

## Evaluation of Oxygen Necessity during Hypothermic Liver Perfusion

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### Abstract

Need of oxygen by the liver during hypothermic perfusion was evaluated using isolated perfusion model. Livers were perfused by a continuous perfusion system with oxygen saturated perfusate or nitrogen saturated perfusate, or simply stored for 12 hours at 5°C. Quality of individual liver was assessed at one hour after normothermic reperfusion. Tissue edema was significant in all experimental groups, but the extent of which was much higher in nitrogen and simple cold storage groups. AST, ALT, LDH and PNP in the perfusate at the end of normothermic reperfusion were significantly higher in nitrogen and simple storage groups and those of oxygen group were similar to the control. Tissue adenine nucleotide and purine catabolite concentration in oxygen group was almost identical to the control at the end of hypothermic preservation, while ATP and energy charge in nitrogen and simple cold storage groups were significantly low. Conjugated dienes before and after reperfusion showed no difference in any groups, indicating no involvement of free radical injury on reperfusion in this asanguineous perfusion model. These results suggest that continuous supply of oxygen is necessary for liver preservation even though the temperature is lowered to inhibit cellular metabolism.

### Introduction

Cause of liver failure immediately after transplantation is multifactorial. It has been attributed to [1] pre-existing injuries before procurement such as warm ischemic insult due to prolonged hypotension or cardiac arrest, [2] warm ischemic insult during surgery in donor and/or in recipient, [3] cold ischemic injury during preservation period, [4] reperfusion injury on restoration of graft circulation<sup>2,45)</sup>, and [5] humoral-type immunological reaction<sup>21)</sup>. Whatever the reasons are, the most important changes exist at peri-transplant period; during cold storage and after graft reperfusion.

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Key words: Liver, Perfusion, Oxygen, Free radical, Preservation

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Abbreviations: ADP, Adenosine diphosphate; ALT, alanine aminotransferase; AMP, Adenosine monophosphate; AN, Adenine nucleotide; AST, aspartate aminotransferase; ATP, Adenosine triphosphate; GSH, reduced glutathione; GSSG, oxidized glutathione; LDH, lactate dehydrogenase; PC, purine catabolite; PNP, purine nucleoside phosphorylase.

During cold storage, hepatocellular swelling caused by reduced activity of  $\text{Na}^+\text{-K}^+$  ATPase from anoxia and/or hypothermia is a major cause of viability loss<sup>30</sup>. Changes of sinusoidal lining cells that relates to microcirculatory disturbance occur at this period. After graft reperfusion, generation of oxygen radicals has been considered to enhance further deterioration of graft function<sup>2,43</sup>. Thus, mechanism of biological events during peri-transplant period is well characterized, but there is no comparative study which one, anoxia, hypothermia or free radicals, is the most important factor that regulates graft viability. In addition, results on the necessity of oxygen during cold preservation are still conflicting<sup>25,43</sup>. In the present study, rat livers were perfused for 12 hours at 5°C with  $\text{N}_2$ -saturated Krebs-Henseleit bicarbonate solution or with  $\text{O}_2$ -saturated solution, or simply stored at the same condition. The effect of oxygenation on cell swelling, sinusoidal lining cell damage, and generation of free radicals before and after reperfusion were determined using isolated liver perfusion model.

### Materials and Methods

#### *Animals:*

Inbred male Lewis rats (Harlan Sprague-Dawley Inc., Indianapolis, IN.) weighing 180–270 g were used. All animals were maintained in conventional animal facilities fed ad libitum with commercial rat chow.

#### *Surgical procedure:*

The animals were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg, Abbott Laboratories, N. Chicago, IL). Through a cruciform abdominal incision, the common bile duct was cannulated with 22 G teflon catheter and 3 mg heparin sodium was injected via the penile vein. Through a 18 G teflon catheter inserted into the portal vein, the liver was perfused with 60 ml air-saturated cooled Krebs-Henseleit bicarbonate solution. The liver was decompressed by cutting and cannulating (14 G teflon) the inferior vena cava (IVC) just below the liver. The perfusion pressure was maintained at 10 cm  $\text{H}_2\text{O}$ . The liver was gently removed and transferred to the perfusion apparatus after weighing.

