

Correlation of Fluorometric Study for Preserved Rat Liver with Energy Metabolism and Bile Excretion

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Introduction

Liver transplantation is now regarded as a common therapy for certain cases of irreversible hepatic diseases⁹⁾. One of the greatest needs in liver transplantation is a method to determine the quality of preserved donor grafts before transplantation. Liver mitochondrial functions are a sensitive index of the viability of donor grafts, since the main metabolic function of the liver depends on the integrity and biologic activity of cellular mitochondria. Recently a fluorometric method has been used to study viability of donor grafts¹⁵⁾. In this experiment we use a new fluorometric device to measure pyridine nucleotide fluorescence in perfused rat liver. In the fluorometric trace, the amplitude between oxidation and reduction (RxA) and the slope or velocity of the trace curve from oxidation to reduction (RxV) were determined by measuring the fluorescence of NAD (P) H. In comparison with the accepted methods and parameters of evaluating viability of grafts, the fluorometric method has the advantage of being a non-invasive way to assess viability in perfused rat liver. This application of fluorometric study still needs to be established and supplemented in combination with other accepted methods and parameters. In common study there are two ways of investigating viability of grafts. One is to observe the survival of recipients or to measure the liver functional indices after transplantation, the other is to imitate the condition of post-transplantation by loading the liver with some nutritive or medial metabolic substance, and then to observe its functioning. This study use preserved rat liver, perfused by Krebs-Henseleit solution with or without taurocholic acid, in order to investigate the changes in cellular ATP level, energy charge, bile excretion and fluorescence of NAD (P) H, which may indicate the viability of donor liver.

Material and Methods

Liver Preservation: Male Wistar rats weighing 270–330 g were anesthetized by an intraperitoneal injection of 30 mg/kg sodium pentobarbital. After ascertaining anesthetization an ab-

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dominal midline incision and a bilateral transverse incision were performed. The liver, portal vein, inferior vena cava (IVC) and right kidney were exposed by moving the alimentary tract to the left. A silk thread was then placed under the portal vein and hepatic artery. When a 16 gauge needle was inserted into the portal vein 15–20 mm away from the hepatic hilus to proximal, the silk thread was fixed with a knot. Immediately after cannulation of the portal vein, the liver was perfused by cold non-oxygenated Euro-Collins' solution (4°C) with a non-recirculating, open end perfusion system driven by a roller pump. The initial flow rate was 20 ml/min for several seconds until the liver changed became pale. Then, the flow was maintained at 5 ml/min until the end of liver harvesting. The perfused livers were preserved by this Euro-Collins' solution at 0 to 4°C for 6, 12 and 24 hours.

Reperfusion: The preserved liver was reperfused by Krebs-Henseleit solution bubbled with mixed 95% O₂ and 5% CO₂ gas at 32°C, through the same portal cannula, at a perfusate rate of 30 ml/min. In the meantime, the Krebs-Henseleit solution, bubbled with mixed gas of 95% N₂ and 5% CO₂, was prepared for non-oxygen perfusion.

Fluorometry: The use of microfluorometry to observe the changes in oxidation-reduction of pyridine nucleotides has been reported by Chance³⁻⁵) and other investigators^{6,11}). The redoximeter is a microspectrofluorometer (Tateishi Life Science Company, Ltd. of Kyoto, Japan) developed for measuring the fluorescence of NAD (P) H at 460 nm with a 366 nm excitation wave length using a 200-watt high-pressure mercury arc as its light source. Fluorescence went an oxidized steady level after perfusion with 95%O₂-5%CO₂ bubbled perfusate as a result of oxidation of pyridine nucleotides, NAD (P). At 30 minutes, when the perfusate was changed from 95%O₂-5%CO₂ to 95%N₂-5%CO₂ bubbling, the fluorescence increased abruptly in accordance with the anaerobic change of NAD (P) to NAD (P) H and became stable until its full reduction state which occurred within a few minutes. Then, the perfusate was changed back to 95%O₂-5%CO₂, and the fluorescence trace recovered gradually to the full oxidation level. The change of fluorescence in graph from (Fig. 1) was obtained by the device's recorder. RxV is the slope or the velocity of the

