Transplant Int (1992) 5 [Suppl 1]: S345–S350

TRANSPLANT
International

Protection by pentoxifylline against graft failure from storage injury after orthotopic rat liver transplantation with arterialization

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Abstract. Destruction of the endothelial cell lining and activation of Kupffer cells after reperfusion limits the safe storage of livers for transplantation surgery. Tumor necrosis factor-alpha (TNF) release by activated Kupffer cells may contribute to graft failure from storage injury. Accordingly, we evaluated whether pentoxifylline, which suppresses macrophage TNF release, would improve graft survival after orthotopic rat liver transplantation with arterialization. Livers from syngeneic Lewis rats were stored for 12–24 h in cold UW solution. Prior to implantation, the livers were flushed with cold Ringer's solution or warm Carolina rinse solution B. With either rinse, pentoxifylline treatment of graft recipients significantly improved graft survival. Combined use of pentoxifylline (50 mg/kg for 5 days) and Carolina rinse solution doubled the safe storage time to 24 h. Acidotic pH and antioxidants were essential components of Carolina rinse solution that acted synergistically with pentoxifylline. Pentoxifylline was also shown to suppress TNF release by lipopolysaccharide (LPS)-stimulated cultured rat Kupffer cells. Thus, pentoxifylline may protect against primary non-function and failure of grafts from storage injury by suppressing excessive TNF release by activated Kupffer cells. However, neutralization of TNF with excess anti-TNF antibody did not improve survival. This may mean that depletion of TNF is as deleterious as excess TNF production. Alternatively, other Kupffer cell secretions [e.g., interleukin-1 (IL-1), interleukin-6 (IL-6) and other cytokines] may be involved in the pathogenesis of graft failure. In conclusion, pentoxifylline could protect against graft failure from storage injury.

Key words: Carolina rinse solution – Endothelium – Kupffer cells – Liver transplantation – Organ preservation – Pentoxifylline – Reperfusion injury – Tumor necrosis factor

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Liver transplantation is an accepted therapy for children and adults with end-stage liver disease and is increasing worldwide. With the development of University of Wisconsin (UW) cold storage solution, human livers can now be preserved under cold ischemic conditions for more than twice the length of time as with Euro-Collins solution which had been used previously [2, 20, 30]. Despite the improvement in UW solution, disturbances in liver function and histology attributable to storage injury commonly occur postoperatively. Moreover, primary graft non-function still occurs in 5–15% of patients [17, 19].

Liver graft failure after prolonged storage involves a reperfusion injury to non-parenchymal cells: sinusoidal endothelial cells lose viability and Kupffer cells become activated by several structural and functional criteria [5, 6, 8, 9, 21, 28]. By contrast, parenchymal structure, function and viability are well maintained. Moreoever, reperfusion injury occurs to liver grafts in vivo even under conditions where long-term graft survival is assured [29]. Nonparenchymal cell injury seems to lead to marked microcirculatory disturbances, including inhomogeneous reflow, hypoperfusion, and leukocyte margination, which may lead to ischemia and inflammation [8, 27]. In addition, Kupffer cells that have been activated by ischemia/reperfusion are likely to release tumor necrosis factor and other cytokines into the general circulation [13]. Release of cytokines may account for severe systemic problems associated with primary non-function, including coagulopathy, pulmonary infiltration, and multiple organ failure [22].

Recently, a new solution, Carolina rinse solution, has been developed to prevent reperfusion injury to livers stored for transplantation surgery [14]. In vitro, Carolina rinse solution prevents reperfusion-induced killing of endothelial cells, and in vivo, it improves graft survival markedly after orthotopic rat liver transplantation [1, 14, 16]. Improved survival has been associated with preservation of endothelial cell ultrastructure, suggesting that protec-