#### *Hypothermic preservation:*

Olympus experimental organ preservation system (OXPS-R10, Olympus Co., Tokyo, Japan) was used for hypothermic perfusion. Masterflex pump controller with N-7016-20 pump head (Cole Parmer Instrument Co., Chicago, IL) was used to circulate the perfusate. Perfusion was with 300 ml Krebs-Henseleit bicarbonate (KHB) solution containing 5.6 mM glucose, 12 mg sulfamethoxazole and 2.4 mg trimethoprin. KHB perfusate was equilibrated with a gas mixture of 95%  $\text{O}_2$ –5%  $\text{CO}_2$  or 95%  $\text{N}_2$ –5%  $\text{CO}_2$  via an oxygenator at a flow rate of 2 L/min. Livers were perfused at a flow rate of 3.0–3.5 ml/g liver/min or simply stored with air saturated KHB solution for 12 hours at 5°C.

#### *Normothermic reperfusion:*

The normothermic recirculating perfusion system was described elsewhere<sup>34</sup>. The livers which were preserved for 12 hours either by perfusion or simple storage were perfused for one hour after 15 minutes equilibrium time with 250 ml oxygenated KHB solution at 37°C at a flow rate of 3.0–3.5 ml/g liver/min.

#### *Experimental groups:*

The livers were perfused for 12 hours at 5°C with oxygen saturated perfusate (oxygen group,

n = 12) or with nitrogen saturated perfusate (nitrogen group, n = 12) or simply stored in air saturated KHB solution (simple storage group, n = 12). Each twelve livers from respective group were subjected to determinations; six at the end of hypothermic preservation, and the remaining six at the end of subsequent one hour normothermic reperfusion. Another six livers were perfused with oxygenated KHB solution which contains 10 mM of ouabain (ouabain group) for 12 hours at 5°C and subjected to determinations at the end of hypothermic perfusion.

*Determinants:*

Perfusate samples were collected from the reservoir (hypothermic perfusion) or from the outflow (normothermic perfusion) at the end of each perfusion. They were used for analysis of potassium, glucose, AST, ALT, LDH and purine nucleoside phosphorylase (PNP). Left lateral lobe of the liver was clamped with precooled plates and immediately immersed in the liquid nitrogen for measurement of adenine nucleotides, purine catabolites and conjugated dienes. The median and right lateral lobes were taken for histology and measurement of water, sodium and potassium content.

Amount of bile production and oxygen consumption were also measured during normothermic preperfusion period.

*Measurements:*

Wet weight of liver was determined on an analytical balance after uniform blotting of each sample with filter paper. Each sample was dried in an oven at 105°C for 24 hours and reweighed to determine the dry weight and tissue water content.

Tissue sodium and potassium concentrations were measured by a flame atomic emission spectroscopy (FAES, Instrumentation Laboratory INC., Lexington, MA) after homogenating tissue in deionized water.

AST, ALT, LDH and glucose in the perfusate were determined by a Technicon RA-500 analyzer (Technicon Instrument Co., Tarrytown, NY) using commercially available kits (Technicon).

PNP in the perfusate was measured by the methods of Hoffee et al.<sup>18)</sup>. The separation of adenine nucleotides and purine catabolites was performed with a single-run high performance liquid chromatography (HPLC) by the modified of Wynants et al.<sup>50)</sup>. Extracted sample was analyzed with a Waters HPLC system (Waters Chromatography Division, Millipore Corporation, Milford, MA; Model 501 pumps, Model 484 absorbance module (path length, 10 mm) and Model 700 WISP system) equipped with Maxima 820 chromatography workstation (Waters). Reversephase column (E. Merk, Darmstadt, Germany; LiChrospher 100 RP-18 (5  $\mu$ m), 4 mm  $\times$  250 mm) was used with a precolumn (Waters; RCSS Guard-PAK). The solvent consisted of two mobile phases; [A], 0.15 M ammonium dihydrogen phosphate buffer, pH 5.7 and [B], acetonitrile and methanol (50/50, v/v) containing 1% of triethanolamine. The analysis was carried out by slow linear gradient from 100% of [A] and 18% of [B] for 20 min. For efficient separation, the first 4 min was isocratic of solvent [A].

Aliquots of the lipids extracted from 25  $\mu$ g of liver were evaporated under nitrogen and dissolved in 2 mol cyclohexane. The second derivative spectrum of each sample was measured from 300 to 220 nm with a model 557 Hitachi two wave length double-beam spectrophotometer (Hitachi, Japan). Tissue concentrations of cis, trans and trans, trans conjugated dienes were determined from the spectra by the method of Coronigiu et al.<sup>7)</sup>.

