Attenuation of Kupffer Cell Activation in Cold-Preserved Livers after Pretreatment of Rats with Methylprednisolone or Its Macromolecular Prodrug

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Purpose. Activation of hepatic Kupffer cells (KCs) during organ preservation and subsequent reperfusion causes release of proinflammatory mediators and is responsible, at least in part, for rejection of transplanted livers. Our hypothesis was that donor pretreatment, before liver harvest, with methylprednisolone (MP) or its dextran prodrug (DMP) would reduce KC activation.

Methods. Adult donor rats were administered a single 5-mg/kg (MP equivalent) IV dose of MP or DMP or saline 2 h before liver harvest. The livers were then stored in University of Wisconsin solution for 24, 48, or 96 h (n = 4/treatment/time). A recirculating perfusion model was used to study, for 180 min, the release of KC activation markers, tumor necrosis factor (TNF)-α and acid phosphatase, and other biochemical indices from the cold-preserved livers.

Results. Cold ischemia–reperfusion resulted in release of substantial levels of TNF-α in untreated groups. Pretreatment of rats with MP or DMP caused a significant ($p < 0.0001$) reduction in TNF-α AUC in the perfusate, with no significant differences between MP and DMP. The maximum inhibitory effect of MP (77.5 ± 10.2%) was observed after 48 h of preservation, whereas DMP showed maximal inhibition of TNF-α AUC at both 24 (74.5 ± 15.8%) and 48 (74.8 ± 12.6%) h of preservation. Similarly, both MP and DMP resulted in a significant ($p < 0.0004$) decrease in acid phosphatase levels of cold-preserved livers. However, neither pretreatment had any substantial effect on the levels of other biochemical markers.

Conclusions. Both MP and DMP pretreatments decreased the release of TNF-α and acid phosphatase from livers subjected to cold ischemia preservation. Therefore, pretreatment of liver donors with MP or its prodrug decreases KC activation by cold ischemia–reperfusion.

KEY WORDS: methylprednisolone; dextran prodrugs; liver preservation; ischemia–reperfusion injury; Kupffer cells.

INTRODUCTION

Recent advances in treatment strategies and development of newer drugs have resulted in a dramatic improvement in the outcome of liver transplantation (1). However, rejection of the grafted liver as a result of primary graft nonfunction poses a major challenge to successful transplantation (1,2). Injury associated with graft harvest, cold preservation ischemia, and subsequent reperfusion is believed to be the main reason for primary graft nonfunction after transplantation (1). Therefore, prevention and/or attenuation of ischemia–reperfusion injury may lead to a decreased incidence of graft rejection.

After procurement of the liver from a donor, the organ is normally preserved in cold (4°C) for several hours before it is transplanted into a suitable recipient. This cold ischemia and subsequent reperfusion of the liver result in an injury in which activated Kupffer cells (KCs) play a major role. The activation of KCs occurs partly in the cold preservation stage and increases substantially during ensuing reperfusion (3). Kupffer cells may also be activated by simple liver manipulation during the organ harvest (4). The activated KCs release a variety of inflammatory mediators such as cytokines [e.g., tumor necrosis factor (TNF)-α and interleukin (IL)-1β], reactive oxygen intermediates (e.g., superoxide and nitric oxide), and eicosanoids (e.g., prostaglandins and leukotrienes) (1,3). Therefore, a reduction in KC activation could potentially improve the chances of graft survival (4,5).

Several approaches are currently under investigation to reduce the injury to the liver during cold preservation and following restoration of the blood flow. One such approach is based on the pretreatment of donors, before the liver is harvested, with drugs that reduce the activation of KCs (4–6). Glucocorticoids, such as methylprednisolone (MP) (7) and prednisolone (8), have been shown to protect the liver from warm ischemia–reperfusion injury resulting from the temporary occlusion of the liver blood supply. However, the effects of these drugs on the cold ischemia–reperfusion damage caused by liver preservation have not been elucidated. Recently (9), we have shown that after *in vivo* administration, a prodrug of MP, dextran methylprednisolone succinate (DMP), preferentially and persistently accumulates in the liver as opposed to indiscriminate distribution of free MP to several tissues. Therefore, in the current study, we used an isolated perfused rat liver model (IPRL) to investigate the effects of both the parent drug (MP) and its prodrug (DMP) on the ischemia–reperfusion-induced KC activation and release of proinflammatory mediators. Many investigators (10–15) have used IPRL as an alternate *ex vivo* model in place of animal transplant experiments. Our hypothesis was that the pretreatment of animals with MP or its prodrug would stabilize KCs and reduce the release of proinflammatory mediators, such as TNF-α, in rat livers subjected to cold preservation and subsequent reperfusion.

MATERIALS AND METHODS

Chemicals

Dextran with an average MW of 73 kDa and polydispersity of <2, 6α-methylprednisolone (MP), sodium taurocholate, and kits for measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and acid phosphatase (ACP) were obtained from Sigma Chemical Co. (St. Louis, MO). 6α-Methylprednisolone 21-hemisuccinate (MPS) was purchased from Steraloids (Wilton, NH). Belzer's University of Wisconsin (UW) solution (Viaspan™) was obtained from Dupont Pharma (Wilmington, DE). Sterile nonpyrogenic lactated Ringer's irrigation solution was obtained from Baxter Healthcare Co. (Deerfield, IL). An ultrasensitive enzyme-linked immunosorbent assay (ELISA) kit for measurement of TNF-α was pur-

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chased from Biosource International, Inc. (Camarillo, CA). Xylazine and ketamine sterile solutions for anesthesia were from Lloyd Laboratories (Shenandoah, IA) and Fort Dodge Animal Health (Fort Dodge, IA), respectively. For chromatography, HPLC grade acetonitrile was obtained from EM Science (Gibbstown, NJ). All other reagents were analytic grade and obtained through commercial sources.

Dextran-methylprednisolone succinate (DMP) was synthesized, purified, and characterized as described before (16). The MP and MPS impurities in the conjugate powder were less than 0.1% (w/w), and the degree of substitution of the powder was 8 mg of MP per 100 mg of powder.

The dosing solutions (5 mg/ml, MP equivalent) of MP and DMP were prepared in a mixture of water:ethanol: PEG400 (17) and distilled water, respectively.

