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Low-temperature fluorometric technique for evaluating the viability of rat liver grafts after simple cold storage

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Abstract. Time-dependent changes in the viability of rat liver graft during cold preservation with Euro-Collins solution were evaluated with NADH fluorometry. Correlation between the fluorometric analysis, 1-week survival rate after liver transplantation, and mitochondrial ATP synthesis activity in the early phase after transplantation was studied. *Fluorometric study:* Rat livers were preserved at 0°–4°C for 0–48 h in Euro-Collins solution and then reperfused for 15 min with oxygenated Krebs-Henseleit solution at 4°C. The amplitude (R × A) between the oxidized and the reduced steady-state NADH fluorometric trace and the velocity (R × V) of the trace were determined to evaluate the mitochondrial respiratory chain. The R × A and R × V remained at levels higher than 90% of control after 6-h preservation, while the R × A of the 9-h preservation group and the R × V of the 12-h preservation group decreased significantly compared with those of the control and the 6-h preservation group. *Survival study:* a 100% survival rate after transplantation was achieved in the 6-h preservation group, whereas the rates were 18.8% and 0% in the 9- and 12-h preservation groups respectively. These survival rates correlated closely with the time-dependent decrease of the fluorometric parameters. *Study of mitochondrial phosphorylative activity and energy charge 3 h after transplantation:* With fresh grafts, the decrease in hepatic energy charge after transplantation was reduced to 0.79 from the control value of 0.86 by a 30% increase in mitochondrial ATP synthesis ability. When the graft was preserved for 12 h, the energy charge dropped to 0.63 due to lack of the enhancement of ATP synthesis ability. The results of this study indicate a possibility of using fluorometric evaluation of the graft to predict post-transplantation mitochondrial ATP synthesis ability and survival rate.

Key words: Viability test, liver, in the rat – Liver transplantation, in the rat, viability test – Fluorometric study, in rat livers

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A method of evaluating the viability of a preserved liver graft prior to transplantation forms an urgent item on the liver transplantation agenda. A potential method was reported by Tokunaga et al. [17], who investigated pyridine nucleotide fluorometry in perfused rat liver after simple cold storage in relation to other metabolic indices. Their results suggested that the fluorometric method provides a practical and noninvasive means of evaluating donor graft viability. However, a comparative study between fluorometric measurement and outcome of liver transplantation has yet to be done.

Previous studies from our laboratory have shown that an enhancement of mitochondrial ATP synthesis is a prerequisite for recovery from metabolic derangements such as those occurring after major hepatectomy [7] and hemorrhagic shock [18], since a high demand for ATP occurs during the period of decreased energy status. However, hepatic energy charge level and mitochondrial phosphorylative activity after liver transplantation were not investigated in Tokunaga's study.

In the present study, the value of fluorometric measurement before transplantation was compared with graft survival rates after liver transplantation and with hepatic energy charge level and mitochondrial phosphorylative activity in the early phase after transplantation. These results were obtained from three separate sets of experiments.

Materials and methods

Male Lewis rats (Charles River Breeding Laboratories, Wilmington, Mass., USA) weighing 180–230 g were used in all three experiments. Following overnight fasting, all animals were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg body weight).

Fluorometric study

Liver procurement and preparation. Preparation of the isolated perfused liver was done according to a previously reported method [17]. Immediately after cannulation of the portal vein, the liver was per-

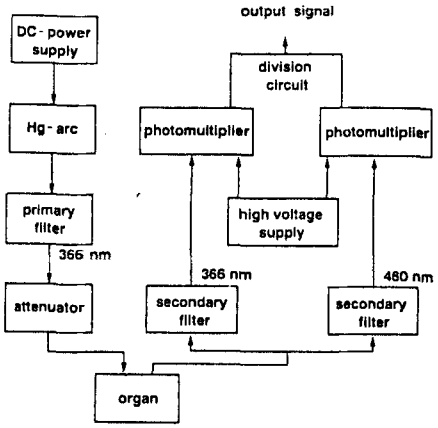

Fig. 1. Essential functional features of the Redoximeter

fused by a non-recirculating, open-end perfusion system driven by a roller pump. The perfusate used was nonoxygenated Euro-Collins solution with 5000 U/l heparin at 0°–4 °C. The initial flow rate was 20 ml/min for 5 s, which was then reduced to 5 ml/min until the end of isolation. The total volume of perfusate used to harvest each organ ranged from 30 ml to 40 ml. The perfused liver was stored at 0°–4 °C for up to 48 h.

