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Why is liver preservation performed at 4 °C?

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Abstract To establish the most suitable temperature for liver preservation, we preserved rat livers at various temperatures (0, 5, 10, and 15 °C) in UW solution and investigated, biochemically, the proton ATPase activity, ATP metabolites in mitochondria, and phosphatidylcholine hydroperoxide (PC-OOH) in liver tissue. Liver specimens were taken every 6 h up to 24 h. The proton ATPase activity and the concentration of ATP, ADP, AMP, and adenosine in livers preserved at 0 °C showed the best results. The total adenine nucleotide (TAN) in livers preserved for 18 and 24 h had significantly higher concentrations compared with those at other temperatures (5, 10, and 15 °C). In the livers preserved at 5 °C, TAN was degraded to hypoxanthine. On the other hand, those preserved at both 10 and 15 °C showed changes from hypoxanthine to xanthine. The concentration of xanthine in both groups preserved at 10 and 15 °C showed high values at 6 and 12 h, respectively, and similar changes in PC-OOH concentrations at both 10 and 15 °C were observed. However, the changes in PC-OOH concentration at various temperatures were not significant for any length of preservation time. In light microscopical examinations, there were no morphological changes in the hepatocytes. From these results, we conclude that the capability of ATP synthesis of mitochondria in livers preserved at 0 °C keep them in the best condition compared with livers preserved at 15, 10, and 5 °C.

Key words Liver preservation · Low temperature · Mitochondrial proton ATPase activity · Mitochondrial ATP metabolites · Phosphatidylcholine hydroperoxide

Introduction

One of the most vital problems in liver transplantation is the preservation of the liver in the best condition. At present, the liver for transplantation is generally preserved at 0–4 °C to inhibit its metabolism. Some investigators have studied the optimal temperature for liver preservation. Attenburrow et al. [1] measured the tissue adenosine nucleotide level in the rat liver and concluded that the optimal temperature was 10 °C. On the other hand, Okouchi et al. [2] investigated the bile flow rate after liver transplantation in the rat at various temperatures and suggested that preservation at 0–5 °C is optimal. The most appropriate temperature, however, for liver transplantation is not clear because of a lack of information concerning the changes in biochemical values at various temperatures during the liver preservation.

Recently, we reported that the deterioration of ATP synthesis in the mitochondria of hepatocytes and the disruption of sinusoidal endothelial cells contributed greatly to liver injury during preservation [3, 4]. Therefore, it is important to prevent these injuries before liver transplantation. To elucidate the optimal temperature

for liver preservation, we studied the changes in mitochondrial function and in the sinusoidal endothelial cells in livers preserved at various temperatures. We have already measured the proton ATPase activity [5] and ATP concentration in mitochondria using livers preserved in UW solution at various temperatures and have considered that the optimal temperature for liver preservation should be 0 °C [6]. To confirm our hypothesis, however, more biochemical information was necessary; for example, the capability of ATP synthesis, the degradation of TAN, and the possibility of phospholipid peroxidation of cell membranes by a generated free radical in the liver.

In this report, we further investigated the consequences of liver preservation at low temperatures by measuring the levels of ATP metabolites in mitochondria and phosphatidylcholine hydroperoxide (PC-OOH) in liver tissue in order to clarify the optimal temperature for liver preservation from the viewpoint of ATP production and phospholipid peroxidation.

Materials and methods

Preservation method

Male Wistar rats weighing 250–300 g were used. Under anesthesia with ether, the liver was flushed with Ringer's lactate solution and then with UW solution via the portal vein. The Ringer's lactate and UW solutions were cooled to various temperatures (0, 5, 10, and 15 °C). The liver was then immediately excised and preserved in UW solution at 0, 5, 10, and 15 °C ($n = 4$ in each group). Liver specimens were examined every 6 h from 0 h to 24 h.

Isolation of mitochondria

Mitochondria were isolated from the liver by the "high-yield" differential centrifugation method [7]. The isolation medium consisted of 0.25 M sucrose buffer (pH 7.4).

Measurement of mitochondrial proton ATPase activity

ATPase activity was measured by a Jasco FP-777 fluorometer (Tokyo, Japan) at 23 °C at an excitation of 625 nm and an emission of 670 nm using 100 µl of mitochondria (4–10 mg/ml protein) isolated by our previously described method [8]. We used diS-C_3 (5) as the fluorescence reagent.

Measurement of concentrations of ATP, ADP, and AMP

The isolated mitochondria in 0.5 N aqueous perchloric acid were smashed by sonication. The extracted medium was injected to a high performance liquid chromatography (HPLC) system using a reverse phase column (Wakosil-II 5C18 HG, 4.6 mm i.d. × 15 cm) using a UV detector (260 nm, Shimadzu SPD-6AV). The eluent was 60 mM phosphate buffer (pH 5.0) and the flow rate was 0.5 ml/min. Protein concentration in the mitochondria was determined by the Bradford method [9].

