Tohoku J. Exp. Med., 1999, **187**, 99-110

Effects of a Phosphate Buffered Extracellular (Ep4) Solution in Preservation and Reperfusion Injury in the Canine Liver

Yoshiyuki Kawashima, Susumu Ohwada, Kazuhiro Sakata, Toshihiro Ohya, Naoki Tomizawa, Izumi Takeyoshi and Yasuo Morishita

The Second Department of Surgery, Gunma University School of Medicine, Maebashi 371-8511

Kawashima, Y., Ohwada, S., Sakata, K., Ohya, T., Tomizawa, N., Takeyoshi, I. and Morishita, Y. *Effects of a Phosphate Buffered Extracellular (Ep4) Solution in Preservation and Reperfusion Injury in the Canine Liver.* Tohoku J. Exp. Med., 1999, **187** (2), 99-110 —— The Ep4 solution, a phosphate buffered extracellular-type solution, is effective in canine lung transplantation following a 96-hour hypothermic (4°C) preservation. In this experiment, we used this solution for liver preservation followed by transplantation. We compared the Ep4 solution with the lactated Ringer's (LR) and the Collins' M (CM) solution (a phosphate buffered intracellular-type solution) in two studies, 1) 48-hour liver preservation, and 2) orthotopic liver transplantation after 5-hour preservation. In the preservation study, purine nucleoside phosphorylase (PNP) levels as a marker of endothelial damage, and alanine aminotransferase (ALT) levels were significantly lower in the livers immersed into the Ep4 solution than in those immersed into other solutions at 36 and 48 hours after preservation. Microscopically, the endothelial injury occurred 24 hours after preservation in the CM solution, and 36 hours after preservation in the LR and Ep4 solutions. In the transplantation study, serum PNP and ALT levels in the livers immersed in Ep4 solution showed a lower tendency compared with those in other solutions at the time of reperfusion, but the histological differences among three groups were not apparent. The present study suggests that the liver can be stored better for a longer time using Ep4 solution than using LR and CM solutions. ——— phosphate buffered extracellular-type solution; Ep4 solution; preservation; reperfusion; liver transplantation © 1999 Tohoku University Medical Press

A phosphate buffered extracellular (Ep4) solution has been developed by Handa et al. (1989) as a solution for lung preservation. They reported success in a 96-hour hypothermic (4°C) preservation of a lung followed by transplantation. While the Ep4 solution demonstrated exellent results in preservation of the lung,

Received October 12, 1998; revision accepted for publication January 27, 1999.

Address for reprints: Yoshiyuki Kawashima, M.D., The Second Department of Surgery, Gunma University School of Medicine, 3-39-15 Showa-Machi, Maebashi, Gunma 371-8511, Japan.

e-mail: ykawashi@sb.gunma-u.ac.jp

there has been no evaluation regarding another organ preservation including the liver for transplantation. We conducted our study in order to assess the efficacy of Ep4 solution in experimental canine liver preservation and transplantation as compared to the Collins' M (CM), a phosphate buffered intracellular-type solution. The preservation and reperfusion injury of the liver were determined through two studies, 1) 48-hour liver preservation and 2) orthotopic liver transplantation after 5-hour preservation. We paid an particular attention to endothelial injury with an analysis of purine nucleoside phosphorylase (PNP) levels as a marker of endothelial damage (Rubio and Berne 1980; Rao et al. 1990) and also microscopical changes.

Materials and Methods

Animals

Adult mongrel dogs of both sexes, weighing 8 to 15 kg, were used in this study. All dogs were housed and had free access to water and standard pellet food. All animals were fasted for 12 hours prior to the experiment and cared for according to the guidelines of animal care in the Gunma University Animal Research Laboratory.

