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Effect of cold aerobic perfusion on nonparenchymal cell viability of rat livers

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Abstract We investigated the effect of oxygen supply on hepatic cellular viability during cold perfusion storage of rat livers. A perfluoro-*N*-methyldecahydroisoquinoline (FMIQ) emulsion is used as an oxygen carrier. The composition of the perfusate containing 20 w/v% FMIQ is essentially the same as the University of Wisconsin (UW) solution except for the exclusion of hydroxyethyl starch. Rat livers were perfused at 4 °C for up to 24 h with either UW solution (group I, oxygenated; group II, unoxygenated) or FMIQ solution (group III, oxygenated; group IV, unoxygenated). After perfusion storage, the livers were reperfused with warm (37 °C) oxygenated or cold (4 °C) unoxygenated Krebs-Henseleit bicarbonate buffer, and nuclear trypan blue uptake was measured as the index of cell death. With warm oxygenated reperfusion, there remained less than 2% noviable parenchymal cells up to 24 h, regardless of perfusate or oxygenation. In UW-perfused livers, the proportion of nonviable nonprenchymal cells (NPC) increased progressively regardless of oxygenation, the values in groups I and II in the periportal field at 24 h being $39.9 \pm 4.7\%$ (mean $\pm$ SD) and $36.5 \pm 4.2\%$, respectively. By contrast, in FMIQ-perfused livers, dye uptake by NPC was significantly reduced with oxygenation ($16.9 \pm 5.7\%$ and $39.4\% \pm 9.1\%$ at 24 h in groups III and IV; $P < 0.001$). With cold unoxygenated reperfusion, livers in groups I, II, and IV showed a significant decrease of nonviable NPC, while those in group III showed no significant changes. These data indicate that oxygen supply during perfusion storage of the liver may ameliorate lethal injury to NPC precipitated during reperfusion.

Key words Cold aerobic perfusion
Liver preservation
Perfluorochemical
Nonparenchymal cell viability

Introduction

The period of safe hypothermic storage of a liver graft has been profoundly extended with the advent of the University of Wisconsin (UW) solution [1]. However, simple cold storage has a specific time limit beyond which the organ is no longer viable [2, 3]. In this regard, continuous aerobic perfusion is theoretically a possible means of obtained a more prolonged preservation time [4, 5].

In recent years, particular attention has been paid to microcirculatory disturbance in the liver graft following cold preservation [6–8]. Graft viability is directly related to the microvascular injury, which may be a consequence of nonparenchymal cell injury [9]. Some investigators have reported that a selective loss occurs among nonparenchymal cells during the cold ischemic interval [8], while others proposed that such lethal injury was precipitated by reperfusion [6, 7]. Although the pathogenesis remains controversial, it is not known why nonparenchymal cells are more vulnerable to such ischemia or reperfusion injury.

Recently, we have formulated a preservation solution containing 20 w/v% perfluoro-*N*-methyldecahydroisoquinoline (FMIQ) as an oxygen-carrying agent [11, 12]. The other component of this solution is prepared in the same way as the UW solution except for the exclusion of hydroxyethyl starch. The purpose of this study was to elucidate whether or not oxygen supply during hypothermic storage alters hepatic parenchymal and nonparenchymal cell viabilities. The results indicate that nonparenchymal cell injury is indeed ameliorated by the use of an oxygen-carrying agent in the perfusate.

Materials and methods

Livers of 12-h-fasted, male Sprague-Dawley rats weighing 200–300 g were used in this study. They rats were anesthetized by intraperitoneal injection of 0.2 mg/g body weight of secobarbiturate. After cannulating the portal vein weith a 6F catheter, 200 units of heparin were administered. Then the liver was excised and perfused via the portal vein at 4 °C with either the UW solution (group 1, oxygenated; group II, unoxygenated) or the perfusate (group III, oxygenated; group IV, unoxygenated) containing 20 w/v% FMIQ emulsion (Green Cross, Japan) at a flow rate of 0.2 ml/mg · min. The perfusion system was similar to that of Kamada et al. [4], consisting of a reservoir, membrane oxygenator, and pulsatile pumps. The basic composition of the FMIQ solution is prepared in the same fashion as the UW solution except for the exclusion of hydroxyethyl starch (Table 1). Oxygenation of the perfusate was provided by a membrane oxygenator using a mixture of 95% O_2 and 5% CO_2. After perfusion storage (12, 18, and 24 h), the livers were reperfused with warm (37 °C) oxygenated or cold (4 °C) unoxygenated Krebs-Henseleit bicarbonate (KHB) buffer, according to the method described by Caldwell-Kenkel et al. [7]. After 8 min of reperfusion, trypan blue (200 μM) was added to the perfusate. After a total of 15 min of reperfusion, the livers were fixed by KHB buffer containing 2% glutaraldehyde and 2% paraformaldehyde. The left lateral lobe was embedded in paraffin and counterstained with either hematoxylin and eosin or eosin alone. For each liver, trypan-blue-positive parenchymal and nonparenchymal cell nuclei in eosin-counterstained sections were counted in five high-power (×400 times) periportal and pericentral fields. The percentage of trypan-blue-positive nuclei was calculated on the basis of total nuclei counted in hematoxylin and eosin-stained sections. In livers stored for 24 h prior to trypan blue staining, aliquots of effluent perfusate were collected at the end of reperfusion with warm oxygenated KHB buffer for the determination of lactate dehydrogenase (LDH). In all groups, trypan blue uptake was determined in 4–6 livers at each preservation time.