Oxygen consumption of the liver during normothermic reperfusion was monitored by the pO<sub>2</sub> (mmHg) measurement with the portal inflow and IVC outflow using a pH-blood gas meter (ABL2

Acid-Base Laboratory, Radiometer, Copenhagen, Denmark).

Tissues for histopathological examination were fixed with Bouin's solution for 24 hours, followed by 80% alcohol and stained with Hematoxylin-Eosin. Severity of histological damage was scored as none, slight, moderate or severe changes of sinusoidal lining cells and hepatocytes. Evaluation was done blindly without knowing the timing of tissue collection and experimental groups.

All data were presented as mean  $\pm$  standard error of the mean (Mean  $\pm$  SEM). Differences among the groups were determined by Mann-Whitney U test. A  $p$  value  $<0.05$  was considered to be significant.

## Results

Tissue edema was assessed by weight gain at the end of hypothermic preservation and by tissue water content at the end of normothermic reperfusion. Ability of the liver to maintain normal intracellular concentrations of potassium and sodium was assessed by potassium sodium ratio in the tissue. Tissue edema was significant in all experimental groups, but the extent of which was much higher in nitrogen and simple cold storage groups (Table 1). Addition of 10 mM ouabain into the oxygenated perfusate (ouabain group) significantly increased liver weight from 5.77% to 13.3% but it was still lower than that of nitrogen and simple storage groups. Tissue potassium sodium ratio in all four experimental groups were much lower than that of control group, reflecting reduced activity

**Table 1** Effect of oxygenation and hypothermia on tissue edema and electrolytes at the end of preservation

|          | Weight gain (%)       | Tissue K/Na           |
|----------|-----------------------|-----------------------|
| Control  |                       | $3.27 \pm 0.22$       |
| Oxygen   | $5.77 \pm 2.18$       | $0.75 \pm 0.18^b$     |
| Ouabain  | $13.3 \pm 1.56^c$     | $0.35 \pm 0.05^{b,c}$ |
| Nitrogen | $22.6 \pm 1.64^{d,e}$ | $0.58 \pm 0.16^b$     |
| Simple   | $24.6 \pm 0.82^{d,f}$ | $0.91 \pm 0.07^{a,e}$ |

Control values were obtained from six fresh livers flushed out by KHB solution.

<sup>a</sup>,  $p < 0.01$  versus control

<sup>d</sup>,  $p < 0.005$  versus oxygen group

<sup>b</sup>,  $p < 0.005$  versus control

<sup>c</sup>,  $p < 0.01$  versus ouabain group

<sup>e</sup>,  $p < 0.05$  versus oxygen group

<sup>f</sup>,  $p < 0.005$  versus ouabain group

**Table 2** Effect of oxygenation and hypothermia on tissue edema and electrolytes after normothermic reperfusion

|          | Tissue water content (Kg/Kg dry weight) | Tissue K/Na           |
|----------|-----------------------------------------|-----------------------|
| Control  | $2.65 \pm 0.10$                         | $2.29 \pm 0.29$       |
| Oxygen   | $2.52 \pm 0.08$                         | $2.11 \pm 0.14$       |
| Nitrogen | $3.36 \pm 0.20^{b,d}$                   | $1.18 \pm 0.11^{a,d}$ |
| Simple   | $3.28 \pm 0.31$                         | $1.19 \pm 0.22^{a,c}$ |

Control values were obtained from six fresh livers after one hour normothermic perfusion

<sup>a</sup>,  $p < 0.05$  versus control

<sup>c</sup>,  $p < 0.05$  versus oxygen group

<sup>b</sup>,  $p < 0.005$  versus control

<sup>d</sup>,  $p < 0.005$  versus oxygen group

of  $\text{Na}^+\text{-K}^+$  ATPase. Highest perfusate potassium and lowest potassium sodium ratio in ouabain group indicated near complete inhibition of  $\text{Na}^+\text{-K}^+$  ATPase.

During one hour normothermic reperfusion, potassium influx and water excretion were seen in all three experimental groups (Table 2). However they did not reach to the control levels except for oxygen group.