Measurement of concentrations of adenosine, inosine, xanthine, and hypoxanthine

The extracted medium, obtained by pretreating mitochondria, was injected to the HPLC system using a reverse phase column (Tosoh TSKgel ODS 80TM, 4.6 mm i.d. × 25 cm). The eluent was 3.5 % acetonitrile in 50 mM phosphate buffer (pH 3.0) and the flow rate was 0.7 ml/min.

Measurement of PC-OOH concentration

PC-OOH in the liver tissue was measured by a chemiluminescence high performance liquid chromatography (CL-HPLC) system applying our improved method. The homogenized solution was centrifuged and extracted with dichloromethane-methanol (2:1) containing 0.002 % 2,6-di-tert-butyl-4-methylphenol [10]. Removal of the dichloromethane phase gave a residue, the total lipids, which was supplemented with methanol. The solution, filtered through a 0.5 mµ Millipore filter, was injected into the CL-HPLC system.

The CL-HPLC system was equipped with a TSK-gel Silica-60 column (5 mµ, 4.6 mm i.d. × 25 cm, Tosoh). Using a Jasco 875-CE UV detector, the flow rate of the mobile phase, dichloromethane-methanol (1:4, v/v), was 1.0 ml/min. After passing through the UV detector, the eluate was mixed with a CL reagent by using a mixing cell. The flow rate of the CL reagent was 1.0 ml/min using a Jasco PU-980i pump. The generated CL was monitored with a single photon counting apparatus, Jasco 825-CL, equipped with a flow spiral quartz cell. The CL reagent was prepared by dissolving 4 µM 2-methyl-6-[p-methoxyphenyl]-3,7-dihydroimidazo [1,2-a] pyrazin-3-one (MCLA) and 10 µM $FeSO_4$ in methanol.

Histological examination

For light microscopy, the rat liver was fixed in 10 % aqueous formalin for 24 h at room temperature, dehydrated, and embedded in paraffin. Sections were stained by hematoxylin and eosin.

Statistical data

All results were expressed as the mean ± standard deviation. Statistical analysis was performed by Student's t-test.

Results

Concentration of ATP metabolites

In our previous paper we reported that the deterioration of proton ATPase activity had a close relationship with the decrement of ATP concentration [6]. The changes in the total adenine nucleotide (TAN) are shown in Fig. 1. The levels of ATP, ADP, and AMP preserved at various temperatures all decreased during preservation. The TAN level in livers preserved at 0 °C kept significantly high values at 18 and 24 h, 10.44 ± 2.19 nmol/mg of mitochondrial protein at 18 h ($P < 0.05$ versus preservation at 5 °C), and 7.38 ± 1.18 nmol/mg at 24 h ($P < 0.05$ versus preservation at 5 °C). The levels of TAN in mitochondria in livers preserved at 5 °C remained the same

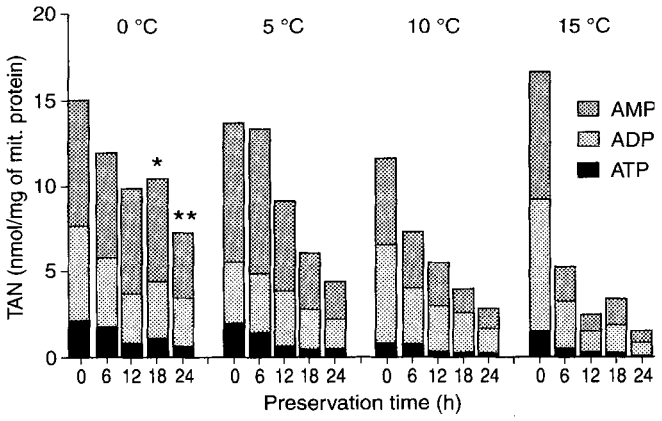

Fig. 1 TAN (ATP + ADP + AMP) in mitochondria during rat liver preservation in UW solution at various temperatures (0, 5, 10, and 15 °C). (* $P < 0.05$ vs other temperatures for 18 h, ** $P < 0.05$ vs other temperatures for 24 h)

as those preserved at 0 °C up to 12 h, but these values deteriorated at 18 h (6.07 ± 1.73 nmol/mg of mitochondrial protein). In livers preserved at both 10 and 15 °C, the level of the energy charge became significantly worse at 12 h.

