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Intravital studies on beneficial effects of warm Ringer's lactate rinse in liver transplantation

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Abstract This quantitative in vivo fluorescence microscopy study investigated the impact of warm versus cold Ringer's lactate (RL) graft rinse on various microvascular manifestations of ischemia-reperfusion injury after liver transplantation in the rat. Syngeneic orthotopic liver transplantation, including arterial revascularization, was performed in male Lewis rats following 24 h of cold storage in University of Wisconsin (UW) solution. In one group ($n = 8$) liver grafts were rinsed with 4 °C (cold) RL, whereas in the other group ($n = 8$) grafts were rinsed with 37 °C (warm) RL immediately prior to revascularization. Hepatic microvascular perfusion, leukocyte-endothelium interaction, and Kupffer cell activation were quantified 30–90 min after graft reperfusion by direct visualization with intravital fluorescence microscopy. Moreover, biliary excretory graft function was analyzed by determination of bile flow and bile salt excretion during the first 90 min after reperfusion. Compared to grafts rinsed with cold RL, acinar and sinusoidal perfusion were found to be significantly increased after rinsing the grafts with warm RL. The amount of nonperfused acini declined from 18.1 % ± 4.0 % to 7.4 % ± 1.6 % ($P < 0.05$), and the total percentage of perfused sinusoids increased from 80.1 ± 1.4 to 88.4 ± 1.2 ($P < 0.001$) after cold and warm rinse, respectively. After rinsing the graft with warm RL, WBC adherence in sinusoids and especially in postsinusoidal venules decreased significantly by 28 % ($P < 0.001$) and 33 % ($P < 0.001$), respectively. Kupffer cell activation was markedly reduced after rinsing with RL at 37 °C, as indicated by a decelerated adherence of latex particles injected 80 min after reperfusion. Excretory graft function was dramatically increased following warm RL rinse during the 90-min observation period. Bile flow was enhanced from 1.04 ± 0.5 to 3.9 ± 0.8 ml/100 g liver per 90 min ($P < 0.01$), with a parallel rise in bile salt excretion from 24.3 ± 5.8 to 128.0 ± 19.8 mmol/100 g liver per 90 min ($P < 0.05$) when compared to cold RL. These data strongly suggest that rinsing liver grafts with warm RL prior to reperfusion represents a simple and inexpensive way to reduce the incidence of primary graft failure secondary to ischemia and reperfusion injury in liver transplantation.

Key words Preservation, liver, warm rinse · Ringer's lactate, warm, liver preservation · Liver, warm rinse, preservation

Introduction

Although marked progress has been achieved since the introduction of modified preservation solutions [3], primary graft dysfunction secondary to injury by ischemia and reperfusion remains a major problem with regard to morbidity and mortality in clinical liver transplantation [8]. As pointed out recently [23], warm ischemia itself results predominantly in damage to hepatocytes, whereas cold ischemia and subsequent reperfusion of liver grafts are accompanied by impaired microvascular hemodynamics due to injury of the sinusoidal lining [20]. The latter is characterized by a loss of endothelial cell integrity [7, 37], Kupffer cell activation, and the subsequent release of lysosomal enzymes [7] and potent mediators [9, 24, 29], which may lead to aggravation of post-transplant liver injury, including secondary damage to hepatocytes [8, 37].

Since commonly used preservation solutions, such as University of Wisconsin (UW) or Euro-Collins solution, both of which have been shown to be effective in minimizing preservation-associated graft damage, contain high concentrations of potassium, these solutions have to be flushed out before reperfusion in order to prevent cardiac complications due to hyperkalemia. For this purpose, Ringer's lactate (RL) or albumin are most often used in clinical practice [3].

