1196

Fluorometric Study of the Viability of Rat Liver Grafts After Simple Cold Storage With UW Solution Versus Euro-Collins Solution

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ONE of the greatest needs in liver transplantation is a method to determine the quality of preserved hepatic homografts in advance of their insertion. To date, there has been no reliable noninvasive method. As a step toward this objective, an interinstitutional study was undertaken. One of the collaborating groups (Kyoto University) has been able to quantitate derangements of hepatic energy metabolism during liver preservation with Euro-Collins (EC) solution using a noninvasive method of pyridine nucleotide fluorometry.[1-3] However, a direct comparative study between fluorometric measurement and outcome of liver transplantation, and a fluorometric study of preservation with University of Wisconsin (UW) solution have not yet been performed. The other collaborating group (University of Pittsburgh) has demonstrated the superiority of the University of Wisconsin (UW) solution[4-7] to EC solution for preservation of canine[8] and human livers.[9,10] For the present study, workers from Kyoto and Pittsburgh joined to see if the superiority of the livers preserved with UW solution could be confirmed with fluorometry in rat livers reperfused at low temperature. Assessment of the recovery of liver adenine nucleotide under conditions of hypothermic reperfusion following static cold storage was first shown to be feasible by Portegnie-Istace and Lambotte,[11] and previously used by us.[1]

MATERIALS AND METHODS

Liver Procurement and Preparation

Male Lewis rats (Charles River Breeding Labs, Wilmington, Mass) weighing 180 to 230 g were allowed access to water and fasted overnight. All animals were anesthetized by IP injection of 30 mg/kg sodium pentobarbital.

Preparation of isolated perfused livers was done according to a method previously described.[1] Immediately after cannulation of the portal vein, the rat livers were perfused by a nonrecirculating, open-end perfusion system driven by a roller pump. The perfusate at 0°C to 4°C was nonoxygenized UW solution or EC solution with 5000 U/L heparin. The initial flow rate was 20 mL/min for 5 seconds. Thereafter, the rate was maintained at 5 mL/min until the end of the perfusion. The total volume of perfusate used to harvest each organ ranged from 30 to 40 mL. The perfused livers were stored at 0°C to 4°C for up to 72 hours (Table 1).

Change of Liver Weight

Livers were weighed immediately after harvesting and at the end of the preservation period (Fig 1).

Reperfusion

At the end of static storage, reperfusion of the preserved liver was done through the same portal cannula using cold (4°C) solution[12] to which 10 mmol/L glucose and 1000 U/L heparin were added and through which 95% o_2 to 5% co_2 were bubbled. The reperfusion flow was at 20 mL/min for 15 minutes. At the end of the 15-minute reperfusion, 1 to 2 minutes were allowed to elapse so that the reperfusate fluid could drain out of the organ. Then, the probe of the redoximeter was brought softly into contact with the liver surface and measurements were made. All of the reperfusion work and preservation was done in a domestic refrigerator equipped with an electric temperature controller to maintain the temperature in the circuit at 4°C.

Fluorometry

The redoximeter is a microspectrofluorometer (Tateishi Life Science Co., Ltd, Kyoto, Japan) developed for measuring the fluorescence of nicotinamide adenine dinucleotide phosphate, reduced form [NAD(P)H], at 460 nm with a 366-nm excitation wave length using a 100-watt, high-pressure mercury arc as the light source. During reperfusion, the fluorescence which measures the amount of nicotinamide adenine dinucleotide, reduced form (NADH), decreases abruptly for 3 or 4 minutes, then more gradually until a relatively steady state is reached after 15 or 20 minutes (Fig 2A). In a liver that has been preserved for a brief period, reperfused and oxygenated with the Krebs–Henseleit solution, the downward slope of the redoximeter tracing after discontinuance of the reperfusion accurately reflects the relative absence of ischemia (Fig 2B). The rate (velocity) of the slope descent is expressed as RxV. The amplitude of this change is expressed as RxA. The RxV and RxA value obtained by freshly harvested liver with UW solution was employed as a control (Fig 3), and RxA and RxV are expressed as percentage change relative to the control.

