia. The histological findings indicated liver damage and apoptosis of hepatocytes in zone 1. In the I/R group, but not in the control and APS groups, serum ALT increased immediately after reperfusion.

**Conclusions.** We succeeded in visualizing the dynamics of both KCs and platelets in the hepatic sinusoids. Liver ischemia induced the adhesion of platelets to KCs in the early period, which could play a key role in reperfusion injury of the liver.

**Keywords:** intravital microscopy (IVM); hepatic ischemia reperfusion; Kupffer cell; electron microscope; adherent platelets
Introduction

Clamping of the portal triad, i.e., the Pringle maneuver, is a standard procedure in hepatic resection and the effects of the interruption of hepatic blood flow have been widely reported [1, 2]. However, if the duration of the Pringle maneuver is prolonged, complications induced by warm ischemia sometimes occur postoperatively [3]. At the time of ischemia-reperfusion (I/R), the hepatic microcirculation is the main target of hepatic injury [4]. The early period of inflammation after hepatic I/R is mainly characterized by activation of Kupffer cells (KCs), which generate reactive oxygen species and aggravate early hepatic injury [5-7].

Platelet–endothelial cell interaction contributes to I/R injury of the liver, as well as to liver regeneration [8-10]. Recently, some studies have focused on the role of platelets in hepatic I/R [11]. Activated platelets produce proinflammatory mediators, i.e., chemokines and cytokines [8, 12]. The platelets likely act in synergy with leukocytes and KCs [13]. We reported that platelet-endothelium interactions occur earlier than leukocyte responses after I/R and that adhesion of platelets requires the presence of activated KCs [14]. In Kupffer cell-depleted rats, the structure of the sinusoidal endothelium was well maintained after I/R, and I/R injury was suppressed [14]. From the results of our previous study, we hypothesized that the interaction between platelets and KCs is indispensable for the early period of I/R injury. Owing to the difficulty of labeling KCs, until now in vivo visualization of the KCs in the sinusoids has not been possible. Recently, however, fluorescein labeling of KCs was enabled by the liposome entrapment method [15]. In this study, we focused on the role of platelets in the early period of I/R and observed KCs in the liver in vivo using this method. Our aim was to evaluate the interaction between platelets and KCs in the liver hepatic microcirculation.
after the early period of I/R with intravital microscopy (IVM).

Materials and Methods

Animals

Male Sprague-Dawley rats, weighing 250 to 300 g, were obtained from CLEA Japan (CLEA Corporation, 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 Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

Experimental Groups

Total warm hepatic ischemia was induced for 20 minutes by clamping the portal triad. The rats were divided into 3 groups: (i) the no-ischemia group (control group; n=6); (ii) the 20-minute ischemia group (I/R group; n=6); and (iii) the 20-minute ischemia + anti-rat platelet serum group (APS group; n=6). Platelet dynamics and the hepatic microcirculation were observed just before ischemia and at 30, 60, and 120 minutes after reperfusion. To investigate the effects of platelets on I/R, the rats were injected intravenously with rabbit anti-rat platelet serum (APS; Inter-Cell Technologies, Hopewell, NJ) 24 hours before induction of ischemia [16, 17]. The APS group
underwent the same procedure as the I/R group (Fig. 1).

**Surgical Procedure**

After induction of anesthesia with intraperitoneal sodium pentobarbital (50 mg/kg), the animals were tracheotomized. To reduce spontaneous breathing, the animals were ventilated mechanically (KN-55; Natsume Seisakusho, 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 saline, polyethylene 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 transverse laparotomy, the ligaments around the liver were dissected to mobilize the left lobe. At the same time, the hepatoduodenal ligament was taped in preparation for clamping later. The left lobe was exteriorized on a plate specially designed to minimize movement caused by respiration, and covered with a cover glass. Surgical procedures were performed under sterile conditions. After 60 minutes of continuous physiological saline infusion, baseline IVM was performed. Hepatic ischemia was then induced by portal triad clamping, i.e., clamping of the 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 times as IVM. At the end of the experiments, liver tissue was taken for histological examination. Finally, the experimental animal was euthanized.
by total blood collection via a catheter.

