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# Reperfusion rather than storage injury predominates following long-term (48 h) cold storage of grafts in UW solution: studies with Carolina Rinse in transplanted rat liver

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**Abstract.** Both storage injury and reperfusion injury have been reported in association with liver transplantation; however, which predominates is not clear. Therefore, these studies were designed to evaluate whether Carolina Rinse, which minimizes reperfusion injury following orthotopic liver transplantation in the rat, would be effective after long-term (48 h) storage of grafts in University of Wisconsin (UW) cold storage solution where sufficient time for development of storage injury exists. Livers were rinsed with either Ringer's solution or Carolina Rinse solution immediately prior to completion of implantation surgery. In the Ringer's group, 30-day survival was high following 24 h of cold storage (4/5) but was very low after 48 h (1/16). Importantly, survival was increased significantly (5/14) when grafts were rinsed with carolina Rinse following 48 h of cold storage. In both groups, parenchymal cells appeared normal by scanning electron microscopy, excluded trypan blue, and released SGOT at values only slightly above the normal range immediately (i.e., less than 5 min) after 48 h of cold storage. However, SGOT values rose steadily during the 1st hour postoperatively following reperfusion in the Ringer's rinse group and reached levels around 1,000 U/l. In addition, nonparenchymal cells were not labelled with trypan blue following storage, but significant labelling occurred within 1 h. Both SGOT release and nonparenchymal cell injury were reduced significantly when grafts were rinsed with Carolina Rinse prior to completion of surgery. Liver injury assessed histologically 24 h postoperatively was also reduced about 50% by Carolina Rinse. Oxidative stress appeared to be involved, since radical adducts, most likely of lipid origin, were trapped during the first 5 min after reperfusion with the spin trapping technique and detected by electron paramagnetic resonance spectroscopy. Lipid radical formation was reduced nearly completely on reperfusion by Carolina Rinse. Since Carolina Rinse improved survival of liver grafts following long periods of cold storage and reduced lipid radical formation and hepatocellular injury, we concluded that a reperfusion injury rather than a storage injury predominates following orthotopic transplantation of livers stored for long periods of time in cold UW solution.

**Key words:** Carolina Rinse – Reperfusion injury – Orthotopic rodent liver transplantation – Lipid radical adducts

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With the development of University of Wisconsin (UW) cold storage solution in the late 1980's [2], the storage time of liver for transplantation was extended from 8–10 h to up to 20–24 h [23]. However, despite this improvement, primary graft non-function still occurs [14].

Several years ago we demonstrated that a selective injury to hepatic nonparenchymal cells occurred following cold storage and reperfusion in vitro and in vivo [7, 8, 21]. Reperfusion injuries have also been demonstrated in many organs, including heart, lung, kidney and pancreas, and have been reported to exacerbate the rejection reaction in liver [15]. Studies on the pathophysiological mechanisms involved in reperfusion injury in liver have focused on damage to endothelial cells [7, 8], activation of Kupffer cells [6, 21], adherence of leukocytes [20], as well as disturbances in the microcirculation [22] and activation of the coagulation system [13]. It is possible that activated Kupffer cells produce toxic mediators such as proteases, toxic radicals, leukotrienes and tumor necrosis factor [18]. Oxygen radicals could be involved, since xanthine and hypoxanthine, substrates for superoxide radical formation, accumulate during cold storage [17] and free radicals could be formed postoperatively when oxygen is reintroduced [9].

Since UW solution increases the time of cold storage, it has been reasoned that injury to the graft occurs during cold storage. In support of this idea, the hepatic ultrastructure of the liver is altered during cold storage. However, recent studies with Carolina Rine indicate that reperfu-

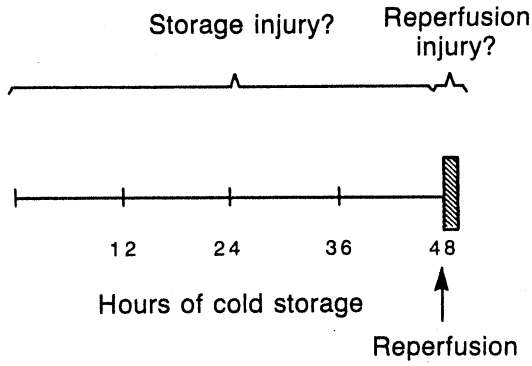


**Fig. 1.** Scheme contrasting storage and reperfusion injury following orthotopic liver transplantation

sion injury also occurs [10, 13, 22]. While it is generally accepted that changes which occur during cold storage probably trigger reperfusion injury, whether reperfusion or storage injury predominates following transplantation has not been clarified (Fig. 1). This is an important distinction, since whether the focus in the future is on storage or rinse solutions depends on the type of injury that predominates. Therefore, these studies were designed to determine whether Carolina Rinse, which minimizes reperfusion injury, would be effective after long-term (48 h) immersion of liver grafts in cold UW solution where storage injury would have sufficient time to develop.