Experimental evidence strongly suggests that not only the type of rinse solution but also the temperature of the rinse solution plays a key role in the reduction of ischemia-reperfusion injury in liver grafts. A dramatically increased 1-week survival rate and increased bile flow have been reported in rat liver transplantation after preservation in normal saline and prewarming the graft to 10 °C prior to implantation [39]. Furthermore, in the same model, significantly increased graft survival and decreased morphologically detectable microvascular injury were observed following a rinse with warm (37 °C) RL prior to reperfusion [35]. Moreover, in morphological studies, Carolina Rinse was shown to protect microvascular integrity more efficiently when used at a temperature of 30 °C rather than 4 °C [2].

Since the impact of rinse solution temperature on liver transplantation has not yet been quantified intravitally despite emphasis on its importance in recent clinical studies [10, 21], the present study was performed to analyze the effect in vivo of warm (37 °C) RL rinse on microvascular perfusion, leukocyte (WBC)/endothelium interaction, and Kupffer cell activation after liver transplantation by direct observation with intravital fluorescence microscopy.

Materials and methods

Surgical procedure

After premedication with atropine (0.1 mg/kg s.c.), syngeneic orthotopic liver transplantation, including arterial reconstruction, was performed under ether anesthesia in 16 male Lewis rats weighing 180–230 g (Charles River Wiga, Sulzfeld, Germany), as described previously in detail [26, 27, 33]. For continuous monitoring of mean arterial blood pressure during the recipient operation and for substitution of plasma volume as well as injection of fluorescent compounds, the left carotid artery and jugular vein were cannulated with polyprophylene catheters. Body temperature was monitored continuously and kept within the range 36.5 °–37.5 °C by a heated operating table. Grafts were harvested after in situ perfusion via the abdominal aorta with 10–15 ml of chilled UW solution (4 °C) at a pressure of 100 cm H_2O, and then with another 6 ml of UW solution ex situ via the portal vein at a pressure of 10 cm H_2O. Subsequently, the liver grafts were stored for 24 h in UW solution at 4 °C. The common bile duct was cannulated in all liver grafts for analysis of bile flow and bile salt secretion. Following intravital microscopy and collection of blood and liver tissue specimens, all animals were sacrified by exsanguination. Experiments were performed in accordance with German legislation on animal protection and with the "Principles of laboratory animal care" (NIH Publication no. 86–23, revised 1985).

Experimental groups

Immediately prior to revascularization, the preservation solution was flushed out of all grafts by rinsing them with 10–20 ml of RL (2 ml/g liver net weight) at a pressure of 10 cm H_2O. In one group of rats ($n = 8$) grafts were rinsed ex situ with 4 °C (cold) RL; in the other group ($n = 8$), grafts were rinsed in situ with 37 °C (warm) RL.

Intravital microscopy

Thirty to ninety minutes after portal reperfusion, the lower surface of the left liver lobe was exposed for in vivo epi-illumination microscopy, as described previously [27]. This exteriorization allows the exposure of more than 2000 single acini per experiment. During the 60-min observation interval, the liver surface was covered with a glass slide, and in-between each single observation field the liver surface was flushed with warm RL in order to protect the hepatic microcirculaton against environmental influences and to increase optical quality of the microscopic recordings. Determination of acinar as well as sinusoidal perfusion was achieved by contrast enhancement of plasma with 1 μmol/kg sodium fluorescein (Merck, Darmstadt, Germany) 30 min after reperfusion. This was repeated twice during the 1-h microscopy episode since this substance is rapidly excreted to the bile. At 45 min after graft reperfusion, intravenous injection of rhodamine 6G (0.1 μmol/kg, Merck, Darmstadt, Germany), a substance that specifically stains WBC and platelets, allowed the quantitative assessment of intrasinusoidal WBC accumulation [27]. Phagocytic activity was assessed by an intra-arterial bolus injection of fluorescence-labelled latex particles 80 min after portal reperfusion [3×10^8 particles/kg, 1.1-μm diameter YG (yellow-green) plain; Polysiences, Warrington, Pa., USA] [25]. Filter combinations of 450–490 nm/greater than 515 nm (excitation/emission) and 530–560 nm/greater than 580 nm were used for visualization of sodium fluorescein/latex beads and rhodamine fluorescence, respectively.