At the end of hypothermic perfusion, AST ( $20.3 \pm 3.09$  IU/L), ALT ( $7.0 \pm 1.98$  IU/L) and LDH ( $130.2 \pm 19.4$  IU/L) in the perfusate of nitrogen group was significantly higher than those of oxygen group ( $7.5 \pm 1.23$  IU/L,  $1.83 \pm 0.60$  IU/L and  $43.2 \pm 3.89$  IU/L, respectively).

At the end of normothermic reperfusion, AST, ALT and LDH showed remarkable increase in nitrogen and simple storage groups (Fig. 1). Those in oxygen group showed no significant difference when compared to the control.

Purine nucleoside phosphorylase (PNP) is located primarily in the cytoplasm of the endothelial and Kupffer cells and used for an indicator of damage to the microvascular endothelial cells of the liver<sup>40</sup>. PNP in nitrogen group ( $11.5 \pm 1.61$  mU/L) was significantly higher than in oxygen group ( $2.67 \pm 0.49$  mU/L,  $p < 0.005$ ) at the end of hypothermic perfusion. After normothermic reperfusion, PNP values in nitrogen and simple storage groups were significantly higher than control or oxygen groups (Fig. 1).

Glucose in the perfusate revealed metabolic activity of liver. Glucose at the end of hypothermic preservation were  $102 \pm 1.2$  mg/dl in oxygen group (same as the initial value), indicating no usage or gluconeogenesis. Glucose in nitrogen group was  $169 \pm 6.22$  mg/dl, reflecting activated anoxic glycogenolysis. This was confirmed by low level of glucose in perfusate in nitrogen group after one hour normothermic perfusion ( $20.2 \pm 6.43$  mg/dl). Glucose level in the perfusate after reperfusion showed no significant differences among control, oxygen and simple storage groups,  $65.3 \pm 10.8$  mg/dl,  $67.3 \pm 8.42$  mg/dl, and  $69.3 \pm 8.6$  mg/dl, respectively.

Bile production during one hour normothermic reperfusion was  $0.99 \pm 0.14$   $\mu\text{l/g}$  liver/min in

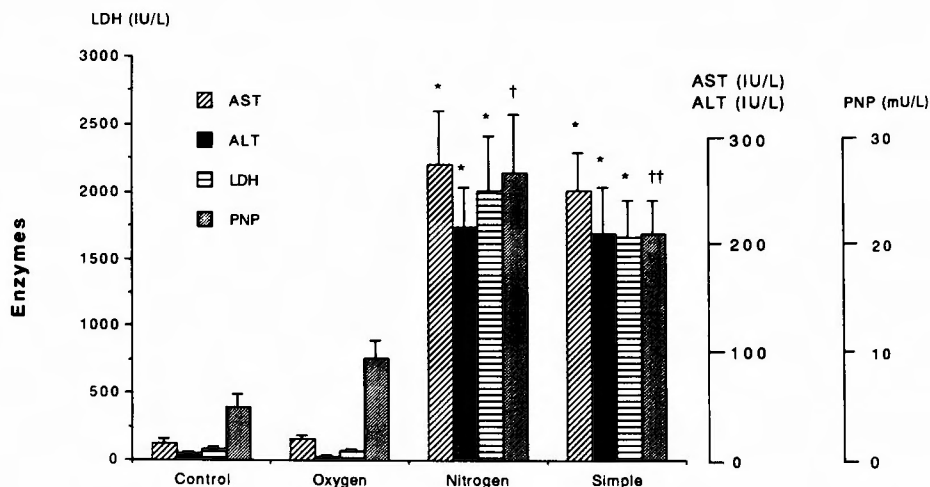


Fig. 1 AST, ALT, LDH and PNP in the perfusate after one hour normothermic reperfusion. Values shown are means with vertical bars equal to  $\pm$  SEM. Control values were obtained from six fresh livers perfused for one hour at  $37^\circ\text{C}$ . \*,  $p < 0.005$  versus control and oxygen groups. †,  $p < 0.05$  versus control and oxygen groups. ††,  $p < 0.005$  versus control,  $p < 0.01$  versus oxygen groups.

the control,  $0.95 \pm 0.09 \mu\text{l/g liver/min}$  in oxygen group,  $0.91 \pm 0.03 \mu\text{l/g liver/min}$  in nitrogen group and  $1.25 \pm 0.15 \mu\text{l/g liver/min}$  in simple storage group. There was no significant difference in bile production among all groups.

