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Microcirculatory disturbances and leucocyte adherence in transplanted livers after cold storage in Euro-Collins, UW and HTK solutions

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Abstract. Integrity of the hepatic microcirculation and maintenance of endothelial cell viability are critical components in preventing primary non-function after liver transplantation. Therefore, hepatic microcirculation and leucocyte-endothelial interaction were studied in rat livers stored for 1 h in Euro-Collins (EC), University of Wisconsin (UW), and histidine–tryptophan–ketoglutarate (HTK) solutions and subsequently transplanted. One hour after transplantation surgery, the livers were exposed under an intravital fluorescence microscope. After injection of the leucocyte marker acridine orange (1 μmol/kg), six pericentral fields were observed for 30 s and experiments were recorded continuously. The percentage of perfused sinusoids was reduced in the livers in the EC group (82.9%) in contrast to the UW (93.2%) and HTK groups (91.0%). Livers in the EC group showed a reduction in the diameters of pericentral sinusoids (7.3 ± 0.2 μm; mean ± SEM) compared with the UW group (9.5 ± 0.2 μm; $P < 0.05$) and HTK group (10.2 ± 0.8 μm; $P < 0.05$), indicating substantial cell swelling in livers stored in EC solution. Permanent adherence of leucocytes was most frequently observed in the EC group ($33.5 \pm 1\%$), while this phenomenon was less pronounced in the UW group ($14.5 + 1.1\%$; $P < 0.05$) and HTK group ($16.3 \pm 0.7\%$; $P < 0.05$). Conversely, temporary adherence of leucocytes was reduced in the EC group ($19.7 + 1.3\%$) compared with the UW group ($30.5 + 2.1\%$) and the HTK group ($34.4 + 0.8\%$). Microcirculatory failure and cell swelling in the EC group might be due to the lack of osmotic substances or oxygen radical scavengers included in UW (allopurinol, glutathione) and HTK (mannitol) solutions. In conclusion, cold storage of livers in UW and HTK solutions results in better preservation of the microcirculation and prevention of adhesion of leucocytes after transplantation compared with the EC solution.

Key words: Cold storage, rat liver, microcirculation – Microcirculation, rat liver – Euro-Collins, microcirculation, rat liver – UW, microcirculation, rat liver – HTK, microcirculation, rat liver – Transplantation rat liver, micro-circulation

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Early failure of the hepatic microcirculation due to ischaemia/reperfusion injury is thought to be a critical factor in the success of the transplantation procedure. Macroscopic observations of transplanted rat livers using a dye perfusion technique [38] as well as intravital microscopic studies of livers transplanted after cold storage in Euro-Collins (EC) solution have demonstrated early dysregulation of the hepatic microcirculation [24]. These observations are supported by the phenomenon that sinusoidal endothelial cells are particularly vulnerable when exposed to cold ischaemia and reperfusion [5, 21, 27]. Moreover, Kupffer cells, also part of the nonparenchymal liver cells and well-known regulators of the hepatic microcirculation, seem to be activated after cold storage and liver transplantation [22]. The release of cytotoxic and regulative mediators (e.g. tumour necrosis factor, prostanoids) by Kupffer cells following ischaemia and reperfusion as reported recently [7] further suggests that this activation might have an impact on the hepatic microcirculation. Intravital fluorescence microscopy of transplanted rat livers demonstrated substantial disturbances of the microcirculation following 1 h and 8 h of liver preservation in EC solution [24]. Additionally, a significant rise in leucocyte adherence to the sinusoidal endothelial wall was observed in transplanted livers following cold storage in EC solution [24].

During the first 20 years of clinical liver preservation and transplantation, Collins (later Euro-Collins) solution was the widely used preservation medium for liver grafts [3, 8]. The introduction of the University of Wisconsin (UW) solution as a cold storage solution by Belzer and coworkers in 1987 [30, 40] allowed significant extension of the liver preservation time form less than 12 h with EC solution to more than 24 h [15, 16]. Meanwhile, UW solution is becoming the most frequently used preservation solution for long-term preservation whereas EC solution is suggested primarily for short-term preservation [28]. In

recent years, the percentage of primary non-function following liver transplantation has ranged from 2–23% [12]. Primary non-function is followed by a high mortality rate even if an immediate retransplantation is possible. The reasons for primary non-function still remain unclear. However, reperfusion injury to endothelial cells, severe microcirculatory disturbances and release of cytokines by macrophages have been suggested as part of the sequelae [12, 21, 27].