Table 1 General data and hepatocellular function. All values represent the mean ± SEM; *P* values were calculated using a two-way nested design ANOVA (*RL*, Ringer's lactate)

	RL 4 °C	RL 37 °C	*P* value
Mean arterial blood pressure (mmHg)	81.1 ± 2.6	78.1 ± 3.3	NS
Cold ischemia time (hours)	25.1 ± 0.24	24.7 ± 0.17	NS
Anhepatic period (min)	18.5 ± 0.9	17.8 ± 0.5	NS
Bile flow (ml/100 g liver per 90 min)	1.04 ± 0.5	3.9 ± 0.8	0.008
Bile salt excretion (mmol/100 g liver per 90 min)	24.3 ± 5.8	128.0 ± 19.8	0.03

Table 2 Microvascular perfusion and leukocyte (WBC) adherence. All values represent the mean ± SEM; *P* values were calculated using a two-way nested design ANOVA (*RL*, Ringer's lactate)

	RL 4 °C	RL 37 °C	*P* value
Acinar perfusion [nonperfused acini (%)]	18.1 ± 4.0	7.4 ± 1.6	0.02
Sinusoidal perfusion [perfused sinusoids within perfused acini (%)]	74.8 ± 1.8	85.4 ± 1.3	0.0001
Permanent WBC adherence in postsinusoidal venules (WBC/mm^2 liver surface)	727 ± 43	485 ± 32	0.0001
Permanent WBC adherence in sinusoids (WBC/mm^2 liver surface)	239 ± 14	172 ± 10	0.0002
Rolling WBC in postsinusoidal venules (% of all moving WBC)	21.3 ± 1.9	29.6 ± 2.9	0.02

Video analysis

Quantification of microhemodynamics, WBC adherence, and phagocytosis of latex beads was performed off-line by frame-to-frame analysis of videotaped images. At 30 and 90 min after portal reperfusion, the left liver lobe was scanned at lower magnification (× 240) for evaluation of acinar perfusion in 50–100 acini. According to the observed sinusoidal flow behavior and distribution of fluorescent compounds, the acini were graded as perfused or nonperfused, and the results were expressed as the percentage of nonperfused acini with respect to the total number of acini.

Furthermore, between 60 and 80 min after graft reperfusion, microvascular reperfusion injury was assessed in sinusoids of 10–15 randomly selected, individual acini per experiment (a minimum of 75 acini per group). At high magnification (× 600), sinusoidal perfusion was quantified as the percentage of completely perfused sinusoids of all observed sinusoids at each subacinar zone (portal, midzonal, and central). WBC adherence was determined as the number of rhodamine-stained cells adhering to hepatic sinusoids as well as to postsinusoidal venules without any movement for an observation period of at least 20 s (standardized in sinusoids per mm^2 liver surface and in venules per mm^2 endothelial surface area, calculated by $\pi \times$ diameter × length of vessel segment under observation). Rhodamine-stained cells within venules moving with less than 30 % of the center line velocity were defined as "rolling WBC" and were expressed as the percentage of all moving leukocytes.

Phagocytic activity was subsequently analyzed during the first 5 min after intra-arterial injection of fluorescence-labelled latex beads within 10–20 randomly selected microscopic fields (405 × 540 μm) per experiment. Phagocytosis was quantified as the percentage of moving versus all visible beads within the field during an observation period of 8–15 s. Beads in pre- or postsinusoidal vessels were excluded from measurements.

Determination of bile acid secretion

Determination of the total amount of unsulfated, unglucuronidated bile acids secreted over the 90-min period was determined using a steroid 3α-dehydrogenase assay [36].