Oxygen consumption at normothermic reperfusion was slightly diminished in nitrogen group ( $1.85 \pm 0.07 \mu\text{mol/g liver/min}$ ), however, there was no significant difference among nitrogen, oxygen ( $1.92 \pm 0.10 \mu\text{mol/g liver/min}$ ) and simple storage groups ( $2.15 \pm 0.09 \mu\text{mol/g liver/min}$ ).

Energy status was assessed by tissue concentration of adenine nucleotide (AN) and purine catabolite (PC). Values were expressed as  $\mu\text{mol/g dry weight}$  instead of wet weight since livers preserved in these experimental protocols gained their weight to different extent at the end of hypothermic preservation as previously described.

All measurements of AN and PC in oxygen group were almost identical to the control at the end of hypothermic preservation (Table 3). ATP and energy charge in nitrogen and simple storage groups were significantly lower than control and oxygen groups. AmP and hypoxanthine in nitrogen and simple storage groups were significantly higher than control and oxygen groups.

At the end of normothermic reperfusion, There was no difference in the measurements of AN and PC except for lower ATP in oxygen and nitrogen groups (Table 4). When compared among three experimental groups, no differences were obtained in all measurements including ATP values.

Conjugated dienes were measured to assess the lipid peroxidation by free radicals on restoration of circulation. At the end of hypothermic preservation, higher values of conjugated dienes were ob-

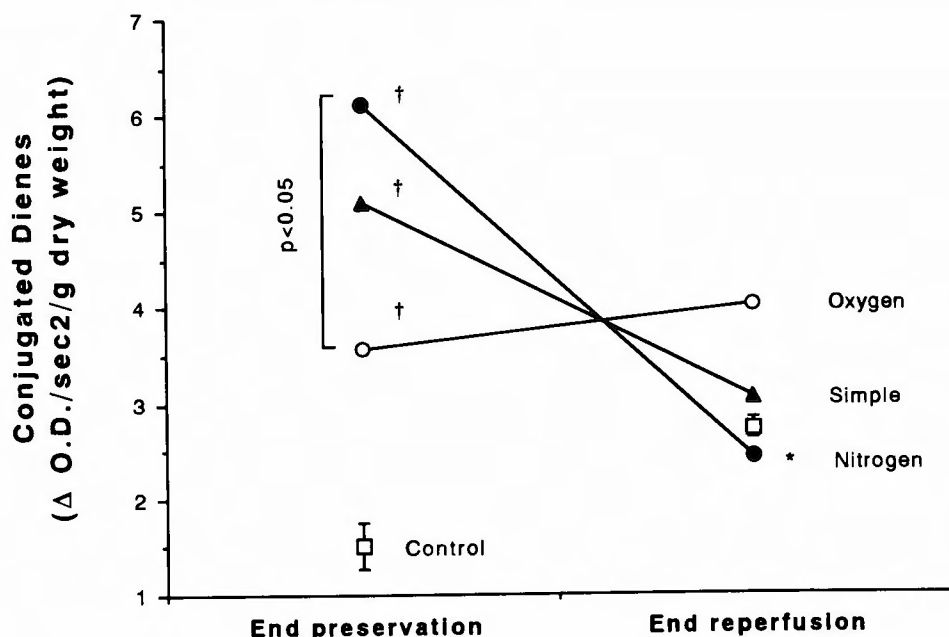


Fig. 2 Conjugated dienes concentrations before and after normothermic reperfusion expressed by  $\Delta \text{O.D./sec}^2/\text{g dry weight}$ . Control value at the end of preservation was obtained from six fresh livers, and control value at the end of reperfusion was obtained from six fresh livers perfused for one hour at  $37^\circ\text{C}$ . Values shown are means for oxygen, nitrogen and simple storage groups and means  $\pm$  SEM for control group.  $\dagger$ ,  $p < 0.05$  versus control value.  $*$   $p < 0.05$  versus prevalue (at the end of preservation) in the same group. No significant increase of conjugated dienes after normothermic reperfusion was detected in all three experimental groups.