Animals and Treatments

All procedures involving animals used in this study were consistent with the guidelines set by the National Institute of Health (NIH publication #85-23, revised 1985) and approved by our Institutional Animal Care and Use Committee. Adult, male Sprague–Dawley rats were purchased from a commercial source and housed in a light- and humidity-controlled animal facility at least 2 days before experiments. The animals had free access to food and water.

A total of 40 rats (weight range 227–280 g) were divided into 10 groups, each consisting of four animals. Three groups were used for testing the effects of MP pretreatment on livers following 24 (MP-24), 48 (MP-48), and 96 (MP-96) hours of cold preservation. Similarly, three groups (DMP-24, DMP-48, and DMP-96) were used for testing the effect of DMP pretreatment following identical durations of cold preservation. Corresponding untreated control groups (Control-24, Control-48, and Control-96, respectively) were run as a reference at each time point. Finally, to determine baseline levels of markers measured, a control group with no cold preservation (Control-0) was included in the study.

The number of animals for each individual group was selected *a priori*, based on power analysis for a 3 × 3 factorial ANOVA design (three drug treatments × three ischemia times) using a Java applet (18). Because the main objective of the study was to investigate the KC activation, the calculations were based on the variability of KC markers (TNF-α and acid phosphatase levels). Literature data on the serial measurements of these two factors under conditions similar to our study were lacking. Therefore, based on the data reported for single-point estimates of the markers in the perfusate of isolated livers subjected to cold ischemia (19), a variability of ~40% was used. Additionally, a type I error of 0.05 (two-tailed) and a power of 80% for detection of a 30% difference between the treatment groups were assumed. This calculation resulted in an estimated sample size of 4 rats for each of the nine individual groups or cells.

The MP and DMP groups received single 5-mg/kg doses (MP equivalent) of MP or DMP, respectively, via their tail veins 2 h before liver isolation. The control groups received similar volumes of sterile saline solution instead. The selection of the dose was based on our previous study (9) on the hepatic concentrations of preformed and regenerated MP after a 5-mg/kg dose and pharmacodynamic studies (20) showing near complete occupancy of the glucocorticoid receptors in the liver of rats treated with a dose of 2 mg/kg of MP.

Liver Harvest, Preservation, and Reperfusion

Two hours following drug or saline administration, rats were anesthetized with an intramuscular injection of ketamine:xylazine mixture (80:12 mg/kg). The techniques used for isolation and cannulation of livers have been a minor modification of methods reported by us before (21,22). Briefly, the bile duct, hepatic portal vein (inlet), and the suprahepatic vena cava (outlet) were cannulated while the liver was perfused with cold (4°C) UW solution. The perfusion was continued for 15 min at a flow rate of 5 ml/min, following which the liver was gently excised and cleaned in an ice-cold saline solution to remove the blood and other tissue debris on the surface. To simulate cold preservation, the inlet and outlet catheters were closed with a clip, and the harvested liver was stored in 50 ml of UW solution at 4°C.

Following 24, 48, or 96 h of cold preservation, the livers were washed with 20 ml of lactated Ringer's solution to remove the UW solution. Then, the livers were mounted onto a water-jacketed, all-glass perfusion system (Radnoti Glass Technology Inc., Monrovia, CA) kept at 37°C. The perfusion was performed in a recirculating manner with 150 ml of perfusate for 3 h. The perfusate was Krebs–Henseleit bicarbonate buffer (pH 7.4) fortified with 1.2 g/L glucose and 75 mg/ml of sodium taurocholate, which was oxygenated with a 95:5 oxygen:carbon dioxide mixture. The perfusate flow rate was maintained at 30 ml/min (3–4 ml/min/g of liver weight). Following commencement of reperfusion, the livers were allowed to stabilize for ~10 min before start of the experiment.

Sample Collection

At the end of cold preservation period, the initial 1-ml samples of the effluent produced during the wash with lactated Ringer's solution were collected for analysis of TNF-α. Thereafter, samples (1.5 ml) of the perfusate were collected at 0 (before start), 15, 30, 45, 60, 80, 100, 120, 150, and 180 min following the start of the recirculating perfusion. An aliquot (1 ml) of each sample was stored at –80°C for measurement of TNF-α, AST, and ACP, and the remaining volume (0.5 ml) was kept at 4°C for measurement of ALT and LDH within a week. Inlet pressure was monitored continuously with a pressure transducer and was recorded at each sampling point. Bile was collected in preweighed microcentrifuge tubes at 30-min intervals. Following reperfusion for 3 h, the liver was blotted dry, weighed, and stored at –80°C for measurement of DMP and/or free MP concentration. Bile samples were also stored at −80°C.

Sample Analysis

The concentrations of TNF-α in the perfusate samples were quantified using a commercial ultrasensitive ELISA kit. The TNF-α assay uses a 100-μl sample and has a minimum detectable limit of 0.7 pg/ml with an intra- and interassay precision coefficient of variation (CV) of <7%. The ALT, AST, ACP, and LDH levels in the perfusate were all quantified using commercially available kits based on the instructions of the manufacturers.

The livers were homogenized with 3 volumes of 2% (v/v) acetic acid, and the homogenate was used for the HPLC analysis of DMP and/or free MP. The concentrations of free MP were determined using a reversed-phase HPLC method

(23) modified for measurement of MP in tissue homogenates (9). The assay had a limit of quantitation of 0.1 μg/ml of homogenate based on a 0.5-ml homogenate sample. The measurement of DMP in the liver homogenate was done by a minor modification (22) of a size-exclusion assay described before (9). The lower limit of quantification of this assay was 1 μg/ml of homogenate based on a 100-μl homogenate sample.

Data Analysis

The perfusate concentrations of TNF-α and other indices of graft injury (namely ACP, ALT, and AST) were plotted against the time of perfusion. A similar plot was also constructed for the inlet pressure readings. The areas under the curves (AUCs) for these plots were then calculated by linear trapezoidal rule. The percentage inhibition of marker levels as a result of MP or DMP pretreatment was estimated for each preservation time according to the following equation:

$$\%\text{Inhibition} = \frac{AUC_{Control} - AUC_{MP/DMP}}{AUC_{Control}} \times 100$$

where $AUC_{Control}$ and $AUC_{MP/DMP}$ refer to the AUC of the marker in livers harvested from untreated animals and animals treated with MP or DMP, respectively. The average portal resistance during the 0- to 180-min perfusion period (PR) was estimated from the following equation:

$$\text{PR (mmHg} \cdot \text{min} \cdot \text{g/ml)} = \frac{\text{Average Portal Pressure (mmHg)}}{\text{Perfusate Flow Rate (ml/min/g liver)}}$$

where the average portal pressure during the perfusion period (0–180 min) was first estimated by dividing the pressure AUC by the perfusion time (180 min).

