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Effects of portal vein clamping time on rat liver microcirculation following extended cold preservation and transplantation

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Abstract Orthotopic rat liver transplantation (ORLT) following extended cold preservation in University of Wisconsin (UW) solution has been shown to induce alterations of the hepatic microcirculation, mainly characterized by areas of no-reflow. The present study was performed to determine whether these alterations were related to the portal vein clamping time (PVCT), shown to be the main determinant of survival after ORLT. The hepatic microcirculation was evaluated using the multiple-indicator dilution curve (MIDC) technique after ORLT following 24-hour cold ischemia in UW solution. Two groups of rats were studied: one with PVCTs of less than 14 min (survival conditions) and one with PVCTs of more than 18 min (nonsurvival conditions). Four hours after ORLT, only long PVCTs were associated with small, but significant, nonperfused areas, about 10% of the liver not being perfused by water; however, in both survival and nonsurvival conditions, the sinusoidal sieving function was well-maintained in perfused areas. In addition, liver viability parameters and hepatocyte function were similarly and minimally altered. The hepatic microcirculation is minimally altered 4 h after ORLT following extended cold preservation in UW solution, whatever the survival condition. Although only found after long PVCTs, the low magnitude of areas of no-reflow should not be associated with lethal injury of the transplanted liver, a finding further supporting the concept that survival after ORLT following 24-hour cold preservation in UW solution is mainly influenced by extrahepatic factors.

Key words Liver microcirculation · Orthotopic rat liver transplantation · Cold preservation · Portal vein clamping time

Introduction

The incidence of "primary liver nonfunction", leading to graft failure and early retransplantation, has decreased considerably with the use of the University of Wisconsin (UW) solution; however, "poor initial liver function" remains a major clinical problem, leading to increased intensive care unit and hospital stays [1, 5, 27]. Up to now, the foremost cause of both conditions was thought to be directly related to the severe sinusoidal endothelial cell (SEC) changes found after cold ischemia-reperfusion and leading to microcirculatory disturbances [2–4, 15, 21, 23, 28]. Indeed, most morphological investigations using either transmission electron or scanning electron microscopy have shown marked alterations of SECs following cold ischemia, with retraction and detachment of cell bodies progressing to an almost complete denudation of the sinusoidal lining during reperfusion [4, 23, 28]. Whether SECs die early during warm reperfusion or during cold ischemia remains controversial [3, 4, 12, 23, 28–30].

Experimentally, the clinical consequence of the SEC alterations has mainly been ascertained by the survival rate after orthotopic rat liver transplantation (ORLT)

following 24-hour cold preservation in UW solution, considered as a severely compromising condition. However, under this preservation condition, reported survival rates varied widely, ranging from 0 to 100% [16, 33, 34, 36–38], suggesting that other factors than 24-hour cold preservation in UW solution might be involved in the postoperative course. Indeed, using the same preservation condition, we have recently reported that the postoperative survival was mainly dependent on the portal vein clamping time (PVCT): short PVCTs (< 14 min) being associated with a 100% survival, while long PVCTs (> 18 min) were associated with a 20% survival [39]. Long PVCTs induced an endotoxin-like syndrome more related to a warm intestinal ischemia than to a cold ischemia injury of the liver [39]. Using the multiple-indicator dilution curve (MIDC) technique in the isolated perfused rat liver model, we have previously reported that nonlethal hepatic microcirculatory disturbances could be found after ORLT with or without 24-hour preservation of the liver in cold UW solution [18]; these disturbances were mainly characterized by areas of no-reflow, with an apparent normal sieving function of the sinusoidal lining in perfused areas [18]. However, in that study, ORLTs were performed using intermediate PVCTs (about 16 to 18 min) [18]. The present study was thus designed to evaluate the effects of short and long PVCTs on the hepatic microcirculation after ORLT following 24-hour liver preservation in cold UW solution, i.e., under survival and nonsurvival conditions.