Reperfusion. The preserved liver was reperfused via the same portal cannula with Krebs-Henseleit solution [10] at 4 °C to which 10 m*M* glucose and 1000 U/l heparin were added and through which a mixture of 95 % O_2: 5 % CO_2 was bubbled. The rate of reperfusion flow was maintained at 20 ml/min for 15 min, after which 1–2 min was allowed to elapse so that the reperfusion solution could drain from the organ. The probe of the fluorometric device (Redoximeter, Tateishi Life Science Inc., Kyoto, Japan) was then carefully brought into contact with the liver surface and the measurements were made. All the reperfusion work and preservation was done in a domestic refrigerator equipped with an electric temperature control device to maintain the temperature in the circuit at 4 °C.

Fluorometry. The Redoximeter is a microfluorometer which measures the fluorescence of the reduced forms of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) at 460 nm with a 366-nm excitation wavelength [17]. Its essential features are shown in the diagram in Fig. 1. The light source used is a 100-watt high-pressure mercury arc lamp (USH-1020, Ushio Electronic Inc., Japan) with its own high-voltage DC power source. The illumination and detection system consists of 36 optical fibers (single mode silica fiber, 400 μm core diameter, Mitsubishi Wiring Inc., Japan) bundled in a probe 4.2 mm in diameter: 12 of them are for transmitting the excitation light, 6 for detecting the reflected excitation light, and 18 for detecting the emitted fluorescence. The light from the mercury arc is filtered by a primary filter (03FIM028, Mells Griot International Inc.) so that ultraviolet light with a peak at 366 nm is transmitted through the optical fibers to illuminate the organ surface. The probe is brought softly into contact with the liver surface and held in position by supporting shafts. In a preliminary experiment, it was confirmed that any lobe could be used for measurement as long as it was well perfused and oxygenated. Two photomultipliers (R647-04, Hamamatsu) receive the emitted fluorescence and the reflected light through filters (IFW type, Vacuum Optics Inc., and 03FIM028, Mells Griot International Inc., respectively). The signals obtained from the photomultipliers are calibrated to a standard signal by adjusting the photomultipliers' dynode voltage, which is obtained from the high-voltage power supply. The output of each photomultiplier is fed into a chart recorder to monitor the signal changes at 366 nm and 460 nm simultaneously. The final output signal is monitored as the corrected fluorescence obtained from the former two outputs to compensate for changes in fluorescence due to stray light and absorption of excitation light and so on, as shown in Fig. 1, enabling the Redoximeter to provide exact information on the oxidation-reduction status of the perfused liver.

In the preserved liver reperfused and oxygenated with Krebs-Henseleit solution at 4 °C, the downward slope of the Redoximeter trace after discontinuation of reperfusion accurately reflects the development of ischemia. The fluorescence, a measure of the amount of NADH, increases abruptly for 3–4 min, and then gradually declines until a relatively steady state is reached after 15–20 min. The velocity of the slope descent in the rapid phase is expressed as R × V, and the amplitude of this change is expressed as R × A. The R × V and R × A values obtained from the freshly harvested liver stored in Euro-Collins solution were employed as a control and values in the experimental livers were expressed as percentage change compared to the control.

Adenine nucleotides and energy charge of the perfused liver. Specimens were taken from nonpreserved livers and frozen by tongs preimmersed in liquid nitrogen for the measurement of adenine nucleotides (ATP, ADP, and AMP) at four separate times during perfusion: (1) at preperfusion, (2) at fully oxidized state, (3) at half-reduced state, and (4) at fully reduced state on the fluorometric trace. Adenine nucleotides were measured by high-performance liquid chromatography [15]. Hepatic energy charge levels were calculated by the formula by Atkinson [2] as follows: Energy charge = (ATP + 0.5 ADP)/(ATP + ADP + AMP).

Study of liver transplant survival

In a separate set of experiments, 44 orthotopic liver transplantations were performed on male Lewis rats using the cuff technique of Kamada and Calne [6] with grafts preserved in Euro-Collins solution after preservation for 0–12 h. Survivors were defined as recipients who survived for 7 days or more.

Study of mitochondrial phosphorylative activity and energy charge 3 h after transplantation

In another separate set of experiments liver tissue was sampled at 3 h after transplantation or sham operation. The energy charge and phosphorylative activity of isolated mitochondria were compared between livers preserved for 12 h with Euro-Collins solution and nonpreserved livers. The sham operation consisted of laparotomy and closure of the abdominal wall after ascertaining the anesthetized state of the rats by the same methods as used in liver transplantation. Mitochondria were prepared from liver tissue according to the method of Ozawa et al. [14]. Mitochondrial oxidative and phosphorylative activities were measured polarographically with glutamate as a substrate [7]. Mitochondrial protein concentration was determined by the method of Lowry et al. [9] using crystallized bovine serum albumin as a standard.