Preservation solutions and groups

Table 1 shows details of each test solution. The liver immersed in the lactated Ringer's (LR) solution was used as a control study. The Ep4 and CM solutions have three similar components, containing electrolytes, phosphate buffer and oncotic agents. The former has extracellular-type electrolytes with high sodium (142 mEq/liter) and low potassium (26 mEq/liter), and the latter has intracellular-type electrolytes with high potassium (89 mEq/liter) and low sodium

Table 1. *Composition of preservation solutions*

(mmol/liter)	LR	Ep4	CM
Na^+	131	142	9
K^+	4	26	89
Cl^-	110	97	19
Mg^+		4	
$H_2PO_4^-$		5	10
HPO_4^{2-}		60	30
Lactate	28		
LMD (%)		2	
Mannitol			4.25
Osm (mOsm/L)	270	360	360
pH	6.8	7.5	7.4

LMD, low molecular dextran.

(9 mEq/liter). The Ep4 solution developed by Tohoku University for lung preservation has a much higher concentration of phosphate buffer compared with the CM solution. The CM solution was originally made by Chiba University for kidney preservation.

Anesthesia

After a subcutaneous administration of ketamine hydrochloride (10 mg/kg of body weight), anesthesia was induced and maintained with an intravenous administration of pentobarbital sodium (20 mg/kg). Ventilation through an intratracheal tube was provided with oxygen (2 liter/minutes) by a Harvard type respirator (SN-480-3, Shinano Co., Ltd., Tokyo) and muscular relaxation was obtained with pancronium bromide (0.1 mg/kg).

Preservation experiment

This experiment was conducted to evaluate the effect of each solution for long time (48 hours) liver preservation, because Ep4 solution succeeded 96-hour lung preservation. Heparin sodium (300 U/kg) was administered intravenously during harvest of the liver. In order to compare the three solutions under similar conditions, a whole liver was divided into three segments. The procedure was as follows. After cross-clamping the ascending aorta, a total hepatectomy was immediately performed. The resected liver was sharply divided into three pieces along the lobulated line (Fig. 1). The vessels and biliary ducts were tied at the cutting plane. Because the bile juice disturbed the measurement of PNP activities (Nishida et al. 1989), the right medial and quadrate lobes, which were attached to the gallbladder, were abandoned. Two other divided livers were used in this study. A 20 cm-long cannula was inserted into the portal vein of the divided livers. The blood was then flushed out of vascular beds using a cold (4°C) preservation solution (30 ml/g liver weight) delivered at one meter of height.

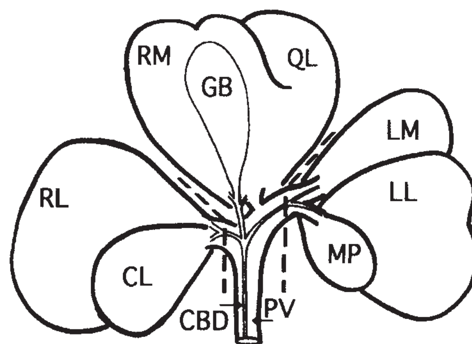

Fig. 1. The schema of canine liver. The liver is divided into three pieces along the interrupted line in the preservation experiment (Hepatic artery isn't figured). LL, left lateral; LM, left medial; RL, right lateral; RM, right medial; CL, caudate lobe; MP, mastoid process; QL, quadrate lobe; GB, gall bladder; CBD, common bile duct; PV, portal vein.

This procedure was done within 15 minutes and the warm ischemic time was less than 15 minutes. Immediately after flushing, each divided liver were immersed into the same cold (4°C) flushing solution for 48 hours. Every 12 hours, the liver was weighed and flushed via the portal vein using a cold preservation solution (0.2 ml/g liver weight) delivered from 20 cm of height. The effluent from the hepatic vein was collected for the measurement of PNP and ALT activities. At the same time, a liver specimen was taken and examined microscopically using hematoxylin-eosin (HE) staining. The experiment was divided into three groups according to the preservation solution; the LR group ($n=9$) with divided livers immersed into the LR solution, the CM group ($n=7$) with divided livers immersed into the CM solution, and the Ep4 group ($n=7$) with divided livers immersed into the EP4 solution.