Adenine Nucleotides and Energy Charge

Liver tissue of nonpreserved livers after 15-minute reperfusion at 4°C was freeze-clamped by tongs pre-immersed in liquid nitrogen at oxidized state, half-reduced state, and reduced state in the fluorometric trace for the measurement of adenine nucleotides (ATP, ADP, and AMP). Adenine nucleotides were measured by

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Supported by Research Grants from the Veterans Administration and Project Grant No. DK 29961 from the National Institutes of Health, Bethesda, Maryland.

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0041-1345/91/$3.00/+0

Table 1. Changes in Values of Fluorometric Trace

	UW Group			EC Group		
	% Rx*A*	% Rx*V*	*n*	% Rx*A*	% Rx*V*	*n*
0 hours	100	100	(7)	91.7 ± 2.5	96.9 ± 8.2	(6)
6 hours	90.4 ± 6.9	99.5 ± 7.9	(6)	88.8 ± 2.5	88.1 ± 3.5	(6)
9 hours				77.0 ± 1.8†	87.8 ± 6.9	(6)
12 hours	88.0 ± 3.5‡	98.0 ± 3.8‡	(6)	71.3 ± 3.4†	80.6 ± 3.5†	(7)
24 hours	87.5 ± 1.8*‡	98.0 ± 5.1‡	(7)	65.9 ± 3.9†	64.8 ± 8.7†	(8)
48 hours	84.9 ± 4.0*‡	87.6 ± 7.5†	(7)	59.8 ± 3.4†	39.8 ± 9.8†	(6)
72 hours	79.7 ± 6.8*	73.3 ± 9.4*	(6)			

Results shown are expressed as means ± SEM.
*Significantly different from 0 hour of the UW group, $P < .05$.
†Significantly different from 0 hour of the UW group, $P < .01$.
‡Significantly different from the EC group, $P < .01$.

high performance liquid chromatography.[13] Hepatic energy charge levels were calculated by formula by Atkinson[14] as follows: energy charge = (ATP + 0.5 ADP)/(ATP + ADP + AMP).

Histology

At the end of the experiment, liver sections were obtained from the median lobe fixed in formalin, and stained with hematoxylin and eosin.

Liver Transplantation

In 47 separate experiments, orthotopic liver transplantation was performed using Kamada's cuff technique[15] with male Lewis grafts preserved for 6 to 24 hours. Recipients were male Lewis, and survival credit was limited to 1 week. The objective was to make a simple comparison of the survival after transplantation using UW and EC livers that had comparable times of preservation.

Statistical Analysis

All results were expressed as mean ± SE. Statistical significance was determined by Student's *t* test. $P < .05$ was considered significant.

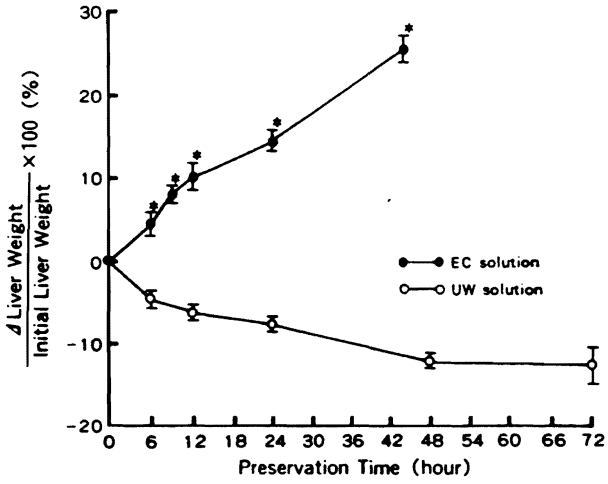

Fig 1. Percentage change in liver weight during the preservation period. Livers preserved in EC solution gained significantly more weight than those preserved in UW solution, $P < .01$.

RESULTS

Liver Weight

The livers preserved in UW solution lost weight during storage, whereas those in EC solution gained weight (Fig 1). The changes correlated with the duration of preservation and were significant at all times (Fig 1).