As a consequence of these considerations, detailed knowledge of the hepatic microcirculation and reperfusion injury following liver transplantation is necessary to further understand these mechanisms. Previous studies have demonstrated significant but not complete liver injury at 1 h after graft reperfusion and 1 h of cold storage [24]. Therefore, the purpose of this study was to assess hepatic microcirculation and leucocyte–endothelial interactions in transplanted rat livers following cold storage in different preservation solutions. To achieve this goal, livers were investigated by intravital fluorescence microscopy 1 h after orthotopic liver transplantation and cold storage of livers for 1 h. Because long-term storage of rat livers in EC solution may result in partial destruction of the liver (e.g. after 8 h [24]), a short preservation time (1 h) was used to avoid difficulties in interpretation of the parameters of the microcirculation. In addition to EC and UW solutions, histidine–tryptophan–ketoglutarate (HTK) solution, extensively studied by Bretschneider and coworkers [4], was used for liver preservation. HTK solution, originally designed for cardioplegia [33], has been used recently for kidney and liver preservation [13, 29]. Since these three preservation solutions represent different concepts and understanding of organ preservation, we intended to compare them with regard to postoperative microcirculation and leucocyte–endothelial interaction.

Methods

Animals

A total of 36 inbred female Lewis rats (Han, Hannover, FRG) weighing 200–230 g were used as donors and recipients. Rats were anaesthetized with ether during surgery and were allowed to take water postoperatively. One hour after completion of transplantation surgery, rats were reanaesthetized with pentobarbital sodium (30 mg/kg i.p.) for intravital microscopy. At the end of the experiments, rats were killed by an overdose of pentobarbital sodium. All experiments were performed after approval by the responsible ethics committee.

Preservation solutions

EC solution (Fresenius, Bad Homburg, FRG), UW solution (DuPont, Waukegan, Ill., USA) and HTK solution (Dr. Köhler Chemie, Alsbach, FRG) were used in three experimental groups with six liver transplantations per group. Liver grafts were stored in 100 ml of the appropriate solution. For detailed information of the ingredients of the preservation solution, see references 2, 4 and 8.

Liver transplantation technique

Orthotopic liver transplantations were performed according the technique originally described by Zimmermann et al. [42] and Kamada and Calne [18]. Briefly, following dissection of all adhesions, insertion of an intraluminal stent into the bile duct and ligation of the hepatic artery, livers were flushed with 10 ml of the appropriate preservation solution (0–4 °C). While the livers were stored for 60 min in the preservation solution at 0–4 °C, cuffs were prepared onto the portal vein and infrahepatic vena cava. In the recipient rats, livers were explanted and the suprahepatic vena cava was connected by a running suture. Prior to the connection of the portal vein and the infrahepatic vena cava using the 'cuff technique', livers were flushed via the portal vein with 10 ml of Ringers lactate. Then transplantation surgery was completed by anastomosing the bile duct over an intraluminal stent and closure of the laparotomy. All recipient rats received 2 ml Ringers lactate and 3 ml human albumin 5% intravenously via a tail vein during surgery.

Fluorescence microscopy

One hour after transplantation surgery, the midline laparotomy was reopened and the left liver lobe was exteriorized under the intravital microscope (Leitz, Wetzlar, Fluotar 10×/0.30; final magnification ×290) under pentobarbital anaesthesia. Drying of the left liver lobe was prevented by superfusion of Ringers lactate and covering the lobe with plastic film [34]. Following i.v. injection of acridine orange (1 μmol/kg; Sigma, 8024 Deisenhofen, FRG) which stains leucocytes [14], perfusion of sinusoids and flow behaviour of leucocytes were observed for 30 s in six to eight pericentral fields (0.163 mm^2). Experiments were recorded using a CCD camera (Cohu, FK6990, San Diego, USA) and a Umatic video recording system (Sony, Tokyo, Japan) with time projection onto the monitor using a video time–date generator as described recently [24].