**Table 3** Effect of oxygenation and hypothermia on adenine nucleotides and purine catabolites at the end of preservation

|          | ATP                      | ADP                    | AMP                      | Adenosine | Inosine   | Hypoxanthine           | Xanthine                 | Energy charge            |
|----------|--------------------------|------------------------|--------------------------|-----------|-----------|------------------------|--------------------------|--------------------------|
| Control  | 12.0±0.47                | 4.76±0.25              | 0.82±0.11                | 0.13±0.06 | 1.00±0.06 | 0.35±0.05              | 0±0                      | 0.82±0.01                |
| Oxygen   | 12.7±0.98                | 4.48±0.69              | 0.83±0.11                | 0.21±0.10 | 0.63±0.25 | 0.43±0.12              | 0.05±0.05                | 0.83±0.02                |
| Nitrogen | 2.88±0.35 <sup>c,e</sup> | 4.10±0.54              | 5.33±0.68 <sup>c,e</sup> | 0.62±0.26 | 0.68±0.17 | 2.61±0.17 <sup>d</sup> | 0.65±0.63                | 0.40±0.04 <sup>c,e</sup> |
| Simple   | 1.03±0.28 <sup>c,e</sup> | 2.91±0.52 <sup>a</sup> | 8.49±1.26 <sup>c,e</sup> | 0.23±0.09 | 1.51±0.36 | 2.77±0.85 <sup>a</sup> | 2.19±0.73 <sup>b,d</sup> | 0.21±0.04 <sup>c,e</sup> |

Values are expressed as  $\mu\text{mol/g}$  liver dry weight.

Energy charge=(ATP+ADP/2)/(ATP+ADP+AMP)

Control values were obtained from six fresh livers.

<sup>a</sup>, p<0.05 versus control

<sup>d</sup>, p<0.05 versus oxygen group

<sup>b</sup>, p<0.01 versus control

<sup>e</sup>, p<0.005 versus oxygen group

<sup>c</sup>, p<0.005 versus control

**Table 4** Effect of oxygenation and hypothermia on adenine nucleotides and purine catabolites after normothermic reperfusion

|          | ATP                    | ADP       | AMP       | Adenosine | Inosine                | Hypoxanthine | Xanthine  | Energy charge |
|----------|------------------------|-----------|-----------|-----------|------------------------|--------------|-----------|---------------|
| Control  | 12.9±0.59              | 6.21±0.48 | 1.68±0.38 | 0.52±0.07 | 1.75±0.11              | 0.66±0.11    | 0±0       | 0.77±0.02     |
| Oxygen   | 8.19±0.51 <sup>b</sup> | 5.21±0.37 | 2.2±0.4   | 1.24±0.92 | 1.33±0.13 <sup>a</sup> | 0.73±0.19    | 0±0       | 0.70±0.03     |
| Nitrogen | 8.89±1.04 <sup>a</sup> | 5.61±0.41 | 2.08±0.46 | 0.42±0.15 | 1.26±0.32              | 0.75±0.16    | 0±0       | 0.70±0.04     |
| Simple   | 8.74±1.52              | 5.47±0.44 | 2.34±0.44 | 0.3±0.11  | 0.64±0.23 <sup>b</sup> | 1.88±0.88    | 0.17±0.17 | 0.68±0.04     |

Values are expressed as  $\mu\text{mol/g}$  liver dry weight.

Energy charge=(ATP+ADP/2)/(ATP+ADP+AMP)

Control values were obtained six fresh livers after one hour normothermic perfusion.

<sup>a</sup>, p<0.05 versus control

<sup>b</sup>, p<0.005 versus control

**Table 5** Histological study at the end of preservation

|          | Endothelial cell damage |                    | Hepatocellular damage |        |          |        |               |        |          |        |
|----------|-------------------------|--------------------|-----------------------|--------|----------|--------|---------------|--------|----------|--------|
|          |                         |                    | discoloration         |        |          |        | Vacuolization |        |          |        |
|          | none to slight          | moderate to severe | none                  | slight | moderate | severe | none          | slight | moderate | severe |
| Oxygen   | 6                       |                    | 6                     |        |          |        | 4             | 2      |          |        |
| Nitrogen | 2                       | 4                  | 6                     |        |          |        | 4             | 1      | 1        |        |
| Simple   | 6                       |                    | 6                     |        |          |        | 4             | 2      |          |        |

Results are expressed by slide numbers determined to have histological findings classified in two or four severities as shown in the table

