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Reperfusion injury to endothelial cells after cold storage of rat livers: protection by mildly acidic pH and lack of protection by antioxidants

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Abstract Lethal reperfusion injury to sinusoidal endothelial cells occurs after cold ischemic storage of livers and may be responsible for liver graft failure from storage injury. Here, we evaluated potential mechanisms underlying this reperfusion injury. In rat livers stored in Euro-Collins solution for 24 h and reperfused with Krebs-Henseleit bicarbonate buffer, nonparenchymal cell killing showed periportal predominance as assessed by nuclear staining with trypan blue. In livers reperfused in the retrograde direction, the lobular distribution of cell killing was reversed, indicating that cell killing was more rapid in oxygen-rich upstream regions. However, antioxidants, including allopurinol, desferrioxamine, catalase, superoxide dismutase, superoxide dismutase plus catalase, and U74006F, did not reduce cell killing. Similarly, reperfusion with anoxic buffer did not prevent lethal injury. Antioxidants and anoxic reperfusion also did not improve cell viability in livers stored in UW solution. Nevertheless, superoxide generation, as identified by formazan formation from nitroblue tetrazolium, was increased in Kupffer cells after lives storage and reperfusion as compared to unstored livers. Acidification of the reperfusion buffer from pH 7.4 to pH 7.15 reduced overall nonparenchymal cell killing from about 40 % to 10 %. Moreover, a pH gradient developed across the liver lobule during reperfusion with the effluent 0.2–0.4 pH units more acidic than the influent. This intralobular pH gradient appears to account for the relative sparing of cells in more acidic downstream regions of the lobule. Lower temperatures of reperfusion also reduced lethal injury. In conclusion, Kupffer cells generated superoxide after perfusion of stored rat livers, but formation of oxygen free radicals did not appear to contribute to lethal reperfusion injury to endothelial cells. Rather, mildly acidotic pH was protective against lethal injury. Thus, hydrogen ion concentration may be a critical determinant of reperfusion injury to sinusoidal endothelial cells.

Key words Preservation, endothelial cells, rat liver
Endothelial cells, preservation, rat liver · Reperfusion injury, rat liver
Liver, rat, reperfusion injury

Introduction

Donor livers for transplantation surgery fail after transplantation when stored longer than about 8 h in Euro-Collins solution [3]. Loss of graft viability correlates with lethal injury to sinusoidal endothelial cells and activation of Kupffer cells [9–11]. Cell killing is specific to endothelial cells and occurs as a consequence of reperfusion. Other hepatic cell types, including Ito, Kupffer, parenchymal, and bile duct epithelial cells, retain viability. In particular, hepatocyte structure and function are well preserved [10, 11]. Similar changes occur in endothelial cells and Kupffer cells of livers stored in University of Wisconsin (UW) cold storage solution, an improved preservation medium, but are observed after longer times of storage [10, 11].

Several factors may contribute to reperfusion-mediated endothelial cell killing in stored livers. First, reoxygenation of cold ischemic livers initiates formation of free radicals that may be toxic to endothelial cells [12]. In particular, activated Kupffer cells may generate toxic oxygen radicals, as demonstrated recently in cultured Kupffer cells during reoxygenation after warm anoxia [30]. Endothelial cells lack the robust antioxidant defenses of hepatocytes and may therefore be more vulnerable to the toxic effects of oxygen radicals [35]. Second, cell killing in several models of warm hypoxia is markedly pH-dependent [4, 14, 18]. The naturally occurring acidosis of ischemia delays the onset of cell death, but the return to normal pH after reperfusion actually accelerates lethal cell injury [4, 5, 14]. Third, lethal injury may result from the stress of resumption of normothermic metabolism in endothelial cells weakened by prolonged ischemic storage. Accordingly, we evaluated the effects of antioxidants, pH, and temperature on reperfusion-induced endothelial cell killing in rat livers stored for transplantation surgery. Our studies indicate that endothelial cell killing is a function of reperfusion pH and temperature but apparently not of oxygen free radical formation.

Materials and methods

Rat livers were stored and reperfused as previously described [9–11]. Briefly, livers from male Sprague-Dawley rats (250–300 g) were perfused via the portal vein at 3–4 ml/min per gram liver with Krebs-Henseleit bicarbonate buffer saturated with 95 % O_2, 5 % CO_2 at 37 °C. After 20 min, the livers were rinsed for 2 min with ice-cold storage solution and immersed in this fluid inside plastic containers that were placed in an ice-water bath for 8–24 h. After storage, the livers were reperfused with buffer for 15 min at 37 °C. The initial flow rate was 1 ml/min per gram, which was gradually increased over 5 min to 3–4 ml/min per gram. Some livers were reperfused at 4 °C and 18 °C, in which case the maximum flow rate was 1.5 ml/min per gram because livers reperfused at 3–4 ml/min per gram swelled at lower temperatures. Livers perfused at 4 °C and 18 °C were adequately oxygenated due to decreased metabolic demand and increased oxygen-carrying capacity of the buffer. Oxygen and LDH concentrations were measured in effluent perfusate as described previously [10]. In some experiments, effluent pH was measured with a glass electrode.

The storage solution was Euro-Collins solution containing 10 mM $NaHCO_3$, 200 mM glucose, 15 mM KCl, 43 mM K_2HPO_4, and 15 mM KH_2PO_4, pH 7.2, or UW solution. The reperfusion buffer was Krebs-Henseleit bicarbonate buffer containing 118 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 4.7 mM KCl, and 1.3 mM $CaCl_2$, pH 7.4. Additions and deletions to the storage and reperfusion solutions are noted for individual experiments. In some experiments, livers were reperfused in the retrograde direction as described previously [25].