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Differences were considered significant for *P* values less than 0.05. Comparisons between groups were performed using an analysis of variance (ANOVA) after rank transformation of non-normally distributed data (dependent on results of the Shapiro-Wilk statistics of normality). For all microscopy data with multiple measurements within a single experiment, a nested design two-way ANOVA was used (group and single experiment within group). Furthermore, effects of time in repeated measurements within a single experiment were determined by a multivariate ANOVA. Adherence kinetics of injected latex particles were calculated using a general linear model with log-transformed percentages of moving beads as the dependent variable. Independent variables in the model were time, time^2, time^3, group, time × group interactions, and animal nested within group. Calculations were performed following the SAS procedures Univariate and GLM (SAS Institute, Cary, N. C., USA).

Results

General data

With respect to mean arterial blood pressure, cold ischemia time, and anhepatic period (Table 1), as well as to delay of arterial revascularization (6.75 ± 0.42 min with cold RL vs 6.75 ± 0.38 min with warm RL) and graft weight (8.11 ± 0.4 g with cold RL, 8.18 ± 0.3 g with warm RL), no significant differences were evident between transplant recipients with liver grafts rinsed with 37 °C or 4 °C RL. In none of the animals did the cross clamping time exceed 25 min, which is considered to be the maximum tolerable limit in rats without a portosystemic shunt [15].

Microvascular perfusion

Acinar perfusion

Disturbances in acinar perfusion were observed in both groups (Table 2, Fig. 1). However, in liver grafts rinsed with warm RL prior to reperfusion, the number of nonperfused acini was found to be markedly reduced in comparison to liver grafts exposed to a cold RL rinse. In particular, the percentage of nonperfused acini was

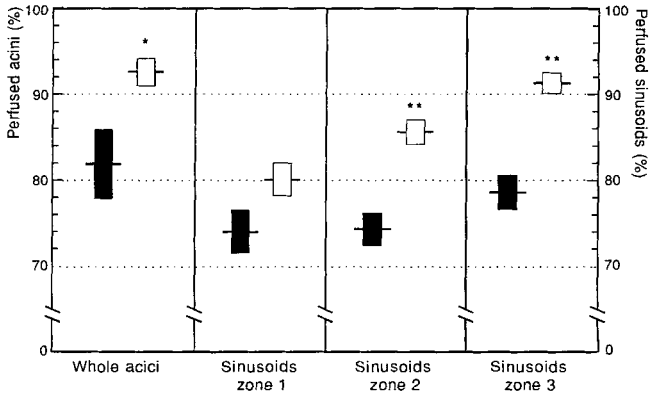

Fig. 1 Acinar and sinusoidal perfusion. Percentage (mean ± SEM) of perfused acini and sinusoids after rinsing liver grafts with cold (■) or warm (□) Ringer's lactate (*RL*). The number of nonperfused sinusoids was significantly reduced after warm RL. $*P < 0.05$ and $**P < 0.001$ vs grafts rinsed with cold RL

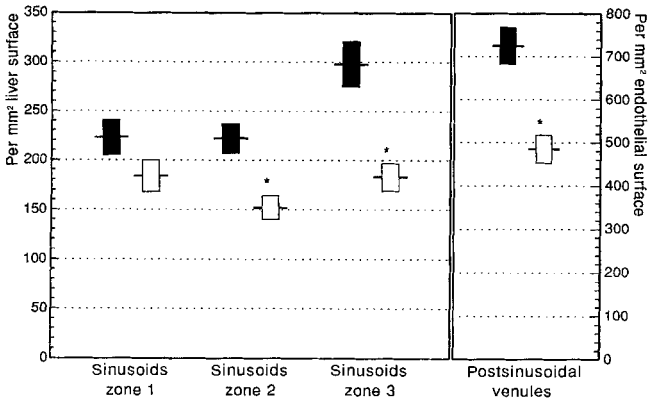

Fig. 2 WBC adherence. WBC adherence (mean ± SEM) for more than 20 s of observation, 60 min after portal reperfusion in grafts rinsed with cold (■) or warm (□) Ringer's lactate (*RL*). WBC adherence was significantly reduced in midzonal and pericentral subacinar areas, as well as in postsinusoidal venules. $*P < 0.001$ vs grafts rinsed with cold RL

found to be 18.1 ± 4.0 after the cold rinse, whereas after the warm rinse this number was significantly lower (7.4 % ± 1.6 %; $P < 0.05$).