Materials and methods

Inbred male Lewis rats (Charles River, Canada) were purchased to exclude immunologic interference. Rats weighing 275–300 g were used as liver donors, and others weighing 300–325 g were used as recipients at transplantation. Animals were housed in a controlled environment with a 12 h light/dark cycle. Donor rats had access to water only for 12 h before organ harvesting, while recipient rats had free access to normal rat chow and water before surgery. The experiments described in this report were conducted according to the *Guide for the Care and Use of Laboratory Animals.*

Orthotopic rat liver transplantation

Liver transplantation was performed according to Kamada's cuff-technique [19], as previously reported [18, 39], under halothane and nitrous oxide anesthesia. Before liver harvesting, 300 units of heparin with 1.2 ml saline were injected via the penile vein, and the donor liver was perfused directly in situ via the portal vein with 10 ml of cold (4 °C) UW solution (DuPont Merck Pharma, Mississauga, Ontario, Canada). The excised liver was placed in a bath of cold UW solution and was perfused with another 10 ml of cold UW solution through the portal vein before cuff installation. Following cuff preparation, livers were stored in a beaker containing 30 ml of UW solution at 4 °C for 24 h. At the end of the storage period, livers were slowly flushed with 20 ml of cold (4 °C) Ringer's lactate and transplanted orthotopically into recipient animals with randomly predetermined PVCTs, as will be described later. The hepatic artery was not reconstructed since, as previously reported, its reconstruction does not modify changes in the hepatic microcirculation induced by ORLT [17]. After surgery, rats received 2 ml of 5% dextrose through the penile vein. No further treatment was given. Animals had free access to normal rat chow and water after surgery. Recipient rats were randomly assigned PVCTs of less than 14 min (mean 9.5 ± 0.4 min; group 1; $n = 8$), corresponding to survival conditions, or PCVTs of 20 min (20.0 ± 0.1 min; group 2; $n = 8$), corresponding to nonsurvival conditions [39]. All rats recovered and were conscious within 15 min after surgery. However, their clinical conditions deteriorated within 2 to 4 h after surgery, particularly in the case of rats from group 2 (long PVCTs), which became markedly weak and lethargic at that time. We chose to explore the hepatic microcirculation at 4 h after transplantation since, according to our experience, it is after this time that survivors recover spontaneously and progressively within the following 24 h while nonsurvivors deteriorate rapidly and die during the same period [39].

Isolated perfused rat liver

Four hours after revascularization, animals were anesthetized with pentobarbital and, following laparotomy, cannulated in situ: the bile duct, portal vein and suprahepatic inferior vena cava were cannulated after ligation of the inferior vena cava as previously described [18, 39]. Livers were perfused for 40 min with oxygenated Krebs buffer containing bovine red blood cells (RBCs; 20%), albumin (20 g/l), and dextrose (1 g/l), under fixed perfusion flow (20 ml/min) through the portal vein in a closed circuit [40]. A loading dose of unlabeled taurocholic acid (TC; mixed with tracer doses of ^{14}C-TC) was added to the reservoir to attain a theoretical plasma concentration of 11.5 ng/ml, followed by continuous infusion to maintain these levels during each perfusion study [18, 39]. The following formula for measuring TC clearance was used to assess hepatocyte function:

TC clearance = Q × TC extraction, with TC extraction = (Ci–Co)/Ci,

in which Q is the perfusate flow and Ci and Co are ^{14}C-TC plasma levels at inflow and outflow. Intrahepatic resistance, oxygen consumption, bile secretion, and LDH release were used to evaluate liver viability parameters. Data were calculated as the mean values obtained between the 20th and 40th min of each perfusion period, and intrahepatic resistance was recorded at 5, 20 and 40 min of reperfusion

Multiple-indicator dilution curves

At the end of the liver function study, the hepatic venous outflow was diverted to avoid recirculation of radioactive materials; then, the MIDCs were obtained following a 0.1 ml injection of radioactive tracer mixture consisting of the following substances: ^{51}Cr-RBC (10^5 dpm), ^{99m}Tc-albumin (2×10^5 dpm), ^{14}C-sucrose (2×10^5 dpm), and ^{3}H-water (3×10^5 dpm) in a solution of Krebs-Henseleit buffer adjusted to a hematocrit matching that of the perfusate containing albumin (20 g/l). The total hepatic venous outflow was collected in serial tubes at the rate of 1 tube/s for 90 s. An aliquot from each tube was used to determine gamma and beta ray activity and processed as previously described [40]. The background contamination by ^{14}C-TC contained in the perfusate

