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Assessment of hepatic viability during cold ischemic preservation

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Abstract A reliable and easy method for assessing the viability of a cold ischemia-preserved donor liver prior to transplanation into the recepient is needed. Based on an earlier study, we hypothesized that liver free fatty acid (FFA) leakage into the preservation fluid may be a useful, atraumatic indicator of irreversible ischemic injury. The aim of the present study was to determine the time course and magnitude of liver FFA release into the preservation solution and its correlation with the duration of cold ischemic preservation compatible with survival after transplantation. Rat livers (n = 48) were flushed and preserved with University of Wisconsin (UW) solution at 4 °C for 0, 12, 24, and 48 h. Thereafter, half of the livers were analyzed for preservation fluid FFA (gas-liquid chromatography) and protein. The other half were perfused with Krebs-Henseleit (KH) solution at 37 °C for 1 h. Bile secretion and liver enzyme release (SGOT, SGPT, and LDH) were measured in addition to perfusate FFA and protein. Total FFA in the preservation fluid was 24 μg/g wet tissue after 12 h; it increased sharply 2.6-fold after 24 h and 3.7-fold after 48 h of preservation. Bile production was normal after 12 h of preservation but fell by 20 % and 54 % after 24 h and 48 h, respectively. LDH release rose from a value of 20 U/l at 0 time to 120 U/l and 260 U/l after 24 h and 48 h of preservation. These results suggest that liver viability declines sharply between 12 and 24 h of cold ischemic preservation, which corresponds with a sharp decrease in the 1-week survival from 100 % to 33 % after 12 h and 24 h, respectively, of cold ischemic preservation. We conclude that measuring FFA and LDH in the preservation solution of donor livers may be a useful means of assessing the quality of the cold-preserved liver before insertion into the recipient. We also speculate that a „threshold" FFA level in the UW preservation fluid indicating irreversible damage may be in the order of 35 μg total FFA/g liver. Studies on the clinical applicability of our findings are currently under way.

Key words Liver transplantation, viability testing · Viability testing, liver transplantation

Introduction

To avoid the complications of a failed graft simply because of the poor quality or nonviability of a donor liver, a method to quantitatively assess the viability of a donor liver before transplantation is urgently needed [8]. In an earlier study, we suggested that free fatty acid (FFA) accumulation in the liver during cold ischemic preservation may be indicative of deteriorating hepatic viability [7] and, in a subsequent study, we compared

phospholipid (PL) degradation and FFA accumulation in the rat liver during ex vivo warm (37 °C) and cold (0°–4 °C) ischemia followed by reperfusion at 37 °C [5]. Liver tissue FFA increased sharply two- and threefold after 12 h and 24 h of cold preservation, respectively, followed by a marked decrease at 48 h. The decline in liver tissue FFA coincided with a four- to fivefold increase in FFA release upon reperfusion at 37 °C after preservation. Thus, we hypothesized that tissue FFA leakage into the preservation solution after 24–48 h of cold ischemia may signal irreversible liver damage. In the present study, we examined the relationship between FFA and protein levels in University of Wisconsin (UW) preservation solution and cold ischemia time to determine whether the release of FFA into the preservation fluid may correlate with the limits of cold ischemic preservation of the rat liver. One week after orthotopic transplantation in our laboratory, survival is reportedly 100 % after up to 12 h of preservation in UW solution but only 66 % and 33 % after 18 and 24 h of preservation, respectively [8].

Materials and methods

This protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. „Principles of laboratory animal care" (NIH publication No. 86–23, revised 1985) and specific national laws were followed in the conduct of these studies. All rats were kept on a 12-h light/dark cycle with free access to standard rat chow and water.