## Methods

*Transplantation.* Liver transplantation was performed under ether anesthesia using a technique essentially as described by Kamada [19]. Syngenic female Lewis rats (175–200 g) were used to eliminate rejection. For electron paramagnetic resonance (EPR) spectroscopy, inbred female Sprague-Dawley rats (175–200 g) were used. Briefly, 0.5 ml Ringer's solution with 100 units of heparin was injected into the donor vena cava and the liver was flushed with 3–5 ml of cold UW solution. Subsequently, cuffs were placed on the portal vein and subhepatic vena cava of the donor liver. Grafts were stored at 0–4 °C for 24 or 48 h in UW solution and were rinsed either with 3–5 ml Ringer's solution or Carolina Rinse. The composition of Carolina Rinse is as follows: NaCl (115 ml), KCl (5 mM), $CaCl_2$ (1.3 mM), $KH_2PO_4$ (1 mM) $MgSO_4$ (1.2 mM), allopurinol (1 mM), desferrioxamine mesylate (1 mM), glutathione (3 mM), nicardipine (2 μM), adenosine (1 mM), fructose (10 mM), glucose (10 mM), hydroxyethyl starch (50 g/l), insulin (100 U/l), MOPS (20 mM), pH 6.5, mOsm/l 290–305 [10, 13]. Subsequently, livers were implanted by connecting the suprahepatic vena cava with a running suture, inserting cuffs into appropriate vessels without rearterialization, and anastomosing the bile duct with an intraluminal splint. The explantation required less than 6 min, and the ischemic interval due to clamping of the portal vein during implantation did not exceed 15 min. Surviving animals were sacrificed after 30 days for histology.

*Serum enzymes.* Blood samples were drawn from the vena cava at 0, 5, 15, 30, 60, and 180 min after the clamp on the portal vein was removed. Sera were separated by centrifugation and kept at −20 °C for enzyme measurements. Serum glutamic oxaloacetic transaminase (SGOT) was assayed by standard enzymatic procedures [3].

*Tissue ultrastructure.* Rats were sacrificed 24 h postoperatively and livers were fixed with 1% paraformaldehyde in Krebs-Henseleit buffer, embedded in paraffin, and processed for light microscopy. Sections were stained with hematoxalin and eosin. Liver damage was scored using a scale of 0–5 based on the degree of necrosis and 0–2 based on six structural parameters: cellular swelling, acidophilic inclusions, nuclear pyknosis, cellular deposition, cytoplasmic vacuolization, and sinusoidal dilatation (maximal score = 17). Where indicated, the vital dye trypan blue was infused into livers for 5 min at the end of experiments, followed by fixation of the liver with 1% paraformaldehyde in Krebs-Henseleit buffer. Livers were processed for light microscopy and sections were stained with eosin only so that nuclei of dead cells could be identified [1].

For scanning electron microscopy, livers fixed with 2% paraformaldehyde: 2% glutaraldehyde were cut into 1 cm cubes and placed overnight in cold secondary fixative containing 2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The tissue was then washed in water, dehydrated in graded ethanol, and critical-point dried in carbon dioxide. The dried tissue was cut manually with a razor blade, mounted on an aluminum stub, coated with gold-palladium using a sputter evaporator, and viewed in a JEOL 820 scanning electron microscope [16].

*Spin trapping of free radicals.* Following cold storage in UW solution, the liver was implanted and an $O_2$-saturated solution of α-phenyl N-tert-butyl nitrone (PBN; 15 mM) in saline, or Carolina Rinse was infused into the portal vein at a rate of 0.3 ml/min immediately after the clamp on the portal vein was opened. Four to five 1 ml samples of blood were collected immediately from the suprahepatic vena cava, and serum was separated by centrifugation and frozen in liquid nitrogen.