Off-line frame-by-frame analysis of recorded experiments allowed quantitation of the following parameters:

- Percentage of perfused sinusoids.

- Permanently adherent leucocytes, defined as leucocytes adherent for more than 20 s to the sinusoidal wall. Permanently adherent leucocytes were expressed as a percentage of the total number of stained circulating and adherent leucocytes during the 30 s observation period per field.

- Temporary adherent leucocytes during the 30 s observation period per field. Temporary adherent leucocytes were expressed as a percentage of the total number or stained leucocytes during the 30 s observation period per field.

- Average diameter of pericentrally located sinusoids at 90 μm prior to entering the central vein. On average, diameters of 60–120 sinusoids in six to eight fields per experiment were measured.

Statistical analysis

Data were expressed as mean ± SEM. Differences between the groups were determined by analysis of variance and using Student's *t*-test. Differences were considered significant for $P < 0.05$.

Results

Intravital microscopy of transplanted livers 1 h following completion of transplantation surgery exhibited preservation of the hepatic angio-architecture to different degrees depending upon the preservation solution used. In livers

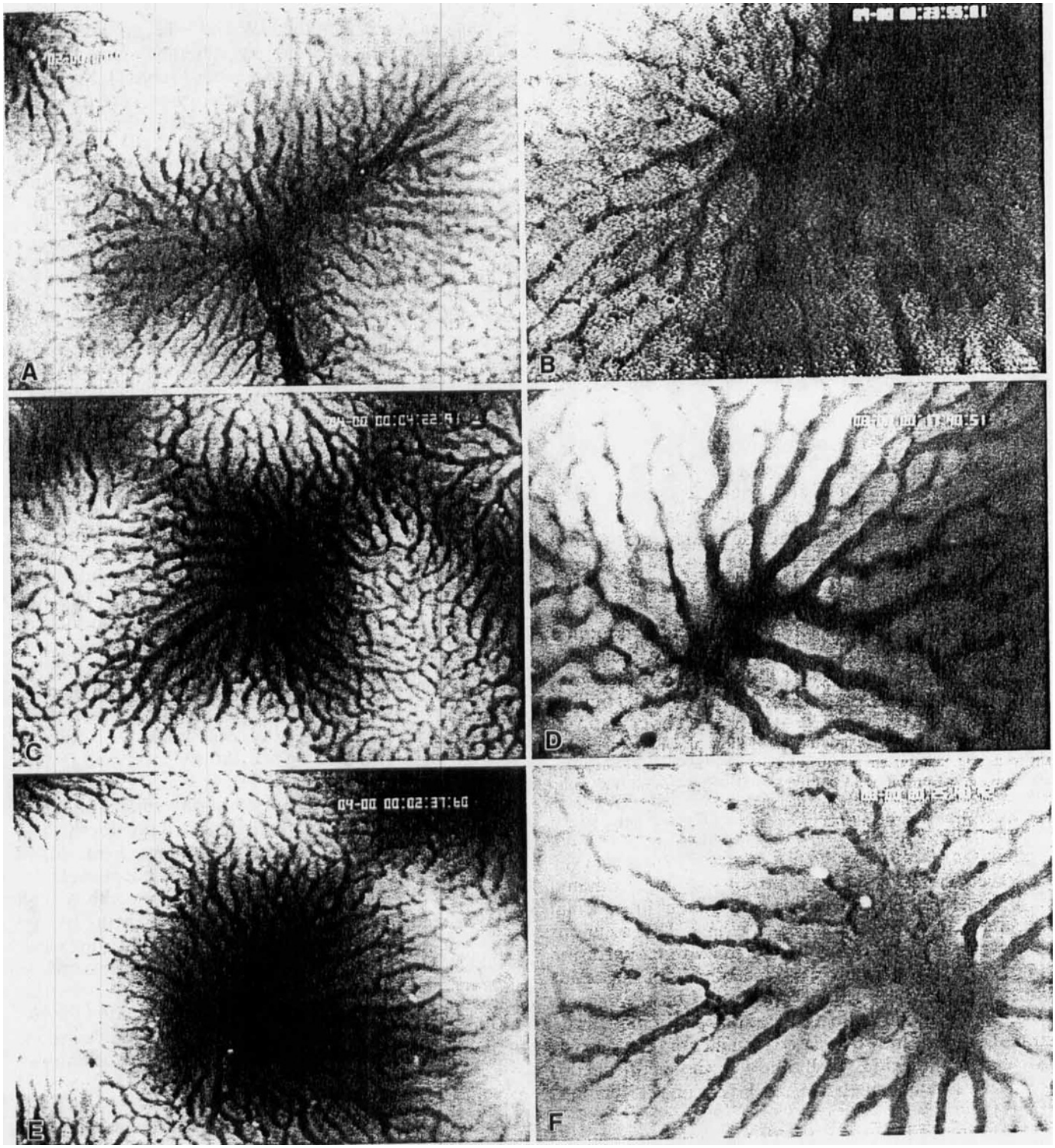

Fig. 1 A–F. Characteristic photographs of pericentral liver fields following injection of acridine orange. Livers were stored for 1 h in the indicated solution and subsequently transplanted. Intravital microscopic observations were performed 1 h after completion of transplantation surgery. **A** Preservation with EC solution. Pericentral liver lobule with oedema formation and significant leucocyte adherence (white spots) in the central vein and pericentral sinusoids. ×130. **B** Preservation with EC solution. Pericentral liver lobule. Pericentral sinusoids are narrow in diameter. Note large area of hemorrhage in the upper right