Sinusoidal perfusion

In parallel, quantitative analysis of the sinusoidal perfusion within perfused acini revealed a significantly ($P < 0.001$) lower number of non-perfused sinusoids after the warm rinse than after the cold rinse. After reperfusion, about 25 % of all sinusoids remained unperfused in cold-rinsed liver grafts. In contrast, after the warm rinse, only 15 % of all visible sinusoids were not perfused (Table 2). This difference between the groups was most pronounced in midzonal (zone 2) and pericentral (zone 3) subacinar areas, whereas in the periportal area (zone 1) statistical significance was not reached ($P = 0.054$; Fig. 1).

Sinusoidal as well as acinar perfusion disturbances basically remained unchanged during the 90-min observation period. No significant influence of observation time was found in the multivariate analysis, and the differences between the groups remained unchanged between 30 and 90 min after reperfusion.

WBC Adherence

Intravital microscopy revealed WBC accumulation within sinusoids in all subacinar zones as well as in postsinusoidal venules in both experimental groups (Fig. 2). However, rinsing liver grafts with warm RL markedly reduced the number of WBC adhering for longer than 20 s in postsinusoidal venules ($P < 0.001$) by approximately 30 % (Table 2, Fig. 2) in comparison to cold-rinsed grafts. An equal effect was also observed in sinusoids. Specifically, separate analysis within the three zones revealed that no significant difference was present in periportal areas ($P = 0.22$), whereas warm RL reduced WBC adherence in both midzonal and pericentral zones significantly ($P < 0.001$) compared to cold RL (Fig. 2). The percentage of rolling WBC in postsinusoidal venules was found to be significantly higher in grafts rinsed with warm RL than in cold-rinsed grafts (Table 2).

Phagocytic activity

As in previous studies [25], rapid clearance of intra-arterially injected fluorescent latex particles by liver sinusoidal cells was observed in grafts transplanted after 24-h storage in 4 °C UW solution and rinsing with cold RL. This rapid adherence of latex particles was markedly ($P < 0.05$) reduced at 1–5 min after bolus injection when the transplant organ was rinsed with warm RL (Fig. 3).

Hepatocellular excretory function

Both bile flow and bile salt excretion, indicators of hepatocellular excretory function, were found to be significantly enhanced after liver grafts were flushed with warm RL prior to reperfusion. Bile flow increased more than three-fold in grafts rinsed with warm RL compared to those rinsed with cold RL. Concurrently, bile salt excretion increased fivefold (Table 1).

Discussion

These results provide evidence that various manifestations of reperfusion injury after 24 h of cold ischemia

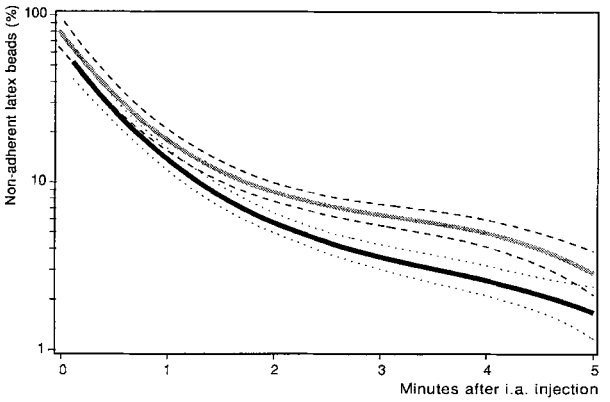

Fig. 3 Kinetics of phagocytic activity. Logarithmic scale representation of nonadherent latex particles as percentage of the total number of visible particles within microscopic fields observed for 10 s each. Values are equal to corresponding points on linear regression curves (mean) at 1, 2, 3, 4, and 5 min after injection of latex particles. *Dashed curves* represent the 95% confidence limit of the mean, calculated using a general linear model. After the 4°C RL rinse (*black curve*), particles adhere markedly more rapidly than after the 37°C RL rinse (*grey curve*), indicating attenuated activation of Kupffer cells

are considerably attenuated by rinsing liver grafts with 37°C rather than 4°C RL. Microvascular perfusion, intrahepatic WBC accumulation, Kupffer cell activation, and hepatocellular excretory function may, therefore, depend not only on the type of rinse solution but, in particular, on the temperature of the rinse solution.