Serum was extracted based on methods used in studies of reperfusion injury to heart [4, 5]. Samples in chloroform were placed in a 3.0 mm i.d. quartz EPR tube, bubbled with nitrogen for 5 min, and placed in the EPR cavity. A varian E-109 spectrometer equipped with a $TM_{110}$ cavity was used. Instrument conditions were 20-mW microwave power, 0.68-G modulation amplitude and 80-G scan width for all analyses. Scan time was 1.0 h with an 8-s time constant.

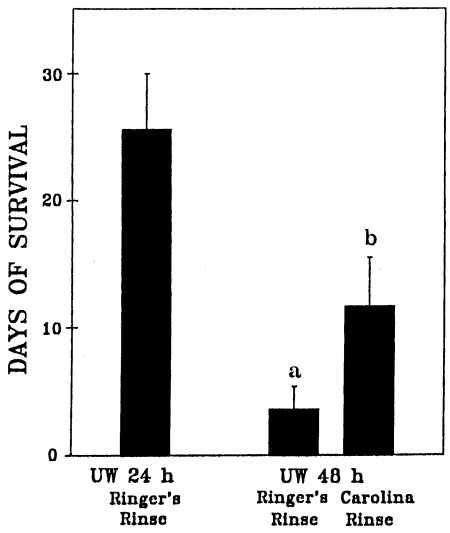


**Fig. 2.** Effect of Carolina Rinse on postoperative survival of livers stored in University of Wisconsin (UW) cold storage solution for 48 h. Livers were rinsed and stored in UW cold storage solution at 0–4 °C for 24 h (survival conditions) or 48 h (non-survival conditions). Following storage, livers were rinsed with 3 to 5 ml of either cold Ringer's or Carolina Rinse solution and implanted immediately. Survival was assumed to be permanent when rats were alive 30 days postoperatively. **a** $P < 0.05$ for comparison with livers stored under survival conditons; **b** $P < 0.05$ for comparison with Ringer's group of livers stored under non-survival conditons. Mean ± SEM for 5 to 16 livers in each group

## Results

*Calronina Rinse increases survival following long-term storage in UW solution.* The purpose of these studies was to determine whether liver graft injury occurs predominantly during cold storage (i.e., while in cold UW solution) or following reperfusion after implantation (Fig. 1). Carolina Rinse was designed to minimize reperfusion injury and was used to address this question. Average survival time of livers stored in UW solution 24 h prior to implantation and rinsed with Ringer's solution was approximately 25 days (i.e., survival conditions; Fig. 2). In this model, survival for 30 days is considered permanent. In contrast, when the time of storage in UW solution was extended to 48 h, the length of survival declined dramatically to less than 3 days when grafts were rinsed with Ringer's solution (i.e., nonsurvival conditions). However, when Carolina Rinse was substituted for Ringer's as the rinse solution, average survival time increased significantly by approximately 3-fold (Fig. 2).

*Comparison of hepatocellular injury following cold storage with cold storage plus reperfusion.* Liver injury was also assessed in these studies by release of transaminases into the blood. At the initiation of reperfusion, SGOT levels were low in livers stored for 48 h in UW solution and rinsed with either Ringer's solution or Carolina Rinse (Fig. 3). However, enzyme release increased sharply to values around 1000 U/l during the 1st hour of reperfusion in the Ringer's group and reached peak values of over 1200 U/l. When Carolina Rinse was substituted for Ringer's solution, maximal transaminase release was diminished approximately 2-fold.

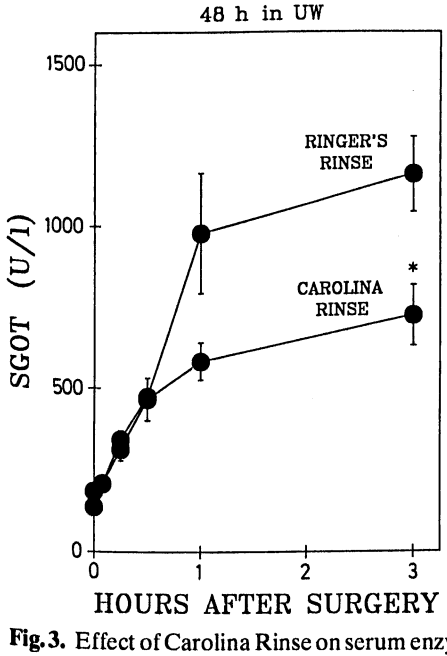


**Fig. 3.** Effect of Carolina Rinse on serum enzyme release from livers stored under non-survival conditions. Blood samples were collected via the inferior vena cava at 0, 5, 15, 30, 60 and 180 min postoperatively. SGOT activity was measured as described in Methods. Blood pressure was maintained by infusion of up to 3 ml of 5% albumin in Ringer's solution via the tail vein. Mean ± SEM for 4 livers in each group