corner. ×325. **C** Preservation with UW solution. Regular angio-architecture of the liver with pericentral lobule in the centre and periportal lobules around (bright areas). Only occasional adherence of leucocytes (white spots). ×130. **D** Preservation with UW solution. Pericentral liver lobule. No oedema formation and only a few leucocytes adherent to the sinusoidal wall. ×325. **E** Preservation with HTK solution. Rat liver with central vein in the centre. Normal angio-architecture with a few leucocytes adherent to the sinusoidal wall. ×130. **F** Preservation with HTK solution with basically normal width of pericentral sinusoids. No oedema formation and little adherence of white blood cells. ×325

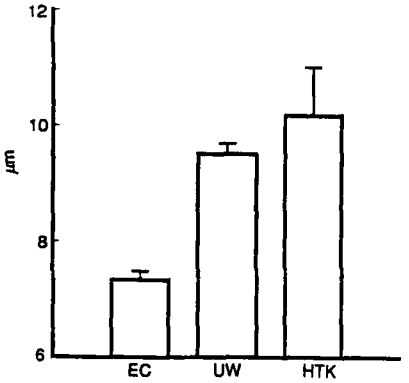

Fig. 2. Mean diameters of pericentrally located liver sinusoids 90 µm prior to draining into the central veins. On average, 10–15 sinusoids were measured in six to eight fields per liver. Both the *UW* and *HTK* group diameters are significantly greater than the *EC* group diameters ($P < 0.05$). Mean ± SEM

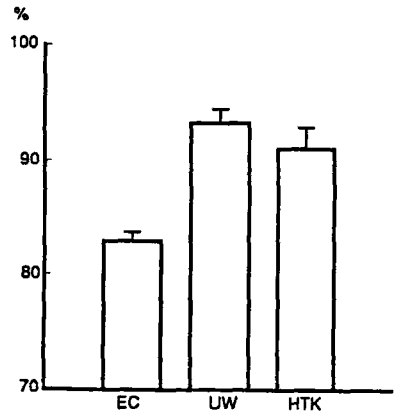

Fig. 3. Percentage of perfused sinusoids following cold storage in EC UW, and HTK solutions. Perfusion of sinusoids was determined in six to eight pericentral fields per liver. Both the *UW* and *HTK* group percentages are significantly greater than the *EC* group percentage ($P < 0.05$). $n = 6$ transplants per group; mean ± SEM

preserved with EC solution relatively small sinusoids and focal areas without perfusion were observed (Fig. 1 A and B). In contrast, livers stored in UW and HTK solutions only occasionally revealed focal areas without perfusion (Fig. 1 C–F). Furthermore, the diameters of the sinusoids did not appear as small as observed in the EC group. Quantitation of the width of pericentral sinusoids demonstrated a significantly decreased diameter of the sinusoids in livers preserved in EC solution, while no significant difference was found between the UW and HTK groups (Fig. 2).