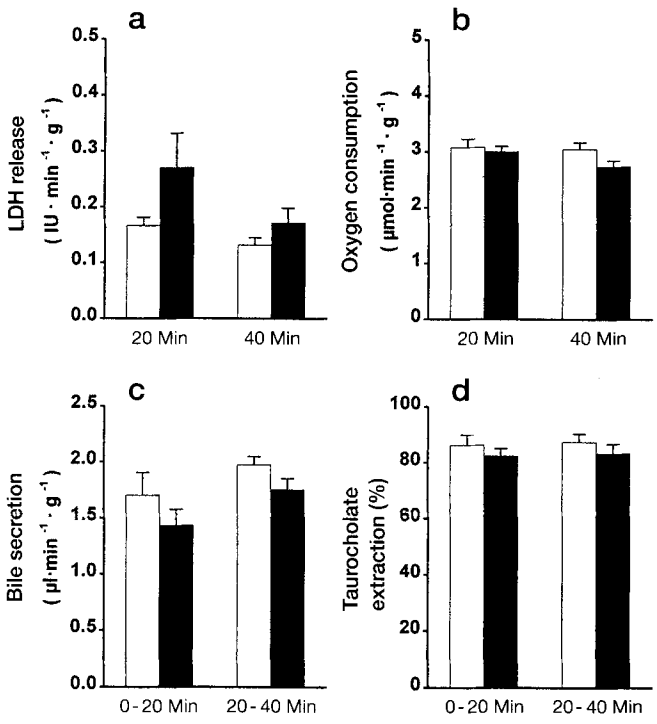

Fig. 1 LDH release (**a**), oxygen consumption (**b**), bile secretion (**c**), and taurocholate extraction (**d**) measured at 20 and 40 min after reperfusion in livers harvested 4 h after transplantation with short [□] and long [■] portal vein clamping times. P = NS between different parameters for both groups

to ^{14}C-sucrose was negligible (less than 0.01 % of the peak value of the labeled sucrose curve). The outflow activity (disintegrations per minute per milliliter) was divided by the total amount injected (disintegrations per minute) in order to yield a normalized outflow fraction recovery per milliliter of blood, thus providing the basis for comparison of each tracer.

In order to calculate the sinusoidal volume, data were then analysed according to Goresky's flow-limited model [13]. This model states that labeled RBCs delineate the vascular space, whereas the interstitial space indicators (high molecular weight or labeled albumin, and low molecular weight or labeled sucrose) as well as the whole organ tracer (labeled water) undergo flow-limited distribution from the sinusoid into Disse's space and hepatic cellular space, respectively, in a manner characterized by a delayed wave type of behaviour. Therefore, the dilution curve of each diffusible substance can be superimposed on that of RBCs if every point is corrected by a constant factor to its corresponding point on the RBC curve using the following equation:

$$C_{dif}(t') = C_{RBC}(t)/(1 + \gamma), \text{ in which } t' = (t-t_0)(1 + \gamma) + t_0$$

and C_{RBC} and C_{dif} are the RBC and diffusible substance (dif) concentration values at corresponding arbitrary points in time of t(RBC) and t'(dif), respectively; t_0 is the large vessel transit time delay, and γ is the ratio of extravascular to vascular volume of distribution of diffusible substance. The values of t_0 and γ were determined as those yielding the least sum of the square of deviations between the RBC curves and the diffusible substance curves when the latter is transformed linearly to be superimposed on the first. The optimization procedure was guided by a least-square, minimization algorithm programmed in Turbo Pascal and Lotus 123 on a Hewlett-Packard computer (Vectra 286 series; Pala Alto, Calif.). The results of the nonlinear fitting procedure were examined by visual inspection of the fitting curve [26] and by measuring the coefficients of variation [14] and determination [11] of the fit. Once γ and t_0 were obtained, the vascular (sinusoidal) volume (V_{sin}) was calculated as

$$V_{sin} = Q \times (t_{RBC}-t_0),$$

in which Q is the perfusate flow and t_{RBC} is the corrected mean transit time of RBC calculated according to Meier and Zierler [24].

Extravascular volumes accessible to albumin (E_{Valb}) and sucrose (E_{Vsuc}) were calculated as a model-independent parameter according to the transit time method [6] as

$$E_V = Q \times (1\text{–Hematocrit}) \times (t_{dif}-t_{RBC}),$$

in which t_{dif} is the corrected mean transit time of diffusible substances (albumin and sucrose).