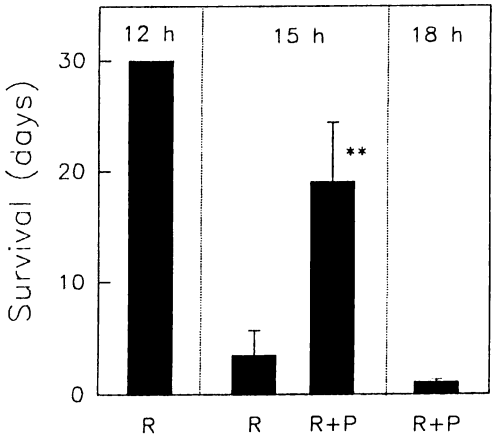

Fig. 1. Improvement by pentoxifylline in graft survival after orthotopic rat liver transplantation. Rat livers were stored for 12–18 h in UW solution and transplanted as described in Materials and methods. Liver grafts were rinsed with Ringer's solution and implanted into untreated recipient rats *(R)* or rats pretreated with a single dose of pentoxifylline *(R + P)*. ** $P < 0.01$ by Students *t*-test compared to *R*. Mean survival times ± SE from 6–13 experiments per group

tion is mediated, at least in part, by prevention of lethal reperfusion injury to endothelial cells.

Pentoxifylline is a drug which has long been used to treat peripheral vascular disease [31]. More recently, pentoxifylline has been shown to suppress tumor necrosis factor-alpha (TNF) release by macrophages [25], to inhibit TNF-mediated inflammatory action [26], and to be effective clinically in graft-versus-host disease after bone marrow transplantation [3]. Accordingly, we evaluated the effect of pentoxifylline on graft survival after rat liver transplantation in order to test the hypothesis that the release of TNF by Kupffer cells contributes to graft failure from storage injury. Our results indicated that pentoxifylline is indeed protective. Significantly, protection was achieved through treatment of the recipient animal, without any change in organ harvesting and storage conditions.

Materials and methods

Orthotopic rat liver transplantation with arterialization – surgery. Rat livers were transplanted under ether anesthesia essentially as described by Steffen et al. [24]. To avoid immunologic interference, syngeneic male Lewis rats (250–300 g) were used. In the donor operations, donor livers were flushed via the portal vein with chilled UW solution, cuffs were attached, and the explants were placed in an ice water bath immersed in storage solution. At the end of storage, donor livers were rinsed with 30 ml of cold Ringer's solution or Carolina rinse solution B at 28–30 °C. Carolina rinse solution B contained 115 mM NaCl, 5 mM KCl, 1.3 $CaCl_2$, 1 mM KH_2PO_4, 50 g/l modified hydroxyethyl starch, 1 mM allopurinol, 1 mM desferrioxamine mesylate, 3 mM glutathione, 2 µM nicardipine, 200 µM adenosine, 10 mM fructose, 10 mM glucose, 100 U/l insulin, and 20 mM MOPS [3-(N-morpholino)propanesulphonic acid] buffer, pH 6.5 [1]. In some experiments, Carolina rinse solution was modified by adjusting its pH to 7.4 or by omitting the antioxidants (allopurinol, desferrioxamine, and glutathione). Implantation surgery required 60 min. During this time the portal vein was clamped for 15 min and the inferior vena cava for not more than 20 min. Rats were given food and water ad libitum postoperatively. Average days of survival (with 30 days as a maximum) was used as an index of experimental outcome.

Treatment of recipient rats with pentoxifylline and anti-TNF antisera. Pentoxifylline was injected intraperitoneally (i.p.) into recipient animals in doses of 50 mg/kg, 1 h preoperatively. In some experiments, the dosage was repeated daily for 4 more days. In preliminary experiments, we compared recipient treatment with donor plus recipient treatment and donor treatment alone. Donor treatment produced no improvement in survival, whereas donor plus recipient treatment was no different than recipient treatment alone.

In other experiments, recipient animals were administered antisera via the penile vein under anesthesia at the beginning of the implantation operation. Two antisera were used: rabbit anti-mouse TNF polyclonal antibody and hamster anti-mouse TNF monoclonal IgG. Both antisera were obtained from Genzyme Corp. (Boston, Mass.) and cross reacted with rat TNF. Polyclonal antibody (1 ml) was diluted into 1 ml RPMI before injection. Monoclonal antibody (0.1 ml of a 2 mg/ml solution) was diluted into 0.5 ml RPMI before use.