Trypan blue (200–500 μM) was added to the reperfusion buffer during the last 5–7 min of each perfusion to label the nuclei of nonviable cells. Livers were then fixed by infusion of 2 % paraformaldehyde, 2 % glutaraldehyde in 0.1 M NaPi buffer, pH 7.4, embedded in glycol methacrylate, and sectioned with a glass knife [10]. Trypan blue uptake by nonparenchymal cell nuclei in eosin-stained sections and total nonparenchymal cell nuclei in methylene blue-acid fuchsin-stained sections were counted by positioning small branches of portal and central veins just adjacent to fields of a 60X oil immersion lens and counting all cells. Five periportal and pericentral fields selected at random were counted for each specimen. Slides were coded so that the identity of the experimental group was not known during counting. To calculate the percentage of nonviable nonparenchymal cells, the number of trypan blue-positive nuclei was divided by total nonparenchymal cell nuclei. Periportal and pericentral percentages were then averaged to obtain values for whole livers. Mean values for various treatment groups were compared using Student's *t*-test with a P level below 0.05 as the criterion for significance. When appropriate, one-tailed tests were used to test unidirectional hypotheses. All data were from new experiments not previously reported.

In some experiments, livers were reperfused with buffer containing 0.05 % nitroblue tetrazolium, but without trypan blue. This reagent reacts with oxygen radicals to form insoluble formazan [28]. The livers were then fixed as described above, embedded, sectioned, and examined unstained.

Allopurinol, catalase, nitroblue tetrazolium, superoxide dismutase, and trypan blue were obtained from Sigma (St. Louis, Mo.). Desferrioxamine mesylate was obtained from Ciba Pharmaceutical (Summit, N.J.). U74006F and UW solution (Viaspan) were gifts of Upjohn (Kalamazoo, Mich.) and DuPont/Merck Pharmaceuticals (Wilmington, Del.).

Results

Flow-dependence of the intralobular gradient of cell killing

After 24 h of storage in Euro-Collins solution and reperfusion with oxygenated Krebs-Henseleit buffer, overall loss of nonparenchymal cell viability was 38 % by the criterion of trypan blue uptake (Fig. 1 A, Table 2). Previously, we have shown that virtually all these nonviable nonparenchymal cells are endothelial cells [10, 11]. By light microscopy, nonviable endothelial cells were detached from sinusoidal walls and displayed pyknotic trypan blue-positive nuclei (Fig. 1 A). Parenchymal cells, in contrast, excluded trypan blue (i. e., they remained viable).

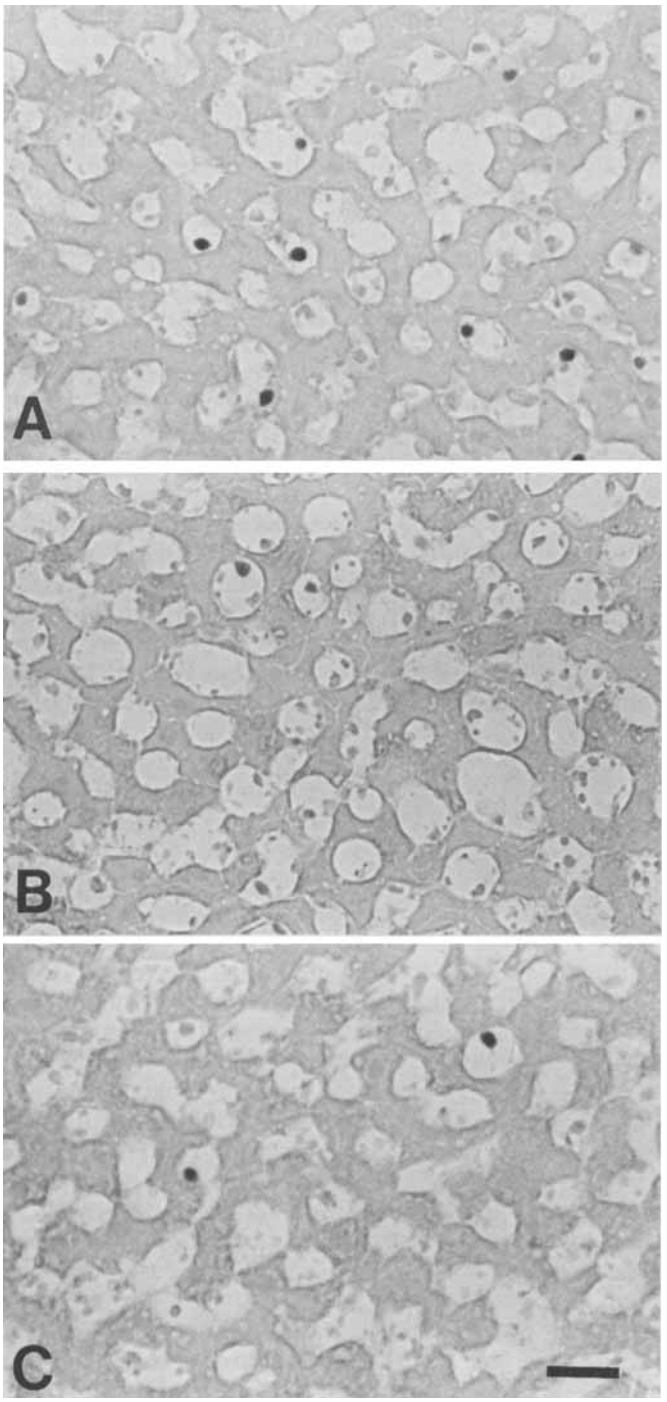

Fig. 1 A–C Trypan blue uptake by endothelial cells after storage and reperfusion. Rat livers were stored for 24 h in Euro-Collins solution and reperfused for 15 min: **A** a liver reperfused with Krebs-Henseleit bicarbonate buffer at 37 °C (pH 7.4). Note trypan blue-positive (darkly labeled) endothelial cell nuclei; **B** drug vehicle for U74006F was added to the storage and reperfusion solutions. Note decreased trypan blue uptake; **C** a liver reperfused at 4 °C. Trypan blue uptake is decreased. *Bar* is 40 μm. Plastic sections were countersteined with eosin