The effects of preservation time (24, 48, or 96 h) and drug pretreatment (Control, MP, or DMP) on the estimated parameters were examined statistically using a two-way ANOVA. For the bile flow rates, because of the presence of three factors (drug treatment, time of cold ischemia, and collection intervals), the effect of treatment and collection intervals was determined at each time of preservation using a two-way ANOVA. The effect of duration of preservation on the hepatic concentrations of DMP and regenerated MP in DMP groups was determined using a one-way ANOVA. In all significant cases, *post-hoc* analysis using Scheffe's *F* test was used to test the pairwise comparison of means. All tests were performed at a significance level (α) of 0.05. Data are presented as mean ± SD.

RESULTS

The concentrations of TNF-α in the effluent samples obtained during the initial flush of livers with lactated Ringer's solution, which are related to cold ischemia damage, are shown in Fig. 1. The TNF-α concentrations in the flush solution of unpreserved control livers (Control-0) were close to zero. However, measurable concentrations of TNF-α were found in all the preserved livers (Fig. 1). Generally, an increase in the preservation time resulted in a significant ($p < 0.0001$, two-way ANOVA) increase in the TNF-α concentrations in the flush solution (Fig. 1). Pairwise comparison of the preservation time groups, however, showed that although the large differences between the 96-h group and both of the other two time groups were significant ($p < 0.001$), the relatively smaller differences between the 24- and 48-h preserved livers did not reach statistical significance (Fig. 1). Additionally, DMP treatment showed a trend toward a reduction in TNF-α concentrations at all the time points, whereas MP pretreatment showed a similar trend only at 24 and 48 h of preservation (Fig. 1). Nevertheless, because of substantial variability in the data, in particular for the 96-h preserved group, the differences among the three treatment groups did not reach statistical difference ($p = 0.1647$; two-way ANOVA).

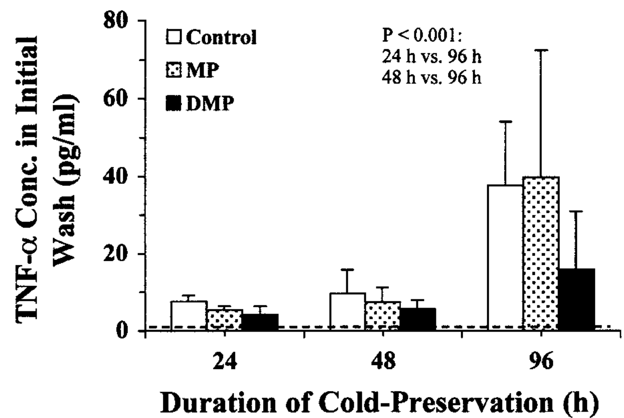

Fig. 1. The concentrations of TNF-α in the effluent samples obtained during the initial flush of cold-preserved livers with lactated Ringer's solution following 24, 48, or 96 h of cold preservation. The rats were pretreated intravenously, 2 h before liver harvest, with a single 5 mg/kg dose (MP equivalent) of MP or DMP or with saline (Control) (n = 4/group). The dashed line indicates the baseline value in unpreserved livers (Control-0). Columns and bars represent mean and SD values, respectively. Statistical comparisons are based on two-way ANOVA with subsequent Scheffe's *F* test.

The plots of perfusate concentrations of TNF-α against the perfusion time are shown in Fig. 2. The release of TNF-α during the perfusion is related to the reperfusion damage. The TNF-α perfusate concentrations of unpreserved livers (Control-0) were relatively low (<30 pg/ml) during the entire perfusion period (Fig. 2). After 24 h of preservation of untreated livers (Control-24), reperfusion resulted in a delay of ~30 min before the perfusate concentrations of TNF-α started to rise, reaching a plateau of ~130 pg/ml at 100 to 180 min (Fig. 2, top). A similar profile was seen for the control livers preserved for 48 h (Fig. 2, middle), albeit both the time when the concentration of TNF-α started to rise (45 min) and the time to reach plateau (120 min) were delayed relative to untreated livers preserved for 24 h (Fig. 2, top). However, for control livers subjected to 96 h of cold preservation (Fig. 2, bottom), the time course of TNF-α in the perfusate had a different shape: there was no delay in the appearance of measurable perfusate concentrations of TNF-α after the reperfusion started (Fig. 2, bottom). Additionally, the TNF-α concentrations increased almost linearly without an apparent plateau during the entire period of perfusion (Fig. 2, bottom). In all three preservation time points, pretreatment of animals with MP or DMP appeared to reduce the concentrations of TNF-α in the perfusate, although the degree of reduction was different for MP and DMP and was dependent on the preservation time (Fig. 2).

The AUCs of the plots of TNF-α perfusate concentration vs. perfusion time are presented in Fig. 3. The baseline TNF-α