Kupffer cell culture and measurement of TNF. Kupffer cells were isolated from Sprague-Dawley rats (300–350 g) by collagenase digestion and purified by counter-flow elutriation by modification of the technique described by Irving et al. [18]. Purified Kupffer cells were cultured overnight in 96-well microtiter plates (500000 cells/well) with RPMI-1640 media supplemented with 20% fetal calf serum and 10 mM HEPES [4-(2-hydroxyethyl)-piperazine ethanesulfonic acid] at 37 °C in humidified air/5% CO_2. The next day, cultures were incubated for 1 h with various concentrations of pentoxifyline before the addition of 400 ng/ml lipopolysaccharide (LPS). After 8 h, supernatants were removed and stored at −70 °C. TNF of thawed samples was measured using an ELISA kit (Genzyme, Boston, Mass.) and was expressed as pg equivalents of mouse recombinant TNF-alpha.

Materials. Nicardipine and modified hydroxyethyl starch (Pentafraction) were gifts of DuPont/Merck Pharmaceuticals (Wilmington, Del.) Pentoxifylline was the gift of Hoechst-Roussel Pharmaceuticals, (Somerville, N.J.). Lipopolysaccharide from *E. coli* was purchased from Sigma Chemical (St. Louis, Mo.). Other reagents were obtained from standard commercial sources.

Results

Protection by pentoxifylline against graft failure. For liver grafts stored 12 h in UW solution and rinsed with cold Ringer's solution, long-term graft survival after transplantation approached 100% (Fig. 1). In contrast, after 15 h storage, no recipient rat survived longer than a day. This poor survival improved dramatically when recipient rats were pretreated with a single dose of pentoxifylline (50 mg/kg, i.p., 2 h preoperatively). However, pentoxifylline could not prevent graft failure after 18 h storage.

Survival was also much improved when grafts were rinsed with Carolina rinse solution instead of Ringer's solution (Fig. 2). After 18 h storage in UW solution, average survival time was more than 10 times that of the Ringer's-rinsed group (compare with Fig. 1). After 24 h storage, however, survival once again was lost. In contrast to our findings with Ringer's rinse, single dose pentoxifylline did not significantly improve survival of Carolina rinse-treated livers after 24 h storage. However, when the preoperative dose of pentoxifylline was followed by daily in-

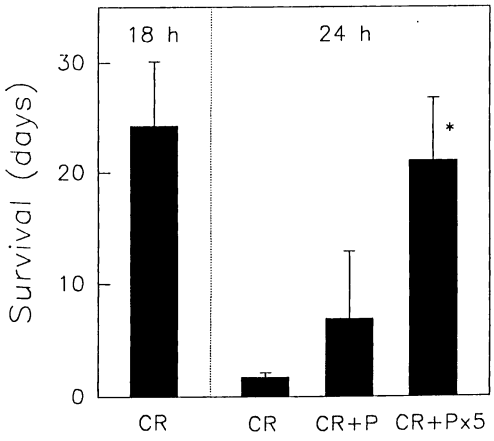

Fig. 2. Survival of liver grafts stored in UW solution and rinsed with Ringer's solution. Liver grafts were rinsed with Carolina rinse solution B after 18 or 24 h cold storage in UW solution and implanted into untreated recipient rats *(CR)*, rats pretreated with a single dose of pentoxifylline *(CR + P)*, or rats treated with five daily doses of pentoxifylline *(CR + Px5)*. * $P < 0.05$ by Student's *t*-test compared to *CR*. There were five to six experiments per group

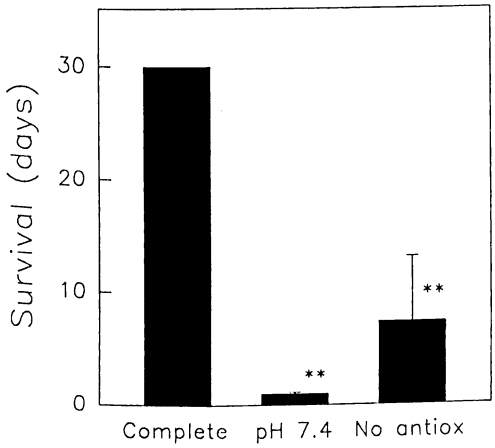

Fig. 3. Loss of survival at pH 7.4 or after omission of antioxidants from Carolina rinse solution. Liver grafts were stored for 18 h in UW solution and transplanted into recipient rats pretreated with a single dose of pentoxifylline. Prior to implantation, grafts were rinsed with complete Carolina rinse solution B *(Complete)*, Carolina rinse solution adjusted to pH 7.4 *(pH 7.4)*, or Carolina rinse solution containing no allopurinol, desferrioxamine or glutathione *(No antiox)*. ** $P < 0.01$ by Student's *t*-test compared to Complete. There were five to six experiments per group