Redoximeter Comparisons

A large change in fluorescence amplitude (Rx*A*) as well as a high velocity of this change (Rx*V*) when anoxia is imposed, are characteristics of a well-preserved liver[1] (Fig 2). These results with energy charge and fluorometric trace are almost identical to those after discontinuance of reperfusion in control nonpreserved livers (Fig 3) in which the hepatic energy charge levels decreased rapidly from 0.88 to 0.78 at a half-reduced point concomitant with rapid downward slope of the redoximeter trace, and then to 0.64 at the reduced steady state. In UW livers, preserved for 48 hours, more than 85% of the expected (zero time) Rx*A* was retained compared to 60% in EC livers (Fig 4). At the same 48-hour time, Rx*V* retention was 88% of expected with UW livers versus 40% with EC livers (Fig 5). These differences between the 2 groups were significant after 12-, 24-, and 48-hour preservation ($P < .01$).

Histopathology

With light microscopic examination, the hepatocytes appeared to be almost normal in both groups, except for rare mild vacuolization in the 24- and 48-hour EC-preserved livers.

Liver Transplantation

The livers preserved with EC solution were satisfactory for only 6 hours, whereas UW livers permitted 100% survival after preservation for 12 hours and a 65% success rate after 18 hours (Table 2). These results confirmed the superiority of UW that has been reported in dogs and humans.[8–10] However, the redoximeter readings did not accurately predict the quality of the UW-preserved livers in that seemingly satisfactory Rx*A* and Rx*V* values were

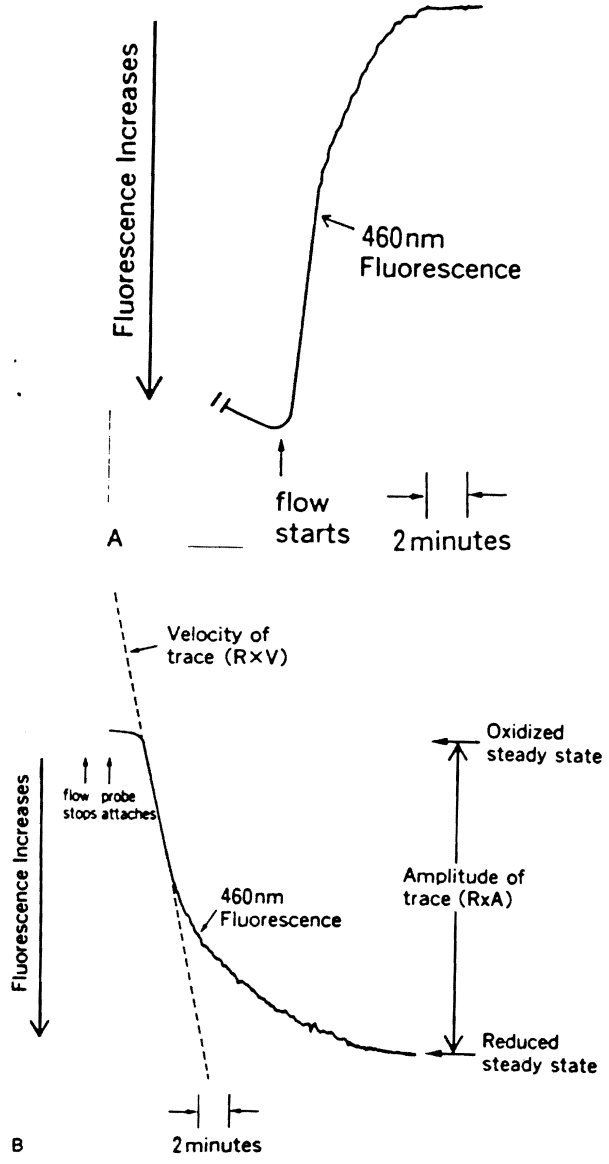

Fig 2. **(A)** Decrease of NADH and establishment of steady state during reperfusion after brief period of preservation of UW liver. **(B)** Redoximeter trace and its measurement in the reperfused liver. Rx*A*: the amplitude between the plateaus of oxidized and reduced steady state; Rx*V*: slope or velocity of the trace curve from oxidized state to reduced one.

retained long after the organs ceased to be able to support life after transplantation.