Table 1 Intralobular distribution of nonparenchymal cell killing during reperfusions of stored rat livers in the anterograde and retrograde direction. Rat livers were stored in cold Euro-Collins solution for 24 h and reperfused in either a retrograde or anterograde direction. Data are expressed as means ± SE

Direction of reperfusion	Loss of nonparenchymal cell viability (%)		
	Periportal	Pericentral	*(n)*
Anterograde	44.5 ± 4.0	26.3 ± 2.8*	(12)
Retrograde	14.7 ± 3.6	38.7 ± 7.0**	(4)

* $P < 0.01$ versus periportal; ** $P < 0.05$ versus periportal

Table 2 Effects of antioxidants on reperfusion injury to nonparenchymal cells. Rat livers were stored in cold Euro-Collins solution for 16 or 24 h and reperfused with warm oxygenated buffer. Antioxidants were added to both storage solutions and reperfusion buffers as indicated. Data are means ± SE from the number of experiments shown in parentheses. None of the treatments caused a statistically significant reduction in cell killing

Treatment	% Nonviable cells
24-hour storage	
None	38.0 ± 3.3 (12)
Allopurinol (5 mM)	38.4 ± 2.4 (6)
Desferrioxamine (0.1 mM)	29.6 ± 4.4 (9)
Desferrioxamine (1.0 mM)	37.8 ± 3.4 (4)
Catalase (500 U/ml)	45.3 ± 10.5 (4)
Catalase (2000 U/ml)	34.5 ± 3.4 (6)
Superoxide dismutase (25 U/ml)	42.0 ± 3.3 (4)
Superoxide dismutase (25 U/ml) plus catalase (500 U/ml)	31.1 ± 6.0 (4)
16-hour storage	
None	32.3 ± 7.4 (6)
Desferrioxamine (1.0 mM)	29.0 ± 4.5 (6)

As we observed earlier [10], loss of nonparenchymal cell viability during reperfusion of stored livers was greater in periportal areas than in pericentral regions (Table 1). To test the hypothesis that this intralobular gradient of cell killing resulted from a flow-dependent gradient of oxygen or other metabolite across the liver lobule, livers were stored and reperfused in a retrograde direction to reverse flow-dependent gradients. In these livers, the intralobular gradient of cell killing was indeed reversed, and endothelial cell killing predominated in pericentral regions (Table 1). Thus, upstream lobular regions with a higher oxygen content exhibited greater endothelial cell killing in both anterograde and retrograde perfusions.

Effects of antioxidants on nonparenchymal cell killing

Since formation of toxic oxygen species from molecular oxygen may contribute to a loss of endothelial cell viability during reperfusion, several antioxidants were evaluated as potential protective agents: allopurinol

Table 3 Effects of anoxic reperfusion on nonparenchymal cell killing. Rat livers were stored in Euro-Collins solution for 8 or 24 h and reperfused for 15 min with oxygen or nitrogen-saturated buffer. Values are means ± SE from the number of experiments in parentheses

Treatment	% Nonviable cells
8-hour storage	
Oxygen	5.7 ± 1.3 (7)
Nitrogen	5.8 ± 1.0 (5)
24-hour storage	
Oxygen	38.0 ± 3.3 (12)[a]
Nitrogen	45.8 ± 6.1 (4)

[a] From Table 2

Table 4 Effect of U74006F and drug vehicle on reperfusion injury to nonparenchymal cells: protection by mildly acidic pH. Rat livers were stored for 24 h in Euro-Collins solution and reperfused with Krebs-Henseleit bicarbonate buffer. U74006F (10 μg/ml) was added to both storage and reperfusion solutions. Vehicle for U74006F added 0.134 mM citric acid, 0.0214 mM trisodium citrate, 0.517 mM NaCl and 0.5 % bovine serum albumin, and decreased the pH of both Euro-Collins solution and Krebs-Henseleit bicarbonate buffer to 7.15 unless readjusted with NaOH. Values are means ± SE from the number of experiments in parentheses

Treatment	Reperfusion pH	% Nonviable cells *(n)*
None	7.40	38.8 ± 3.3 (12)[a]
U74006F	7.15	8.5 ± 2.0 (6)*
Vehicle	7.15	9.9 ± 2.3 (4)*
pH-adjusted vehicle	7.40	33.9 ± 10.9 (3)**

* $P < 0.0001$ compared to "None" by one-tailed *t*-test; ** $P < 0.05$ compared to "Vehicle" by one-tailed *t*-test; $P < 0.001$ compared to combined "U74006F" and "Vehicle" data at pH 7.15
[a] From Table 2

(5 mM), desferrioxamine (0.1–1.0 mM), U74006F (10 μg/ml), catalase (500–2000 U/ml), superoxide dismutase (25 U/ml), and superoxide dismutase plus catalase. Antioxidants were added to both storage and reperfusion solutions, and endothelial cell killing was evaluated after 24 h of storage. Except for U74006F, none of these treatments reduced endothelial cell killing after reperfusion (Table 2). Desferrioxamine was also evaluated after 16 h of storage to determine if protection might be observed after shorter periods of storage when reperfusion injury is less severe. Again, no protection was observed (Table 2). LDH release and oxygen consumption were also measured during reperfusion. Release of LDH remained low, in the range reported previously after 24 h of storage in Euro-Collins solution [9, 10], and oxygen consumption in all groups fell within ± 10 % of one another (data not shown).