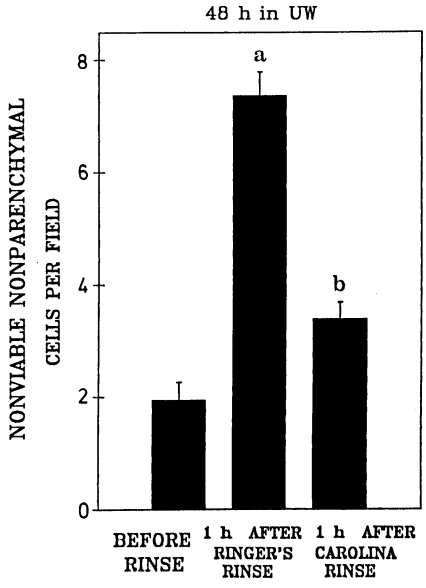


**Fig. 4.** Effect of Carolina Rinse on nonparenchymal cell injury following long-term cold storage and reperfusion. Livers were reperfused with oxygenated Krebs-Henseleit bicarbonate buffer (pH 7.4, 37 °C) either before implantation or 1 h postoperatively. The flow rate was initially 15 ml/min and was increased to 30 ml/min over 2 min. After 5 min of reperfusion, trypan blue (0.5 mM) was added and perfusion was continued for 7 min. Livers were flushed with buffer for 5 min, and tissue was fixed and stained as described in Methods. Trypan blue-positive nonparenchymal cell nuclei in eosin-stained sections were counted in 8 random microscopic fields. Total cells were determined in H & E sections. **a** $P < 0.05$ for comparison with nontransplant group. **b** $P < 0.05$ for comparison with either nontransplant or Ringer's groups. Mean ± SEM for 4 to 6 livers per group

Infusion of the vital dye trypan blue allows easy identification of irreversibly damaged cells. Liver stored for 48 h in UW solution and fixed immediately sustained minimal cell injury (i.e., only about 3% of parenchymal cells were stained with trypan blue; data not shown). Nonparenchymal cell injury was also minimal immediately following surgery (Fig. 4). However, nonparenchymal cell injury increased 3 to 4-fold 1 h following surgery if the graft had been rinsed with Ringer's solution. This nonparenchymal cell injury was reduced dramatically by Carolina Rinse (Fig. 4).

Liver damage was also assessed 24 h postoperatively on a histological score as described in Methods. In livers stored for 48 h in UW solution and rinsed with Ringer's solution, the liver damage index 24 h postoperatively was around 7.5 (Fig. 5). In livers stored under similar conditions but rinsed with Carolina Rinse, however, the index was reduced significantly by almost 50%.

To evaluate the effect of reperfusion on hepatic ultrastructure, scanning electron microscopy was performed. Livers were stored for 48 h in UW solution, rinsed either with Ringer's solution or Carolina Rinse, fixed 15 min postoperatively and processed for electron microscopy. As expected, at this early time point following reperfusion, parenchymal cells exhibited near normal morphology (i.e., cell death was minimal; data not shown).

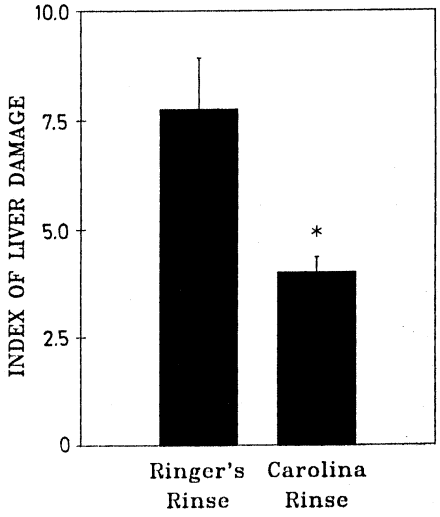


**Fig. 5.** Effect of Carolina Rinse on postoperative liver damage. Index of liver damage was determined 24 h postoperatively as described in Methods. Mean ± S.E.M. for 4 livers in each group. * $P < 0.05$ compared to the Ringer's rinse group