DISCUSSION

Because the maintenance of biologic function depends on a continuous supply of ATP, its absence causes metabolic and physiologic dysfunction in the vital organs. Studies from the Kyoto group have focused on the metabolic derangements in the energy balance necessary for maintaining cellular viability after major hepatectomy, jaundice, shock, and other conditions.[16–21] It was shown that there is a positive correlation among several parameters of energy metabolism, such as mitochondrial redox state (NAD+/NADH), energy charge, and arterial blood ketone

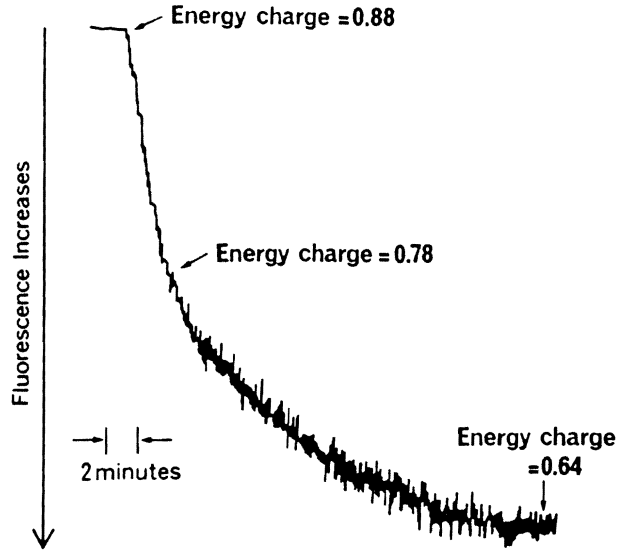

Fig 3. Changes in energy charge and fluorometric trace of a nonpreserved liver after stopping 15-minute reperfusion at 4°C. Energy charge rapidly decreases concomitant with downward slope of the fluorometric trace, indicating virtual absence of ischemic injury. Note similarity to slope in Fig 2.

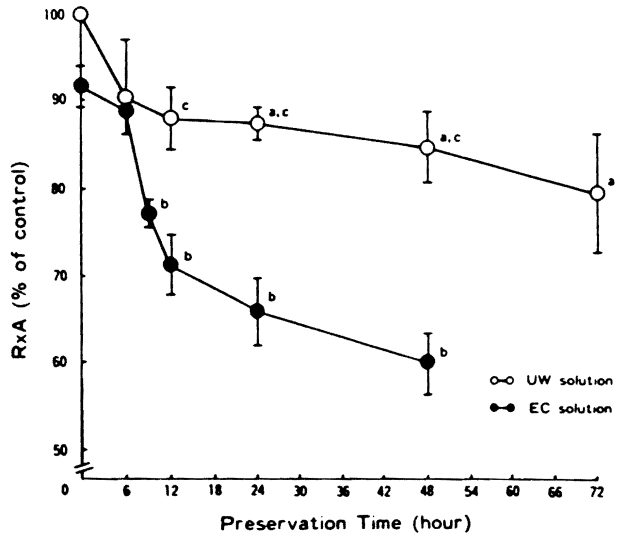

Fig 4. Changes in percentage of Rx*A* (% Rx*A*) in the fluorometric trace. % Rx*A* decreases proportionally to the duration of preservation period in both groups, and shows significantly higher levels in the UW group than in the EC group after 12, 24, and 48 hours of preservation. (a) Significantly different from 0 hour of the UW group, $P < .05$; (b) $P < .01$; (c) significantly different from the EC group, $P < .01$.