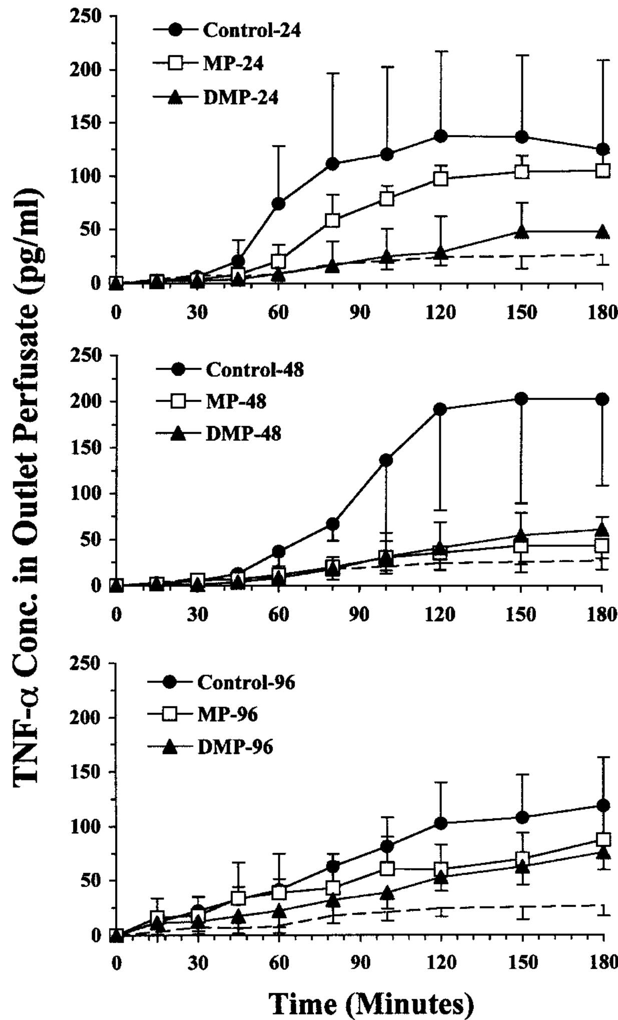

Fig. 2. The concentration–time courses of TNF-α in the outlet perfusate of livers following 24 (top), 48 (middle), or 96 (bottom) h of cold preservation. The rats were pretreated intravenously, 2 h before liver harvest, with a single 5 mg/kg dose (MP equivalent) of MP or DMP or with saline (Control) ($n = 4$/group). The dashed lines indicate the baseline values in unpreserved livers (Control-0). Symbols and bars represent mean and SD values, respectively.

AUC value (2,970 ± 1,050 pg·min/ml) in unpreserved control livers (Control-0) was substantially (four- to sixfold) lower than those for the control livers subjected to preservation (Fig. 3), indicating that the high concentrations of TNF-α in the perfusate of preserved livers resulted from cold preservation followed by reperfusion. Overall, the preservation time (24, 48, or 96 h) did not significantly affect the TNF-α AUC values ($p > 0.90$; two-way ANOVA). However, pretreatment of rats with either MP or DMP significantly ($p < 0.0001$, two-way ANOVA) reduced the TNF-α AUC compared with their respective control groups (Fig. 3). Although DMP appeared to have a higher degree of inhibitory effect than MP at 24 and 96 h (Fig. 3), the differences between the two groups were not significant ($p > 0.05$). After 24 h of preservation, MP and DMP pretreatment, respectively, resulted in 33.9 ± 7.7% and 74.5 ± 15.8% decreases in the TNF-α AUC. The inhibitory percentages for MP and DMP, respectively, were 77.5 ± 10.2% and 74.8 ± 12.6% for the 48-h group and 28.9 ± 35.8% and 44.3 ± 17.3% for the 96-h group.

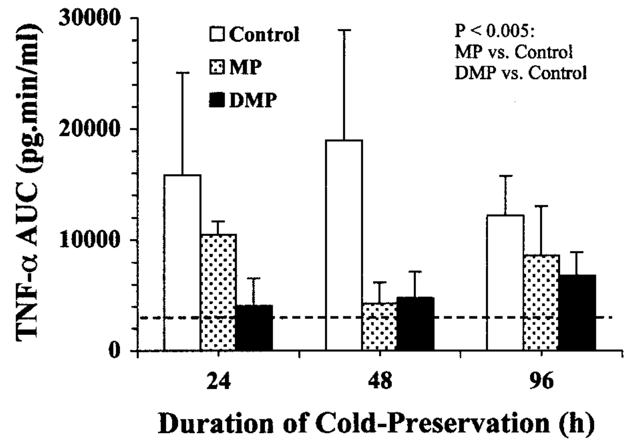

Fig. 3. Perfusate AUC of TNF-α vs. duration of cold preservation for control, MP, and DMP groups following 24, 48, or 96 h of cold-preservation. The rats were pretreated intravenously, 2 h before liver harvest, with a single 5 mg/kg dose (MP equivalent) of MP or DMP or with saline (Control) ($n = 4$/group). The dashed line indicates the baseline value in unpreserved livers (Control-0). Columns and bars represent mean and SD values, respectively. Statistical comparisons are based on two-way ANOVA with subsequent Scheffe's F test.

The AUC values of acid phosphatase, which is another KC marker (13), during the perfusion time (0–180 min) are shown in Fig. 4. Also depicted in the figure is the AUC for the unpreserved control livers (Control-0), which was 246 ± 88 IU·min/ml. Generally, an increase in the preservation time resulted in a significant ($p < 0.001$) increase in the AUC of this enzyme. However, only the enzyme AUCs in the 96-h group were markedly higher than those for the other two groups (Fig. 4). Additionally, pretreatment of the animals had a significant ($p < 0.0004$) effect on the AUC of the enzyme. The highest degree of reduction in the acid phosphatase AUC was observed at 96 h (65.1 ± 12.2%) for MP and at 24 h (44.4

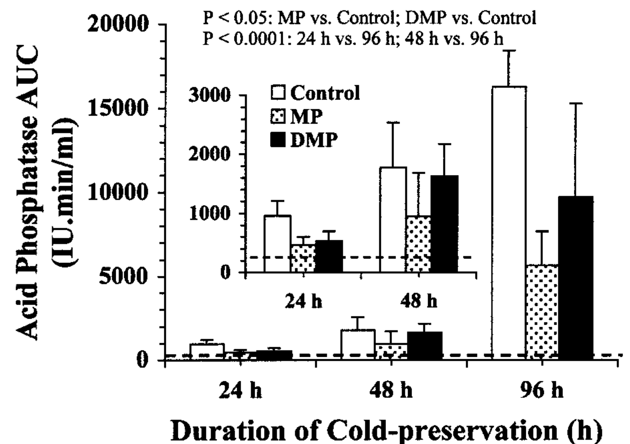

Fig. 4. Perfusate AUC of acid phosphatase vs. duration of cold preservation for control, MP, and DMP groups following 24, 48, or 96 h of cold preservation. The rats were pretreated intravenously, 2 h before liver harvest, with a single 5-mg/kg dose (MP equivalent) of MP or DMP or with saline (Control) ($n = 4$/group). The dashed line indicates the baseline value in unpreserved livers (Control-0). For clarity, the data for the 24- and 48-h groups are also presented in the inset. Columns and bars represent mean and SD values, respectively. Statistical comparisons are based on two-way ANOVA with subsequent Scheffe's F test.

± 17.5%) for DMP pretreated groups (Fig. 4). Whereas significant differences were found between the MP or DMP pretreatment groups and the control group, there were no significant differences between the MP and DMP groups with regard to the inhibition of acid phosphatase AUC.