jections for an additional 4 days, survival of Carolina rinse-treated livers increased significantly after 24 h storage.

Importance of acidotic pH and antioxidants in Carolina rinse solution for graft survival. Single dose pentoxifylline did not improve survival of liver grafts rinsed with Carolina rinse solution. To explore the hypothesis that pentoxifylline might be acting in the same way as components of Carolina rinse solution, we investigated the consequence of deleting specific components of Carolina rinse on survival of pentoxifylline-treated graft recipients. Previously, we have found that the acidotic pH of Carolina rinse solution was critically important in preventing lethal reperfusion injury to endothelial cells after storage and reperfusion of isolated rat livers [5, 13]. To determine whether acidotic treatment remained important when pentoxifylline was used, we rinsed 18 h-stored livers with Carolina rinse solution adjusted to pH 7.4 prior to implanting livers into pentoxifylline-treated recipient rats (Fig. 3). Under these conditions, long-term survival went from 100% to 0% (Fig. 3). Similarly, we investigated the importance of antioxidants in Carolina rinse solution, since in vitro and in vivo studies have shown oxygen free radical generation by Kupffer cells after long-term storage and reperfusion [5, 12]. When antioxidants (allopurinol, desferrioxamine, glutathione) were deleted from Carolina rinse solution, survival of pentoxifylline-treated recipient rats again fell drastically (Fig. 3). Thus, we conclude that the beneficial effects of pentoxifylline seemed unrelated to prevention of pH-dependent endothelial cell killing or to suppression of oxygen free radical formation.

Suppression by pentoxifylline of TNF release by Kupffer cells. To explore the possibility that pentoxifylline suppresses TNF release by Kupffer cells, we exposed cultured rat Kupffer cells to LPS. This treatment caused TNF release into the medium to increase more than 25-fold over untreated cells (Fig. 4). Pentoxifylline suppressed LPS-stimulated TNF release by more than 70% in a dose-dependent fashion. Half-maximal suppression of TNF release was achieved with about 10 μM pentoxifylline.

Lack of protection by TNF antisera against graft failure after liver transplantation. To test further the hypothesis that pentoxifylline-sensitive TNF release contributes to graft failure from storage injury, transplant recipients

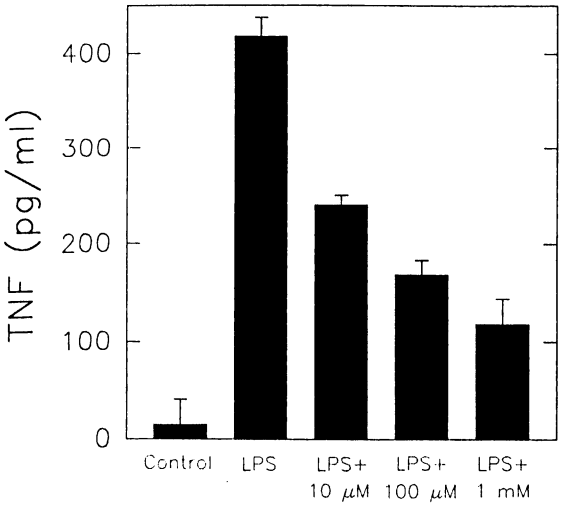

Fig. 4. Suppression by pentoxifylline of TNF release by LPS-stimulated cultured rat Kupffer cells. Kupffer cells cultured overnight were incubated for 8 h as described in Materials and methods. Cells were exposed to LPS (400 ng/ml) and 10–1000 μM pentoxifylline as indicated