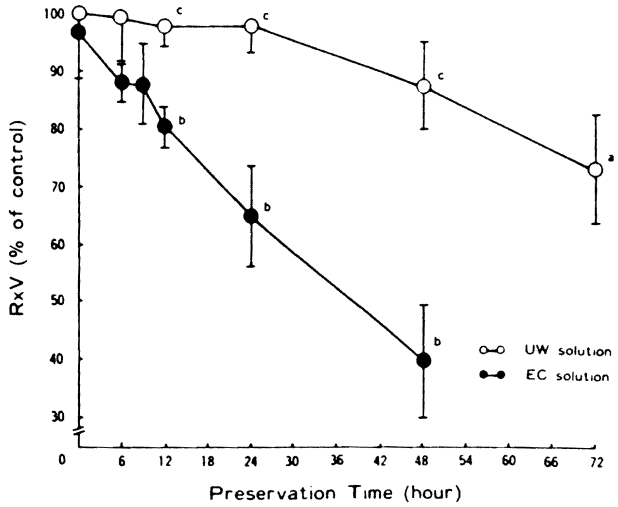

Fig 5. Changes in percentage of Rx*V* (% Rx*V*) in the fluorometric trace. % Rx*V* decreases proportionally to the duration of preservation period in both groups, and shows significantly higher levels in the UW group than in the EC group after 12, 24, and 48 hours of preservation. (a) Significantly different from 0 hour of the UW group, $P < .05$; (b) $P < .01$; (c) significantly different from the EC group, $P < .01$.

body ratio (acetoacetate/3-hydroxybutyrate). Previous reports also showed that hepatic failure after transplantation was linked to a marked decrease in hepatic energy charge, which is associated with the metabolic derangements of the graft.[22,23] Tokunaga et al[1] investigated the mitochondrial redox state of perfused rat liver after simple cold storage with EC solution at low temperature, using the microfluorometric device which was developed in principle by Chance et al[24–26] and applied to experimental animals by the Kyoto group.[1–3] The fluorometric measurements deteriorated steadily with time over 48 hours of preservation and correlated with tissue concentration of NAD, total adenine nucleotides, energy charge level, and mitochondrial phosphorylation rate.[1] However, direct comparative study between fluorometric measurement and outcome of liver transplantation has not yet been performed with either EC or UW solution.

The conditions of fluorometric measurement involved

Table 2. One Week Survival Rate After EC and UW Solution Preservation

Preservation Solution	Preservation Time (h)	Survival Rate at 7 Days (%)
EC	6	6/6 (100)
	9	2/13(15.4)
	12	0/7 (0)
UW	9	6/6 (100)
	12	3/3 (100)
	18	2/3 (66.7)
	24	3/9 (33.3)

reperfusion at 4°C. The possibility of measuring the recovery of energy charge levels during hypothermic reperfusion was demonstrated by Pontegnie-Istace and Lambotte[11] who used blood at 20°C. Our perfusate was asanguinous at the much lower temperature of 4°C. These conditions were accepted after demonstrating that the respiratory chain of liver mitochondria is still active at 4°C (see Fig 2A). The latter demonstration defined the practical possibility in clinical practice of testing livers during cold reperfusion without warming the organs from their static preservation temperature.

The correlation between the redoximeter results and the adequacy of the livers actually transplanted was good when static preservation was done with EC solution. The Rx*A* and Rx*V* remained within normal for 6 hours of preservation, but with rapid deterioration after 9 and 12 hours. In separate experiments, transplantation could be performed successfully with livers preserved for up to 6 hours, but not thereafter.

The same correlation was not seen with livers preserved for long periods with UW solution. As measured by fluorometry, these livers did not deteriorate at all in the first 24 hours, and changes were minor even as late as 3 days. This remarkable maintenance of a nearly normal redox state did not mean that the livers were satisfactory for transplantation, in fact, most of the livers preserved for 24 hours could not support recipient life. This means that redoximeter measurements would not be useful at present in guiding judgment about liver suitability after simple cold storage with UW solution except with relatively short preservation times.