The perfusate AUC values of AST and ALT, along with the LDH concentrations at the end of perfusion and average portal resistance values for different groups of livers, are presented in Table I. For comparison, the baseline values in the unpreserved livers are also presented in the table. An increase in the preservation time was associated with a significant ($p < 0.0001$) increase in the AUC of both ALT and AST and the perfusate concentrations of LDH, regardless of drug treatment group (Table I). Pretreatment with MP or DMP did not have any significant effects on the perfusate levels of these enzymes (Table I). As for portal resistance, the presented data are average values between zero and 180 min of perfusion. In all cases, the portal resistance decreased with an increase in the perfusion time (data not shown). However, no significant differences were observed among the preservation times or the treatment groups with regard to this parameter (Table I).

The plots of bile flow rates against the collection intervals are demonstrated in Fig. 5 for livers subjected to 24 (top), 48 (middle), or 96 (bottom) h of cold preservation. Similar plots for the unpreserved livers are also depicted for comparison purposes. Interestingly, the bile flow rates in the control groups were significantly higher than those for the MP or DMP groups subjected to 24 or 48 h of cold preservation (Fig. 5). The lowest bile flow rate was noticed for the DMP group. However, after 96 h of cold preservation, the trend was partially reversed, with the bile flow rates being the lowest for the control group and the highest for the MP-treated group (Fig. 5, bottom). Nevertheless, bile flow rates after 96 h of cold preservation were very low (Fig. 5, bottom).

The hepatic concentrations of DMP and regenerated parent drug following cold preservation and subsequent perfusion are presented in Table II for the DMP group. Although the livers were harvested 2 h following the administration of DMP in all the cases, there were significant ($p < 0.02$) differences in the concentrations of DMP among the livers subjected to different lengths of preservation. Generally, the concentrations of DMP decreased with an increase in the preservation time. However, pairwise comparison of the means showed a significant difference only between the 24- and 96-h preserved groups (Table II). Although the regenerated MP was detectable in the liver of DMP-injected rats, its concentration was not significantly different among the three preservation time groups (Table II). Following injections of equivalent doses of the parent drug, the concentrations of MP in the liver were below the limit of quantification of the assay (<0.1 μg/ml) for all the MP-treated groups.

DISCUSSION

Donor pretreatment is a relatively new approach among efforts to reduce preservation injury to the liver. Based on this approach, liver donors are pretreated with a variety of agents that are capable of protecting the liver during the harvest and subsequent cold ischemia–reperfusion damage (4,11,15,24). Glucocorticoids such as MP have long been used as immunosuppressants administered to the recipients after the liver transplantation for prevention of graft rejection and/or in the treatment of acute rejection (25,26). We hypothesized that MP or its prodrug administered to donors before liver harvest may reduce the KC-mediated preservation damage. Indeed, our results (Figs. 1–3) with regard to TNF-α, a cytokine mainly released from the activated KCs of the liver, are in agreement with this hypothesis. Both MP and DMP appeared to reduce the TNF-α levels in both the initial wash (Fig. 1) and after reperfusion (Figs. 2 and 3), although only the TNF-α levels after reperfusion reached statistical significance. Additionally, the inhibitory effect of MP or DMP on the levels of ACP, a marker for KC activation (13), further supports our hypothesis (Fig. 4).

Relative to the substantial effects of MP or DMP pretreatment on the TNF-α levels after liver perfusion (Figs. 2 and 3), the pretreatment effects on the release of the cytokine in the initial flush (Fig. 1) were minor, if any. This is not surprising because cold ischemia *per se* has minimal effects on the activation of KCs. In contrast, reperfusion, preceded by cold ischemia, causes pronounced activation of these cells (3,11,14). Consequently, agents that attenuate KC activation, such as MP, are expected to exhibit their main effects during the reperfusion phase (Figs. 2 and 3). Consistent with our results in cold ischemia–reperfusion, MP pretreatment in a warm ischemia model improved hepatic protein synthesis only during the reperfusion period and not in the ischemic phase (7). Nevertheless, the source of TNF-α in the flush solution (Fig. 1) may be shedding from its transmembrane form (27) rather than KC activation.

Despite the positive effects of MP or DMP pretreatment on KC activity markers (Figs. 1–4), the enzyme levels attributed to parenchymal cell viability were not significantly altered by the pretreatments (Table I), suggesting that these pretreatments do not improve the viability of parenchymal cells. However, it is generally believed that the ischemia–reperfusion injury resulting in graft dysfunction after transplantation is mostly, if not totally, caused by the damage to the nonparenchymal cells (14,28). For example, using an experimental liver transplantation model, Marzi *et al.* (28) demonstrated that cold ischemia–reperfusion damage to the nonparenchymal liver cells, and not to the hepatocytes, was predictive of liver transplantation outcome. These authors further showed that nonparenchymal cells lose their viability early following ischemia–reperfusion, as opposed to relatively late damage to the parenchymal cells. Therefore, they suggested that for successful liver transplantation, preservation of nonparenchymal cells, such as endothelial cells and KCs, is critical (28).

In addition to KCs, endothelial cells play a major role in the cold ischemia–reperfusion damage to the transplanted livers (1,3). It has been reported that the cold ischemia causes detachment of these cells, which correlates with the length of cold preservation. Subsequent reperfusion of the stored livers causes endothelial cell death by apoptosis and necrosis (11). Deaciuc *et al.* (12) showed that there is intercellular communication among the liver cells, where activated KCs could reduce the viability and function of endothelial cells. Additionally, agents that inhibited the KC activation were shown to reduce the endothelial cell damage via KCs (12). In our studies reported here, the beneficial effects of donor pretreatment with MP or DMP on the ischemia–reperfusion activation of KCs are clearly shown. However, the possible effects

Table I. The Average (SD) Values of Cellular and Hemodynamic Markers in Rat Livers following Pretreatment with a Single 5 mg/kg (MP Equivalent) Dose of MP or DMP or with Saline (Control) and Cold Preservation for 24, 48, or 96 h (n = 4/Group)

Marker	Unpreserved control	Duration of preservation (h)[d]								
		24			48			96		
		Control	MP	DMP	Control	MP	DMP	Control	MP	DMP
ALT AUC[a]	1,800 (529)	4,490 (2,190)	8,890 (6,020)	6,280 (1,950)	7,310 (2,080)	7,190 (1,970)	10,400 (2,260)	13,600 (2,460)	14,700 (1,360)	15,600 (2,320)
AST AUC[a]	2,420 (981)	6,700 (2,520)	7,610 (1,660)	12,500 (4,400)	12,800 (4,100)	8,890 (2,700)	19,300 (1,620)	21,200 (8,030)	21,400 (6,050)	22,300 (8,860)
LDH Conc.[b]	128 (36)	532 (212)	316 (113)	781 (212)	1,550 (587)	2,370 (1,410)	3,780 (1,260)	5,980 (4,500)	5,140 (1,750)	5,300 (1,780)
Portal resistance[c]	0.424 (0.195)	1.16 (0.49)	0.593 (0.273)	1.20 (0.60)	0.895 (0.445)	1.40 (1.19)	0.960 (0.633)	1.34 (0.74)	0.768 (0.510)	1.15 (0.52)

[a] IU · min/ml.
[b] IU/ml.
[c] mmHg · min · g/ml.
[d] For ALT and AST, the 24- and 48-h data were significantly (<0.003) different from the 96-h data. For LDH, all the preservation time groups were significantly (<0.04) different from each other.