**Table 6** Histological study after normothermic reperfusion

|          | Endothelial cell damage |                    | Hepatocellular damage |        |          |        |               |        |          |        |
|----------|-------------------------|--------------------|-----------------------|--------|----------|--------|---------------|--------|----------|--------|
|          |                         |                    | discoloration         |        |          |        | Vacuolization |        |          |        |
|          | none to slight          | moderate to severe | none                  | slight | moderate | severe | none          | slight | moderate | severe |
| Oxygen   | 5                       | 1                  | 5                     | 1      |          |        | 2             | 4      |          |        |
| Nitrogen | 2                       | 4                  | 1                     | 3      | 1        | 1      |               | 3      | 3        |        |
| Simple   | 3                       | 3                  | 2                     | 1      | 2        | 1      | 1             |        | 4        | 1      |

Results are expressed by slide numbers determined to have histological findings classified in two or four severities as shown in the table



tained in all three experimental groups when compared to the control, and that of nitrogen group was significantly higher than oxygen group. However, when compared to the prevlaue of each group, no increase of conjugated dienes was detected after reperfusion.

At the end of hypothermic preservation, sinusoidal lining cells changes were often seen in nitrogen group, but were rate in oxygen and simple storage groups (Table 5). Hepatocytes showed no significant changes in all three groups.

After normothermic reperfusion, moderate to severe endothelial lining cell damages were evident in nitrogen and simple storage groups (Table 6), which were also accompanied with ischemic features, vacuolization and pyknotic nuclei at centrilobular hepatocytes. Histology of the oxygen group was well preserved than the others.

### Discussion

In a previous study, we measured oxygen consumption of rat livers at different temperatures<sup>9)</sup>. Livers perfused with KHB solution at 37°C utilized  $2.4 \pm 0.06 \mu\text{mol/g liver/min}$  of oxygen. When temperature was dropped, oxygen consumption decreased to  $0.40 \pm 0.02 \mu\text{mol/g liver/min}$  at 10°C and  $0.28 \pm 0.01 \mu\text{mol/g liver/min}$  at 5°C, which were still 16% and 12% of the amount at 37°, respectively. This impelled us to re-evaluate the oxygen necessity of liver at low temperature.

In this study, livers in all groups were preserved at 5°C. Thus, insult imposed upon livers by hypothermia or low temperature was consequently identical in all groups. Only difference was an accessibility to oxygen during the preservation period. Oxygen group received free oxygen, nitrogen group had no oxygen supply, and simple storage group used oxygen dissolved in initial flushing solution.

At physiological condition, cell volume is mainly regulated by  $\text{Na}^+\text{-K}^+$  ATPase and activity of which depends on an energy supply<sup>38)</sup> and a temperature<sup>30)</sup>. In present study, the influence of oxygen depletion was remarkable on tissue dema. Although tissue edema in oxygen group was higher than the control, those in oxygen deprived groups, nitrogen and simple storage groups, were much more higher than the oxygen group. The tissue edema in oxygen group might attribute to hypothermia as energy status in oxygen and control groups were identical. More striking tissue edema in nitrogen and simple cold storage groups might be derived from low energy status as the temperature was identical to all these three groups. Further more, results obtained from ouabain administration revealed some involvement of other volume regulating mechanisms which also need oxygen. Ouabain was used as a specific inhibitor of  $\text{Na}^+\text{-K}^+$  ATPase<sup>13)</sup> and the concentration of which (10 mM) is considered to be high enough to inhibit the activity of  $\text{Na}^+\text{-K}^+$  ATPase almost completely<sup>26)</sup>. The activity of  $\text{Na}^+\text{-K}^+$  ATPase was reduced to the same extent, or rather lowered in ouabain group, however, tissue edema was still lower in ouabain group than nitrogen and simple cold storage groups. This suggests that other mechanisms to maintain cell volume could spare the activity of  $\text{Na}^+\text{-K}^+$  ATPase when oxygen was supplied<sup>4,8,10-12,23,27,41,42,46,47,49)</sup>. These findings clearly ndicate that tissue edema is caused primarily by ischemia.

This harmful effect of oxygen depletion was also reflected in enzyme release at both before and after normothermic reperfusion. AST, ALT and LDH in oxygen deprived groups were 3 to 60 times higher than the control or oxygen groups, suggesting severe damage of hepatocytes.

