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Application of a Novel Method for Subsequent Evaluation of Sinusoids and Postsinusoidal Venules after Ischemia-Reperfusion Injury of Rat Liver

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Ischemia-reperfusion liver injury
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Intravital fluorescence microscopy
Repetitive observation of microvasculature

Abstract
Although several intravital fluorescence microscopic studies demonstrated that microcirculatory derangement is induced during liver ischemia-reperfusion, these data were obtained from randomly selected microvascular areas and microvessels. Repeated observation of the identical microvessels has not been performed yet. Using a specially designed cover glass, it is now possible to relocate desired sites of observation repeatedly over the whole reperfusion time. The aim of this study was to determine the impact of reperfusion time on hepatic microvascular perfusion state. Twenty minutes of ischemia induced a significant decrease in sinusoidal perfusion rate (29.1 ± 10.2%) as compared with baseline values (98.0 ± 0.3%). At 30, 60, and 120 min of reperfusion, the percentage of perfused sinusoids recovered to 62.8 ± 6.6, 67.5 ± 5.7, and 77.2 ± 5.4%. The number of stagnant leukocytes in the same sinusoids was 6.2 ± 1.9/lobule at baseline and increased to 22.3 ± 3.6/lobule at 120 min of reperfusion. The number of leukocytes adhering within postsinusoidal venules was 53.5 ± 12.5/mm^2 before ischemia and increased to 414.2 ± 62.5/mm^2 at 120 min of reperfusion. We have demonstrated that during 120 min of reperfusion, there was a steady increase in both sinusoidal and venular leukocyte adhesion along with an attenuation of the initially severely depressed sinusoidal perfusion. A no-reflow phenomenon at an early phase of reperfusion and subsequent reflow were proven.

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Introduction

Intravital fluorescence microscopy (IVM) has provided in vivo evidence that ischemia-reperfusion leads to microvascular reperfusion failure. However, these findings are not based on continuous observations prior to and after induction of ischemia in each animal. The observation was performed once at the time of reperfusion [1–4], or at different time points [5–8] in single animals rather than derived from the identical fields of observation. Hence it has not been clarified how the microcirculatory status changes immediately after ischemia, or whether sinusoidal perfusion recovers during the time of reperfusion. During reperfusion time, one study suggested recovery of sinusoidal flow [6], while another suggested progressive reduction of sinusoidal flow [8]. This difference may be due to the variation of experimental design, the method of induction of liver ischemia and lack of repeated observations of identical microvessels. Successive observations of identical microvessels of the liver have been attempted [5], but not yet been realized. Chun et al. [5] mentioned that it was not possible to exactly relocate the identical fields of interest repeatedly.

We therefore developed a new method which enables us to observe identical areas of microvenules before and after the induction of ischemia. The purpose of this study was to determine the impact of the duration of reperfusion on the hepatic microcirculation after induction of ischemia.

Materials and Methods

Animal Model and Interventions

After overnight fasting with free access to tap water, 12 male Sprague-Dawley rats (Charles River Wiga, Sulzfeld, Germany; mean body weight ± SEM, 280 ± 13 g) were anesthetized with pentobarbital (50 mg/kg, i.p.) and tracheotomized. The animals were ventilated mechanically (Harvard rodent ventilator, model 683, South Natick, Mass., USA). The absence of spontaneous breathing was confirmed every 5 min. When spontaneous breathing interfered with mechanical ventilation, additional pentobarbital (10 mg/kg) was given (approximately every 30–40 min). The animals were placed in supine position on a heating pad in order to keep body temperature at 37.0 °C. After dissection of the neck, polyethylene catheters (PE-50, 0.58 mm/0.96 mm inside/outside diameter; Portex, Kent, UK) were inserted into the left carotid artery and left jugular vein. These catheters were used for blood pressure monitoring, arterial blood sampling, continuous infusion of Ringer's solution, and injection of fluorescent dyes for microscopy. PaO_2 and $PaCO_2$ were maintained at 100–120 and 35–40 mm Hg, respectively, through manipulating the inspiratory O_2 fraction (35–38%), tidal volume (0.8–1.2 ml 100 g body weight^{-1}), and respiratory rate (50–60 min^{-1}). 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 for the preparation of further clamping. After waiting to confirm the stability of arterial blood pressure and blood gases, the left liver lobe was exteriorized and fixed to an adjustable stage, avoiding the movements caused by respiration.