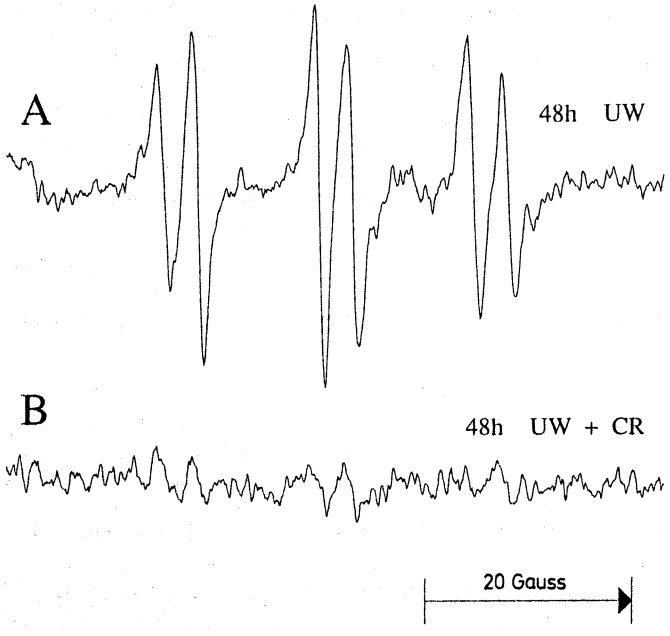


**Fig. 6 A, B.** Representative EPR spectra of radical adducts in chloroform extracts of serum from transplanted livers stored in cold UW solution for 48 h. Livers were transplanted, spin trap was infused, blood was collected for 5 min, and EPR analysis was performed as described in Methods. **A** livers stored for 48 h in UW solution and rinsed with Ringer's solution before implantation surgery. **B** liver stored for 48 h in UW solution and rinsed with Carolina Rinse solution

*Inhibition of radical adduct formation by Carolina Rinse.* Since oxygen and oxygen radicals have been implicated in the mechanism of reperfusion injury [13], experiments utilizing electron paramagnetic resonance (EPR) spectroscopy were designed to determine if free radicals were formed as a result of reperfusion following orthotopic liver transplantation. When livers were stored for 48 h in UW solution and reperfused with oxygenated blood con-

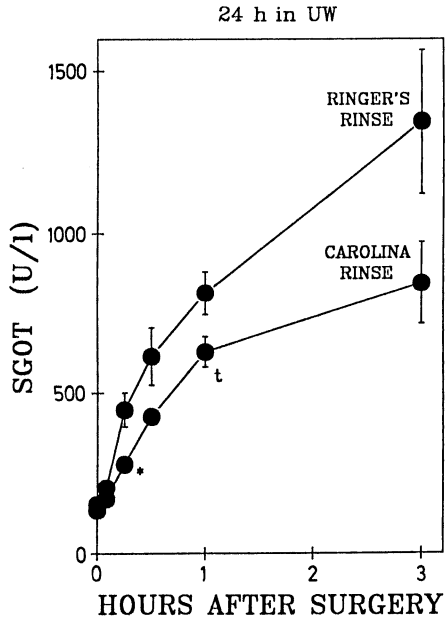


**Fig. 7.** Effect of Carolina Rinse on serum enzyme rlease by livers stored under survival conditions. Conditions as in Fig. 3 except that livers were stored in cold UW solution for 24 h (survival conditions). Mean ± SEM for 6 to 10 livers in each group

taining the spin trap PBN, the immediate effluent leaving the liver contained high levels of PBN radical adducts, as indicated by the appearance of a robust six-line EPR signal (Fig. 6). Computer simulation of the data indicated that two carbon-centered radical adducts were present, and the hydrophobic behavior of these species in extraction and sample preparation suggested strongly that they were lipid-derived radicals. Carolina Rinse, which contains reagents which inhibit radical formation, suppressed the radical adduct signal nearly completely (Fig. 6).

*Effect of Carolina Rinse on SGOT release following 24 h of storage in UW solution.* In the clinic, most human livers are stored for less than 24 h before transplantation; therefore, we compared the effect of Ringer's Rinse, which is used currently to remove cardiotoxic potassium contained in UW solution, with the effect of Carolina Rinse on transaminase release during reperfusion following 24 h of storage in UW solution. SGOT release increased following reperfusion with Ringer's rinse, increasing gradually from approximately 150 to 1200 U/l over 3 h. Carolina Rinse tended to reduce values at all time points examined (Fig. 7).