It has previously been thought that the period of warm ischemia during liver grafting should be kept as brief as possible in order to avoid hepatotoxic effects. Despite this known damage associated with a prolonged warm ischemia, liver grafts showed increased microvascular perfusion after rinsing with 37°C RL. This observation is in accordance with previous reports in nonarterialized liver grafts, where significantly decreased general hepatic resistance, reduced enzyme release, enhanced excretory function, and improved survival were observed following prewarming of rinse solutions [35, 39]. In addition, it is well known from isolated kidney [4], perfused heart [1], and perfused rat mesenteric artery [17] that raising the temperature of the perfusate decreases organ perfusion resistance, and vice versa. On the other hand, injury to the sinusoidal lining cells with the subsequent microvascular perfusion deficit is considered to be one of the crucial mechanisms in ischemia-induced liver graft dysfunction [37]. Because of this problem, recently developed strategies have focused predominantly on reducing ischemia-reperfusion injury in order to prevent damage to sinusoidal lining cells during ischemia and organ rinse and to preserve hepatic microvascular circulation during reperfusion [3, 8, 37]. Using intravital microscopy we were able to demonstrate that rinsing liver grafts with warm (37°C) RL not only reduces resistance to flow of the whole organ [35], but also increases acinar and sinusoidal perfusion and, therefore, provides a more homogeneous and enhanced nutritive hepatic blood flow. In accordance with our intravital observation, positive effects have been reported for warm rinse solutions by examining the ultrastructural integrity with light and scanning electron microscopy [2, 35].

Adherence of WBC to microvascular endothelium and their subsequent activation, accompanied by the release of reactive oxygen intermediates and various mediators, are thought to be a characteristic phenomenon during the early stage after reperfusion following warm and cold ischemia of the liver [12, 13, 19, 24]. According to our present understanding, the interaction between WBC and endothelial cells is dual in nature, consisting of two distinct kinds of interaction, temporary ('rolling') and secondary permanent ('sticking') adherence. Both processes are mediated in three or more steps [6] by the expression of selectins (step 1) and integrins (step 2 and step 3), adhesion molecules that are present on the surface of leukocytes and/or endothelial cells [34, 38]. In addition, WBC have to be activated by chemoattractants or other activating factors (step 2/3, i.e., released leukotrienes, platelet-activating factor, C5a, formyl peptides, interleukins-2 and -8). Previous studies by others and by our own group have demonstrated that the extent of WBC/endothelium interaction in sinusoids and postsinusoidal venules is strongly dependent on the type of rinse solution used before graft reperfusion [27]. Interestingly, although RL is not known to have any direct effects on intercellular adhesion molecules, permanent WBC adherence in sinusoids and postsinusoidal venules was found to be reduced following warm RL rinse. This indicates that decreased WBC/endothelium interaction is influenced secondary to changes in microvascular perfusion. In contrast, the number of leukocytes rolling in postsinusoidal venules increased after warm rinse. This observation is not necessarily inconsistent since it has been shown that an increase in microvascular perfusion results in a considerable rise in the shear stress acting on adherent WBC. This rise, in turn, leads to a decrease in the number of permanently adherent WBC [32]. This observation appears likely, since de-adhesion of sticking leukocytes was frequently detected by direct observation by intravital microscopy. According to the abovementioned three-step model [6], one possible explanation might be that the primary adhesion pathways via selectins (step 1) remain unaffected by the mechanisms induced by warm rinse of the graft and, in contrast, processes that mediate firm leukocyte adherence (step 2 and step 3) are suppressed by this regimen. Thus, 'rolling' of leukocytes may only appear to be enhanced, since permanent WBC adherence is inhibited to a certain extent and the rolling periods for individual WBC are prolonged.