Before induction of normothermic hepatic ischemia, intravital fluorescence microscopy was performed as a baseline study. Then, liver ischemia was induced by clamping the hepatic artery, portal vein, and bile duct by means of a micro clip (Aesculap, Tuttlingen, Germany) for 20 min (ischemia group, n = 6). IVM was performed again immediately after ischemia and after 30, 60 and 120 min of reperfusion.

Sham-operated animals underwent identical operative procedures without induction of ischemia (control group, n = 6).

Cover Glass

Following exteriorization, the left liver lobe was covered with a specially designed glass coverslip (Zeiss, Oberkochen, Germany). This coverslip has a grid with a side length of 300 µm etched into its surface. Furthermore, all the 900 areas of the grid are numbered from 1 to 900. In pilot experiments, it has been ascertained that the cover glass attached to the liver surface is kept in place by capillary force during the entire period of baseline measurements and the subsequent ischemia-reperfusion period. Through identification of the grid number, it was possible to relocate a desired region of interest at later

time points, so that during acute experiments the identical acini and venules could be observed repeatedly.

Intravital Fluorescence Microscopy

Using a modified Leitz-Orthoplan microscope with a 100-watt HBO mercury lamp attached to a filter-block, the hepatic microcirculation was recorded by means of a CCD camera (FK6990, Cohu, Prospective Measurements Inc., San Diego, USA) and a video system (S-VHS, AG7330, Panasonic, Tokyo, Japan) for off-line analysis. By means of a water immersion objective (W25 × /0.60, Leitz, Wetzlar, Germany), magnification of ×650 was achieved on the video screen (PVM2042QM, Sony, Tokyo, Japan). To assess sinusoidal perfusion, sodium fluorescein (10^{-6} *M*/kg per animal, Merck, Darmstadt, Germany) was injected via the jugular catheter. Leukocytes were stained in vivo by means of rhodamine-6G (10^{-7} *M*/kg per animal, Merck).

In vivo analysis of the hepatic microvasculature was performed in 10 liver acini and postsinusoidal venules. Every number of their location on the grid was recorded, so that the identical acini and venules could be observed repeatedly. At each time point, the microcirculatory status of the liver lobe was recorded for 30 s.

Microcirculatory Analysis

Evaluation of leukocyte flow dynamics in sinusoids and postsinusoidal venules was performed off-line by frame-to-frame analysis of the videotaped images. The following three parameters were analyzed: sinusoidal perfusion rate (%), given in percentage of sinusoids with flow in observed acini (approximatively 10 sinusoids were observed per acinus); number of stagnant leukocytes, defined as the number of cells located within sinusoids and not moving during an observation period of 20 s (given as cells/lobule); number of adherent leukocytes, defined as the number of cells located within postsinusoidal venules (given as cells/mm^2), and not moving or detaching from the endothelial lining during the observation period of 20 s.

Statistical Analysis

Statistical analysis was performed using Stat View software (Abacus Concepts, Inc., USA). All values are given as means ± SEM. Mann-Whitney test and repeated measures ANOVA test were carried out. A p value less than 0.05 was considered as statistically significant.

Results

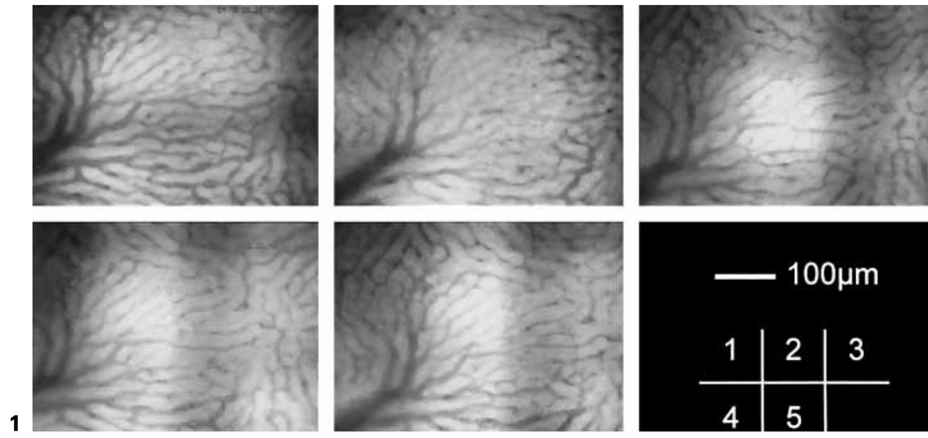
We performed successive observations of identical microvessels over the reperfusion time. In figures 1–3, series of images obtained from identical acini and postsinusoidal venules in the ischemia group are shown.