**Liposome Entrapment Method (Fluorescence Labeling of KCs)**

There are various methods for preparing liposomes [18, 19]. In this study, we applied the method of Watanabe et al [15]. Fluorescently labeled phosphatidylcholine (PC) was incorporated into liposomes consisting of PC. The fluorescent pigment used was 2-(12-[7-nitrobenz-2-oxa 1,3-diazol-4-yl] amino)dodecanoyl-1-hexadecanoly-sn-glycero-3-phosphocholine (NBD-C\textsubscript{12}-HPC; Molecular Probes, Eugene, OR). After intraarterial injection, KCs in the rat liver were stained and were clearly delineated in the fluorescent IVM image. The phagocytic activity of KCs after the administration of liposomes was determined by measurement of the amount of hepatic uptake of intravenously administered fluorescent microspheres. No detrimental influence of the liposomes on the phagocytic activity was observed. Additionally, no histopathological changes were found in the livers of liposome-treated rats [15]. Sixty minutes before induction of hepatic ischemia, liposome-encapsulated fluorescent liposomes (4 mL/kg) were administered via the carotid artery catheter.

**Platelet Preparation**

Platelets were isolated from the 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 [20]. Briefly, the collected blood was diluted with buffer after the addition of prostaglandin E1 and rhodamine-6G. After 2 cycles of centrifugation,
fluorescent platelets were resuspended in PBS. In this study, a total amount of $1 \times 10^8$ fluorescence-labeled platelets, approximately 1% of all circulating platelets in the recipient rat, were injected via the left carotid artery 5 minutes before IVM.

**Intravital Microscopy**

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 with a CCD camera (C5810; Hamamatsu Photonics, Hamamatsu, Japan) and a digital video recorder (GV-D1000 NTSC; Sony, Tokyo, Japan) for offline analysis. Using objective lenses (10×0.3 to 20×0.7; Olympus, Tokyo, Japan), a final magnification of x325 to x650 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 intraarterially just before ischemia and at 30, 60, and 120 minutes after reperfusion, and 10 randomly chosen acini were visualized. Quantitative assessment of the microcirculatory parameters was performed offline using WinROOF imaging software (version 5.0; Mitani Shoji, Tokyo, Japan).

**Microcirculatory Analysis**

The following 3 parameters were analyzed: (i) the zonal distribution of KCs (zones 1, 2, and 3); (ii) the number of adherent platelets, i.e., platelets firmly attached to the sinusoidal endothelium for longer than 20 seconds (The number of adherent
platelets in the scanned acini was counted. The results were expressed as the number of adherent platelets per field [1 field = approximately 0.2 mm²]; and (iii) the number of adherent platelets adhering to KCs.

**Serum Alanine Transaminase (ALT) Levels**

To assess damage to the hepatic parenchyma, serum ALT levels were measured using a Drychem 7000V autoanalyzer (Fujifilm, Tokyo, Japan). A blood sample was taken from the catheter of the carotid artery before induction of ischemia and up to 120 minutes after reperfusion.

**Histological Analysis**

After 120 minutes of reperfusion, liver tissues were obtained from each group, fixed with 10% formaldehyde, and embedded in paraffin. Thin sections (4 μm) were prepared and stained with hematoxylin-eosin (HE). Tissue damage was evaluated in 5 randomly selected high-power fields (x200). To detect apoptotic cells in liver tissue, a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (In situ Apoptosis Detection Kit; Takara Biomedicals, Tokyo, Japan) was performed after 120 minutes of reperfusion. Histological alterations were determined according to (i) the number of vacuolations per acinus and (ii) the palisade arrangement grade of each acinus as follows: grade 0, no damage; grade 1, loss of palisade arrangement in less than half of the sinusoids in the acini; grade 2, loss of palisade arrangement in more than half of the sinusoids in the acini; and grade 3, total loss of
palisade arrangement.

**Protein Extraction and Western Blot Analysis**

Liver tissues kept at -80°C were homogenized in a buffer consisting of 150 mmol/L NaCl, 50 mM TrisCl, 1% NP-40, and proteinase inhibitors. The samples were centrifuged and the supernatants collected for analysis. The samples were separated on 12% SDS-PAGE gels and transferred to nitrocellulose membranes (Millipore, Bedford, MA). Anti-cleaved caspase-3 antibody (9661) (Cell Signaling Technology, Beverly, MA) was used as the primary antibody. Secondary goat anti-rabbit antibody conjugated with horseradish peroxidase was purchased from Zymed Laboratories (San Francisco, CA).