Effect of oxygen on nonparenchymal cell killing

Since formation of oxygen radicals requires the presence of molecular oxygen, stored livers were also reperfused with nitrogen-saturated (95 % N_2, 5 % CO_2) buffer. Anoxic reperfusion did not change nonparenchymal cell killing after either 8 or 24 h of storage (Table 3). Thus, reperfusion injury to endothelial cells was not oxygen-dependent after either short or long periods of storage.

Superoxide formation by Kupffer cells after reperfusion

Kupffer cells become activated after reperfusion of livers stored for transplantation [10, 11]. To determine if Kupffer cells activated by reperfusion produce superoxide, livers were perfused with Krebs-Henseleit buffer containing nitroblue tetrazolium. Nitroblue tetrazolium reacts with superoxide to form insoluble formazan [28]. In unstored control livers, light deposits of formazan overlaid Kupffer cells (Fig. 2 A). In livers reperfused after 24 h of storage, formazan labeled Kupffer cells heavily, indicating increased superoxide formation (Fig. 2 B).

Effect of pH on cell killing

U74006F, an aminosteroid with antioxidant properties [6], appeared at first to provide substantial protection against lethal reperfusion injury to endothelial cells. With 10 μg/ml U74006F, cell killing decreased from 38 % to 8.5 % (Table 4). However, citrate-containing drug vehicle alone reduced endothelial cell death to the same extent (Table 4, Fig. 1 B). Drug vehicle reduced pH of the storage solution from pH 7.2 to pH 7.15 and pH of the reperfusion buffer from pH 7.4 to pH 7.15. When pH was adjusted to original values (pH 7.2 for Euro-Collins solution and pH 7.4 for Krebs-Henseleit buffer), protection by drug vehicle was lost (Table 4). Thus, acidification caused by drug vehicle decreased endothelial cell killing after reperfusion.

During cold ischemic storage, livers become acidotic due to anaerobic glycolysis and hydrolysis of high-energy phosphates. To determine the extent of acidosis and the recovery of pH during reperfusion, the pH of the liver effluent was monitored during reperfusion of stored livers. At an influent pH 7.4, effluent pH was initially 7.0. Effluent pH increased gradually to 7.2 over about 8 min and remained constant thereafter (Fig. 3). In unstored livers, effluent pH was also pH 7.2 during perfusions under identical conditions. Thus, under these perfusion conditions, a pH gradient of at least 0.2 units was maintained across the liver lobule. Since downstream