Aliquots of influent and effluent perfusates were collected for gas determinations after 12 h perfusion storage in each group. Oxygen consumption was calculated from the influent-effluent oxygen concentration difference, flow rate, and wet liver weight. Values were expressed as mean ± SD. Differences between groups were evaluated using the unpaired Student's *t*-test. Differences were considered significant at the $P < 0.05$ level.

Table 1 Perfusate composition (FMIQ perfluoro-*N*-methyldecahydroisoquinoline)

Component	FMIQ solution	UW solution
FMIQ	20 w/v %	–
Hydroxyethyl starch	–	5 w/v %
Lactobionate	100 mM	100 mM
Raffinose	30 mM	30 mM
$NaKH_2PO_4$	25 mM	25 mM
$MgSO_4$	5 mM	5 mM
Na	30 mM	30 mM
K	120 mM	120 mM
Adenosine	5 mM	5 mM
Allopurinol	1 mM	1 mM
Glutathione	3 mM	3 mM
Insulin	40 IU/l	40 IU/l
Dexamethasone	16 mg/l	16 mg/l
pH	7.4	7.4
Osmolarity	320 mOsm/l	320 mOsm/l

Results

Trypan blue uptake in periportal field after warm oxygenated reperfusion

After 12, 18, and 24 h of perfusion storage in group I, the percentages of trypan-blue-positive nuclei of parenchymal cells were 0.3% ± 0.3%, 0.3% ± 0.1%, and 1.2% ± 0.6%, respectively (Fig. 1). The remaining three groups also showed a similar time course of parenchymal cell losses. On the other hand, dye-positive nuclei of nonparenchymal cells increased with time in UW-perfused livers irrespective of oxygen supply, the per-

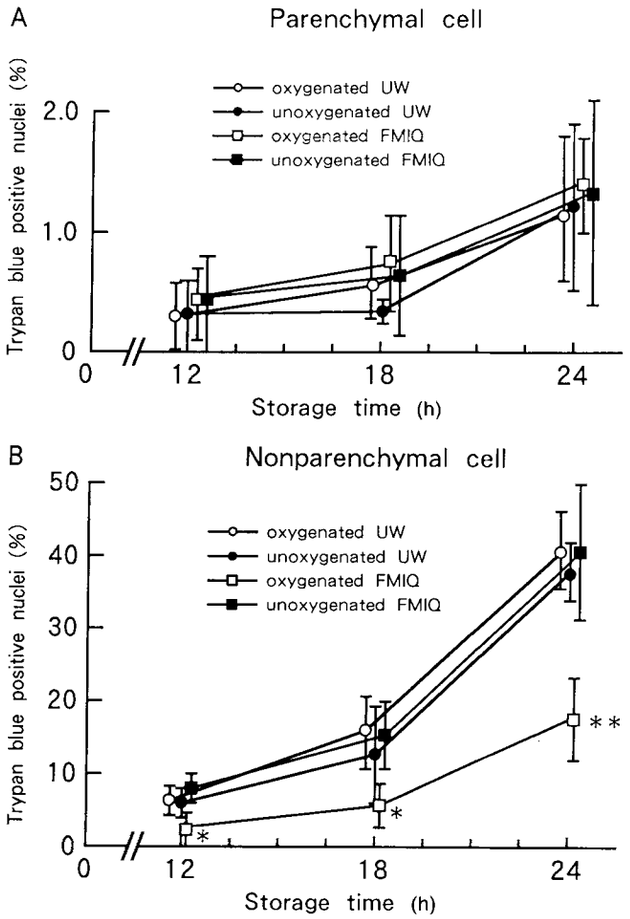

Fig A, B Trypan blue uptake by parenchymal (**A**) and nonparenchymal (**B**) cells in the periportal field after warm oxygenated reperfusion. Values are expressed as mean ± SD. (* $P < 0.01$, ** $P < 0.001$, respectively, regarding group III vs. groups I II, and IV)