Harvesting the liver

Forty-eight male Lewis rats, weighing from 230 to 349 g, were anesthetized with methoxyflurane (Pitmann-Moore, Washington Crossing, N.J.) administered by an open-drop technique. Heparin (400 U) was injected into the penile vein and the peritoneal cavity was opened by a transverse abdominal incision at the level of the xiphoid process. An intramedic PE-10 polyethylene catheter (Clay Adams, Parsippany, N.J.) was inserted into the common bile duct and a 20-gauge catheter into the abdominal aorta. The thoracic cavity was opened, the descending thoracic aorta cross-clamped, and the right atrium incised. Ten milliliters of UW solution at 4 °C was perfused into the abdominal aorta, followed by another 5 ml into the portal vein. The liver was carefully removed and immersed in 100 ml of UW solution.

Preservation and reperfusion protocols

All 48 livers were preserved for 0, 12, 24, and 48 h ($n = 12$ each) in UW solution at 4 °C. Thereafter, 24 livers (6 each after 0, 12, 24, and 48 h of cold ischemic preservation) were assayed for tissue FFA and protein immediately after preservation without reperfusion. The other 24 livers were reperfused for 1 h at 37 °C with Krebs-Henseleit (KH) solution. Measurements were taken of perfusate levels of protein, FFA, serum glutamate-oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT) and lactate dehydrogenase (LDH).

Liver perfusion

The livers were perfused via the portal vein according to the technique used by Mischinger et al. [6] with 250 ml of KH solution containing 5.5 mM glucose, 2 % albumin, and 73 mg/l taurocholic acid for 1 h after 15 min of equilibration. Partial pressures of O_2 and CO_2 and pH of perfusate were measured with an ABL2 blood-gas analyzer (Acid Base Laboratory, Radiometer, Copenhagen, Denmark).

Biochemical measurements

Protein

Protein concentration in the UW and KH solutions was measured following the method of Bradford [1] with a commercial protein assay kit (Bio-Rad protein assay kit, Bio-Rad, Richmond, Calif.) using bovine serum albumin (Sigma Chemical, St. Louis, Mo.) as a protein standard. The total amount of protein found in the solutions was expressed as mg protein/g of liver tissue.

Enzymes

Liver enzymes, SGOT, SGPT, and LDH activities in the preservation solution and perfusate were measured with a Technicon RA-500 analyzer (Technicon Instrument, Tarrytown, N.Y.) using commercially available kits.

Free fatty acid (FFA)

Ten milliliters of the preservation solution and the perfusate were collected for FFA analysis as described by Shiu and Nemoto [10]. Briefly, to avoid oxidation of unsaturated FFA, the samples were immediately extracted with 30 ml of butylated hydroxytoluene (BHT, 10 mg/ml)-containing chloroform-methanol (2:1) containing 200 µg of heneicosanoic acid (Sigma Chemical, St. Louis, Mo.) as an internal standard and homogenized for 30 min at 4 °C with Polytron homogenizer (Brinkmann, Westbury N.Y.). After centrifugation, the organic phase was collected and dried and the lipid dissolved in 1 ml of chloroform-methanol (2:1) for separation on a silica gel thin layer chromatography plate (Whatman LK6DF, Whatman, Clifton, N.J.). Thereafter, the FFA were subjected to methanolysis, dissolved in 50 µl of carbon disulfide, and 1 µl was injected into a gas-liquid chromatograph with a flame ionization detector (Model 3700, Varian, Desplaine, Ill.). A 6' × 1/8" ID glass column packed with SP 2330 on 10/20 Chromosorb (Supelco, Supelco Park, Bellafonte, Pa.) was used.

Statistics

The data were analyzed by an analysis of variance and post-hoc analysis by Fischer's test. The maximum significant P value was 0.05. All the values were expressed as mean ± SD.