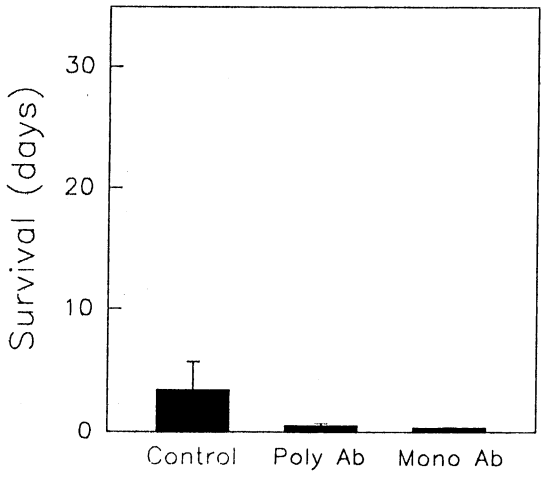

Fig. 5. Lack of protection against graft failure by anti-TNF antisera. Liver grafts were stored for 15 h in UW solution, rinsed with Ringer's rinse, and implanted into untreated rats *(Control)*, rats injected with anti-TNF polyclonal antibody *(Poly Ab)*, or rats injected with anti-TNF monoclonal antibody *(Mono Ab)*. There were two to three experiments per Ab group

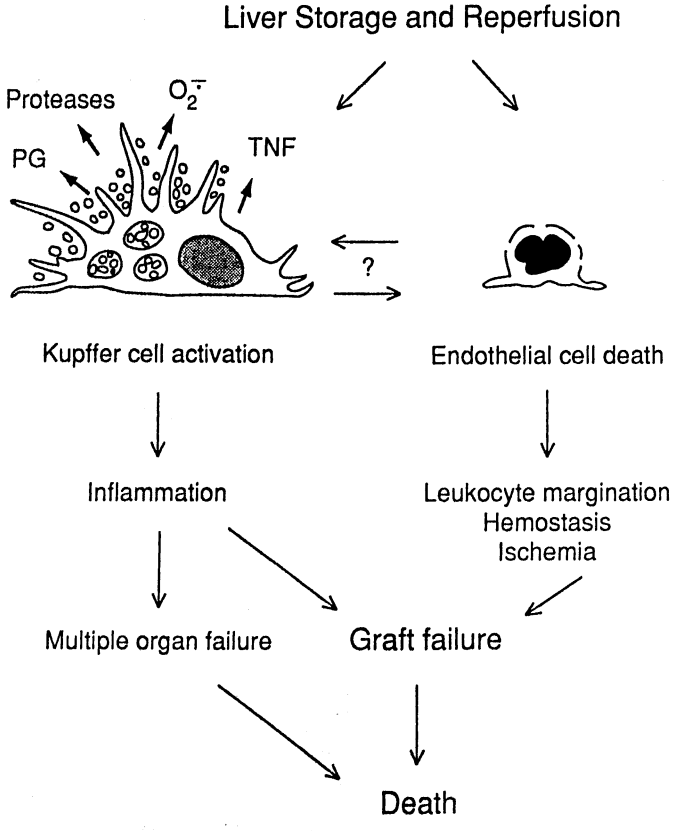

Fig. 6. Schematic picture of storage and reperfusion injury to liver. See text for details. *PG*, prostaglandin; O_2^-, superoxide radical; *TNF*, tumor necrosis factor

were pretreated with anti-TNF antisera. Under the conditions employed (storage for 15 h, rinse with Ringer's solution), neither polyclonal nor amonoclonal antisera improved graft survival (Fig. 5). If anything, average time of survival trended lower.

Discussion

Primary graft failure (primary non-function) remains a serious complication of liver transplantation surgery, occurring in about 5–15 % of cases. Graft failure is generally attributed to suboptimal conditions during harvesting and storage and to pathology in the donor liver, such as fatty infiltration, but the exact mechanisms are unknown. As shown by the present work, storage time is a very important determinant of graft success. Under controlled laboratory conditions employing syngeneic animals, only 3 h difference in storage time determined the success or failure of orthotopic rat livers transplantations (Figs. 1 and 2). In out-bred human populations under far less controlled conditions, it is unlikely that the response to storage time would be so consistent. Thus, mechanisms causing failure of rat livers after prolonged cold ischemic storage may be similar to those underlying idiopathic primary graft failure in human transplantation.