Perfusion of hepatic sinusoids was reduced in all experimental groups as demonstrated in Fig. 3. No difference was found between the perfusion rate in the UW and the HTK group. The percentage of perfused sinusoids, however, was significantly lower in the EC group ($P < 0.05$).

After 1 h of cold storage and subsequent transplantation, the percentage of temporary and permanent adhesion of leucocytes rose substantially in all groups when compared with control values (less than 3% and 10%, respectively [24]). The percentage of permanently adherent leucocytes was significantly increased in the EC group compared with the UW and HTK groups whereas the percentage of temporary adherent leucocytes was higher in the UW and HTK groups (Table 1). No significant differences in adherent leucocytes were found between cold storage in UW and HTK solutions in this study.

Discussion

The combination of the orthotopic rat liver transplantation model and subsequent intravital fluorescence video microscopy is a powerful tool in studying early changes in hepatic microcirculation and leucocyte behaviour. In contrast to histological studies, microhaemodynamic measurements can be performed in vivo, not being influenced by post mortem swelling or fixation and histological processing. Indeed, after storage of rat livers for 1 h in different preservation solutions and subsequent transplantation, different degrees of preservation of the livers were observed. In livers transplanted orthotopically following cold storage in EC solution, the diameters of the sinusoids were significantly smaller than in livers stored in UW or HTK solutions. This may be interpreted as endothelial swelling as reported also by other authors [20]. This conclusion is further supported by the observation that the microscopic image of the livers in the EC group was of lower contrast than those from the other groups (Fig. 1). Contrast and sharpness of the liver images in the UW and HTK groups were similar and the mean diameters of the sinusoids in these groups were comparable. According to these results, cell swelling was largely prevented in UW and HTK solution.

The theory behind the preservation solutions under investigation is quite different as reported previously [2, 4, 8]. The UW solution contains several ingredients added specifically to prevent cell swelling. These include lactobionate, an impermeant anion, raffinose, with a high molecular weight and the stable colloid hydroxyethyl starch, and these may account for the reduction in cell swelling following cold storage and transplantation as demonstrated previously [2] and confirmed in vivo in our model. In contrast, glucose, a main component of the EC solution, to which the liver is relatively permeable in contrast to kidneys, will be metabolized to lactic acid under

Table 1. Leucocyte adherence following liver preservation and transplantation. Temporary adhesion was defined when leucocytes were adherent less than 20 s. Leucocyte adhesion was determined over a 30 s observation period in six to eight pericentral areas of 455 µm diameter per liver, and expressed as a percentage of the total number of acridine-orange-stained leucocytes. $n = 6$ livers per group

Preservation solution	Leucocyte adherence	
	Permanent (%)	Temporary (%)
EC	33.5 ± 1.0	19.7 ± 1.3
UW	14.5 ± 1.1*	30.5 ± 2.1*
HTK	16.3 ± 0.7*	34.4 ± 0.8*

* $P < 0.05$ vs. EC group
Mean ± SEM

anaerobic conditions during cold ischaemia. This results in accumulation of lactate with development of intracellular acidosis. In contrast, HTK and UW solutions do not contain glucose. The rationale of HTK solution, the balanced buffering system of histidine/histidine-HCl, was shown to prevent cell swelling in myocardial endothelial cells and kidneys [4]. In this study, livers stored in HTK solution exhibited minor cell swelling when compared with the EC group.