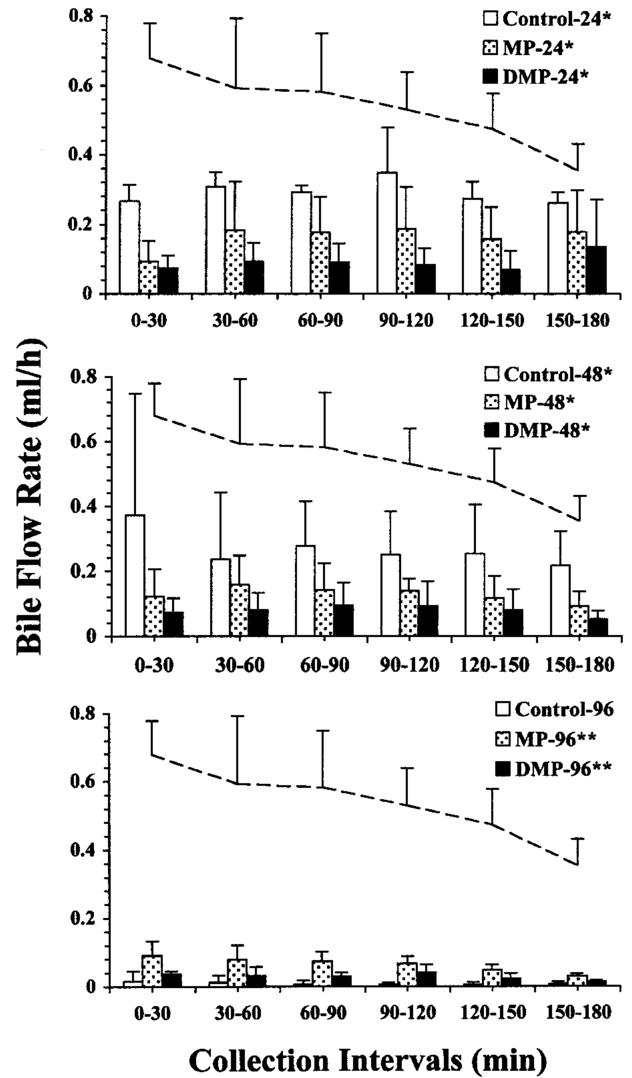

Fig. 5. Bile flow rates at different collection intervals in livers preserved for 24 (top), 48 (middle), or 96 (bottom) h. Rats were pretreated, 2 h before liver harvest, with a 5 mg/kg dose (MP equivalent) of MP or DMP or with saline (Control) (n = 4/group). The dashed lines indicate the baseline values in unpreserved livers (Control-0). Columns and bars represent mean and SD values, respectively. Statistical comparisons are based on two-way ANOVA with subsequent Scheffe's F test: *significant differences among all three treatment groups for the 24- and 48-h preservation times; **significant differences between the treated (MP or DMP) and untreated (Control) groups for the 96-h preservation time.

of these pretreatments on endothelial cells cannot be elucidated from these data. Further studies, using markers specific for the viability of endothelial cells, such as hyaluronic acid uptake by the livers (12), are planned to specifically test the effects of MP or DMP pretreatment on the viability of these cells.

In contrast to the positive effects of MP or DMP pretreatment on KC activation, the pretreatments showed negative effects on bile flow rates of livers preserved for 24 or 48 h (Fig. 5, top and middle); only livers preserved for 96 h showed improved bile flow rates as a result of pretreatments (Fig. 5, bottom). At first glance, these results are unexpected because a previous report (29) using continuous cold perfu-

Table II. The Hepatic Concentrations (Average ± SD) of DMP and Regenerated MP after a Single 5 mg/kg Dose (MP Equivalent) of DMP in Livers Subjected to 24, 48, or 96 h of Cold Preservation and Reperfusion (n = 4/Group)

Duration of preservation	Liver concentration (μg/g of liver)	
	DMP	Regenerated MP
24 h	27.0 ± 4.1[a]	0.165 ± 0.070
48 h	21.6 ± 1.3	0.212 ± 0.045
96 h	19.3 ± 3.0[a]	0.134 ± 0.075

[a] Significantly different from each other based on one-way ANOVA followed by Scheffe's *post-hoc* analysis.

sion for preservation of rabbit livers showed an improvement in the bile flow rate of livers that were harvested from animals pretreated with a single 20 mg/kg dose of MP. However, the improvement was observed after 72 h of preservation, which is in agreement with our data for rat livers preserved for 96 h (Fig. 5, bottom). Additionally, our own recent studies using IPRLs (21,22) may shed some light on the apparent discrepancy between the shorter (24 and 48 h) and longer (96 h) preservation times in terms of pretreatment effect. One of these studies (22) showed that in the presence of taurocholic acid in the perfusate, pretreatment of rats with MP at 2 h before the liver harvest resulted in a significant decline in the bile flow rates of unpreserved livers at all the collection intervals. However, MP pretreatment at ≥5 h before liver harvest did not affect the bile flow rate of the livers. This was attributed to the relatively higher hepatic concentrations of MP at the earlier time point (2 h) (22). On the other hand, our other study (21) showed that in the absence of taurocholic acid, MP pretreatment even at 1 h before liver harvest did not reduce the bile flow, suggesting that the effects of MP are bile-acid dependent. Because bile flow in rats consists of two components, bile-acid-dependent and -independent (30), the above data suggest that MP reversibly inhibits the bile-acid-dependent component of bile flow. Indeed, other studies have shown that corticosteroids are substrates for both the organic anion transporter (OAT) (31) and one of the two hepatic subtypes of the organic cation transporter, namely OCT-2 (32), both of which are also responsible for the uptake of bile acids across the sinusoidal membrane. Because our current perfusate contained taurocholic acid, MP or DMP might have reduced the uptake of this bile acid into the hepatocytes, thus reducing the bile-acid-dependent bile flow in the 24- and 48-h preserved livers. This postulate, however, cannot explain the opposite effects observed for the 96-h preserved livers, although it is possible that the low flow rates observed after 96 h are almost entirely bile-acid-independent, and, therefore, not inhibited by MP or its prodrug. The above hypothesis may also explain the apparently higher inhibitory effects of DMP on bile flow rates compared with those of MP (Fig. 5); the hepatic concentrations of the active parent drug are much higher after DMP administration than after MP injection (9). Nevertheless, the effects of DMP or MP on the biliary flow and excretion of xenobiotics need further evaluation.