In the control group (no ischemia induced), there was no change in sinusoidal perfusion over 120 min of exteriorization. On the other hand, following 20 min of liver ischemia, sinusoidal perfusion rate dropped significantly to 29.1 ± 10.2%. At 30, 60 and 120 min of reperfusion, perfusion recovered to 62.8 ± 6.6, 67.5 ± 5.7 and 77.2 ± 5.4% (fig. 4). Twenty-minute ischemia also induced an increase in the number of stagnant cells in sinusoids. In the ischemia group, the number of stagnant cells increased from 6.2 ± 1.9 at

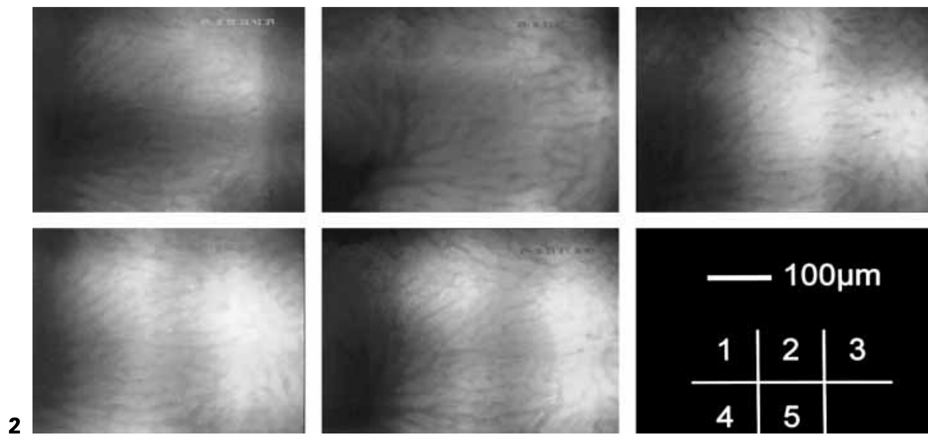
Fig. 1. Successive observations of the identical sinusoid. Contrast enhancement was achieved by administration of Na-fluorescein. Images were acquired from observing identical acini before induction of ischemia (1), at 0 min after reperfusion (2), 30 min after reperfusion (3), 60 min after reperfusion (4), and 120 min after reperfusion (5). Cessation of perfusion was obvious at 60 min.

Fig. 2. Successive observations of the identical sinusoid. Leukocytes were stained by rhodamine 6G. Images were acquired from observing identical acini before induction of ischemia (1), at 0 min after reperfusion (2), 30 min after reperfusion (3), 60 min after reperfusion (4), and 120 min after reperfusion (5). An increase in the number of stagnant cells in the sinusoids was observed at 60 and 120 min.

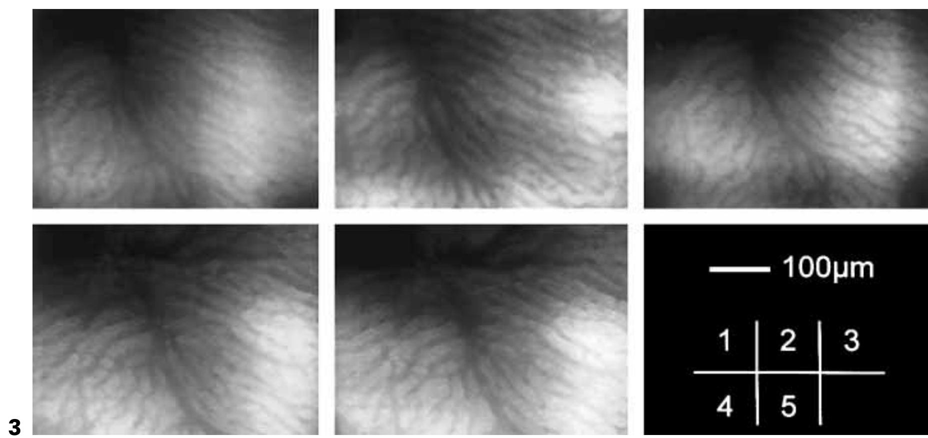
Fig. 3. Successive observation of the identical postsinusoidal venule. Leukocytes were stained by rhodamine 6G. Images were acquired from observing identical postsinusoidal venules before induction of ischemia (1), at 0 min after reperfusion (2), 30 min after reperfusion (3), 60 min after reperfusion (4), and 120 min after reperfusion (5). Dependent on the duration of reperfusion, there is an increase in stagnant cells along the postsinusoidal venule.