**Transmission Electron Microscopy**

Kupffer cells after hepatic I/R were assessed by transmission electron microscopy. After 30 minutes of reperfusion, the livers were quickly resected. Tissue samples from the right hepatic lobe, not observed by IVM, were cut into 1-mm³ cubes and stored in 2.5% glutaraldehyde. The specimens were postfixed with osmium tetroxide, dehydrated through a graded alcohol series, and embedded in an Epon mixture. Ultrathin sections were prepared using an Ultracut S microtome (Leica Aktiengesellschaft, Vienna, Austria) and picked up on copper grids. The sections were treated with uranyl acetate and lead citrate to enhance the contrast. Specimens were examined using a Hitachi H-7000 transmission electron microscope (Hitachi, Tokyo, Japan).
**Statistical Analysis**

All data were expressed as the mean ± standard deviation of the samples. The Mann-Whitney test and analysis of variance (ANOVA) were used, followed by the Scheffé test. *P* values of less than 0.05 were considered statistically significant.

**Results**

**Zonal Distribution of KCs**

There was no significant difference between the control and I/R groups in the distribution of KCs within the hepatic zone. In both groups, approximately 70% of KCs were located in zone 1 during the observation period, while approximately 20% were located in zone 2, and approximately 10% in zone 3.

**Number of Adherent Platelets in Acini**

In the I/R group, the number of adherent platelets increased along with the reperfusion time significantly more than in the control group. On the other hand, in the control group, the number of adherent platelets remained within twice that before the induction of ischemia (Fig. 2A). In both groups, more than 50% of the platelets adhered to KCs, and those platelets were abundant in hepatic zone 1 (Fig. 2B, C).

**Platelet Depletion with Antiplatelet Serum**
Platelet counts in the arterial blood of the APS group declined markedly after the administration of APS. The platelet counts in the I/R, control, and APS groups were 904.5±199.7×10^3/ul, 908.1±163.6×10^3/ul, and 228.7±104.8×10^3/ul ($P<0.001$), respectively.

**Serum ALT Levels**

In the I/R group, serum ALT, reflecting hepatic parenchymal injury, immediately increased after reperfusion, but did not increase in the control or APS group (Table 1).

**Histological Findings**

Light microscopy photographs of the liver tissue, stained with HE, are shown in Figure 3A. In the I/R group, hepatocyte vacuolation and sinusoidal narrowing were observed after 120 minutes of reperfusion. On the other hand, histological alteration was not observed in the control and APS groups.

**Liver Apoptosis**

In the I/R group, TUNEL-positive cells were observed after 120 minutes of reperfusion in hepatic zone 1 (Fig. 3B). They were not observed in the control and APS groups. The expression of cleaved caspase-3 in the I/R group was higher than that in the control group (Fig. 3C).
**Transmission Electron Microscopy**

In the electron microscopy findings, the platelets were mostly adherent to the endothelial cells in the hepatic sinusoids, although in some parts, they were adherent to the KCs (Fig. 4A,B,C).

**Discussion**

Hepatic I/R injury is considered to result from proinflammatory cytokines released by KCs and accumulating leukocytes [21, 22]. In addition to leukocytes, intrahepatic adhesion and accumulation of platelets have also been implicated in liver injury [8, 14, 23, 24]. The early period of hepatic injury, i.e., within 2 hours of reperfusion, is characterized by KC-induced oxidant stress, and the late period, i.e., 6 hours or more after reperfusion, by accumulation of neutrophils [25]. The contribution of KCs during the early period of I/R has been less investigated [14, 26]. KCs and neutrophil interactions were reported to play important roles in I/R injury, whereas the relationship between platelets and KCs remains unclear [27, 28]. Various studies have shown that KCs play a prominent role in hepatic I/R injury. However, the dynamics of KCs in vivo have not been elucidated. In this study, we investigated the dynamics of platelets and KCs in the microcirculation of the liver during the early period of I/R. We clarified that platelets were adherent to KCs and sinusoidal endothelial cells (SECs) in the early period of I/R and that platelets adhering to KCs were involved in the mechanism of hepatic I/R injury.

We previously examined the movement of platelets in a group with KC-inhibition
in similar experiments [14]. The rats in the KC-inhibition group were intravenously injected with liposome-encapsulated dichloromethylene disphosphonate (Cl2MDP) to eliminate KCs [29]. Platelet adhesion in the sinusoids and endothelial cell damage were remarkably suppressed. Moreover, serum alanine aminotransferase levels were also significantly improved. Therefore, we have selected the experimental groups as follows: no-ischemia group, 20-minute ischemia group, and 20-minute ischemia + anti-rat platelet serum group. In this study, we focused on the role of platelets in the early period of I/R.