The level of adenine in livers preserved at 0 °C also remained unchanged for 18 h (1.48 ± 0.66 nmol/mg of protein at 18 h, $P < 0.05$ versus preservation at 5 °C). At other temperatures, the decrement of the adenosine levels was observed. The concentration of inosine remained unchanged at any temperature and for any length of preservation time (Table 1, 2). The concentration of hypoxanthine in livers preserved at 5 °C remained at a high value for any length of preservation time and showed significant change at 12 h, 6.08 ± 2.20 nmol/mg of mitochondrial protein ($P < 0.05$ versus other temperatures). The hypoxanthine level in livers preserved at 15 °C showed a significant increment at 6 h (3.67 ± 0.64 nmol/mg of mitochondrial protein, $P < 0.05$ versus at 0 h). On the other hand, the concentration of xanthine in these livers showed significant increments at 6 and 12 h. The level of xanthine in livers preserved at 10 °C also showed significant increments at 12 h (Fig. 2).

The concentration of PC-OOH (Fig. 3)

The concentrations of PC-OOH remained at low values without significant changes for various preservation times and at all temperatures. The PC-OOH concentration at 0 °C was, however, slightly lower for all lengths of preservation time (0.44 ± 0.62 nmol/100 mg of total lipids in the liver at 0 h, 0.64 ± 0.21 at 12 h, and 0.21 ± 0.29 at 18 h).

Histological findings

The 24-h-preserved livers at any temperature showed increments of intracellular space in comparison with the specimens just after graftectomy. There were, however, no morphological changes in the hepatocytes for any length of preservation times.

Discussion

It is well known that livers are injured by the reperfusion after preservation. We confirmed from our investigations that the mitochondria and sinusoidal lining cells are especially susceptible to injury during preservation [3, 4]. Accordingly, it is important to prevent injury to the mitochondria and sinusoidal cells during liver preservation before liver transplantation. Investigations to develop a preservation solution are constantly being

Table 1 Adenosine in mitochondria during preservation ($n = 4$), measured in nmol/mg mitochondrial protein

Preservation temperature (°C)	0	6	Preservation time (h)		
			12	18	24
0	1.53 ± 0.83	1.66 ± 0.40	1.38 ± 0.74	1.48 ± 0.66	0.84 ± 0.32
5	2.61 ± 1.04	2.36 ± 0.72	1.07 ± 0.90	0.41 ± 0.41	0.48 ± 0.34
10	0.83 ± 0.58	0.71 ± 0.39	0.51 ± 0.22	0.38 ± 0.21	0.18 ± 0.19
15	1.73 ± 0.27	0.71 ± 0.08	0.41 ± 0.09	0.38 ± 0.08	0.29 ± 0.13

Table 2 Inosine in mitochondria during preservation ($n = 4$), measured in nmol/mg mitochondrial protein

* $P < 0.001$ versus 15 °C preservation at 6 h; ** $P < 0.05$ versus 15 °C preservation at 6 h

Preservation temperature (°C)	0	6	Preservation time (h)		
			12	18	24
0	3.64 ± 1.58	4.52 ± 1.13	5.59 ± 1.10	3.72 ± 1.60	5.90 ± 1.69
5	6.80 ± 2.51	6.44 ± 1.76	6.24 ± 1.41	6.99 ± 1.39	6.92 ± 1.30
10	5.49 ± 1.06	5.48 ± 1.65	5.70 ± 0.92	5.41 ± 1.13	4.97 ± 1.25
15	3.57 ± 0.50*	6.83 ± 1.02	5.88 ± 1.32	4.88 ± 0.93	4.41 ± 0.57**

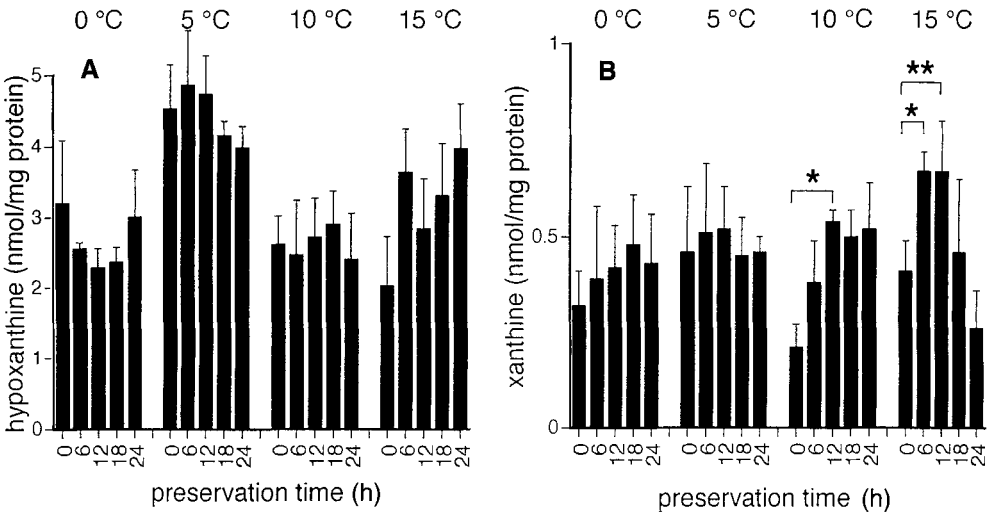

Fig. 2 A, B Hypoxanthine (**A**) and xanthine (**B**) in mitochondria during rat liver preservation in UW solution at various temperatures (0, 5, 10, and 15 °C). ($^{*}P < 0.01$, $^{**}P < 0.05$)