Results

Bile production measured during 1 h of reperfusion with KH solution at 37 °C was reduced ($P < 0.05$) from 74.3 ± 4.0 to 50.9 ± 12.8 µl/g per hour ($x \pm$ SEM), or

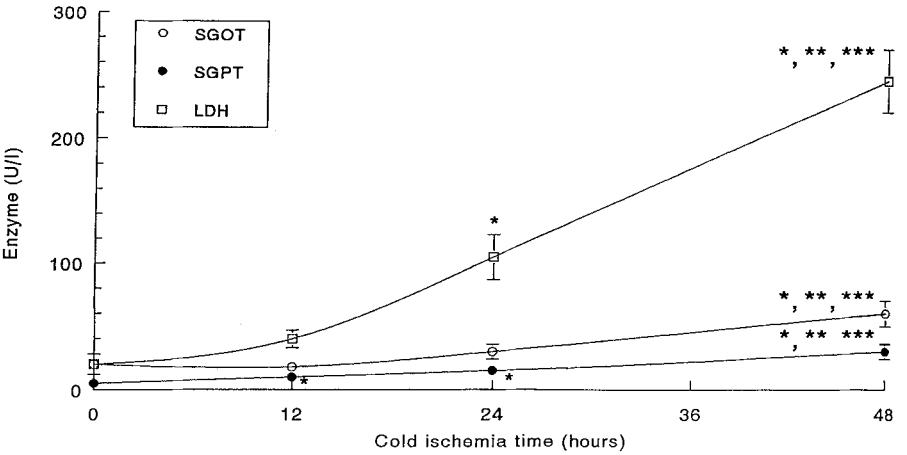

Fig. 1 Hepatocellular enzyme release after 1-h reperfusion of cold-preserved liver. * $P < 0.05$ compared to 0-h value; ** $P < 0.05$ compared to 12-h value; *** $P < 0.05$ compared to 24-h value

about 70 % of normal after 24 h of cold ischemic preservation. After 48 h it fell significantly below the 12-h level to about 50 % of normal (37.2 ± 3.7 µl/g per hour). Thus, liver function was already significantly compromised after 24 h of cold ischemic preservation.

The release of liver enzymes, SGOT, SGPT, and LDH increased progressively during 1 h of reperfusion after longer times of cold ischemic preservation (Fig. 1). Whereas the increase in SGOT and SGPT was relatively modest even after 48 h of cold ischemia, LDH rose linearly by more than tenfold between 12 and 48 h. The earliest significant increase occurred at 12 h for SGPT and not until 48 h for SGOT. The significant increase in LDH at 24 h coincides with the time course of significant reduction in bile production.

Protein release into the UW preservation solution was detectable after 12 h at about 0.08 ± 0.05 mg/g tissue, increased ($P < 0.05$) about tenfold to 0.42 ± 0.08 mg/g tissue, and plateaued at about 0.6 ± 0.09 mg/g liver at 48 h. Protein release into the KH perfusate differed from that seen in the UW solution. With KH at 12 h of perfusion, protein release was highest after 0 h of preservation at 3.0 ± 0.2 mg/g liver, then gradually fell and plateaued at about 2 mg/g liver, where it remained for up to 48 h.

Total FFA release into the UW preservation solution showed a nearly 300 % increase between 12 and 24 h of preservation, and a 350 % increase after 48 h (Table 1). In keeping with the pattern of protein release into the KH perfusate solution, FFA release was maximal immediately after harvesting of the liver, i. e., 0 h preservation at 162.37 ± 58.48 µg/g liver (x ± SD), and fell markedly by 85 % to 26.48 ± 14.52 µg/g liver after 12 h. Thereafter, FFA release into the perfusate progressively rose after 24 and 48 h of preservation to about 90 µg/g liver.