In our previous study, an increment in adherent platelets in the sinusoids was demonstrated with the same ischemia model [14]. In general, the existence of platelets and KCs has been observed by electron microscopy and immunohistochemical methods in a rodent model [30, 31]. However, evaluating the precise dynamics of platelets and KCs in the hepatic sinusoids is difficult. Evaluation of platelet dynamics in the hepatic microcirculation is essential for determining activation of KCs and prognosis of liver injury [14]. In the present study, we demonstrated the interaction of platelets with SECs and KCs using an IVM system. We succeeded in observing the dynamics of platelets and KCs in the hepatic sinusoids on a real-time basis for the first time. The observations revealed that platelets attach to KCs in the sinusoids during the early period of I/R. The number of platelets adhering to KCs and the sinusoidal endothelium increased in proportion to the duration of reperfusion. Until now, in vivo observation of the time-dependent course of phenomena has been very difficult. Only histological findings, i.e., light microscopy and transmission electron microscopy, were used for investigation. The results of our present study indicate that the IVM system would be very effective for the investigation of hematocytes, i.e., platelets, neutrophils, and KCs, in various
kinds of pathophysiology, such as I/R.

In the present study, half of the adherent platelets located were attached to KCs, and these were abundant in hepatic zone 1. What is the physiological meaning of this phenomenon? KCs are distributed over zone 1, the periportal region of the liver lobules, zone 2, the midzonal region, and zone 3, the pericentral region. The ratio of KCs in the rat liver lobule is reported to be 4:3:2 [26]. The periportal KCs are larger and contain higher numbers of lysosomal enzymes with various kinds of activities, i.e., more heterogeneous and higher endocytic activities than those of the midzonal and pericentral segments of the sinusoids [32]. In this study, KCs were found to be distributed throughout zones 1, 2, and 3 of the liver at a ratio of 7:2:1 in proportion to platelet accumulation. By three-dimensional examination, the composite of zone 3 follows the branching pattern of the hepatic vein, whereas the composite of zone 1 envelops the pericentral compartment [33]. In zone 1, the arterial blood supply to the hepatocytes and the oxygenation gradient between a branch of the hepatic artery and branches of the hepatic vein are emphasized. From these reports, hepatocytes of zone 1 likely require a higher oxygen concentration and more nutrients. KCs participate indirectly in the mechanism of elevated oxygen metabolism in hepatic parenchymal cells by producing mediators such as tumor necrosis factor-alpha, interleukin-1 alpha, and interleukin 6 [34, 35]. We previously reported that a large number of adherent platelets were recognized in zone 1 in the early period after hepatic I/R [14]. Therefore, in I/R, platelet adhesion is likely associated with KC activation in zone 1.

The early period of I/R is mainly characterized by activation of KCs, which generate reactive oxygen species and aggravate early hepatic injury [5-7]. KCs also produce cytokines that result in injury following hepatic I/R. However, Sindram et al
reported that without platelets and leukocytes, the function of KCs is suppressed [13]. From these reports, KCs do not seem to induce the hepatic I/R injury by themselves. Previously, we reported that the adhesion reaction of platelets to the SECs occurred in the early period after hepatic I/R and then followed leukocyte accumulation [14]. Platelet-endothelial cell interactions occur earlier than leukocyte responses, and adhesion of platelets requires the presence of activated KCs [14]. In this study, liver dysfunction was reduced after I/R in the APS group. Therefore, the existence of platelets as well as activated KCs is indispensable for the interaction of liver injury.

Platelet–endothelial cell interaction contributes to disturbance of the microcirculation in the early period of I/R injury [8]. Although the number of adherent leukocytes was not significantly increased immediately after reperfusion, a remarkable increment of adherent leukocytes was observed late after reperfusion, and this phenomenon continued in proportion to the duration of reperfusion [28]. In addition, after I/R, KCs secreted a number of proinflammatory cytokines [36-39]. In the present study, we elucidated that platelets were adherent to KCs in the electron microscopic image taken after the early period of I/R.

Matsuo et al reported that direct contact between platelets and hepatocytes is essential to trigger the release of the soluble factors necessary for liver regeneration [10]. In contrast, we showed that the direct contact of platelets to KCs was the cause of I/R damage to the liver. Sindram et al have shown that platelets induce SEC apoptosis and significantly contribute to I/R injury [40]. SEC apoptosis is a central feature of early reperfusion injury. The localization of the platelets suggests that platelets mediate injury by direct interaction with SECs or by local release of mediators [40]. In the present study, half of the adherent platelets were attached to KCs, and those platelets were
abundant in hepatic zone 1. In addition, the histological findings showed that apoptosis of hepatocytes occurred in zone 1. From these results, we indicated the importance of the direct contact of platelets to KCs in causing damage to hepatocytes in zone 1. We suppose that improvements in I/R injury are the result of decreased interaction between platelets and KCs in the early period of I/R as well as interaction between platelets and SECs. Therefore, in the clinical setting, I/R injury may be reduced by administration of drugs that inhibit platelet function, e.g., phosphodiesterase type 3 inhibitors and adenosine diphosphate receptor inhibitor.