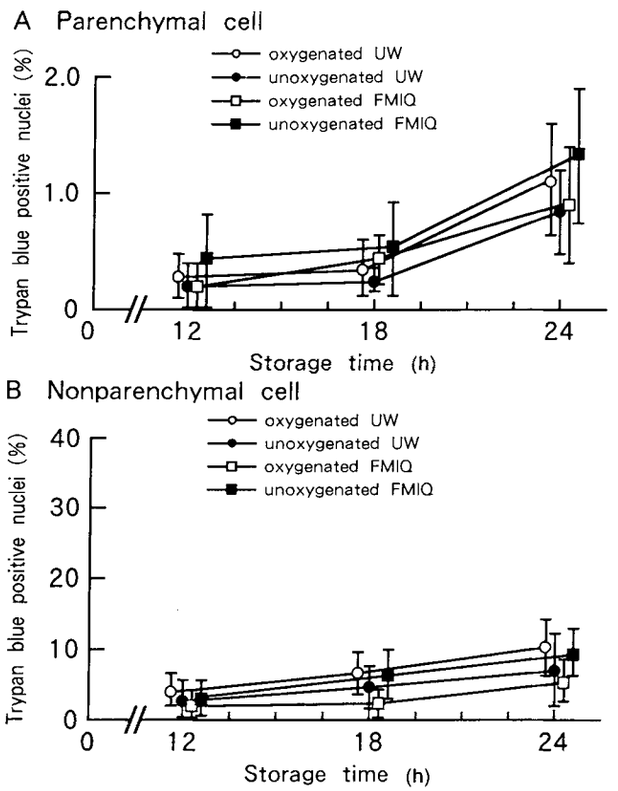

Fig. 2 A, B Trypan blue uptake by parenchymal (**A**) and nonparenchymal (**B**) cells in the pericentral field after warm oxygenated reperfusion. Values are expressed as mean ± SD

Table 2 Trypan blue uptake of nonparenchymal cells in the periportal field after 24 h storage and reperfusion

Group	Perfusate	Reperfusion	Trypan blue uptake (%) of nonparenchymal cells
I	Oxygenated UW solution	Warm oxygenated	39.9 ± 4.7 *
		Cold unoxygenated	17.2 ± 6.3 *
II	Unoxygenated UW solution	Warm oxygenated	36.5 ± 4.2 *
		Cold unoxygenated	15.4 ± 5.8 *
III	Oxygenated FMIQ solution	Warm oxygenated	16.9 ± 5.7
		Cold unoxygenated	16.2 ± 7.6
IV	Unoxygenated FMIQ solution	Warm oxygenated	39.4 ± 9.1 *
		Cold unoxygenated	17.6 ± 9.6 *

* $P < 0.001$ for comparison between warm oxygenated and cold unoxygenated reperfusions

centages at 24 h in groups I and II being 39.9% ± 4.7% and 36.5% ± 4.2%, respectively. The FMIQ solution without oxygenation (group IV) showed similar time-related increases of dye-positive nonparenchymal cells (8.0% ± 2.4%, 14.4% ± 4.9%, and 39.4% ± 9.1% at 12, 18, and 24 h, respectively). In contrast, livers perfused by oxygenated FMIQ solution (group III) had a significant reduction in the amount of nonviable nonparenchymal cells: 2.1% ± 2.0%, 4.5% ± 2.9%, and 16.9% ± 5.7% at 12, 18, and 24 h, respectively ($P < 0.01$ at 12 and 18 h vs.

groups I, II, and IV; $P < 0.001$ at 24 h vs. groups I, II and IV).

Trypan blue uptake in pericentral field after warm oxygenated reperfusion

Dye-positive nonparenchymal cells were significantly less abundant in the pericentral field than in the perioportal field at 24 h in all four groups ($P < 0.01$). Although there was a trend for improvement of nonparenchymal cell viability in group III at 18 and 24 h compared with other three groups, the difference was not statistically significant. For parenchymal cells, the loss of viability remained less than 2% for up to 24 h without any significant difference between the perfusates (Fig. 2).