Statistical analysis

Values were expressed as means ± SEM. Statistical significance was examined using Student's *t*-test. *P* values less than 0.05 were considered to be significant.

Results

Metabolic activity of cold preserved liver as measured fluorometrically

Figure 2 shows the changes in intensity of the Redoximeter trace over time during the reduction and oxidation

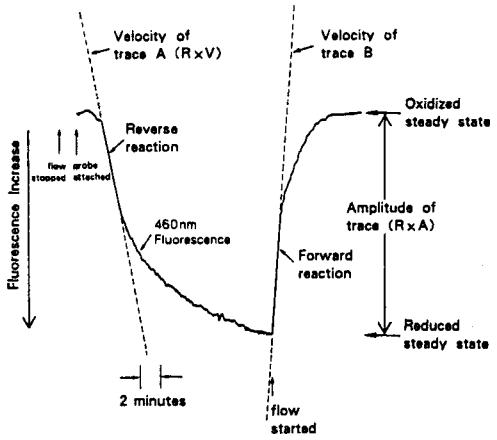

Fig. 2. A typical Redoximeter trace and its measurement in perfused rat liver. *R × A* the amplitude between the plateaux of oxidized and reduced steady state; *Velocity of trace A (R × V)* velocity of the trace curve from oxidized to reduced state; *Velocity of trace B* velocity of the trace curve from reduced to oxidized state

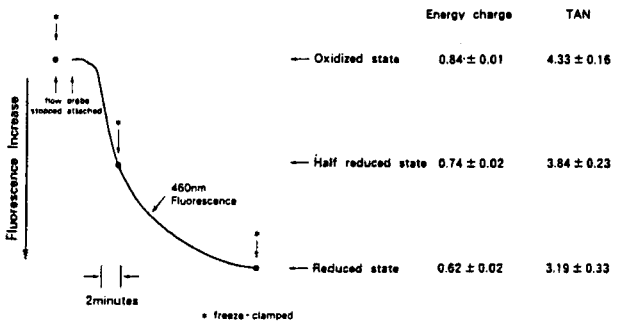

Fig. 3. Changes in energy charge level, total adenine nucleotides (TAN), and fluorometric trace of a non-preserved liver after stopping 15-min perfusion at 4 °C. Results are means ± SEM ($n = 4$)

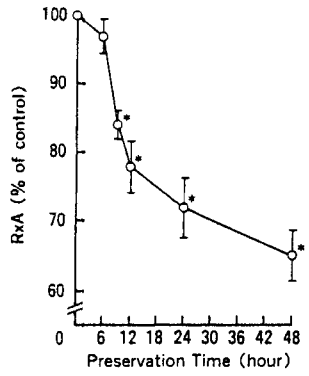

Fig. 4. Changes in percentage of R × A in the fluorometric trace. Results are means ± SEM ($n = 6$). * Significantly different from the control group, $P < 0.01$

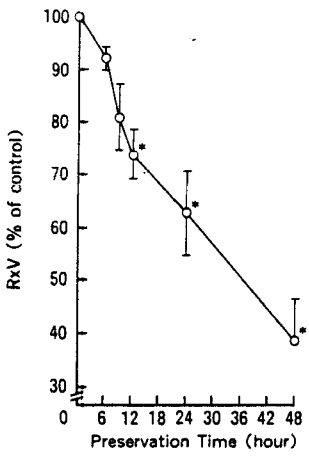

Fig. 5. Changes in percentage of R × V in the fluorometric trace. Results are means ± SEM ($n = 6$). * Significantly different from the control group, $P < 0.01$

reactions of a nonpreserved perfused liver. After the liver was perfused and oyxgenated with Krebs-Henseleit solution at 4 °C for 15 min, the perfusate flow was stopped at the point indicated by the arrow above the left portion of the trace. Fluorescence increased immediately as a result of the reduction of pyridine nucleotides, to reach a reduced steady state of NAD(P)H. When the flow was restarted, the fluorescence decreased abruptly with the shift in oxidized state from NAD(P)H to NAD(P). These fluorometric traces were used to measure R × A and R × V, as discussed in the Methods section of this article.

Figure 3 shows a typical Redoximeter trace during the reduction reaction and the changes in energy charge and total adenine nucleotides (TAN) after discontinuation of perfusion by oxygenated Krebs-Henseleit solution in non-preserved liver at 4 °C. The hepatic energy charge and TAN before perfusion were 0.69 and 3.52, respectively. The energy charge increased to 0.84 at the oxidized state and decreased rapidly to 0.74 concomitant with the rapid downward slope of the Redoximeter trace at the half-reduced state, which further declined to 0.62 when the relatively steady state was reached after 20 min. The Redoximeter trace recorded an abrupt rise of NAD(P)H in liver mitochondria concomitant with the decrease in hepatic energy charge due to the drop in the mitochondrial ability to produce ATP following oxygen deprivation.