Transplantation experiment

This experiment was conducted to evaluate the susceptibility of livers immersed in each solution against reperfusion injury. Five-hour preservation was considered to be safe for liver transplantation using Collins' solution (Caldwell-Kenkel et al. 1988), so that it would be able to compare magnitude of reperfusion injury in each liver without the influence of preservation injury. The donor liver was skeletonized and a cannula was inserted into the portal vein. After an intravenous administration of heparin (300 U/kg), the common hepatic artery was clamped and one litter of cold (4°C) test solution was infused from a hight of 1 meter through the cannula. Topical cooling was obtained using ice slush. After flushing the vascular beds, a total hepatectomy was performed. At back table surgery, the liver was immersed in a metallic bowl filled with a chilled (4°C) preservation solution, vessels of the resected liver were trimmed and catheters were inserted into the hepatic vein and biliary duct for sampling. The liver was then stored in the same cold (4°C) preservation solution until the transplantation procedure. Five hours later, the donor liver was slowly flushed with a 500 ml chilled LR solution and transplanted orthotopically according to the procedures describd elsewhere (Kam et al. 1986). In the anhepatic phase, the blood from the portal vein and the inferior vena cava returned to the superior vena cava by extracorporal bypass using a roller pump (Mera KBM-1, Senko Ika Co., Ltd., Tokyo) and heparin coated tubes (Anthron tubes™, TORAY medical Co., Ltd., Tokyo). Extracorporal circulation was maintained at a maximum flow of 30 ml/kg•min using 0.5 mg/kg of heparin. Blood samples were taken from the hepatic vein at the time of harvest and reperfusion (5 minutes after reperfusion) for measurement of PNP and ALT levels. Liver specimens for histological study were also taken 6 hours after reperfusion. As this experiment used roller pump and heparin, all animals were sacrificed 6 hours after reperfusion. The experiment was divided into three groups according to the test solution; the LR group with seven livers immersed into the LR solution, the CM group with five livers

immersed into the CM solution, and the Ep4 group with six livers immersed into the EP4 solution.

Laboratory parameters

PNP activity was measured by a coupled enzyme assay with xanthin oxidase (Kurashige et al. 1982; Rao et al. 1990). The amount of uric acid produced from inosine was measured by the increase in absorbance at 293 nm using a spectrophotometer (model 100-30, HITACHI Co., Ltd., Tokyo). One unit of PNP activity was determined to be the production of one μmol uric acid from inosine. ALT was measured by enzyme assay with an autoanalyzer (model 736-60, HITACHI Co., Ltd.) in the Department of the Central Laboratory of Gunma University hospital.

Microscopic examination

The enlargement of the space of Disse and/or the endothelial detachment from hepatocytes was considered to be endothelial injury. The presence of cytoplasmic pyknosis, nucleolysis or intracellular vacuoles was considered to be hepatocellular injury (Belzer et al. 1970; Koizumi et al. 1989).

Statistic analysis

All data are expressed as the mean$\pm$the standard error of the mean (S.E.M). The analysis of variance was used with post hoc test for the statistical analysis among the three groups. A p value of less than 0.05 was considered to be significant.

Result

Preservation experiment

The wet liver weight increased time-dependently in each group (Fig. 2). In the LR group, the wet liver weight gradually increased to 140% 48 hours after preservation, and in the CM and Ep4 groups, the wet liver weight gradually increased to 110%. There was a significant difference ($p<0.05$) in the wet liver weight between the LR group and the two other groups at 36 and 48 hours after preservation. PNP levels in the hepatic venous effluent increased time-dependently in each group (Fig. 3a). PNP levels in the CM group were higher than the two other groups until 24 hours after preservation; there was a significant difference ($p<0.05$) between the CM group and two other groups 24 hours after preservation. However, 36 hours after preservation, PNP levels in the LR group steeply increased. In the Ep4 group, PNP levels were significantly ($p<0.05$) lower compared with the two other groups at 36 and 48 hours after preservation. The change of ALT levels showed a similar pattern to the change of PNP levels (Fig. 3b). ALT levels in the CM group were higher than the two other groups at each point after preservation, but there was no significant difference among the