Extravascular water space was calculated using the same formula with the exception that, in the present case, Q equals water flow (Q_w), which needs to be calculated beforehand as

$$Q_w = [Q \times \text{Hematocrit} \times 0.7] + [Q \times (1\text{–Hematocrit}) \times 0.93],$$

in which 0.7 and 0.93 represent the proportion of water (ml/ml) in RBC and plasma, respectively [9]. The hepatic cellular water space (V_{cell}) is consequently [9]:

$$V_{cell} = E_{Vwater}-E_{Vsuc}.$$

Statistical analysis

Data are expressed as mean with SEM. Student's unpaired t-tests were used to compare the two groups. A P value of less than 0.05 was accepted as statistically significant.

Results

Viability parameters

No significant difference was found with respect to liver-to-body weight ratio (0.028 ± 0.001 vs 0.030 ± 0.001), oxygen consumption, bile secretion, LDH release, and TC clearance between short and long PVCT groups in livers perfused 4 h after ORLT (Fig. 1). The intrahepatic resistance was similar in both groups from the beginning to the end of reperfusion (Fig. 2). Viability parameters were only minimally altered when compared to those previously reported in normal control livers or in livers preserved for 24 h in cold UW solution [18].

Multiple-indicator dilution curves

Typical sets of MIDCs are shown in Fig. 3. In all rats from both experimental groups, labeled RBCs emerged first in the outflow, rapidly reaching a peak concentra-

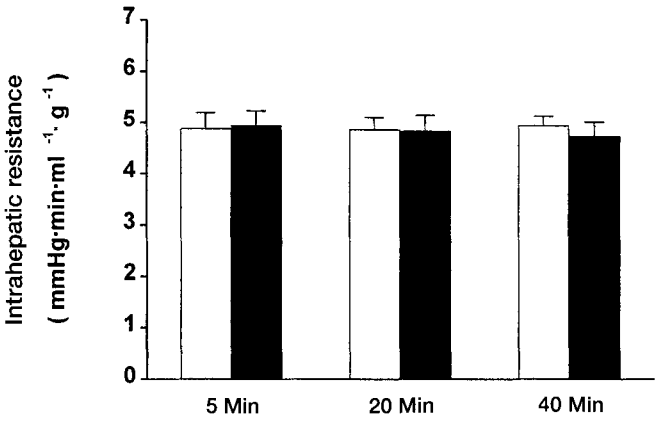

Fig. 2 Intrahepatic resistance measured 5, 20, and 40 min after reperfusion in livers harvested 4 h after transplantation with short [□] and long [■] portal vein clamping times. P = NS between both groups

Table 1 Distribution volumes and total reflow spaces obtained using the multiple-indicator dilution curve technique in isolated perfused rat livers. [a] All values (mean ± SEM) are in ml/g liver weight (*PVCT* portal vein clamping time)

	Group 1 (PVCT < 14 min) (n = 8)	Group 2 (PVCT > 18 min) (n = 8)	P value
Sinusoidal space	0.22 ± 0.01	0.20 ± 0.01	NS
Albumin interstitial space	0.12 ± 0.01	0.11 ± 0.01	NS
Sucrose interstitial space	0.16 ± 0.01	0.14 ± 0.01	< 0.05
Cellular water space	0.50 ± 0.01	0.45 ± 0.01	< 0.01
Total perfused space[b]	0.88 ± 0.02	0.79 ± 0.01	< 0.01

[a] The rat livers were transplanted after 24-hour cold storage in UW solution at 4 °C. Four hours after transplantation, the liver was isolated and perfused for 40 min, after which distribution spaces were measured

[b] Evaluated by the addition of the sinusoidal space, sucrose interstitial space (Disse's space) and cellular water space