Trypan blue uptake in periportal field after 24 h storage and cold unoxygenated reperfusion

As described in Table 2, UW-perfused livers showed a significant decrease in dye-positive nonparenchymal cells after cold unoxygenated reperfusion as compared with warm oxygenated reperfusion, irrespective of oxygenation during storage (group I, 17.2% ± 6.3% in cold vs. 39.9% ± 4.7% in warm, $P < 0.001$; group II, 15.4% ± 5.8% in cold vs. 36.5% ± 4.2% in warm, $P < 0.001$). Similarly, in livers stored with the unoxygenated FMIQ solution, cold unoxygenated reperfusion resulted in a significant decrease in dye-positive nonparenchymal cells (17.6% ± 9.6% in cold vs. 39.4% ± 9.1% in warm, $P < 0.001$). However, in livers stored in the oxygenated FMIQ solution, there was no significant difference between warm oxygenated and cold unoxygenated reperfusion (16.2% ± 7.6% in cold vs. 16.9% ± 5.7% in warm).

LCH release after warm oxygenated reperfusion

LDH release at 24 h in groups I, II, III, and IV was 125 ± 82 IU/l, 106 ± 72 IU/l, 255 ± 90 IU/l, and 175 ± 78 IU/l, respectively. Although LDH release tended to be higher in livers perfused with the FMIQ solution as compared with the UW solution, the difference was not statistically significant.

Oxygen consumption

As shown in Table 3, the oxygen consumption after 12 h perfusion storage with the unoxygenated FMIQ and UW solutions was nearly equal. Oxygenation resulted in a significant increase in the oxygen consumption in both perfusates. However, the oxygen consumption of the oxygenated FMIQ group was almost three times higher than that of the oxygenated UW group.

Table 3 Oxygen consumption of rat livers after 12 h storage

Group	Perfusate	Oxygen consumption (10^{-3} ml/g · min)		
I	Oxygenated UW solution	3.2 ± 0.5	**	*
II	Unoxygenated UW solution	1.5 ± 1.2	*	
III	Oxygenated FMIQ solution	9.4 ± 0.5	*	
IV	Unoxygenated FMIQ solution	1.6 ± 0.8		

* $P < 0.01$, ** $P < 0.05$

Discussion

On a theoretical basis, the metabolic rate is suppressed about 10-fold when the temperature is reduced from 37 °C to 4 °C [2, 3]. However, hypothermia does not stop metabolism. Therefore, oxygen supply seems to be a key to long-term organ preservation. Recently, Kuroda et al. [13, 14] demonstrated that a two-layer [UW solution/perfluorochemical (PFC)] cold storage method supplying sufficient oxygen continuously extends the preservation time of the canine pancreas up to 96 h. Tamaki et al. [5] also reported successful rat liver transplantation after hypothermic aerobic perfusion storage of the liver for up to 48 h using a perfusate containing PFC as an oxygen-carrying agent. In related work, others suggested that continuous hypothermic preservation, using a mixture of UW solution and perfluorotributyl amine (FC-43) emulsion, is better able to preserve the hepatic energy metabolism as assessed by the ketone body ratio, tissue adenine nucleotides, and energy charge levels [15]. These studies strongly indicate the beneficial effects of oxygen supply during preservation on graft function. However, little is yet known about the influence of oxygen supply on the hepatic structural damage following cold preservation.

It is well known that in the kidney, different cells have different susceptibilities to ischemic injury. In this regard, Caldwell-Kenkel et al. [7] reported that selective injury occurred to nonparenchymal cells following cold ischemic storage of the liver according to trypan blue labelling. They stated that microcirculatory disturbances leading to graft failure after transplantation may be a consequence of such nonparenchymal cell injury. McKeown et al. [16] also pointed out that the critical injury to the microcirculation was characterized by the

loss of the sinusoidal lining, rounding up of sinusoidal lining cells, and detachment from underlying hepatocytes during cold ischemia. Therefore, we designed this study to elucidate the influence of oxygen supply on the hepatic microcirculation with special reference to nonparenchymal cell viability.