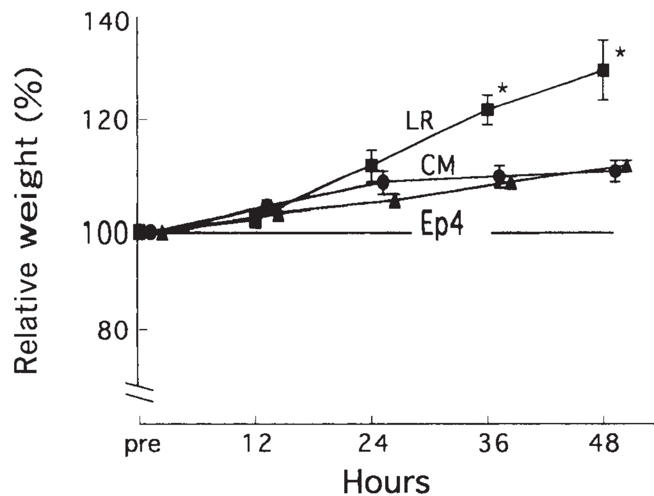

Fig. 2. The wet liver weight percentage change to pre-preservation values during cold (4°C) simple immersed preservation for 48 hours. Values present mean ± S.E.M. obtained from 7 to 9 divided livers. Significant difference between the LR group and the other two groups was shown by $^{*}p<0.05$; LR, lactated Ringer's ($n=9$); CM, Collins' M ($n=7$); Ep4 ($n=7$).

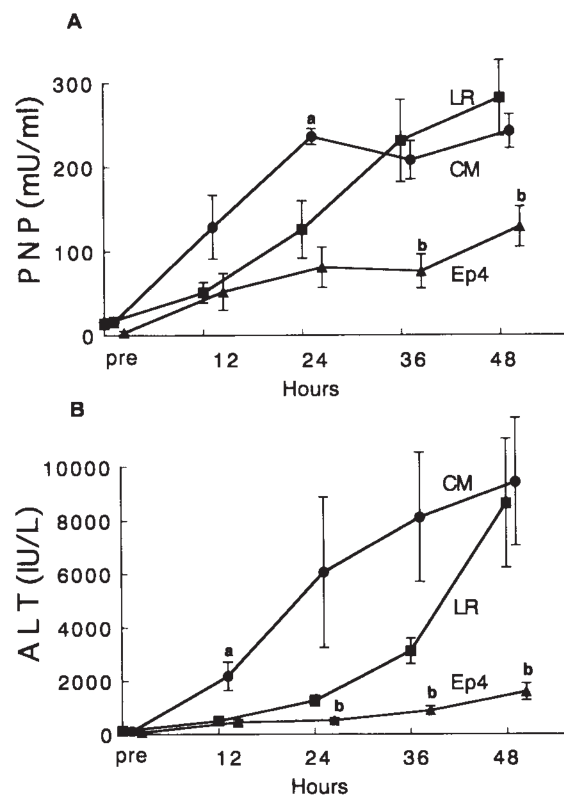

Fig. 3. The change of purine nucleoside phosphorylase (PNP; A) and alanine aminotransferase (ALT; B) levels in the effluent of the hepatic vein during cold (4°C) simple immersed preservation for 48 hours. PNP activity was measured by enzyme assay (Kurashige et al. 1982; Rao et al. 1990). ALT was measured by enzyme assay with an autoanalyzer (model 736-60, HITACHI). Values present mean ± S.E.M. obtained from 7 to 9 divided livers. Significant difference between the Ep4 group and the other two groups was shown by $^{a}p<0.05$, and that difference between the CM group and the other two groups was shown by $^{b}p<0.05$. LR, lactated Ringer's ($n=9$); CM, Collins' M ($n=7$); Ep4, ($n=7$).