1



2



3

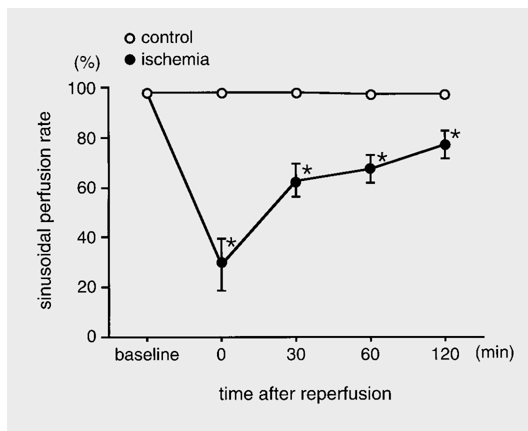


Fig. 4. Sinusoidal perfusion rate (* $p < 0.01$ vs. controls).

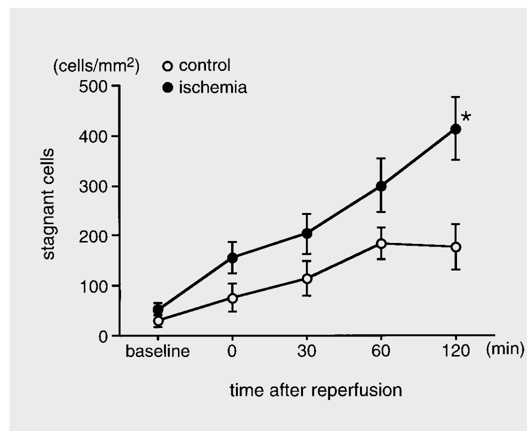


Fig. 6. Number of stagnant cells in postsinusoidal venules (* $p < 0.05$ vs. controls).

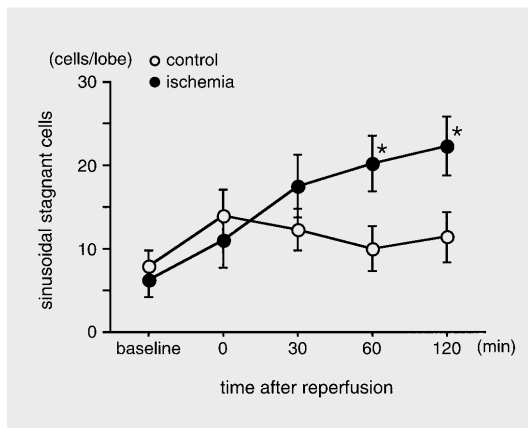


Fig. 5. Number of sinusoidal stagnant cells (* $p < 0.05$ vs. controls).

baseline to 22.3 ± 3.6 cells/lobule at 120 min of reperfusion (fig. 5). There was a steady increase in the number of stagnant cells in postsinusoidal venules, from 53.5 ± 12.6 at baseline to 155.9 ± 32.4, 204.5 ± 40.3, 299.5 ± 53.2 and 414.2 ± 62.5 cells/mm^2 at 0, 30, 60, and 120 min of reperfusion, respectively (fig. 6).