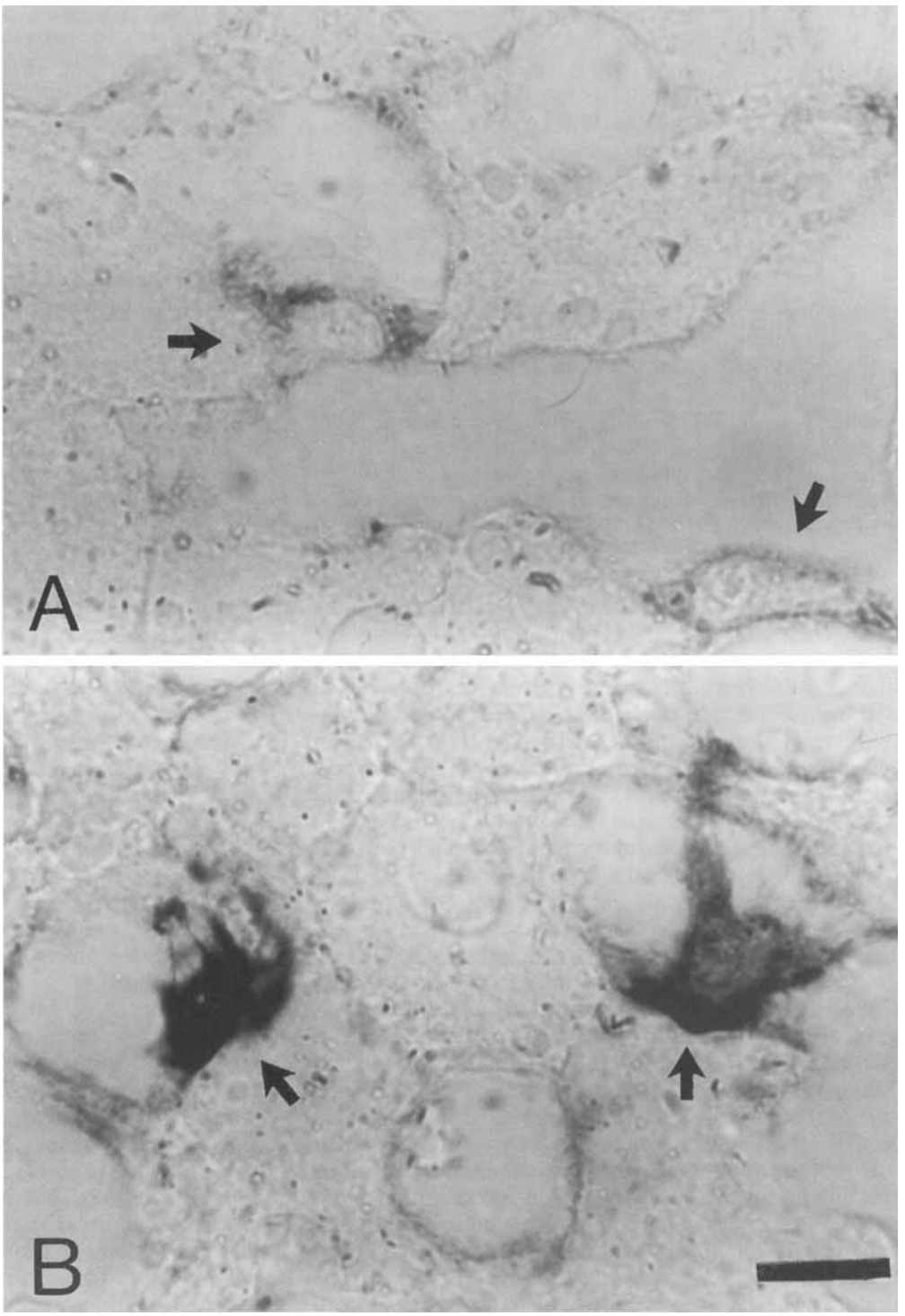

Fig. 2 A, B Formazan deposition over Kupffer cells after perfusion of stored and unstored livers. Rat livers were perfused with 0.05 % nitroblue tetrazolium in Krebs-Henseleit bicarbonate buffer for 15 min: **A** in unstored livers, little formazan deposited over Kupffer cells; **B** in livers stored for 24 h in Euro-Collins solution, formazan deposits were heavy. *Arrows* identify Kupffer cells in unstained plastic sections. *Bar* is 25 μm

regions were more acidic and since acidosis was protective against lethal cell injury, we conclude that the lobular gradient of pH may account for the gradient of cell killing across the liver lobule.

Temperature dependence of lethal reperfusion injury

Stored livers were also reperfused with chilled buffer. Cooling decreased trypan blue labeling of endothelial cells from 38 % at 37 °C to 16 % and 8 % at 18 °C and

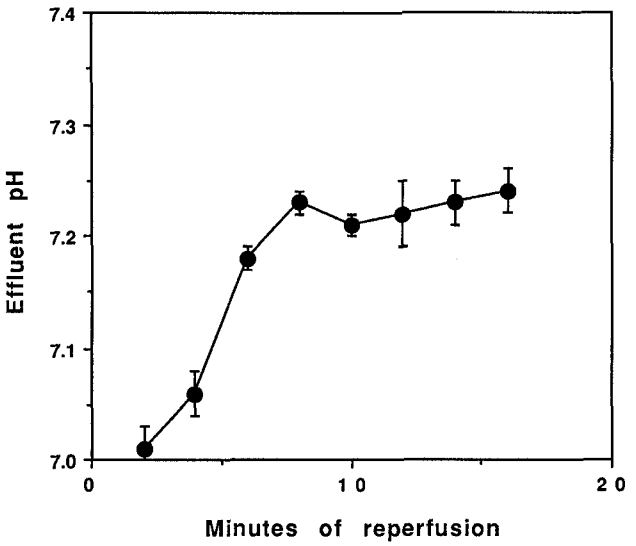

Fig. 3 pH of effluent buffer during reperfusion of stored livers. Rat livers were stored for 24 h in Euro-Collins solution and reperfused for 15 min. pH was measured in the effluent. Points are means ± SE ($n = 3$)

4 °C, respectively (Table 5, Fig. 1 C). However, trypan blue uptake was not reduced in livers first reperfused with warm buffer to cause endothelial cells to lose viability and subsequently reperfused with cold trypan blue-containing buffer to label them (Table 5). Thus, cool temperatures did not prevent trypan blue labeling when nonviable cells were present, and we conclude that lethal reperfusion injury to endothelial cells decreases as temperature falls.

Endothelial cell killing after storage in UW solution

Compared to Euro-Collins solution, UW solution extends the period of storage before reperfusion-induced endothelial cell killing and graft failure occur [3, 10, 11, 32]. After 24 h of storage in UW solution, nonparenchymal cell killing after reperfusion with aerobic Krebs-Henseleit bicarbonate buffer was 27.5 %, which was significantly less than after storage in Euro-Collins solution (Table 6). Antioxidants (desferrioxamine or superoxide dismutase plus catalase) added to the storage and reperfusion solutions did not significantly reduce this cell killing. Similarly, reperfusion with nitrogen-saturated buffer did not reduce nonparenchymal injury and actually increased cell killing after storage in UW solution (Table 6). Thus, as after storage in Euro-Collins solution, we could find no evidence of an oxygen-dependent reperfusion injury to nonparenchymal cells after storage of livers in UW solution.

Table 5 Effect of temperature on reperfusion injury to nonparenchymal cells. Rat livers were stored in Euro-Collins solution for 24 h and reperfused at various temperatures for 15 min. In the last experiment, livers were first perfused for 15 min at 37 °C and subsequently at 4 °C for 7 min more. Values are means ± SE from the number of experiments shown in parentheses

Temperature	% Nonviable cells
37 °C	38.0 ± 3.3 (12)[a]
18 °C	16.3 ± 1.0 (3)*
4 °C	8.4 ± 1.5 (5)*
37 °C, then 4 °C	45.8 ± 14.3 (3)**

* $P < 0.01$ compared to "37 °C"; ** $P < 0.05$ compared to "4 °C"
[a] From Table 2

Table 6 Effects of antioxidants on reperfusion injury to nonparenchymal cells after storage of rat livers in UW solution. Rat livers were stored in cold UW solution for 24 h and reperfused with warm oxygenated Krebs-Henseleit bicarbonate buffer for 15 min. As indicated, antioxidants were added to both storage solutions and reperfusion buffers, or the livers were reperfused with anoxic, nitrogen-saturated buffer instead of oxygen-saturated buffer. Values representing the percentage of nonviable nonparenchymal cells for each condition are means ± SE from the number of experiments shown in parentheses

Treatment	% Nonviable cells
None	27.5 ± 2.7 (17)*
Desferrioxamine (1.0 mM)	23.9 ± 3.2 (5)
Superoxide dismutase (25 U/ml) plus catalase (500 U/ml)	18.5 ± 5.3 (5)
Nitrogen-saturated	42.2 ± 5.0 (5)**

* $P < 0.01$ by one-tailed t-test compared to livers stored in Euro-Collins solution (Table 2); ** $P < 0.05$ compared to "None"