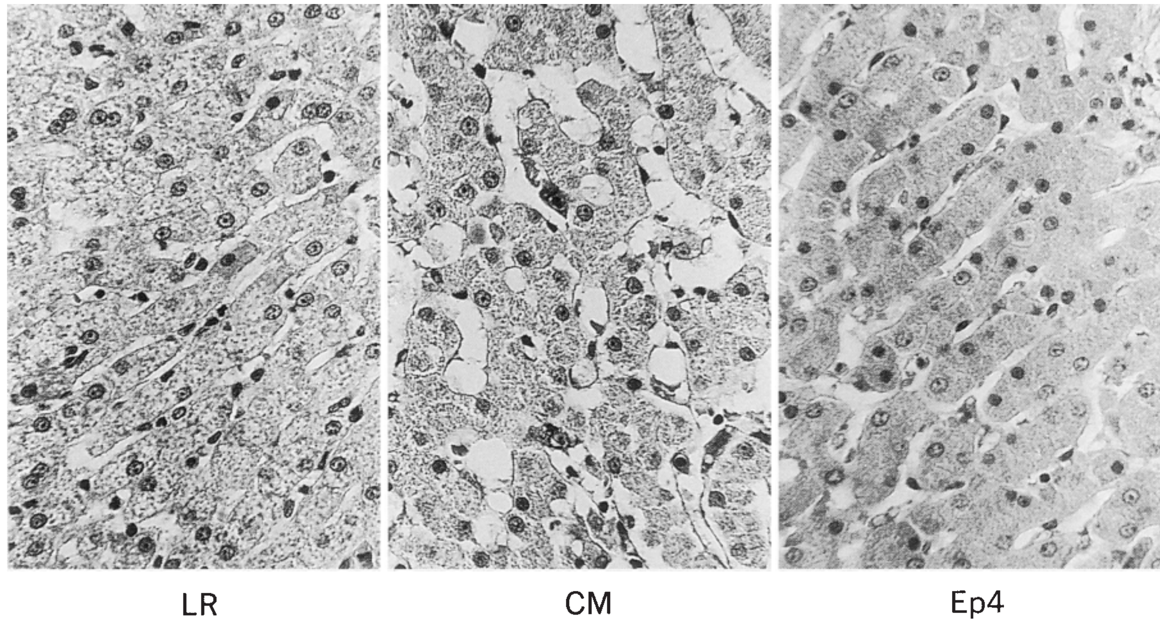

Fig. 4. Histological findings 24 hours after preservation (Hematoxyline-eosin, 200×). LR, lactated Ringer's; CM, Collins' M.