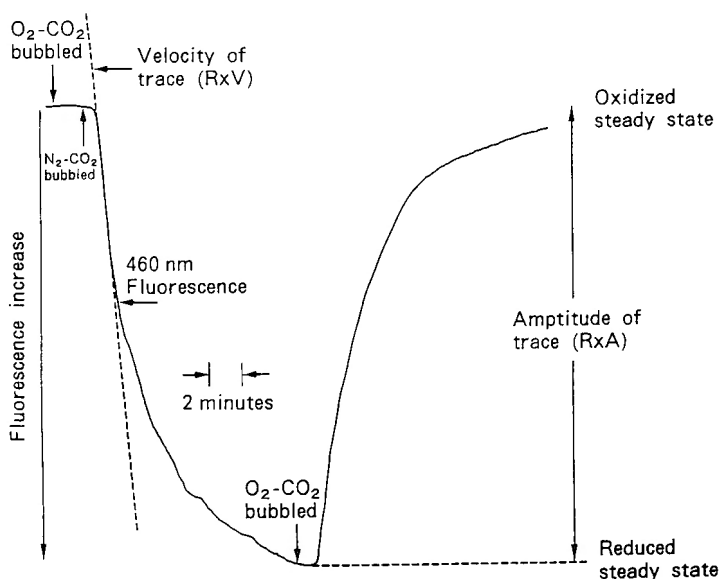


Fig. 1 An example of the Redoximeter trace in perfused rat liver.

fluorometric trace from the oxidation to reduction level; R_{xV} is the amplitude between full oxidation and reduction levels in the fluorometric trace during the anaerobic period. The percentages of R_{xV} and R_{xA} were obtained by comparing them with the control values (no preservation).

Bile Excretion: The livers should be prepared by inserting another thin plastic tube (I.D. 0.28 mm, O.D. 0.61 mm) into the common bile duct, which will enable quantification of bile flow during reperfusion. the perfusate was Krebs-Henseleit solution with 30 μ M of taurocholic acid, bubbled with 95% O_2 and 5% CO_2 .

Tissue Adenine Nucleotides Contents and Energy Charge (EC): After 30 minutes of reperfusion, a piece of liver was sampled with liquid nitrogen, and the amounts of ATP, ADP and AMP

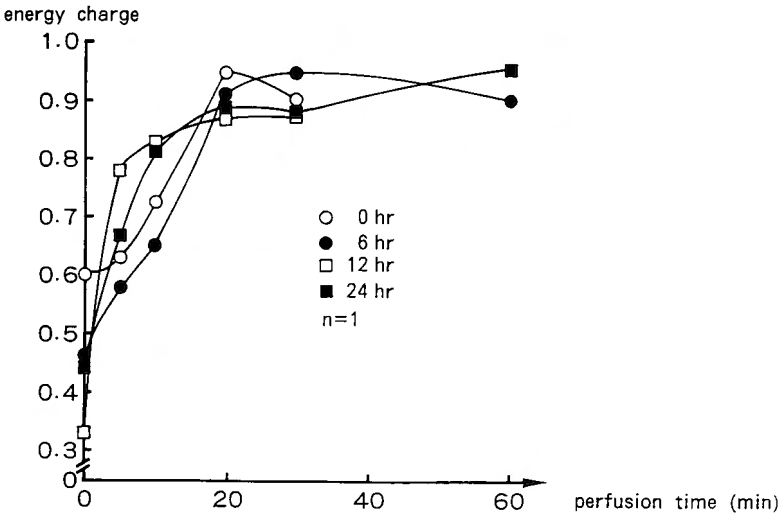


Fig. 2 Energy charge of preserved rat liver with different perfusion times.