The major FFA released into the UW preservation solution were eicosanoic (20:0) stearic (18:0) erucic (22:1) and nervonic (24:1) acids (Table 2 and Fig. 2), which increased significantly after 24 h as compared to

Table 1 Total free fatty acid *(FFA)* release into University of Wisconsin *(UW)* solution during cold ischemic preservation and into Krebs-Henseleit *(KH)* perfusate during 1-h reperfusion at 37 °C. Values are x ± SD in µg/g liver *(n)*

Preservation time (hours)	UW Solution	KH Perfusate
0		162.37 ± 58.48 (6)
12	21.45 ± 9.27 (8)	26.48 ± 14.52 (8)
24	52.56 ± 27.91 (6)	77.29 ± 43.71 (7)
48	73.82 ± 43.60 (7)	90.25 ± 52.53 (5)

Table 2 Free fatty acid (FFA) release into UW solution during cold preservation

FFA	Cold ischemia time (hours)		
	12	24	48
	µg/g liver, mean ± SD (*n*)		
16:0	9.19 ± 5.34 (8)	29.31 ± 5.41 (5)	16.76 ± 8.50 (7)
18:0	3.60 ± 1.98 (8)	15.93 ± 11.56* (6)	24.59 ± 16.67** (5)
20:1	0.03 ± 0.02 (5)	0.28 ± 0.24 (3)	0.45 ± 0.45 (5)*
20:0	1.81 ± 1.03 (8)	13.96 ± 8.76** (6)	19.67 ± 15.05** (7)
22:0	4.03 ± 2.00 (8)	5.26 ± 3.45 (7)	7.92 ± 6.83 (7)
22:1	0.61 ± 0.38 (8)	3.37 ± 2.50* (6)	5.68 ± 4.38** (7)
24:1	0.65 ± 0.31 (8)	3.78 ± 2.46** (6)	3.38 ± 2.48** (7)
22:6	1.54 ± 1.08 (8)	3.45 ± 2.55 (5)	2.91 ± 2.32 (6)

* $p < 0.05$; ** $p < 0.01$ versus 12 h

12 h. Eicosanoic acid increased sevenfold from 1.80 µg/g liver at 12 h to 13.96 µg/g liver at 24 h, and tenfold to 19.67 µg/g liver at 48 h. Stearic acid increased sevenfold between 12 and 48 h from 3.6 to 24.60 µg/g liver. Although erucic (22:1) and nervonic (24:1) acidsalso showed nine- and fivefold increases after 48 h, their contribution was not quantitatively as significant as the saturated FFA (20:0 and 18:0).

The release of FFA into the KH perfusion solution was erratic compared to that observed in the UW preservation solution (Table 3). Again, the release of FFA into

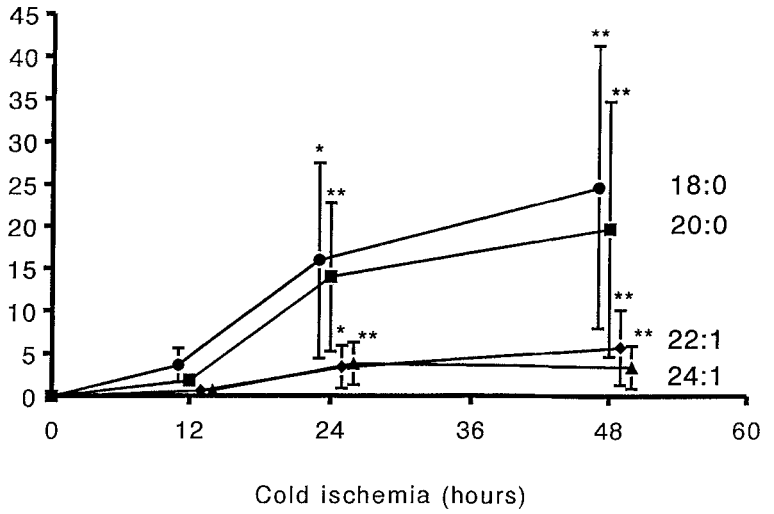

Fig. 2 Release of major free fatty acids (*FFA*) into UW solution during cold preservation. FFA release was expressed as FFA amount from g wet tissue of the liver. * $P < 0.05$ compared to 0-h value; ** $P < 0.01$