Our recent studies (9) have shown that DMP, a macromolecular prodrug of MP, preferentially accumulates in the liver and spleen, as opposed to indiscriminate distribution of the parent drug in the body. Consequently, both the systemic (33) and local (hepatic) (22) immunosuppressive effects of DMP were shown to be substantially more intense and sustained, compared with the effects of the parent drug. Therefore, we expected similar trends in the effects of the prodrug and the parent MP in our current hepatic ischemia–reperfusion model. However, our present data (Figs. 1–4 and Table I) do not suggest a substantial difference between DMP and MP using the *in vitro* model used here. This is despite substantially higher hepatic concentrations of MP after the administration of the prodrug (Table II), as opposed to below detection levels of MP after the parent drug injection. Nevertheless, it should be noted that after the administration of DMP, substantial levels of MP (up to 27 μg/g liver) are still available in the liver in the form of the prodrug (Table II). In a liver transplantation model, this preexisting high level of DMP could slowly release MP after the liver is transplanted in the recipient, contributing to the graft survival. Whether pretreatment of donors with DMP is superior to MP with regard to the outcome of liver transplantations will be tested in future studies in a liver transplantation model.

The introduction of UW preservation solution has allowed a dramatic extension of the cold ischemic time for the clinical liver transplantation; although most of the preservation times with this solution are limited to ≤18 h (34), extended times as long as 35 h have been reported (35). The extension of cold ischemia time beyond the current time frames would allow long distance graft transportation and operation of liver transplantation as a semielective procedure. However, an increase in the preservation time has been associated with an increase in the primary graft nonfunction and retransplantation rate (35). Therefore, we used the extended time frame of 24 to 96 h to magnify the injury to KCs in our IPRL model, allowing for detection of the effects of our pretreatment protocols. Long preservation times of 24 to 96 h have also been used by others (14,29,36) in investigations of the effects of various treatments on the cold ischemia injury in the isolated perfused liver model.

In conclusion, studies in an isolated rat liver model showed that pretreatment of rats with methylprednisolone or its dextran prodrug results in a significant inhibition of KC activation induced by cold ischemia–reperfusion. Because KC activation is one of the major factors affecting the survival following liver transplantation, pretreatment of the donors with MP or DMP before liver harvest may favorably influence the outcome of liver transplantation. Further studies using orthotopic liver transplant models are needed to confirm these results.

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REFERENCES

1. J. J. Lemasters and R. G. Thurman. Reperfusion injury after liver preservation for transplantation. *Annu. Rev. Pharmacol. Toxicol.* **37**:327–338 (1997).
2. D. Adams and J. M. Neuberger. Treatment of acute rejection. *Semin. Liver Dis.* **12**:80–87 (1992).
3. S. N. Lichtman and J. J. Lemasters. Role of cytokines and cytokine-producing cells in reperfusion injury to the liver. *Semin. Liver Dis.* **19**:171–187 (1999).
4. P. Schemmer, B. U. Bradford, M. L. Rose, H. Bunzendahl,