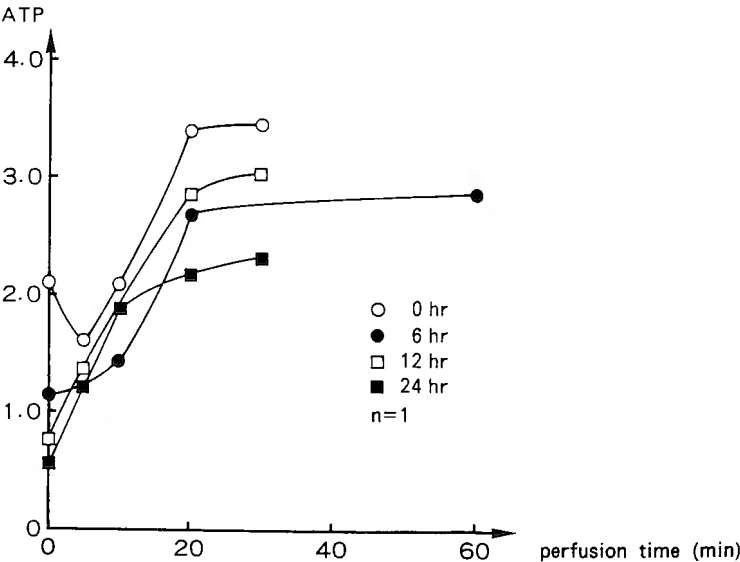


Fig. 3 ATP of preserved rat liver with different perfusion times.

Table 1 Changes in ATP, energy charge (EC), bile excretion (BE) and fluorometry in perfused rat liver after preservation.

preservation time (hr)	Perfusate without taurocholic acid		Perfusate with taurochoilic acid				
	ATP	EC	ATP	EC	BE	fluorometry	
						RxV (%)	RxA (%)
0	2.78±0.31 (4)	0.844±0.04 (4)	2.46±0.15 (8)	0.730±0.02 (8)	2.55±0.25 (11)	100 (5)	100 (5)
6	2.42±0.09 (4)	0.837±0.05 (4)	2.35±0.22 (3)	0.712±0.01 (3)	1.39±0.27 (2)**	80.4±1.4 (3)**	91.0±2.4 (3)*
12	1.98±0.15 (3)	0.837±0.02 (3)	2.04±0.15 (3)	0.717±0.02 (3)	0.98±0.19 (10)**	70.7±6.1 (7)**	89.1±3.5 (7)*
24	1.77±0.44 (3)	0.831±0.03 (3)	1.89±0.44 (4)	1.89±0.44 (4)	0.49±0.09 (6)**	62.9±4.6 (6)**	81.3±6.0 (4)**

*: P<0.05, **: P<0.01 compared with the control (0 hr. preservation).
 Results are expressed in the table as means±SEM with the number of rats in parentheses.
 EC=(ATP+0.5 ADP)/(AMP+ADP+ATP)
 BE.=bile excretion (μl/min./g. liver)

were measured by high-performance liquid chromatography (HPLC)¹²⁾. EC was calculated by the formula of $(\text{ATP} + 0.5 \text{ ADP}) / (\text{AMP} + \text{ADP} + \text{ATP})$, proposed by Atkinson¹⁾.

All results were expressed as means $\pm$ SEM. Statistical significance was determined by student's t-test and values of p less than 0.05 were considered significant.

Results

Figure 2 shows the change of energy charge of rat liver preserved in Euro-Collins' solution at 0–4°C for different lengths of time, then perfused with different lengths of time with Krebs-Henseleit solution at 32°C. Regardless of preservation time, the energy charge increased proportionally with longer, reperfusion, and became stable at 20 to 30 minutes. Figure 3 shows the change of ATP under the same conditions. From 20 to 30 minutes of perfusion the changes were steady. According to this preliminary test, 20 and 30 minutes of reperfusion time were chosen for the flowing study.

Table 1 shows energy charge did not significantly change in rat livers preserved for 0, 6, 12, 24 hours in Euro-Collins' solution at 0–4°C, following reperfusion by Krebs-Henseleit solution at 32°C in each group, with and without taurocholic acid. But the energy charge (0.70–0.73) in the group perfused with taurocholic acid was lower than that (0.83–0.84) in the non-load group ($P < 0.05$). However, the amount of ATP was not statistically different ($P < 0.05$) between the two groups with the same preservation times. the fluometric trace (% RxV and % RxA) and bile excretion decreased greatly between no preservation and preservation, proportional to duration of the preservation period. It can be seen from Table 1 that the values of RxV were 80.4% at 6 hours, 70.7% at 12 hours, 62.9% at 24 hours of preservation, and the values of RxA were 91.0% at 6 hours, 89.1% at 12 hours, 8.13% at 24 hours of preservation compared with that of the controls. Bile excretion decreased from 2.55 $\mu\text{l}/\text{min}/\text{g}$ liver to 1.98 at 6 hours, 0.98 at 12 hours, and 0.49 at 24 hours of preservat-