Why the results were so divergent between the fluorometry results and the actual performance of the long-preserved livers is not known. One possibility is that the present fluorometry methods provide an incomplete assessment of the integrity of the mitochondrial respiratory chain, and cannot predict the capacity of enhancement of mitochondrial oxidative phosphorylation which is necessary to restore ATP synthesis after reperfusion. In addition, the fluorometric readings obtained at 4°C may not have revealed microcirculatory damage that could later prevent restoration of normal circulation after transplantation, thus causing a self-perpetuating reperfusion injury of the revascularized graft. In a similar ex vivo model, in which reperfusion was for 90 minutes at 37°C, Ontell et al[27] showed extensive and progressive disruption of the sinusoidal endothelium. Such observations, as well as even more direct evidence,[28–31] have suggested that injury to the microvasculature of livers as well as kidneys[32] may be far more important than previously realized in the etiology of preservation injury. If so, measurement of the hepatocyte redox state after a brief period of hypothermic reperfusion will not be predictive of the organ's true potential for survival. Further investigation of these possibilities will be necessary.

ACKNOWLEDGMENT

We thank Dr Y. Orii, Dr Y. Shimahara, and Dr K. Mori for their helpful advice.

REFERENCES

1. Tokunaga Y, Ozaki N, Wakashiro S, et al: Transplantation 44:701, 1987
2. Tokunaga Y, Ozaki N, Wakashiro S, et al: Transplantation 45:1031, 1988
3. Ozaki N, Tokunaga Y, Wakashiro S, et al: Surgery 104:98, 1988
4. Jamieson NV, Sundberg R, Lindell S, et al: Transplant Proc 20:945, 1988
5. Kalayoglu M, Sollinger WH, Stratta RJ, et al: Lancet 1:617, 1988
6. Jamieson NV, Sundberg R, Lindell S, et al: Transplantation 46:517, 1988
7. Belzer FO, Southard JH: Transplantation 45:673, 1988
8. Todo S, Podesta L, Ueda Y, et al: Clin Transplantation 3:253, 1989
9. Todo S, et al: JAMA 261:711, 1989
10. Todo S, Tzakis A, Starzl TE: Transplantation 46:925, 1988
11. Pontegnie-Istace S, Lambotte L: J Surg Res 23:339, 1977
12. Meijer DKF, Kleulemans K, Mulder GJ: Meth Enzymol 77:81, 1981
13. Schweinsberg PD, Loot TL: J Chromatogr 181:103, 1980
14. Atkinson DE: Biochemistry 7:4030, 1968
15. Kamada N, Calne RY: Transplantation 28:47, 1979
16. Ozawa K, Tanaka J, Ukikusa M, et al: Eur Surg Res 11:61, 1979
17. Tanaka J, Ozawa K, Tobe T, et al: Gastroenterology 76:691, 1979
18. Ukikusa M, Ozawa K, Shimahara Y, et al: Arch Surg 116:781, 1981
19. Kono Y, Ozawa K, Tanaka J, et al: Transplantation 33:150, 1982
20. Shimahara Y, Ozawa K, Ida I, et al: J Surg Res 33:314, 1982
21. Ozawa K, Aoyama H, Yasuda K, et al: Arch Surg 118: 1245, 1983
22. Jujo H, Tanaka K, Ozawa K, et al: Transplant Proc 9:1088, 1987
23. Morimoto T, Ukikusa M, Taki Y, et al: 20:120, 1988
24. Chance B, Cohen P, Jobsis F, et al: Science 137:499, 1962
25. Chance B, Graham N, Mayer D: Rev Sci Instr 42:951, 1974
26. Chance B, Barlow C, Nakase Y, et al: Am J Physiol 235:H809, 1978
27. Ontell S, Makowka L, Ove P, et al: Gastroenterology 95:1617, 1988
28. Otto G, Wolff H, David H: Transplant Proc 16:1247, 1984
29. Iu S, Harvey PRC, Makowka L, et al: Transplantation 44:562, 1987
30. Thurman RG, Marzi I, Seitz G, et al: Transplantation 46:502, 1988
31. Mekeown CMB, Edwards V, Phillips MJ, et al: Transplantation 46:178, 1988
32. Ueda Y, Todo S, Imventarza O, et al: Transplantation 48:913, 1989