Discussion

The present study demonstrates the impact of time of reperfusion on microcirculatory parameters after the induction of hepatic ischemia. Until now, to estimate the impact of ischemia-reperfusion liver injury, microcirculatory parameters have been obtained from observing randomly selected venules and sinusoids [1, 3, 9]. Clemens et al. [6] established a randomization method. Numbered analysis fields were determined before starting microscopic observation. Then the preparation was moved to these positions without viewing the microcirculation 'to reduce subjectivity' [6]. Other authors followed this randomization method [5, 7, 10]. Although they established the observation method, it was noted that the relocation of the identical fields on the liver surface was not possible at successive time points [5]. By means of our novel method using the grid system, it is possible to relocate exactly the identical observation fields for a long period of time.

Critical duration of organ ischemia resulted in complete cessation or reduction of

blood flow into the previously ischemic organ. Although this so-called 'no-reflow' phenomenon has already been demonstrated for muscle [11], brain [12, 13], and heart [14], it has not been well studied in the liver so far. Koo et al. [8] tried to prove the 'no-reflow' phenomenon after induction of hepatic ischemia. They demonstrated that during the 30-min reperfusion after liver ischemia, the sinusoidal flow immediately returned to normal and then gradually decreased. Other investigators used the term 'no-reflow' phenomenon to imply a cessation of blood flow after a period of ischemia. However, Koo et al. [8]. applied the term no-reflow phenomenon to their gradual decrease in perfusion. In contrast to their results, we demonstrated a severe microcirculatory perfusion failure at the start of reperfusion followed by a partial recovery within 120 min after reperfusion. This cessation of flow, which was present immediately after reperfusion, suggested the presence of a 'no-reflow' phenomenon. Following such a 'no-reflow'phenomenon, subsequent recovery of the blood supply to the previously ischemic tissue has been demonstrated in some organs. The reflow itself may then contribute to the manifestations of injury. Since Menger et al. [15] proposed the term 'reflow-paradox' for an event initiated by postischemic reperfusion and reoxygenation, few attempts have been made to identify its role for postischemic liver injury. This may be due to the difficulty of successive observations of identical liver areas using IVM. We demonstrated that while sinusoidal perfusion recovered during reperfusion, sinusoidal stagnant cells and postsinusoidal venular stagnant cells increased. This reflects the presence of a 'reflow-paradox' as suggested by Menger et al. [15].

Using the specially designed coverslip, the desired sites of interest can be observed repeatedly. Once the microcirculatory sites of interest have been identified, they can be observed also at later points of time. We were able to avoid the subjectivity of selecting the sites of observation, since our data were obtained from analyzing the identical microcirculatory units. Until now, microvascular parameters have been obtained from analyzing randomly selected liver areas [1, 3, 9, 16]. Using our method, we found the sinusoidal perfusion rate (%) to decrease from 97.2 ± 2.9 to 67.5 ± 14 after 60 min of reperfusion. According to the data published by Vollmar et al. [3], the corresponding values were 88.1 ± 1.1 at 60 min of reperfusion and 99.4 ± 0.2 in sham animals. In addition, 60 min of reperfusion induced an increase in venular stagnant cells (cells/mm^2) from 29.7 ± 12 to 299 ± 53 in our study, while in the study by Vollmar et al. [3] it increased from 46 ± 15 to 165 ± 34. Actually, we cannot compare these data directly, because they might be influenced by investigators, techniques, and individual observers. It is, however, obvious that the changes induced by ischemia obtained with our method (sinusoidal perfusion and venular stagnant cells) at baseline and after 60 min of perfusion are greater than those reported by others [3]. Random selection of regions of interest without specification of criteria for evaluation is not free of the risk of bias. Usually, those areas are accepted for video-image analysis presenting optimal criteria in terms of contrast and clarity of the microscopic image, and presence or absence of structures or phenomena to be analyzed. Therefore, equality of observation is not given when random selection is performed several times, particularly when the phenomenon to be identified affects the quality of the optical image as is the case with postischemic injury. Our method excludes the subjectivity of selecting microcirculatory units at each observation period; it reflects the time dependence of local alterations and is therefore superior for quantifying the dynamic changes in leukocyte-endothelial interaction.

Conclusion

In conclusion, using a specially designed cover glass, identical acini and venules could be observed over a prolonged period of time, thus allowing for determination of the impact of duration of postischemic reperfusion on hepatic microvascular perfusion state. We have demonstrated that during 120 min of reperfusion, there was a steady increase in both sinusoidal and venular leukocyte adhesion along with an attenuation of the initially severe sinusoidal nonperfusion.

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