Table 3 Free fatty acid (FFA) release into Krebs-Henseleit (KH) solution during 1 h reperfusion following cold preservation for various times in UW solution

FFA	Cold ischemia time (hours)			
	0	12	24	48
	μg/g liver, mean ± SD (*n*)			
16:0	37.31 ± 24.11 (4)	7.78 ± 2.08* (5)	12.11 ± 6.89 (6)	17.35 ± 12.48 (5)
18:0	27.90 ± 10.81 (5)	8.32 ± 5.35* (7)	22.76 ± 13.73 (6)	25.64 ± 18.32 (4)
18:1	2.92 ± 1.73 (6)	2.65 ± 1.91 (5)	1.39 ± 1.38 (3)	3.29 ± 2.85 (4)
20:0	35.30 ± 22.22 (6)	0.00 ± 0.00 (0)	0.39 ± 0.44** (4)	0.64 ± 0.77* (4)
18:3	0.50 ± 0.43 (3)	1.91 ± 1.28 (4)	24.67 ± 23.17* (6)	20.21 ± 13.64* (5)
22:0	21.67 ± 10.54 (6)	0.00 ± 0.00 (0)	11.73 ± 9.65 (5)	14.09 ± 9.65 (5)
22:1	13.84 ± 9.61 (6)	1.40 ± 1.22** (4)	2.80 ± 1.73* (6)	3.61 ± 2.88 (5)
24:1	15.64 ± 8.75 (5)	1.09 ± 0.98** (8)	6.10 ± 5.56 (6)	3.20 ± 1.93 (5)
22:6	26.24 ± 12.47 (6)	8.81 ± 6.15 (8)	7.04 ± 5.28* (7)	6.66 ± 4.75 (5)

*$p < 0.05$; **$p < 0.01$ compared to 0 h values

the perfusate was greatest immediately after harvesting and perfusion (Table 3). The major FFA released into the perfusate were similar to those found in the UW preservation solution. However, the release of FFA into the KH perfusion solution fell after the initial high values.

Discussion

Our measurements of bile production, liver LDH, and FFA release suggest that during cold ischemic preservation, hepatic viability deteriorates sometime between 12 and 24 h. These results corroborate the earlier findings by Okamura et al. [8] from this laboratory of 100 % survival after 12 h of preservation in UW solution but only 66 % and 33 % survival after 18 and 24 h of preservation indicating significant damage with preservation beyond 12 h [8]. Similarly, Sumimoto et al. [11] reported the survival of 3/8 liver grafts at 10 days post-transplant after 24 h of preservation in UW solution. Clearly, our results suggest that FFA and LDH analyses of the UW storage solution may be clinically applicable indicators of the viability of the preserved liver before insertion into the recipient since both can be done atraumatically and rapidly (within 60 min) by spectrophotometry. Although the deterioration of the liver during preservation is probably not a threshold phenomenon, as indicated by the relationship between duration of preservation and percent survival [8, 11], the point of departure from 100 % survival could be considered a „threshold" duration of preservation for clinical transplantation.

Cellular release of LDH is a commonly used measure of cell death in tissue culture, signaling the loss of cellular membrane integrity, and would also seem a logical candidate as an indicator of hepatic viability. LDH increases significantly, more than twofold from 40 to 100 U/l in UW solution, after 24 h of preservation. However, the apparently linear rate of increase between 12 and 24 h may make it difficult to discern a threshold value in the clinical determination of hepatic viability.

Following a slight rise in FFA in UW solution during the first 12 h of preservation to between 2 and 3 μg/g liver, FFA levels increased dramatically between 12 and 24 h, nearly sevenfold to 15 μg/g liver, followed by a slower rate of increase between 24 and 48 h. Thus, there is a distinct point where the leakage of FFA and protein from the cold preserved liver occurs. These results, like those of liver enzymes, indicate that irreversible ischemic damage to the rat liver commences sometime between 12 and 24 h of preservation, coinciding with an abrupt increase in the 1-week mortality of transplanted rats after cold ischemic preservation. A „threshold" FFA level in the UW preservation may be estimated to lie somewhere midway between 20 and 50 μg/g of liver, or about 35 μg/g liver. However, the validity of this estimate would have to be verified with clinical trials.