Discussion

Role of free radical generation in reperfusion injury to endothelial cells

Free radical formation is implicated in the mechanism of reperfusion injury to ischemic heart, intestine, and transplanted liver [8, 12, 23, 34]. Previous work has demonstrated that sinusoidal endothelial cells of stored livers are especially vulnerable to a reperfusion injury in vitro and in vivo [2, 9–11, 17]. Formation of oxygen free radicals during reperfusion thus seems a likely mechanism for this injury. Holloway et al. [21] reported that antioxidants failed to protect against morphological alterations to sinusoidal lining cells during cold storage. In those studies, injury was evaluated histologically before reperfusion. Since ischemic liver is anoxic, formation of free radicals from molecular oxygen cannot contribute to endothelial cell killing until after reperfusion. Here, we evaluated antioxidants for protection against lethal nonparenchymal cell injury after reperfusion and we determined that allopurinol, desferrioxamine, U74006F, superoxide dismutase, catalase, and superoxide dismu-

tase plus catalase did not prevent reperfusion-induced nonparenchymal cell killing (Tables 2, 6). Importantly, the concentrations of antioxidants used were shown previously to reduce oxygen radical-mediated injury in perfused liver, isolated hepatocytes, and other systems [8, 19, 25, 36]. Moreover, reperfusion with anoxic buffer, which should prevent the formation of oxygen free radicals, also failed to reduce endothelial cell killing during reperfusion (Tables 3, 6). In this respect, there was no difference between livers stored in Euro-Collins solution and those stored in UW solution. We conclude, therefore, that oxygen free radical formation does not contribute to lethal injury to sinusoidal endothelial cells occurring after reperfusion of livers stored for transplantation surgery.

Superoxide formation by activated Kupffer cells

In previous work, Kupffer cells became activated during reperfusion by the criteria of changes of cell structure, release of lysosomal enzymes, and increased rates of phagocytosis [9–11, 32]. Here, we showed that Kupffer cells activated by storage and reperfusion also produce superoxide radicals, which is consistent with findings by Rymsa et al. [30], who showed increased superoxide formation by cultured Kupffer cells after warm anoxia and reoxygenation. In unstored livers, we observed that little deposition of formazan occurred after infusion of nitroblue tetrazolium. However, after cold storage, heavy deposits of formazan formed over Kupffer cells during reperfusion (Fig. 2). Although oxygen radicals did not appear to contribute to endothelial cell killing, formation of oxygen radicals by activated Kupffer cells may nonetheless contribute to graft failure in vivo through such mechanisms as neutrophil recruitment and initiation of an inflammatory response [24, 26, 33]. Indeed, reperfusion-induced injury to hepatocytes in vivo after orthotopic rat liver transplantation was shown to be oxygen-dependent [34]. Thus, graft failure from storage/reperfusion injury cannot be ascribed entirely to endothelial cell injury, and a role for toxic oxygen species in the pathogenesis of primary graft failure in vivo should not be discounted.

Periportal regions are relatively enriched in Kupffer cells. Since Kupffer cells were activated by reperfusion (Fig. 2) [10, 11], toxic mediators released by activated Kupffer cells might cause the periportal predominance of reperfusion-induced nonparenchymal cell killing. However, when livers were reperfused in a retrograde direction, the zonal distribution of cell killing was reversed, which does not support the concept of Kupffer cell-mediated cytotoxicity to endothelial cells.

pH and reperfusion injury

In confirmation of our earlier report [10], reperfusion-induced nonparenchymal cell killing showed periportal predominance (Table 1). However, reperfusion in the retrograde direction reversed this intralobular gradient of cell killing. Initially, our hypothesis was that cell killing was linked to oxygen radical formation. In upstream areas where oxygen concentration is higher, oxygen radical production and, hence, cell killing would be expected to be greater. However, subsequent experiments did not support a linkage of oxygen radical formation to cell killing. Since mild acidification of the reperfusion buffer reduced endothelial cell killing (Table 4, Fig. 1 B), the explanation for upstream predominance of cell killing appears to be the lobular gradient of pH. Due to hydrogen ions generated by metabolism, downstream regions of the liver lobule are more acidic than upstream regions. The magnitude of the lobular pH gradient was at least 0.2 units (Fig. 3), and reductions in influent pH of this magnitude reduced endothelial cell killing substantially. Thus, the intralobular pH gradient can account for the decreased endothelial cell killing observed in downstream regions. Previously, acidosis was shown to be markedly protective against lethal cell injury in models of hypoxic, ischemic, and toxic injury to isolated hepatocytes, perfused livers, and cultured cardiac myocytes [4, 5, 14, 18, 29]. Moreover, the return of pH from acidotic to physiologic is a factor that precipitates lethal reperfusion injury [4, 5, 14]. This paradoxical cell killing ("pH paradox") upon the return to physiologic pH may also underlie reperfusion-induced injury in livers stored for transplantation.

The loss of endothelial cell viability during reperfusion of stored livers was reduced greatly at lower reperfusion temperatures (Table 5). Thus, rewarming is another factor contributing to lethal reperfusion injury. This finding supports the hypothesis that the processes causing endothelial cell destruction are both pH and temperature-dependent. Such processes might be the action of hydrolytic enzymes, such as phospholipases and proteases, as postulated previously for hypoxic and toxic injury to hepatocytes [7, 20] and for graft failure from storage/reperfusion injury [32].