Parallel to adhesive and triggered WBC, activated Kupffer cells are one, if not the most important, source

of reactive oxygen intermediates and various mediators involved in the pathophysiology of hepatic reperfusion injury after cold ischemia [7, 9, 12, 24, 29]. Moreover, activation of Kupffer cells is accompanied by inceased phagocytic activity with inversely reduced hepatic biliary excretion [25] and decreased survival after liver transplantation [18]. The present study clearly demonstrates that rinsing liver grafts with warm RL is an efficient method for reducing Kupffer cell phagocytic activity after graft reperfusion. An explanation for this observation could be that early re-established homogeneous perfusion after cold ischemia and reperfusion following a warm RL rinse may have a significant impact on the activation state of Kupffer cells and, thereby, on the release of reactive oxygen intermediates and deleterious mediators.

Analysis of bile production is known to provide more accurate information on general graft function than serum levels of hepatocellular enzymes [5, 11, 14] since serum levels rise without regularity in the early reperfusion period after transplantation and tend to be underestimated in severely damaged grafts [30]. Therefore, in the present study, bile flow and bile acid excretion were analyzed. In accordance with former observations by our group, both bile acid-dependent and bile acid-independent mechanisms are similarly affected by reperfusion injury after transplantation [25], in contrast to the results in a model of warm ischemia of the isolated perfused liver [16]. Moreover, since warm RL rinse resulted in increased bile flow and bile salt excretion, our results reconfirm previous data from our group and other studies [39] where close correlations between bile production and microvascular perfusion, WBC/endothelium interaction, and Kupffer cell activation after liver transplantation were demonstrated [25, 27]. Taking the abovementioned results into account, together with the fact that increased survival was reported after a prewarming of the rinse solution [37, 39], it is highly likely that a warm rinse has a considerable capacity to prevent early graft dysfunction.

Since the simple physical modification of warming the rinse solution results in a similar protection against cold ischemia-induced reperfusion injury in liver grafts, as has been demonstrated for complex rinse solutions, which additionally induce a vasodilatation and reduce the formation of oxygen reactive intermediates, the question is: through what mechanism does warm RL act? First, based on fundamental studies on membrane thermodynamics [31], the beneficial effect of a warm rinse on microvascular nutritive blood flow during reperfusion was explained by reduced membrane rigidity after exposure to warmth, thereby decreasing vascular resistance [35]. In addition, increased and more homogenous microvascular perfusion after warm RL rinse could contribute to a rise in driving pressure during the initial reperfusion. As has been shown previously by our group, during this time detached endothelial cells and hepatocellular blebs must be cleared from sinusoidal lumina; an increased driving pressure would help to facilitate this clearance process [27, 28]. Second, increased and more homogeneous perfusion might reduce acinar and subacinar low-flow/reflow or, even worse, no-reflow states [22], including local stasis, warm ischemia, and subsequent activation of sinusoidal lining cells. Furthermore, this improved perfusion should help to provide adequate nutritional as well as oxygen supply to hepatic tissue. Moreover, since reactive oxygen intermediates are predominantly liberated during the first minutes of graft reperfusion [9], it could be hypothesized that hepatic intracellular antioxidant enzymes, which generally function best at a temperature optimum of 37 °C rather than at 4 °C, could already be present in an active state before reperfusion and thus limit the formation of oxygen radicals. To elucidate the exact mechanisms of simple warm rinse solutions on a molecular basis, further studies are certainly needed.

In conclusion, the data presented in this study provide further evidence that by rinsing liver grafts in warm (37 °C) RL, one can reduce the manifestations of microvascular reperfusion injury. Since earlier studies demonstrated a significant increase in post-transplant survival in the rat following the use of prewarmed rinse solutions [35, 39], this simple physical modification of the reperfusion technique could represent an inexpensive and very effective way to protect donor organs against early graft failure in clinical liver transplantation.

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