It is well known that continuous perfusion incurs the risk of greater vascular injury. In this regard, we employed a relatively low flow rate (0.2 ml/g · min) to minimize such injury, while fulfilling the theoretical oxygen demand at 4 °C. FMIQ has recently been developed as a second-generation blood substitute with an estimated half-life of 9 days [17]. This half-life is much shorter than that of FC-43, hence it has a better potential for clinical use. The oxygen-carrying capacity of the FMIQ emulsion is essentially the same as that of the FC-43 emulsion, which has been used in previous studies by others [4, 5, 15]. The amount of oxygen dissolved in 20 w/v% FMIQ emulsion is a linear function of PO_2 and is about 12.6 ml/dl at a PO_2 of 760 mmHg and 4 °C [18]. Thus, the oxygen delivery of the oxygenated FMIQ solution is estimated to be about 25×10^{-3} ml/g · min at the flow rate of 0.2 ml/g · min. In the present study, the oxygenated FMIQ-perfused livers consumed 9.4×10^{-3} ml of oxygen, which corresponds to 38% of the estimated oxygen delivery. Based on the data reported by others [19], this is approximately 10–20% of the hepatic oxygen consumption under normothermic conditions. Previously, it was demonstrated that, even at a flow rate of 0.5 ml/g · min, rat liver showed a satisfactory metabolic function when perfused with Euro-Collins solution containing 10% FC-43 emulsion [20]. Thus, it may be assumed that the oxygen supply by the oxygenated FMIQ solution at a flow rate of 0.2 ml/g · min is satisfactory for the actual oxygen demand at 4 °C. On the other hand, oxygenation of the UW solution resulted in an increase of oxygen utilization of the stored livers. However, the level was much lower than that of the oxygenated FMIQ-perfused livers and did not reach the theoretical oxygen demand at 4 °C.

The major finding of the present study is that a sufficient oxygen supply during cold perfusion using an oxygen-carrying agent indeed ameliorates nonparenchymal cell injury as assessed by trypan blue labelling. We also observed that lethal injury occurs almost exclusively to nonparenchymal cells. In addition, such injury is predominant in the periportal region regardless of the perfusate employed. According to the data collected by others [7], the loss of nonparenchymal cell viability was nearly 50% in the periportal region, whereas it was about 30% in the pericentral region with warm oxygenated reperfusion after 24 h of simple cold storage in UW solution. They suggested that the observed difference could be explained by an oxygen free radical mechanism because the periportal region is oxygen-rich compared with the pericentral region. Our results under anaerobic perfusion storage with UW and FMIQ solutions were thus consistent with their results in support of this hypothesis. Moreover, it is interesting to note that the effect of aerobic perfusion storage with FMIQ solution on the nonparenchymal cells is more remarkable in the periportal region than in the pericentral region. This implies that nonparenchymal cells in the oxygen-rich periportal region benefit more by the oxygen supply during cold storage than those in the pericentral region. Previously, it has been demonstrated in various organs that the provision of a high oxygen tension does improve the synthesis of high energy nucleotides [15, 20]. In this setting, it is hypothesized that the mitochondrial oxidative phosphorylation capacity is well preserved, conversion of xanthine dehydrogenase into xanthine oxidase is reduced, and hypoxanthine as a purine precursor in the salvage pathway produces a further improvement in high energy phosphate concentrations [2, 21]. As a consequence, the accumulation of substrates such as hypoxanthine for O_2-radical generation via xanthine oxidase may be minimized with oxygen supply during cold storage. This hypothesis is also supported by our observation that cold unoxygenated reperfusion in livers stored with the oxygenated FMIQ solution did not show any further improvement of nonparenchymal cell viability, while the remaining three groups evidenced a significant reduction of dye-positive nonparenchymal cells when reperfused in the same manner. The loss of viability was not affected by the type of perfusate during cold storage but was affected by warm oxygenated reperfusion. In our study, temperature and oxygenation appear to be both involved during reperfusion. However, other investigators clearly demonstrated that a low temperature (0–4 °C) by itself did not change the trypan blue labelling of nonviable cells [7]. Therefore, it is suggested that oxygen supply during cold preservation prevents an oxygen-dependent reperfusion injury to nonparenchymal cells. On the other hand, there were no significant differences in parenchymal cell viability between the UW and FMIQ solutions, oxygenated or unoxygenated. However, according to light microscopic observations 24 h after perfusion storage, cell swelling tended to be more marked with the FMIQ solution compared with the UW solution, regardless of oxygenation. LDH release after warm reperfusion also tended to be higher in the FMIQ solution, although not statistically significantly so. These findings could be

explained by the fact that the colloidal osmotic pressure of the FMIQ solution is lower than that of the UW solution due to the lack of hydroxyethyl starch as an impermeant.

In summary, our data indicate that nonparenchymal cells are more susceptible to cold ischemia and subsequent reperfusion injury, and that oxygen supply during the storage period plays an important role in minimizing such microcirculatory injury precipitated during reperfusion.

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