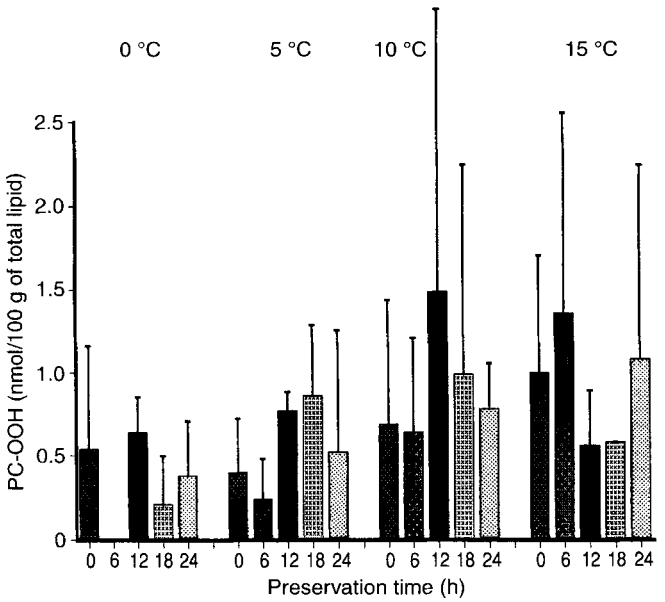

Fig. 3 Phosphatidylcholine hydroperoxide (PC-OOH) in liver tissue during rat liver preservation in UW solution at various temperatures (0, 5, 10, and 15 °C)

preformed. However, we considered the influence of temperature on preservation and investigated the change of mitochondrial function under various temperatures to clarify the best temperature for liver preservation.

The decrement of TAN is generally attributed to the natural degradation of ATP. We suspected that the TAN level in livers preserved at 0 °C remained high in comparison with those at other temperatures because the lowest temperature for preservation will slow the rate of its degradation. We also measured the change in the levels of adenosine, inosine, hypoxanthine, and xanthine to elucidate the limit of TAN degradation at the various temperatures during preservation. In livers preserved at 0 °C, the degradation of TAN was limited to adenosine; the values of the other components remained low. In those preserved at 5 °C, the degradation of TAN was limited to hypoxanthine because the levels of inosine and hypoxanthine were higher than at other temperatures and the levels of xanthine were very low. On the other hand, the levels of xanthine in livers preserved at 10 °C increased at 12 h, and in those preserved at 15 °C increased at 6 h. From these results, we suggested that the ATP degradation is prevented at a lower temperatures and the best temperature for liver preservation is 0 °C.

To clarify the possibility of free radical generation induced by abundant oxygen in the UW solution, we measured PC-OOH in the liver tissue. Measurement of PC-OOH by CL-HPLC has been developed by Miyazawa et al. [11]. In this system, however, since the eluent (methanol-chloroform) and reaction solution (borate buffer) when mixed generate chemiluminescence, so the reproducibility of this system is disrupted by the generation of foam. Consequently, we investigated and developed an improved method which uses methanol medium including a highly sensitive chemiluminescence reagent, MCLA, and $FeSO_4$ as the reagent for the Fenton reaction. The non-metallic pump was used for the elution of reactive solution. The detection limit of PC-OOH using our improved method was 50 pmol (S/N ratio > 3). Thus, we utilized our improved method to measure the concentration of PC-OOH because of its high sensitivity.

The phospholipid peroxidation by oxygen-derived free radicals is well investigated and it is well known that the microsomal membrane is easily injured by free radicals. Although a significant change of PC-OOH concentration for any length of preservation at various tem-

peratures was not observed, it was shown that the the change of xanthine levels is similar to the change of PC-OOH concentration at 10 and 15 °C. However, the degree of these changes was small and we suggest that the influence of the phospholipid peroxidation by free radicals is little during the preservation at various temperatures.

In spite of the significant differences in the mitochondrial function depending on the various temperatures, there were no morphological changes to the hepatocytes for any length of preservation time. Light microscopical study is incapable of demonstrating the condition of the sinusoidal endothelial cells precisely [4]. It is necessary to scan the microstructure of sinusoidal cells by transmission of electron microscopy.

In summary, we confirm that the deterioration of mitochondrial function in liver preservation is caused by TAN degradation and of proton ATPase activity by low temperature. In this study, we can conclude that the optimal temperature for liver preservation is 0 °C. For more precise information, electron microscopical study of sinusoidal endothelial cells is required.

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