In order to obtain some idea of the relative amounts of FFA leaking from the tissue, we calculated an FFA leak ratio: the ratio of the amount of FFA released into the UW solution in (μg/g tissue) to the sum of UW solution FFA plus tissue FFA (also in μg/g tissue). The increase in this ratio between 12 and 48 h of cold ischemic preservation for 16:0, 18:0, and 20:0 were 26 %–36 %, 14 %–46 %, and 11 %–47 %, respectively, again suggesting that liver membranes release FFA sometime between12 and 24 h of cold ischemic preservation; this coincides with the tolerable duration of cold ischemic preservation of the rat liver compatible with survival.

Eight different FFA were consistently detectable in the UW preservation solution. Among them, three FFA, namely, 16:0, 18:0, and 20:0, occurred in greatest abundance while four FFA, 20:0, 22:1, 18:0, and 24:1, showed the most dramatic increase during cold ischemia. In rat liver, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) account for 80 % of total PL [2, 9]. Molecular species analysis of these PL showed that 16:0, 18:0, 18:2, 18:3, and 20:4 were most common in PC and PE [3, 4]. Therefore, the origin of these major FFA are apparently derived from PC and PE.

The FFA measured in the liver during cold ischemic preservation in our previous study [5] differ from those detectable in the UW solution. The tissue FFA detectable during cold ischemia, in addition to the saturated FFA, 16:0 and 18:0, included substantial increases in oleic, linoleic, and arachidonic acids, which were inconsistently detectable in the UW solution and were, therefore, not presented in the tables. One obvious reason for the discrepancy between the FFA measured in the liver during cold ischemia and the FFA measured in the UW solution is auto-oxidation in the oxygenated UW solution. This may account for the inconsistent measurements of FFA, namely, oleic, linoleic, and arachidonic acids, as well as for the detection of 22:1, 24:1, and 22:6 in the UW solution, which were undetectable in the liver in our previous studies.

After normothermic reperfusion following cold ischemia, 16:0, 18:0, 20:0, 22:0, 22:1, 24:1, and 22:6 were detectable in the perfusate. Compared with our previous report [5], about 30 % of released FFA leaked out of the cells during reperfusion. The immediately reperfused liver (0 h group) released the largest amount of FFA, and this was followed by an 85 % decrease in FFA release into the perfusate after 12 h of cold ischemia. However, there was a tendency toward an increase in FFA release into the perfusate after 24 and 48 h of cold ischemic preservation to levels of 90 μg/g liver, or about 50 % of the levels released immediately after harvesting. These results suggest that harvesting the liver is a tremendous metabolic shock to the liver, rendering membranes highly permeable. The massive release of FFA immediately after harvesting and reperfusion may be due to the UW solution, which is of intracellular composition and could cause a massive release of FFA tied to the depolarization of the transmembrane potential by the high potassium levels. Accordingly, harvesting the liver with KH buffer, which is of extracellular ionic concentration compared to UW solution, eliminated the large increase in FFA [5].

In summary, without knowing the origin or mechanism of FFA and protein release into the preservation solution, the time course of this process relative to the maximum tolerable duration of cold ischemic preservation of rat livers compatible with survival after transplantation indicates that these variables may be reliable indicators of hepatic viability. The use of a colorimetric method, rather than TLC and GLC for total FFA in the preservation fluid, could improve the rapidity of the analyses to within 1 h for clinical application to determine whether the preserved liver is adequately preserved and viable. Clinical studies to assess the usefulness of FFA analysis in the preservation fluid and to assess the quality of the preserved liver are currently in progress.

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