## Discussion

*Definition of storage and reperfusion injury.* Storage injury is defined as any alteration in the liver that occurs *during* the period of cold storage (see Fig. 1). This is sometimes referred to erroneously as a preservation injury since preservation means "to keep safe, alive, or free from injury". To avoid this contradiction in terms, storage injury is used as a more appropriate term to describe damage which occurs during cold storage. On the other hand, reperfusion

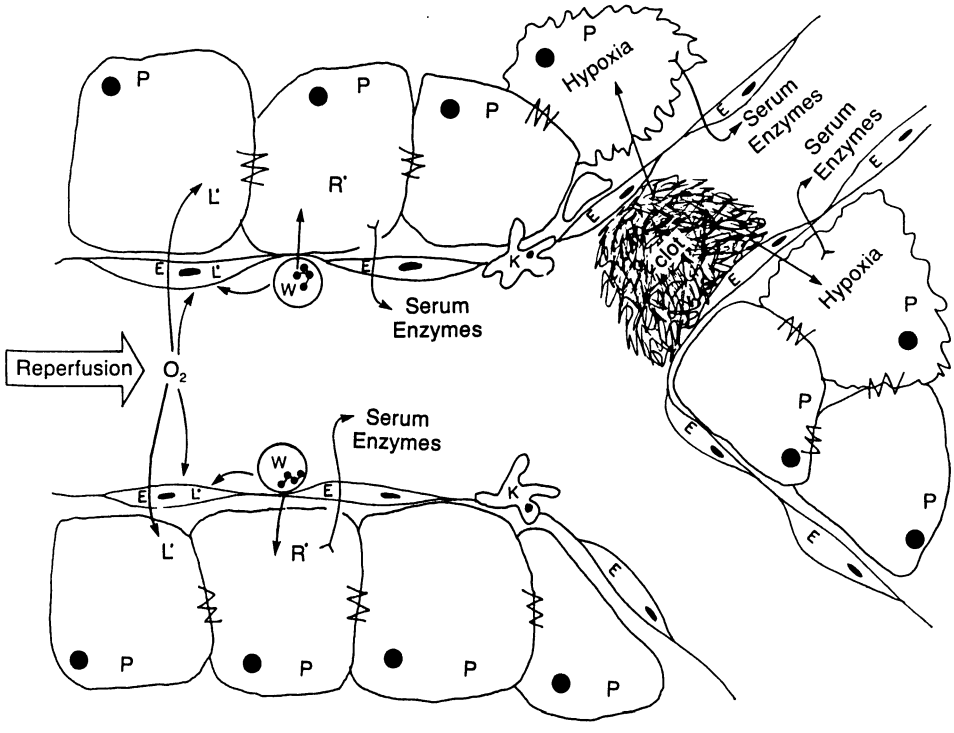


**Fig. 8.** Scheme depicting possible mechanisms of reperfusion injury to the graft following orthotopic liver transplantation. *P*, parenchymal cell; *E*, endothelial cell; *K*, Kupffer cell; *W*, white blood cell; *L*· and *R*·, lipid and other free radicals

injury is broadly defined as any pathophysiological alteration in the liver which occurs *after* surgery but narrowly refers to radical-mediated events which occur upon reflow which may be due to oxygen [22]. Obviously, changes that occur in the cold most likely trigger the subsequent reperfusion injury.

There is considerable variation in times of cold storage which allow maximal survival. In the rat, times range from 4 to 24 h [12, 20] for reasons that may relate to the time of ischemia during surgery. In this study, 24 h in UW solution resulted in complete survival (Fig. 2). Thus, the rat can be as suitable a model as the dog and human where maximal cold storage times which result in 100% survival are also around 24 h.

*Reperfusion injury rather than storage injury predominated after 48 h in UW solution.* As mentioned in the introduction, an oxygen-dependent reperfusion injury has been described in the orthotopic rat liver transplantation model [22]. Further, since cold storage time has been extended significantly over times for Euro-Collins by the introduction of UW solution and because alterations in hepatic ultrastructure have been observed immediately following cold storage [23], it has been assumed that injury occurs during cold storage. As mentioned above, 48 h of cold storage was used in this study to allow sufficient time for injury to develop. However, based on several criteria of hepatic viability assessed in this study, it was clear that significant damage did not occur during the 48 h of cold storage in UW solution but rather developed during the 1st hours after reperfusion (i.e., it was a reperfusion injury). Specifically, cells excluded trypan blue and had near normal SGOT values immediately after 48 h of cold storage in UW solution, indicating that they were not injured irreversibly during cold storage (Figs. 3, 4). However, massive injury was detected 1 h following reperfusion (e.g., serum enzymes increased gradually over about 1 h postoperatively). This observation was very important since it defined the time course of the reperfusion injury and led to the inescapable conclusion that a reperfusion injury occured following long-term cold storage in UW solution only after oxygenated blood reentered the organ following transplantation.