Figures 4 and 5 show the changes in R × A and R × V of the cold preserved liver. Both R × A and R × V decreased in accordance with preservation time. Both R × A and R × V values remained at levels of higher than 90 % of the controls during 6-h preservation. The R × A values after preservation of more than 9 h were significantly decreased from the values of both controls and after 6-h preservation. The R × V values after preservation for more than 12 h were significantly decreased compared with the values both of controls and after preservation for 6 h.

Survival of liver transplants

Table 1 shows that successful transplantation using livers preserved with Euro-Collins solution was satisfactorily achieved only in the 6-h preservation group, whereas the 9-h preservation group had only 18.8 % survival and the 12-h preservation group 0 % survival.

Liver transplantation and enhanced mitochondrial phosphorylative activity

Table 2 shows the changes in hepatic energy charge and oxidative phosphorylation activity of the isolated mitochondria from the graft at 3 h after liver transplantation in freshly harvested liver and liver preserved for 12 h.

The respiratory control ratio, state 3 respiration, and phosphorylative activity at 3 h after transplantation of freshly harvested liver were significantly enhanced compared with those in the sham-operated control. Maximal ATP synthesis ability was significantly increased by 33 %, while hepatic energy charge was decreased to 0.79 from

Table 1. Effect of preservation time on survival rates after liver transplantation

Preservation time (h)	No. of rats	No. of survivors[a]	Survival rates (%)
0	8	8	100
6	8	8	100
9	16	3	18.8
12	12	0	0

[a] Survival for more than 7 days

the control value of 0.86. This suggests that mitochondrial phosphorylative activity was enhanced to restore the decreased hepatic energy charge level. By contrast, such enhancement of mitochondrial function was not observed in livers preserved for 12 h in Euro-Collins solution.

Discussion

Studies from our laboratory have focused on the compensatory responses of liver mitochondria to metabolic derangements such as those occurring after major hepatectomy [7], hemorrhagic shock [18], and warm ischemia [8]. These results have shown that the arterial blood ketone body ratio (acetoacetate/3-hydroxybutyrate; AKBR) correlates positively with the hepatic energy charge levels, and that an enhancement of mitochondrial phosphorylative activity is a prerequisite for recovery from various types of borderline injuries. Furthermore, the hepatic energy crisis has been shown to contribute to hepatic failure, hepatic coma, and multiple organ failure [12, 13]. Other reports from our laboratory have also shown that the AKBR provides a clear indication of the hepatic energy status in the critical post-transplantation period [1, 16]. These measurements, however, cannot be applied to the evaluation of harvested grafts, since liver tissue or arterial blood is required.

On the other hand, the mitochondrial redox state after reperfusion was estimated by the microfluorometry technique developed on the basis of principles reported by Chance et al. [4, 5] and applied in experimental studies by our Kyoto group [11, 17]. For the noninvasive method of evaluating donor graft viability, Tokunaga et al. [17] investigated pyridine nucleotide fluorometry in the perfused rat liver at low temperature after simple cold storage in relation to other metabolic indices. Furthermore, as to larger animals, Ozaki et al. [11] reported on the fluorometric technique in perfused porcine liver after simple cold storage at low temperature by administration of fluorocarbon emulsion. These results suggested that this spectrofluorometer might also have potential application in evaluating the viability of a larger organ.

Regarding the reperfusion temperature, the present experiment in low-temperature fluorometry showed that, when the graft liver harvested with cold Euro-Collins solution was perfused with Krebs-Henseleit solution at 4 °C for 15 min, its energy charge level increased from 0.69 to 0.84, followed by a rapid decrease after discontinuance of perfusion (Fig. 3). This indicated that respiratory activity still takes place even at 4 °C. Hence, we proposed the use of low-temperature fluorometry to evaluate graft liver function.

In the fluorometric study, the R × A and R × V of 6-h-preserved livers were similar to those of the controls, and all recipients transplanted with grafts preserved for 6 h survived. By contrast, the R × A of the 9-h preserved liver and the R × V of the 12-h preserved liver decreased significantly, compared with those of the control and the 6-h preserved liver. The 9-h preservation group had 18.8% survival and the 12-h preservation group had 0% survival. These results showed that the values of fluorometric study were in close correlation with these survival rates.