Relation of nonparenchymal cell killing to graft survival in vivo

The magnitude of nonparenchymal cell injury that is sufficient to cause graft failure is not precisely known. After 24 h of storage in Euro-Collins solution, nonparenchymal cell killing is about 40 % and grafts fail [3, 9, 10]. After 8 h of storage, when cell killing is less than 10 %, grafts can still survive. In vivo during implantation, a period of warm ischemia occurs as the unper-

fused liver explant sits in the abdomen during the vascular anastomoses. This period of warm ischemia likely accelerates the deleterious processes occurring during cold storage. Thus, survival rates after orthotopic rat liver transplantation are highly dependent on the skill and experience of the surgeon and the details of the surgical technique. In the present work, we examined cold storage without any period of subsequent warm ischemia. Most of our work involved a 24-h period of storage in Euro-Collins solution that earlier studies showed to be the minimum storage time causing maximal reperfusion injury to nonparenchymal cells [9, 10]. Where we made comparisons with UW solution or shorter periods of storage, the results were similar except that the initial magnitude of cell killing was less (Tables 2, 3, 6). Thus, it seems likely that mechanisms causing killing of small numbers of endothelial cells when grafts can still survive, are the same as those causing more complete cell killing and graft failure.

Possible mechanisms of reperfusion injury to livers stored for transplantation

Solutions used clinically to preserve livers contain high concentrations of K^+, which must be removed prior to restoration of blood flow to avoid adverse effects on cardiac function. Since reperfusion causes endothelial cell killing and Kupffer cell activation in stored livers, modification of the conditions of reperfusion might reduce the extent of injury. Recently, a new solution, named Carolina rinse solution, was designed to counteract various potential mechanisms leading to reperfusion injury. Carolina rinse solution contains electrolytes similar to plasma, oncotic support against interstitial edema, antioxidants, vasodilators, substrates to regenerate ATP, and mildly acidic pH [13]. In vitro, reperfusion with Carolina rinse solution reduced nonparenchymal cell killing from 25 % to less than 1 % in rat livers after 24 h of storage in UW solution [13]. Use of Carolina rinse solution to rinse stored livers prior to implantation also prevented graft failure in vivo after orthotopic rat liver transplantation [1, 2, 16, 17] and improved liver function tests after human transplantation [31]. Consistent with the present findings, the protective effect of Carolina rinse solution in preventing graft failure after prolonged storage is lost when its pH is increased from pH 6.5 to pH 7.4 [1]. A controversial issue is whether the critical injury limiting storage of livers for transplantation surgery is a reperfusion injury as we have maintained [9–11], or whether this injury occurs during storage prior to reperfusion [22, 27]. The efficacy of Carolina rinse solution both in vitro and in vivo, together with the new data presented here, provide convincing evidence that the critical events leading to endothelial cell killing and graft failure are indeed a reperfusion phenomenon.

The efficacy of Carolina rinse solution in preventing graft failure after orthotopic rat liver transplantation is dependent upon each of at least three classes of components: acidotic pH, antioxidants, and adenosine [1, 2, 16]. Here, we show that reperfusion at acidotic pH prevents endothelial cell killing. However, antioxidants (Table 2) and adenosine (Currin RT and Lemasters JJ, unpublished observation) did not prevent reperfusion-induced endothelial cell killing. It is likely that antioxidants and adenosine target Kupffer cells for their protective effect. Antioxidants may block the effects of increased oxygen radical formation by Kupffer cells (Fig. 2), whereas adenosine may suppress cytokine formation by stimulated Kupffer cells, as recently shown in cultured Kupffer cells [15]. Thus, endothelial cell killing and Kupffer cell activation may both contribute independently to liver graft failure from storage/reperfusion injury.

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References

1. Bachmann S, Caldwell-Kenkel JC, Currin RT, Lichtman SN, Steffen R, Thurman RG, Lemasters JJ (1992) Protection by pentoxifylline against graft failure from storage injury after orthotopic rat liver transplantation with arterialization. Transpl Int 5 [Suppl 1]: S345–S350
2. Bachmann S, Caldwell-Kenkel JC, Oleksy I, Steffen R, Thurman RG, Lemasters JJ (1992) Warm Carolina rinse solution prevents graft failure from storage injury after orthotopic rat liver transplantation with arterialization. Transpl Int. 5: 108–114
3. Belzer FO, Southard JH (1988) Principles of solid organ preservation by cold storage. Transplantation 45: 673–676
4. Bond JM, Herman B, Lemasters JJ (1991) Protection by acidotic pH against anoxia/reoxygenation injury to rat neonatal cardiac myocytes. Biochem Biophys Res Commun 179: 798–803
5. Bond JM, Chacon E, Herman B, Lemasters JJ (1993) Intracellular pH and calcium homeostasis during the pH paradox of reperfusion injury to cultured neonatal rat cardiac myocytes. Am J Physiol 265: C129–C137
6. Braughler JM, Pregenzer JF, Chase RL, Ducan LA, Jacobsen EJ, McCall JM (1987) Novel 21-amino steroids as potent inhibitors of iron-dependent lipid peroxidation. J Biol Chem 262: 10438–10440
7. Bronk SF, Gores GJ (1993) pH-dependent nonlysosomal proteolysis contributes to lethal anoxic injury of rat hepatocytes. Am J Physiol 264: G744–G751
8. Burton KP (1985) Superoxide dismutase enhances recovery following myocardial ischemia. Am J Physiol 248: H637–H643