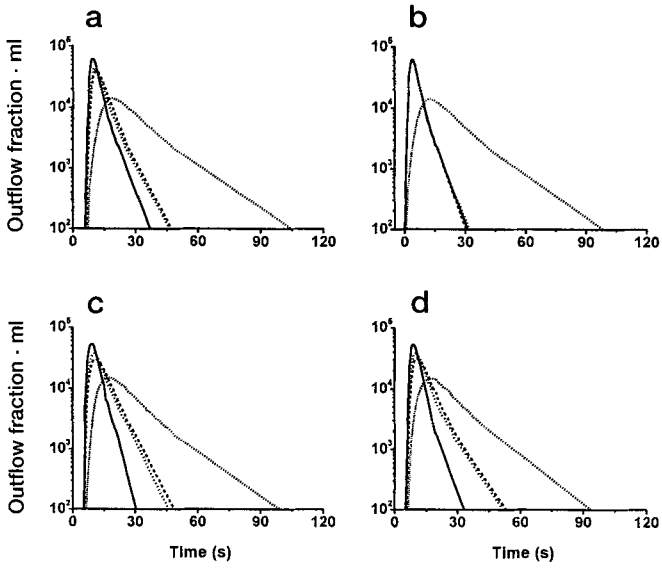

Fig. 3 Representative examples of multiple-indicator dilution curves (**a**) in a control rat (reproduced with permission by [21]), (**c**) in a rat with transplantation and short portal vein clamping time, and (**d**) in a rat with transplantation and long portal vein clamping time. In (**b**), theoretical curves that should be obtained if complete destruction of the sinusoidal lining had occurred. ____ ^{51}Cr RBC; . . . . . ^{99m}Tc albumin; —— ^{14}C sucrose; . . . . .^{3}H water

tion and declining monoexponentially with time. Curves for labeled albumin, sucrose, and water showed lower peaks and appeared later in the outflow with patterns comparable to the one obtained in normal livers (Fig. 3). In all cases, these curves could be transformed linearly and properly superimposed on RBC curves, indicating that diffusion of these substances to their respective distribution space was always flow-limited, irrespective of the experimental groups, as occurs in normal livers [18].

The RBC, albumin, sucrose, and water distribution volumes are summarized in Table 1. The sinusoidal and albumin interstitial spaces were slightly but not significantly smaller in group 2 than in group 1, whereas sucrose interstitial and cellular water spaces were significantly decreased in group 2 rats.

When using the MIDC technique, the addition of the sinusoidal volume, Disse's space (sucrose interstitial volume), and the water cellular spaces should be an estimate of the total space perfused by water, approximately 0.9 ml/g of liver under normal conditions [18]. In group 1, the total space perfused by water was similar to values obtained under normal conditions [18]; in contrast, this value was significantly reduced by 10% in group 2 ($P < 0.01$; Table 1), indicating that nonperfused spaces, assumed to be areas of no-reflow, could only be found in rats undergoing ORLT with prolonged PVCTs.

Discussion

The objective of the present study was to evaluate liver microcirculatory disturbances occurring after cold ischemia-reperfusion of the transplanted liver when operated with short and long PVCTs, i.e., under survival and nonsurvival conditions. To this end, the liver microcirculation was evaluated using the MIDC technique, which is probably one of the best approaches to hepatic microcirculation because it reveals the whole internal liver structure without dissection. Another approach has been proposed to study *in vivo* liver microcirculation using fluorescence microscopy with epi-illumina-

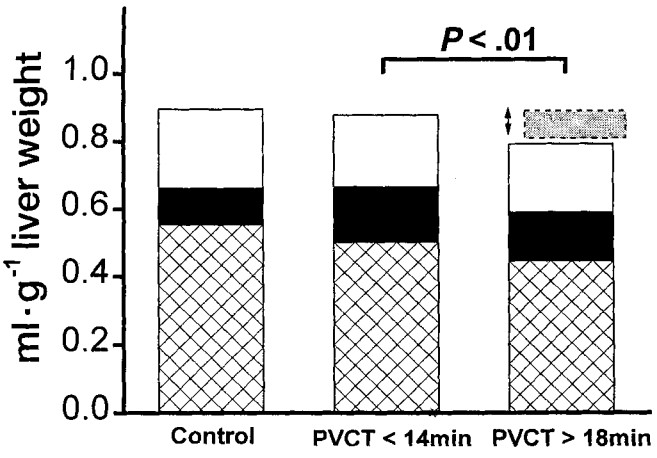

Fig. 4 Addition of sinusoidal, Disse, and cellular spaces in animals operated with short (< 14 min) and long (> 18 min) portal vein clamping times (*PVCT*). In rats operated with short PVCTs, these additions reflected complete perfusion of all liver structures as occurred in control rats (reproduced with permission by [21]). In rats operated with long PVCTs, the total space perfused by water was significantly reduced by 10% when compared to rats operated with short PVCTs. The nonperipheral spaces were assumed to be areas of no-reflow. □ Sinusoidal space; ■ Disse's space; ⊠ cellular space; ▒ areas of no-reflow

tion [35]. However, only a few selected sinusoids beneath the liver capsule can be explored at the same time, and it is not known if data can be extrapolated to the whole internal liver structure. In addition, this approach cannot assess the respective contribution of various vascular, extravascular, and cellular spaces to the sinusoidal architecture as does the MIDC technique.