A similar effect of oxygen on sinusoidal endothelial cells was demonstrated in present study. Recently the importance of sinusoidal lining cell damage in cold preservation of liver has been focused

<sup>3,31,32</sup>). Sinusoidal endothelial cells are considered to be more sensitive to cold ischemic insult than hepatocytes. In present study, higher value of PNP was detected at both before and after normothermic reperfusion in nitrogen and simple cold storage groups. Integrity of sinusoidal endothelial cell was well maintained solely in oxygen group assessed by PNP and histology.

The influence of oxygen depletion was also manifest in energy status at the end of hypothermic preservation. However, it was obscure after one hour normothermic reperfusion. The importance of preserving a high energy status during simple cold storage has been reported in reference to the post transplant function of a variety of organs<sup>6,16,17,22,33</sup>). In present study, ATP and energy charge in oxygen deprived groups were significantly lower than oxygen group at the end of hypothermic preservation, however, no significant differences were obtained in all measurements of AN and PC in all three groups after one hour normothermic reperfusion. Rapid and sufficient recovery of mitochondrial function in ischemia/reperfusion model has been reported at normothermia<sup>48</sup>) and at hypothermia<sup>39</sup>) in liver. A similar results were obtained in canine liver transplant model in our laboratory (unpublished data). These findings suggest that low energy status during hypothermic preservation might be caused by mere shortage of energy source (oxygen), and energy producing systems (particularly mitochondria) might be much more resistant to ischemic injury than plasma membrane system<sup>35,36</sup>).

Contrary to the study in lung<sup>44</sup>) and small bowel<sup>14</sup>), there still exists a controversy about the role of free radicals in ischemically insulted liver. In warm ischemia model, free radical theory is supported by the findings of ameliorative effects of antioxidants or free radical scavengers<sup>1,5,15,24,28,29,37</sup>), while no involvement of free radical is reported by measuring tissue GSH and GSSG<sup>20</sup>). In cold ischemia, Thurman et al.<sup>45</sup>) claimed oxygen free radicals as a major injury at peri-transplant period assessed by trypan blue uptake. However, Holloway et al.<sup>19</sup>) doubted an influence of free radicals assessed by futility of SOD, catalase and allopurinol. In present study, accumulation of hypoxanthine in oxygen deprived groups, and some extent of lipid peroxidation in all three groups (especially in nitrogen group) occurred during hypothermic preservation. Thus, ready status of reperfusion injury was established at the end of hypothermic preservation. Nevertheless, no increase of conjugated dienes after normothermic reperfusion was detected. Conjugated dienes were used as a specific marker of lipid peroxidation by free radicals<sup>7</sup>). These findings suggest no involvement of free radicals on restoration of circulation in this asanguinous model. However, as shown by enzyme release and histology, cell injury was remarkably exaggerated after reperfusion in oxygen deprived groups. This suggests that an important additional process might be involved in the pathogenesis of ischemia/reperfusion injury. Further study is needed in this field.

We used a standard electrolyte perfusion solution without cell components which might be important in free radical generation and we perfused the liver for only rather short period, prolongation of which might lead to substantial damage of mitochondria. However, our results clearly demonstrate a beneficial effect of oxygen supply to hypothermically preserved liver based on tissue edema, enzyme release and histological findings. Such results explain the limitation of preservation period with simple cold storage techniques. To establish better preservation method, oxygen supply should be considered, possibly using a machine perfusion.

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## 和文抄録

## 低温肝灌流における酸素の必要性について

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肝移植における保存臓器の障害が, 低温, 虚血, 再灌流時の活性酸素のいずれの要因によるものかを調べるため, ラット肝灌流モデルを用いて実験を行った. ラット肝を Simple Cold Storage 法にて保存もしくは, 酸素で飽和した灌流液または窒素により酸素を置き換えた灌流液をもちいて 5 度で 12 時間持続灌流し, 37 度 1 時間再灌流にて評価した. 組織浮腫は窒素加および Simple Cold Storage 群で著明であった. 再灌流後の灌流液中 AST, ALT, LDH, および PNP は窒素加および Simple Cold Storage 群で有意に高く, 酸素加群で

はコントロールと有意差を認めなかった. 低温保存後の組織中 Adenine Nucleotide Profile は酸素加群とコントロール群がほぼ一致し, 窒素加および Simple Cold Storage 群においては ATP 量, Energy Charge が有意に低値を示した. Conjugate Dienes は再灌流前後において有意の増加を示さず, 再灌流時の活性酸素の関与は認められなかった. これらの結果より保存中, および再灌流直後の障害は主に低酸素によることが示唆された.