A second argument that a reperfusion injury predominated comes from studies with Carolina Rinse. Carolina Rinse was developed specifically to prevent reperfusion

**Table 1.** Time course of pathophysiologic changes on reperfusion

| System | Detection after reperfusion (min) | Reference |
|---|---|---|
| Free radical formation | 5 | Fig. 6; Connor et al. (1991) Transplantation, submitted |
| Clotting time decreased and platelet count down | 5 | Gao et al. (1991) Transplantation, in press |
| Endothelial cell death | 7–15 | Caldwell-Kenkel et al. (1988) Hepatology 10: 292–299 |
| Leukocyte adhesion | 15 | Takei et al. (1991) Transplantation 51: 959–65 |
| Kupffer cell activation | 30 | Caldwell-Kenkel et al. (1991) Cells of the hepatic sinusoid, in press |
| Hepatocyte injury | 15–60 | Fig. 3 |

injury and contains adenosine [11] as well as antioxidants and radical scavengers [13]. In this study the postoperative increases in trypan blue labelling of nonparenchymal cells and serum enzyme release were diminished significantly by Carolina Rinse (Figs. 3, 4). Thus, it was concluded that survival was affected by what occured during reperfusion, making the evaluation of cold storage solutions based on survival outcome questionable.

*Radical-mediated endothelial cell killing rather than parenchymal cell death correlated with graft survival.* Interestingly, in livers stored under survival conditions (24 h; Fig. 7), the time course of SGOT release postoperatively (i.e., parenchymal cell injury) was identical in livers stored under non-survival (48 h) conditions. Thus, reperfusion injury to *parenchymal cells* did not correlate directly with survival. This is important clinically, since serum enzymes are a common measure of postoperative outcome. On the other hand, survival did correlate with nonparenchymal cell injury (Fig. 4). More appropriate indices related specifically to survival will need to be identified in the future.

It is accepted that hypothermia is the basic principle on which existing liver preservation methods depend. By reducing the metabolic demand of the tissue for nutrients and oxygen, the period during which anoxic tissue retains viability is lengthened. However, cold ischemia causes alterations in the organ which most likely are responsible for reperfusion injury. For example, ATP degradation leads to high cellular concentrations of xanthine which could enhance superoxide production and thus injure the cell when oxygen is reintroduced. Indeed, on reoxygenation, free radicals are formed in minutes (Fig. 8) followed by endothelial cell injury ([9]; Table 1), Kupffer cell activation [6] and leukocyte adhesion. Only much later does loss of parenchymal cell function occur. In support of this hypothetical sequence of events, Connor et al. [9] have demonstrated that free radical formation in the early postoperative minutes correlates with survival.

Alternatively, the clotting system may be involved (Fig. 8). In a previous study, we have demonstrated that clotting time decreased as soon as 5 min postoperatively under nonsurvival conditions [13]. Further, in this study massive microthrombi were observed by electron microscopy 15 min after revascularization. On the other hand, SGOT values did not increase maximally until about 1 h postoperatively. Thus, one alternative explanation for early graft injury is that damaged endothelial cells stimulate platelet aggregation. This could stimulate clotting, block the microcirculation and lead to hypoxia which could ultimately kill parenchymal cells.

*Can reperfusion injury be treated with Carolina Rinse?* Carolina Rinse contains antioxidants and was shown in this study to diminish free radical formation and endothelial cell death and to decrease parenchymal cell injury dramatically following long-term cold storage in UW solution (Figs. 3, 6). The efficacy of Carolina Rinse is apparently due to its ability to prevent free radical formation in the liver during the 1st minutes following surgery [9] as well as an apparent extrahepatic effect of adenosine on survival [11]. Thus, Carolina Rinse might be a valuable adjunct to UW solution in reducing postoperative graft injury clinically. Currently, a limited clinical trial to evaluate Carolina Rinse is underway at the Mayo clinic.

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