Overall, the liver microcirculation was minimally altered under both experimental conditions. Following ORLT with short PVCTs (group 1), the various vascular, extravascular, and cellular spaces, as well as the total space perfused by water, were similar to those reported in normal livers and in livers following cold preservation alone (24 h in UW solution), reflecting complete perfusion of all liver structures and a normal sieving function of the sinusoidal lining [18]. These findings were unexpected, particularly when considering that most morphological studies using either transmission electron or scanning electron microscopy have shown marked alteration of SECs following extended cold ischemia with retraction and detachment of cell bodies progressing to an almost complete denudation of the SEC lining during reperfusion [4, 23, 28]. Indeed, if such were the case, the whole extracellular space would be available to labeled RBCs, albumin, and sucrose without limitation to their extravascular diffusion and thus without their separation during the passage through the altered sinusoids: the RBC, albumin, and sucrose curves would be superimposed on each other (Fig. 3).

The discrepancy between the present MIDC findings and morphologic data obtained in previous electron microscopy studies is difficult to explain. It seems unlikely that a fixation artifact occurred during perfusion of cold-preserved livers with glutaraldehyde/formaldehyde or by using high perfusion-fixation pressure, conditions which generally induce a marked widening of endothelial fenestrae but no retraction and/or detachment of SECs. Another explanation can be put forward, i.e., that substances already existing in Disse's space, such as collagen, laminin, fibronectin, proteoglycans etc., (or accumulating during cold ischemia-reperfusion) are more important than SECs with regard to the sieving function in liver sinusoids, as assessed using the MIDC technique. These substances can act as a semipermeable gel in which distribution of diffusible substances occurs according to their molecular weight. This question remains to be answered in a future study.

In livers reperfused after ORLT with long PVCTs, the sieving function of sinusoids was also well-maintained with near-normal diffusion patterns. By contrast, and in these livers only, areas of no-reflow could be identified, with significant reduction in the sucrose interstitial space and cellular water space when compared to the group operated with short PVCTs. These areas (about 10% of the whole liver) were of the same magnitude as those previously reported using intermediate PVCTs (16 to 18 min) with and without extended cold preservation in UW solution [18]. Similarly, a no-reflow phenomenon was observed with in vivo fluorescence microscopy during normothermic and cold ischemia-reperfusion of the liver [20, 22, 31, 32]. These alterations of the hepatic microcirculation are most probably nonlethal, although only observed in animals operated with long PVCTs (therefore under nonsurvival conditions), since similar areas of no-reflow were also found in rats undergoing ORLT without extended preservation and a 100% survival [18].

The use of long PVCTs during ORLT has been shown to be associated with an endotoxemia-like syndrome caused by a confounding warm intestinal ischemia and not related to the cold ischemia of the liver [39]. Under such conditions, stimulation of Kupffer cells may exert their deleterious effect by releasing reactive oxygen radicals and/or toxic cytokines, such as tumor necrosis factor α [39], and mediating the adherence and accumulation of whole blood elements, such as leukocytes and/or platelets within the sinusoids [7, 10], resulting in a defective perfusion of the sinusoidal bed.

In conclusion, the present investigation indicates that liver microcirculation is only minimally altered after ORLT, irrespective of the length of the PVCT, and that the areas of no-reflow, only found with long PVCTs, should not be held responsible for the postoperative

clinical deterioration of animals. Indeed, liver viability parameters and hepatocyte function (as evaluated by taurocholate elimination) were well-maintained 4 h after ORLT with either short or long PVCTs. The present findings further support the concept that survival after ORLT with 24-hour cold preservation in UW solution is mainly influenced by extrahepatic factors, such as pulmonary injury [8] or endotoxin-related shock [25], with secondary hepatic dysfunction [39].

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