Perfusion of sinusoids, which may be influenced by endothelial cell swelling and intrahepatic regulation processes [26], was significantly reduced in the EC group. The percentage of perfused sinusoids in UW- and HTK-stored livers was significantly higher, but did not reach a regular perfusion rate, which has been reported to be more than 98% [24]. The improved perfusion rate in the UW and HTK groups can be explained largely by prevention of cell swelling, expressed by the diameters of the pericentral sinusoids, reaching almost control values [39]. Furthermore, Kupffer cells have been shown to play an important role in the regulation of the hepatic microcirculation [26]. Cold storage of livers in EC solution was found to activate Kupffer cells upon reperfusion [22]. The mechanism of this activation still remains unclear. However, free radical injury to endothelial cells as well as a rise in intracellular calcium are likely mechanisms [22, 37, 41]. Cold storage of livers in UW solution has the potential to reduce Kupffer cell activation significantly during reperfusion after transplantation (unpublished results). Thus, UW solution may improve the sinusoidal perfusion rate due to a reduction in cell swelling, prevention of Kupffer cell activation and free radical mediated injury. The preservation of the hepatic perfusion rate by HTK solution might also be due to a reduction in cell swelling, particularly endothelical cell swelling [4]. We are not aware of detailed studies concerning Kupffer cell activation following storage in HTK solution. However, the addition of mannitol, a well-known hydroxyl radical scavenger [36], to HTK solution may reduce free-radical-mediated endothelial injury.

In recent years, the role of leucocytes in reperfusion injury has been studied in several models. Briefly, it has been demonstrated that primary reperfusion injury due to generation of free radicals occurs following ischaemia [25]. Subsequently, due to endothelial cell injury and release of chemotactic mediators by macrophages or endothelial cells, increased adherence of leucocytes to the endothelial wall has been demonstrated [10, 11]. The precise mechanism of mediation and induction of leucocyte adhesion has not been clarified sufficiently. However, increased expression and regulation of adhesion sites on endothelial cells (e.g. ICAM-1 and ELAM-1 receptors [31]) or leucocytes (Mac-1 (CD11/18) [17, 19, 32]) are involved in this process [9, 19, 31]. In consequence, further endothelial cell injury seems to be due to release of cytotoxic mediators and free radicals by activated and adherent leucocytes [1], accompanied by subsequent microvascular perfusion failure.

Leucocyte adhesion following liver transplantation seems to be triggered by oxygen-derived free radicals, as demonstrated by the prevention of leucocyte adherence after superoxide dismutase application [23]. Thus, oxygen-derived free radicals generated during reperfusion injury may be involved in regulation of leucocyte adherence [35]. In this study, permanent adherence of leucocytes was most pronounced after EC solution preservation, in which reperfusion injury to endothelial cells has been described [21, 38]. The significant reduction in permanent leucocyte adherence in UW solution might, therefore, be due to scavenging of free radicals with the antioxidant glutathione and the xanthine oxidase inhibitor allopurinol. Prevention of permanent leucocyte adherence by HTK solution might also be explained by the component mannitol, a hydroxyl radical scavenger.

In addition, prevention of Kupffer cell activation by UW and HTK solutions might be likely, thereby reducing the release of adhesion-promoting mediators (e.g. leukotrienes; [6]). In contrast to the reduction of permanent leucocyte adherence in the UW and HTK solutions, the percentage of temporary adherent leucocytes increased significantly in both groups compared with the EC group. Conversion of permanent to temporary leucocyte adhesion in our study might indicate a change in the adhesion force or a regulation of adhesion sites for temporary adhesion (e.g. Mel-14) and permanent adhesion e.g. Mac-1/CD18 complex) [19]. Thus, reduction of permanent leucocyte adhesion might result first in conversion to temporary leucocyte adhesion as observed in this study. Complete prevention of leucocyte–endothelial interaction should abolish both types of adhesion, as demonstrated by application of selective monoclonal antibodies in other models (e.g. MoAb 60.3; [32]) Further studies with preservation solutions should include selective monoclonal antibodies to elucidate the leucocyte–endothelial adhesion mechanisms.

In conclusion, this study demonstrates that intravital fluorescence microscopy is a powerful method for assessing the microcirculation of liver grafts after liver preservation with various cold storage solutions. The results demonstrate a significantly better microcirculation in liver grafts stored in UW or HTK solution than in those stored in EC solution. Microcirculatory failure and increased leucocyte adherence following cold storage in EC solution may be caused by endothelial cell swelling and injury due to oxygen-derived free radicals.

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