J. A. Raleigh, J. J. Lemasters, and R. G. Thurman. Intravenous glycine improves survival in rat liver transplantation. *Am. J. Physiol.* **276**:G924–G932 (1999).
5. K. Kozaki, H. Egawa, L. Bermudez, E. B. Keefe, S. K. So, and C. O. Esquivel. Effects of pentoxifylline pretreatment on Kupffer cells in rat liver transplantation. *Hepatology* **21**:1079–1082 (1995).
6. M. Kukan, K. Vajdova, J. Horecky, A. Nagyova, H. M. Mehendale, and T. Trnovec. Effects of blockade of Kupffer cells by gadolinium chloride on hepatobiliary function in cold ischemia–reperfusion injury of rat liver. *Hepatology* **26**:1250–1257 (1997).
7. J. Fornander, A. Hellman, and P. O. Hasselgren. Effects of methylprednisolone on protein synthesis and blood flow in the postischemic liver. *Circ. Shock* **12**:287–295 (1984).
8. M. Wang, M. Sakon, K. Umeshita, M. Okuyama, K. Shiozaki, H. Nagano, K. Dohno, S. Nakamori, and M. Monden. Prednisolone suppresses ischemia–reperfusion injury of the rat liver by reducing cytokine production and calpain mu activation. *J. Hepatol.* **34**:278–283 (2001).
9. X. Zhang and R. Mehvar. Dextran-methylprednisolone succinate as a prodrug of methylprednisolone: plasma and tissue disposition. *J. Pharm. Sci.* **90**:2078–2087 (2001).
10. I. Ahmed, M. S. Attia, N. Ahmad, J. P. A. Lodge, and D. J. Potts. Use of isolated perfused rat liver model for testing liver preservation solutions. *Transplant. Proc.* **33**:3709–3711 (2001).
11. G. E. Gondolesi, N. Lausada, G. Schinella, A. M. Semplici, M. S. Vidal, G. C. Luna, J. Toledo, P. M. deBuschiazzo, and J. C. Raimondi. Reduction of ischemia–reperfusion injury in parenchymal and nonparenchymal liver cells by donor treatment with DL-alpha-tocopherol prior to organ harvest. *Transplant. Proc.* **34**:1086–1091 (2002).
12. I. V. Deaciuc, G. J. Bagby, M. R. Niesman, N. Skrepnik, and J. J. Spitzer. Modulation of hepatic sinusoidal endothelial cell function by Kupffer cells: an example of intercellular communication in the liver. *Hepatology* **19**:464–470 (1994).
13. H. Nakano, K. Boudjema, E. Alexandre, P. Imbs, M. P. Chenard, P. Wolf, J. Cinqualbre, and D. Jaeck. Protective effects of N-acetylcysteine on hypothermic ischemia–reperfusion injury of rat liver. *Hepatology* **22**:539–545 (1995).
14. J. C. Caldwell-Kenkel, R. G. Thurman, and J. J. Lemasters. Selective loss of nonparenchymal cell viability after cold ischemic storage of rat livers. *Transplantation* **45**:834–837 (1988).
15. T. Higa, M. Shiraishi, S. Hiroyasu, H. Tomori, Y. Okuhama, T. Kusano, and Y. Muto. Effect of exogenous L-arginine for hepatic ischemia–reperfusion injury in an isolated rat liver *in vitro*. *Transplant. Proc.* **30**:3728–3729 (1998).
16. R. Mehvar. Simultaneous analysis of dextran-methylprednisolone succinate, methylprednisolone succinate, and methylprednisolone by size-exclusion chromatography. *J. Pharm. Biomed. Anal.* **19**:785–792 (1999).
17. A. N. Kong and W. J. Jusko. Disposition of methylprednisolone and its sodium succinate prodrug *in vivo* and in perfused liver of rats: nonlinear and sequential first-pass elimination. *J. Pharm. Sci.* **80**:409–415 (1991).
18. R. V. Lenth. Some practical guidelines for effective sample size determination. *Am. Stat.* **55**:187–193 (2001).
19. H. Itasaka, T. Suehiro, S. Wakiyama, K. Yanaga, M. Shimada, and K. Sugimachi. The mechanism of hepatic graft protection against reperfusion injury by prostaglandin E_1. *Surg. Today* **29**:526–532 (1999).
20. E. Mishina, R. Straubinger, N. Pyszczynski, and W. Jusko. Enhancement of tissue delivery and receptor occupancy of methylprednisolone in rats by a liposomal formulation. *Pharm. Res.* **10**:1402–1410 (1993).
21. R. Mehvar and X. P. Zhang. Development and application of an isolated perfused rat liver model to study the stimulation and inhibition of tumor necrosis factor-α production *ex vivo. Pharm. Res.* **19**:47–53 (2002).
22. A. P. Chimalakonda and R. Mehvar. Dextran-methylprednisolone succinate as a prodrug of methylprednisolone: local immunosuppressive effects in liver after systemic administration to rats. *Pharm. Res.* **20**:198–204 (2003).
23. R. Mehvar, R. O. Dann, and D. A. Hoganson. Simultaneous analysis of methylprednisolone, methylprednisolone succinate, and endogenous corticosterone in rat plasma. *J. Pharm. Biomed. Anal.* **22**:1015–1022 (2000).
24. O. Le Moine, H. Louis, A. Demols, F. Desalle, F. Demoor, E. Quertinmont, M. Goldman, and J. Deviere. Cold liver ischemia–reperfusion injury critically depends on liver T cells and is improved by donor pretreatment with interleukin 10 in mice. *Hepatology* **31**:1266–1274 (2000).
25. R. Wiesner, J. Ludwig, R. Krom, J. Steers, M. Porayko, G. Gores, and J. Hay. Treatment of early cellular rejection following liver transplantation with intravenous methylprednisolone. The effect of dose on response. *Transplantation* **58**:1053–1056 (1994).
26. M. S. Cattral, L. B. Lilly, and G. A. Levy. Immunosuppression in liver transplantation. *Semin. Liver Dis.* **20**:523–531 (2000).
27. M. Lutterova, Z. Szatmary, M. Kukan, D. Kuba, and K. Vajdova. Marked difference in tumor necrosis factor-alpha expression in warm ischemia- and cold ischemia–reperfusion of the rat liver. *Cryobiology* **41**:301–314 (2000).
28. I. Marzi, Z. Zhong, J. J. Lemasters, and R. G. Thurman. Evidence that graft survival is not related to parenchymal cell viability in rat liver transplantation. The importance of nonparenchymal cells. *Transplantation* **48**:463–468 (1989).
29. R. Sundberg, S. Lindell, N. V. Jamieson, J. H. Southard, and F. O. Belzer. Effects of chlorpromazine and methylprednisolone on perfusion preservation of rabbit livers. *Cryobiology* **25**:417–424 (1988).
30. J. A. Handler, D. C. Kossor, and R. S. Goldstein. Assessment of hepatobiliary function *in vivo* and *ex vivo* in the rat. *J. Pharmacol. Toxicol. Methods* **31**:11–19 (1994).
31. X. Bossuyt, M. Muller, B. Hagenbuch, and P. J. Meier. Polyspecific drug and steroid clearance by an organic anion transporter of mammalian liver. *J. Pharmacol. Exp. Ther.* **276**:891–896 (1996).
32. X. Wu, R. Kekuda, W. Huang, Y. J. Fei, F. H. Leibach, J. Chen, S. J. Conway, and V. Ganapathy. Identity of the organic cation transporter OCT3 as the extraneuronal monoamine transporter (uptake2) and evidence for the expression of the transporter in the brain. *J. Biol. Chem.* **273**:32776–32786 (1998).
33. R. Mehvar and D. A. Hoganson. Dextran-methylprednisolone succinate as a prodrug of methylprednisolone: immunosuppressive effects after *in vivo* administration to rats. *Pharm. Res.* **17**:1402–1407 (2000).
34. T. Yamada, H. Hirose, Y. Mori, E. Sasaki, A. Onitsuka, M. Hayashi, S. Senga, N. Futamura, K. Sakamoto, T. Sago, M. Yasumura, and H. Iwata. An experimental estimation of the maximum period of liver preservation using dielectric parameters. *Transplant Proc.* **34**:1098–1104 (2002).
35. H. Furukawa, S. Todo, O. Imventarza, A. Casavilla, Y. M. Wu, C. Scotti-Foglieni, B. Broznick, J. Bryant, R. Day, and T. E. Starzl. Effect of cold ischemia time on the early outcome of human hepatic allografts preserved with UW solution. *Transplantation* **51**:1000–1004 (1991).
36. K. Claesson, S. Lindell, J. H. Southard, and F. O. Belzer. Chlorpromazine, quinacrine, and verapamil as donor pretreatment in liver preservation, tested in the isolated perfused rat liver. *Cryobiology* **28**:422–427 (1991).