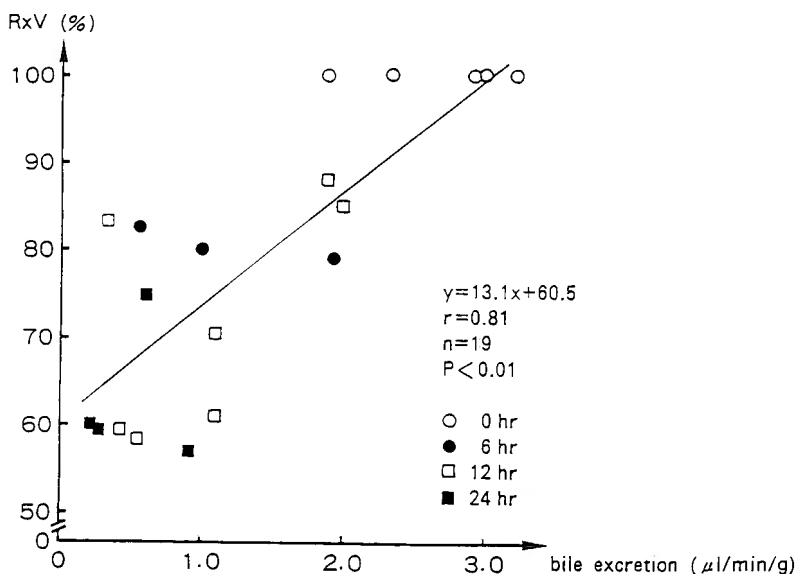


Fig. 4 Correlation between bile excretion and percentage of RxV in fluorometric trace.

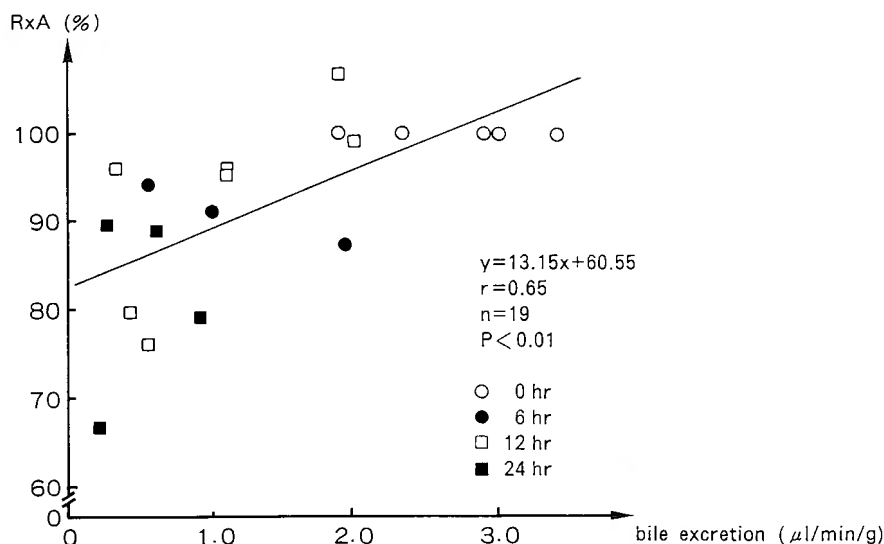


Fig. 5 Correlation between bile excretion and percentage of RxA in fluorometric trace.

ion. Bile excretion also positively correlated with %RxV (Fig. 4, $r=0.81$, $p<0.01$) and with %RxA (Fig. 5, $r=0.65$, $p<0.01$).

Discussion

One of the greatest needs in liver transplantation is a method to determine the cellular viability of preserved liver. This can only be done through revascularization and reoxygenation, either in a recipient animal after transplantation or in an extracorporeal perfusion system. The failure rate after transplantation with preserved liver becomes high after 6 hours of simple cold storage in Euro-Collins' solution and the longer of preservation, the poorer the survival. Investigators^{13,14} were unable to obtain any survivors using Euro-Collins' solution after 12–16 hours of preservation.