In conclusion, we succeeded in visualizing for the first time the cellular dynamics of KCs and platelets in the hepatic sinusoids. In the early period of I/R, half of the adherent platelets were located around KCs. Ischemia-reperfusion induced the adhesion of platelets to KCs, and this interaction plays a role during the early period of I/R injury of the liver.

References


Table 1. Serum ALT levels before ischemia and after reperfusion

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<td>23.0±4.0*</td>
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<tr>
<td>I/R group</td>
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<td>178.2±133.5</td>
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<td>APS group</td>
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<td>40.2±14.1*</td>
<td>56.8±24.9*</td>
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</table>

I/R, ischemia-reperfusion; APS, anti-platelet serum. mean ± SD (IU/L); n=6; *P<0.001 vs I/R group.
Figure Legends

Fig. 1 Experimental groups
Total warm hepatic ischemia was performed for 20 min by clamping the portal triad in the subject rats. The rats were divided into 3 groups: (1) the control group, (2) the I/R group, and (3) the APS group. A total of $1 \times 10^8$ fluorescence-labeled platelets, approximately 1% of all circulating platelets in the recipient rat, was injected via the left carotid artery 5 min before IVM. The APS group underwent the same procedure as that in the I/R group.

Fig. 2 Platelet dynamics in the hepatic sinusoids
(A) In the I/R group, the number of adherent platelets was significantly more increased than in the control group at 30 min after reperfusion and concomitant with the duration of reperfusion. Mean±SD; n=6. *$P<0.001$ versus the control group. (B) Video images of Kupffer cells (KCs) and platelets in acini 30 min after reperfusion. The field is approximately 0.2 mm$^2$. The left figure shows the acini of fluorescently stained KCs, and the right figure, the acini of fluorescently stained platelets. White circles indicate adhesion to KCs and platelets in the same place. (C) In the I/R group, the number of platelets adhering to KCs was significantly more increased than in the control group at 30 min after reperfusion and concomitant with the duration of reperfusion. Mean±SD; n=6. *$P<0.001$ versus the control group.

Fig. 3 Histological findings and Western blot analysis
(A) In the I/R group, histological examination showed vacuolation of hepatocytes
(arrowhead) and loss of palisade arrangement after 120 min of reperfusion. These findings were not observed in the control and APS groups. (B) In the I/R group, TUNEL-positive cells were observed in zone 1 after 120 min of reperfusion. These findings were not observed in the control and APS groups. (C) Western blot analysis of cleaved caspase-3. The expression of cleaved caspase-3 in the I/R group was higher than in the control group.

**Fig. 4 Transmission electron microscopy findings after 30 min of I/R in the I/R group**

The arrowheads indicate platelets in the hepatic sinusoids. (A) The platelets are directly adhered to the sinusoidal endothelium. (B) The platelets are in close vicinity of the KCs. (C) The platelets are directly adhered to the KCs.
Fig. 1

- **control group** n=6
  - -80 min
  - Liposome injection
  - 0 min
  - 30 min
  - 60 min
  - 120 min
  - intravital microscopy & platelet injection

- **I/R group** n=6
  - -80 min
  - Liposome injection
  - -20 min
  - 0 min
  - 30 min
  - 60 min
  - 120 min
  - intravital microscopy & platelet injection
  - 20-min ischemia of liver

- **APS group** n=6
  - -24 hr
  - APS injection
  - -20 min
  - 0 min
  - 30 min
  - 60 min
  - 120 min
  - arterial blood sampling
  - 20-min ischemia of liver
**Fig. 2**

**A**

![Graph showing the number of adherent platelets in sinusoid over time.](image)

**B**

**Kupffer cells**

![Images showing Kupffer cells and their distribution.](image)

**Platelets**

![Images showing platelets adherence to sinusoids.](image)

**C**

![Graph showing the number of platelets adherent to Kupffer cells over time.](image)
Fig. 3

A

control group ×200  I/R group ×200  APS group ×200

central vein  portal vein

B

control group ×400  I/R group ×400  APS group ×400

portal vein

central vein

C

cleaved caspase-3

β actin

Control group  I/R group
Fig. 4

A

B

C

Top / T. Tamura
<table>
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