The results given in Table 2 for the successfully transplanted nonpreserved grafts show that the ATP synthesis capacity of the mitochondria in these livers was enhanced significantly, enabling the liver to restore its reduced energy charge level to 0.79 at 3 h, whereas no similar enhancement was observed in the unsuccessfully transplanted 12-h-preserved livers at 3 h. This indicated that the enhancement of mitochondrial ability to synthesize ATP plays an important role in determining whether a liver graft can be successfully transplanted or not. It is known that the catabolic processes are accelerated immediately after rewarming in liver transplantation, due to accumulation of the so-called hepatodepressant factors in the blood, such as lactate and amino acids, during the anhepatic period of liver transplantation. To metabolize these substances rapidly requires the consumption of energy which the enhanced mitochondrial ATP synthesis

Table 2. Changes in energy charge and phosphorylative activity of isolated mitochondria of the transplanted liver (means ± SEM)

	Sham operation ($n = 8$)	Nonpreserved liver ($n = 4$)	Liver preserved for 12 h ($n = 4$)
Energy charge	0.86 ± 0.02	0.79 ± 0.04	0.63 ± 0.03[d,e]
Total adenine nucleotides (mmol/g wet weight)	4.74 ± 0.13	4.55 ± 0.38	2.99 ± 0.32[d,e]
State 3 respiration rate (natom O/min mg protein)	41.1 ± 1.3	56.0 ± 3.8[b]	42.0 ± 2.1[e]
Respiratory control ratio[a]	5.3 ± 0.5	8.8 ± 0.7[b]	6.4 ± 0.3[f]
P/O ratio[b]	2.69 ± 0.09	2.64 ± 0.08	2.76 ± 0.18
Phosphorylation rate[c]	110.3 ± 4.1	147.1 ± 8.4[b]	115.5 ± 7.6[f]

[a] Respiratory control ratio: state 3 respiration rate/state 4 respiration rate
[b] P/O ratio: moles ATP formed per atom of oxygen consumed
[c] Phosphorylation rate: nmol ATP formed per minute per milligram protein
[d] Significantly different from the sham-operated group, $P < 0.01$
[e] Significantly different from the nonpreserved group, $P < 0.01$
[f] Significantly different from the nonpreserved group, $P < 0.05$

can hardly afford to spare. This results in a metabolic overload in the delicate energy balance of the graft liver. An enhancement of mitochondrial phosphorylative activity is thus prerequisite for restoring the reduced energy charge.

As is apparent from Fig. 2, the reduction rate from NAD to NADH (the "reverse" reaction of electron transfer) is rather slow compared with the oxidation rate from NADH to NAD (the "forward" reaction). The reversal of electron transport in the respiratory chain was first verified by Chance and Hollunger [3] to explain the extensive reduction of NAD observed in mitochondria upon addition of non-NAD-linked substrates such as succinate, glycerol phosphate, and fatty acids. The respiratory control is the most important example of the reversibility of the oxidative phosphorylation. Comparative studies on the mitochondria of various organs have shown that this type of NAD reduction is a characteristic shared by intact mitochondria from all sources. The studies make apparent the key role that the reversal in oxidative phosphorylation plays in explaining the puzzling facts surrounding the redox state of mitochondrial NAD. This leads us to think that the reverse reaction of electron transfer in the absence of oxygen may be a clearer indication of the cellular integrity for electron transfer than the forward reaction. The reason for this is that, from the viewpoint of the efficiency of electron transport, the reverse electron transfer requires the full energy of the oxidation-reduction reaction, whereas the forward reaction needs to expend only part of the free energy change required, and hence the reverse electron transfer activity reflected by $R \times A$ and $R \times V$ is more critically dependent on the integrity of the electron carrier state. In the present study, therefore, the values of $R \times A$ and $R \times V$ of the reverse reaction might reflect the function of the respiratory chain.

These results suggest that the significant decrease in $R \times A$ and $R \times V$ indicates derangement of the respiratory chain and lack of enhancement of the mitochondrial ATP synthesis ability after liver transplantation. From the results shown in Figs. 4 and 5, since the 12-h-preservation group had 0% survival, the thresholds for $R \times A$ and $R \times V$ could be regarded as around 80% and 75% of the control values, respectively.

Although almost all rats with nonpreserved grafts survived after fluorometry, the survival rates in those with preserved liver grafts became worse after this measurement than without it. The fluorometry might have had some influence on the organ viability. It is hoped that further improvements to this technique will make it truly noninvasive. The present study leads us to suggest that this spectrofluorometric method has potential for evaluating graft livers by predicting the post-transplantation mitochondrial ATP synthesis ability and the survival rate prior to transplantation.

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