Studies from our laboratory have focused on metabolic derangements in the energy charge balance, which is necessary for maintaining cellular viability, and have shown that positive correlation exists among several parameters of energy metabolism, such as mitochondrial redox state (NAD/NADH), energy charge and arterial blood ketone body ratio (acetoacetate/3-hydroxybutyrate) after major hepatectomy, jaundice, shock and animal liver transplantation.

Our results showed that ATP level and energy charge decreased at the end of preservation and clear differences between preservation durations were not observed. Pontegnie-Istace S. and Lambotte L. also found that ATP level at the end of the anoxic period in Collin's preservation does not seem to be a reliable criterion of liver viability before transplantation¹⁰. The ATP level and energy charge are gradually increased by continuous perfusion with Krebs-Henseleit solution at 32°C, then reach a plateau after 20 minutes of perfusion. The recovery of the cellular ATP level on reoxygenation was also shown to be associated with the restoration of many hepatic functions. The change of ATP and energy charge in each group cannot tell us much more about differences in enhancement of mitochondria after different durations of preservation in Euro-Collins' solution. This study showed that recovery of the cellular ATP level and energy charge with perfusion did not correlate to the sur-

vival rate after transplantaion.

Many investigators report that bile flow rate is a practical and reliable index of hepatic function in studies of preservation of donor liver as well as in experimental perfusion⁷⁾. Kamiike⁸⁾ reported that the extent of hepatic injury could be assessed by monitoring the rate of bile excretion, which reflects the cellular level of ATP. In this study, the bile flow rate decreased proportionally with time: 6, 12 and 24 hours of preservation. The ATP level also shows potential decrease with 6, 12 and 24-hour preservation although results were not significant. However, ATP decreased from 2.78 to 2.42, 1.98, 1.77 μ mol/g wet liver in the perfusion group without taurocholic acid and from 2.46 to 2.35, 2.04, 1.89 μ mol/g wet liver in the perfusion group with taurocholic acid.

The mitochondria redox state (NAD/NADH) after reperfusion was estimated using microfluometry developed on the principle held by Chance et al. The redox state of NAD (P) H of the whole cell can be measured with this technique, although it is difficult to distinguish the fluorescence of NADH or NADPH. Fluorometry reflects not only the oxidation-reduction state of pyridine nucleotides but also the energy-yielding potential of mitochondria⁹⁾. This study shows fluorometric traces (%R_xV and %R_xA) and bile excretion decrease greatly between no preservation and preservation, proportionally to the duration of preservation. Bile flow rate also correlates positively with the %R_xV and %R_xA. It is reasonable to assume that fluorometry, particularly the velocity and amplitude of the fluorometric trace in anaerobic change, accurately indicates the viability of the donor gragt. This fluorometric study can provide an efficient non-invasive methos of evaluating the viability of the donor graft, and may solve the problem of how to assess the primary nonfunctioning liver, one of the major difficulties by surgeons in recent clinical liver transplatation.

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和文抄録

NADH 測定法による保存肝 viability の評価 —肝エネルギー代謝および胆汁排泄量との相関—

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雄性ウイスターラットを用いて, 4°C ユーロコリンズ液にて wash out 後, 同液内で単純浸漬保存肝を作製した。保存時間は 0, 6, 12, 24 時間とした。保存終了時では, ATP 値およびエネルギーチャージは低く, それぞれの間に有意差がなかった。保存肝を保存後 32°C, 酸素飽和 Krebs-Henseleit 液で毎分 30 ml で灌流した。灌流後徐々に ATP 値およびエネルギーチャージは上昇し 20 分~30 分後にプラトーに達した。20 分~30 分後の ATP およびエネルギーチャージにはそれぞれの間に有意差がなかった。同灌流液にタウロ

コール酸 30 μ M 入れて胆汁排泄量を保存時間で比較した。保存時間の延長とともに有意に胆汁排泄量が減少した。

366 nm 励起光による肝表面からの 460 nm 蛍光を測定し, in vivo におけるミトコンドリアの redox state を測定する方法 (Redoximeter) では, 酸化から還元への Velocity (RxR) と Amplitude (RxV) で, 保存時間に有意差があり, 胆汁排泄量とよく相関した。NADH の蛍光測定 (Redoximeter) はラット保存肝の viability 評価する上で有用で非侵襲的方法である。