9. Caldwell-Kenkel JC, Thurman RG, Lemasters JJ (1988) Selective loss of nonparenchymal cell viability after cold ischemic storage of rat livers. Transplantation 45: 834–837
10. Caldwell-Kenkel JC, Currin RT, Tanaka Y, Thurman RG, Lemasters JJ (1989) Reperfusion injury to endothelial cells following cold ischemic storage of rat livers. Hepatology 10: 292–299
11. Caldwell-Kenkel JC, Currin RT, Tanaka Y, Thurman RG, Lemasters JJ (1991) Kupffer cell activation and endothelial cell damage after storage of rat livers: effect of reperfusion. Hepatology 13: 83–95
12. Connor HD, Gao W, Nukina S, Lemasters JJ, Mason RP, Thurman RG (1992) Evidence that free radicals are involved in graft failure following orthotopic liver transplantation in the rat – an electron paramagnetic resonance spin trapping study. Transplantation 54: 199–204
13. Currin RT, Thurman RG, Toole JG, Lemasters JJ (1990) Evidence that Carolina rinse solution protects sinusoidal endothelial cells against reperfusion injury after cold ischemic storage of rat liver. Transplantation 50: 1076–1078
14. Currin RT, Gores GJ, Thurman RT, Lemasters JJ (1991) Protection by acidotic pH against anoxic cell killing in perfused rat liver: evidence for a 'pH paradox'. FASEB J 5: 207–210
15. Currin RT, Reinstein LJ, Lichtman SN, Thurman RG, Lemasters JJ (1993) Inhibition of tumor necrosis factor release from cultured rat Kupffer cells by agents that reduce graft failure from storage injury. Transplant Proc 25: 1631–1632
16. Gao W, Hijioka T, Lindert KA, Caldwell-Kenkel JC, Lemasters JJ, Thurman RG (1991) Evidence that adenosine is a key component in Carolina rinse responsible for reducing graft failure after orthotopic liver transplantation in the rat. Transplantation 52: 992–998
17. Gao W, Takei Y, Marzi I, Lindert KA, Caldwell-Kenkel JC, Currin RT, Tanaka Y, Lemasters JJ, Thurman RG (1991) Carolina rinse solution – a new strategy to increase survival time after orthotopic liver transplantation in the rat. Transplantation 52: 417–424
18. Gores GJ, Nieminen AL, Fleishman KE, Dawson TL, Herman B, Lemasters JJ (1988) Extracellular acidosis delays onset of cell death in ATP-depleted hepatocytes. Am J Physiol 255: C315–C322
19. Gores GJ, Flarsheim CE, Dawson TL, Nieminen AL, Herman B, Lemasters JJ (1989) Swelling, reductive stress, and cell death during chemical hypoxia in hepatocytes. Am J Physiol 257: C347–C354
20. Harrison DC, Lemasters JJ, Herman B (1991) A pH-dependent phospholipase A_2 contributes to loss of plasma membrane integrity during chemical hypoxia in rat hepatocytes. Biochem Biophys Res Commun 174: 654–659
21. Holloway CMB, Harvey PRC, Mullen JB, Strasberg SM (1989) Evidence that cold preservation-induced microcirculatory injury in liver allografts is not mediated by oxygen free radicals or cell swelling in the rat. Transplantation 48: 179–188
22. Holloway CMB, Harvey PRC, Strasberg SM (1990) Viability of sinusoidal lining cells in cold-preserved rat liver allografts. Transplantation 49: 225–229
23. Itoh M, Guth RH (1985) Role of oxygen-derived free radicals in hemorrhagic shock-induced gastric lesions in the rat. Gastroenterology 88: 1162–1167
24. Koo A, Komatsu H, Tao G, Inoue M, Guth PH, Kaplowitz N (1992) Contribution of no-reflow phenomenon to hepatic injury after ischemia-reperfusion: evidence for a role for superoxide anion. Hepatology 15: 507–514
25. Marotto ME, Thurman RG, Lemasters JJ (1988) Early midzonal cell death during low-flow hypoxia in the isolated perfused rat liver: protection by allopurinol. Hepatology 8: 585–590
26. Marzi I, Knee J, Buhren V, Menger M, Trentz O (1992) Reduction by superoxide dismutase of leukocyte-endothelial adherence after liver transplantation. Surgery 111: 90–97
27. McKeown CMB, Edwards V, Phillips MJ, Harvey PRC, Petrunka CN, Strasberg SM (1988) Sinusoidal lining cell damage: the critical injury in cold preservation of liver allografts in the rat. Transplantation 46: 178–191
28. Mochida S, Ogata I, Ohta Y, Yamada S, Fujiwara K (1989) In situ evaluation of the stimulatory state of hepatic macrophages based on their ability to produce superoxide anions in rats. J Pathol 158: 67–71
29. Nieminen A-L, Dawson TL, Gores GJ, Kawanishi T, Herman B, Lemasters JJ (1990) Protection by acidotic pH and fructose against lethal injury to rat hepatocytes from mitochondrial inhibitors, ionophores and oxidant chemicals. Biochem Biophys Res Commun 167: 600–606
30. Rymsa B, Wang JF, Groot H de (1991) $O_2 \cdot$ -release by activated Kupffer cells upon reoxygenation. Am J Physiol 261: G602–G607
31. Sanchez-Urdazpal L, Gores GJ, Lemasters JJ, Thurman RG, Steers JL, Wahlstrom HE, Hay EI, Porayko MK, Wiesner RH, Krom RAF (1993) Carolina rinse solution decreases liver injury during clinical liver transplantation. Transplant Proc 25: 1574–1575
32. Takei Y, Marzi I, Kauffman FC, Currin RT, Lemasters JJ, Thurman RG (1990) Increase in survival time of liver transplants by protease inhibitors and a calcium channel blocker, nisoldipine. Transplantation 50: 14–20
33. Takei Y, Marzi I, Gao W, Gores GJ, Lemasters JJ, Thurman RG (1991) Leukocyte adhesion and cell death following orthotopic liver transplantation in the rat. Transplantation 51: 959–965
34. Thurman RG, Marzi I, Seitz G, Thies J, Lemasters JJ, Zimmerman F (1988) Hepatic reperfusion injury following orthotopic liver transplantation in the rat. Transplantation 46: 502–506
35. Warren JS, Ward PA (1986) Review: oxidative injury to the vascular endothelium. Am J Med Sci 292: 97–103
36. Zhong Z, Lemasters JJ, Thurman RG (1989) Role of purine and xanthine oxidase in reperfusion injury in perfused rat liver. J Pharmacol Exp Ther 250: 470–475