The present study demonstrated, for the first time, that pentoxifylline treatment of recipient rats could protect against graft failure from storage injury (Figs. 1 and 2). The only other drug showing possible efficacy against primary graft failure is prostaglandin E1 [17]. Since it is well documented that pentoxifylline is safe to humans, there is a possible use for the drug as a routine pre- and postoperative medication to minimize the incidence of primary non-function. Such a prophylactic use might have other benefits, as pentoxifylline also reduces the nephrotoxicity of cyclosporin and amphotericin, agents typically given to transplant patients [4, 32].

The beneficial effects of pentoxifylline were synergistic with Carolina rinse solution. Together, recipient treatment with pentoxifylline and rinsing of implants with Carolina rinse solution doubled safe preservation times (Figs. 1 and 3). Pentoxifylline is unlikely to act as an antioxidant, since pentoxifylline-treated recipient rats died when antioxidants were removed from Carolina rinse (Fig. 3). Similarly, pentoxifylline may not protect against pH-related injury to endothelial cells, since no animal survived when the pH of Carolina rinse solution was increased from pH 6.5 to pH 7.4.

A likely scenario is that pentoxifylline suppresses cytokine formation by Kupffer cells activated by storage and reperfusion. In cultured Kupffer cells, pentoxifylline strongly suppressed TNF release after stimulation with LPS (Fig. 4). Moreover, serum TNF increases in transplant recipients with failing liver grafts ([23], and data not shown). Against this hypothesis is the observation that neutralization of TNF with excess antibody did not improve graft survival (Fig. 5). This may mean that depletion of TNF was as deleterious as excess TNF production. Alternatively, other Kupffer cell secretions (e.g., IL-1, IL-6 and other cytokines) may be involved in the pathogenesis of graft failure.

Pentoxifylline is also a hemorrheologic agent which improves microcirculation in vasoocclusive disease [31]. It improves hepatic function and reduces enzyme release after hypothermic preservation of isolated rat livers in vitro, an effect associated with increased blood flow and reduced vascular resistance [10]. Since microcirculatory

disturbances are prominent in failing liver grafts [15, 27], pentoxifylline may enhance graft survival by improving hepatic blood flow. By whatever mechanism, the benefit of pentoxifylline extends beyond the initial period of reperfusion, since pentoxifylline was more effective given daily over 5 days than when given as a single preoperative does (Fig. 2).

The present work also extended our in vivo analysis of the ingredients of Carolina rinse solution that contribute to its success. Previously, we have shown that Carolina rinse solution loses efficacy if adenosine is omitted [1, 15, 16]. The present work showed that efficacy was also lost if either antioxidants or acidotic pH were removed (Fig. 3). These findings suggested strongly that multiple mechanisms underlie liver graft failure from storage injury. As illustrated in Fig. 6, liver storage and reperfusion led to endothelial cell killing and Kupffer cell activation. Endothelial cell death promotes leukocyte margination, hemostasis, microcirculatory disturbances, and ischemia. Kupffer cell activation leads to inflammation, release of toxic soluble mediators, and possibly multiple organ failure. These parallel mechanisms both contribute to graft failure and death of recipient animals. It seems probable, therefore, that prevention of endothelial cell killing (acidotic pH), neutralization of oxygen free radicals (antioxidants), suppression of Kupffer cell release of TNF (pentoxifylline), and vasodilation (adenosine) together account for the efficacy and synergism of pentoxifylline and Carolina rinse solution.

In conclusion, pentoxifylline and Carolina rinse solution used together doubled storage times of rat livers in UW solution. Such treatments may become useful clinically in extending storage times of human livers, in reducing the incidence of primary graft failure, and improving graft function postoperatively.

Acknowledgements. We thank Mr. John Williams of Genzyme Corporation for helpful discussions and access to anti-TNF antisera. This work was supported, in part, by grant DK 37034 from the National Institutes of Health. J. C. C.-K. was the recipient of Individual National Research Service Fellowship DK 08094 from the National Institutes of Health.

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