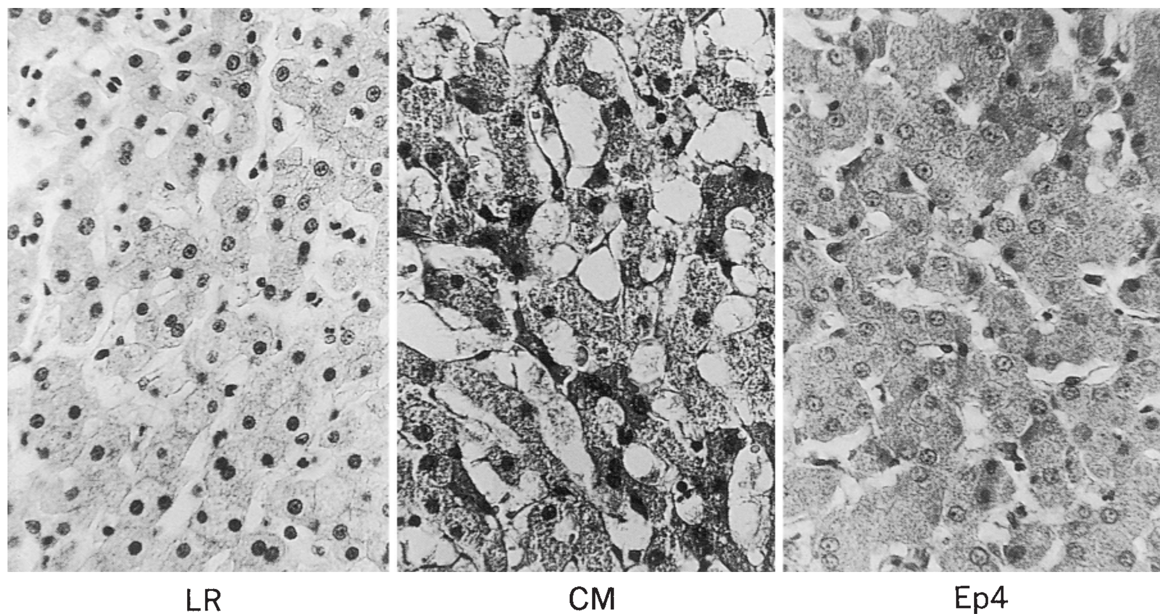

Fig. 5. Histological findings 36 hours after preservation (Hematoxyline-eosin, 200×). LR, lactated Ringer's; CM, Collins' M.

three groups.

Microscopical damage was not observed soon after flushing of the liver using each solution (Fig. is not shown). Microscopically, endothelial injuries, such as enlargement of space of Disse and/or detachment of endothelium from the hepatic cord, were observed at 24 hours of preservation in the CM group, and these changes were compatible with the findings reported by Koizumi et al. (1989). In both the LR and Ep4 groups, endothelial changes were seen 36 hours after preservation, but the degree of these changes seemed to be less in the Ep4 group than in the LR

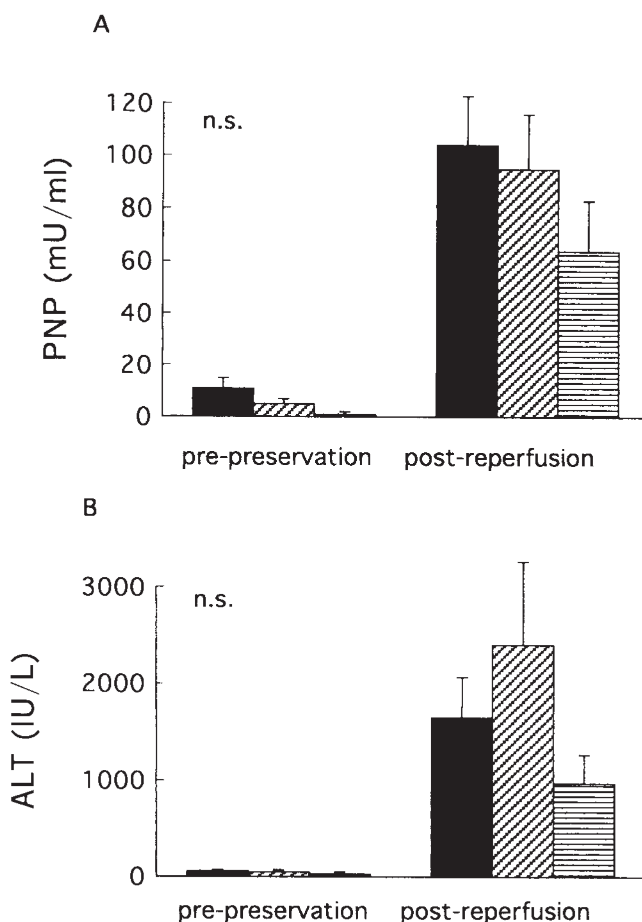

Fig. 6. The change of purine nucleoside phosphorylase (PNP; A) and alanine aminotransferase (ALT; B) activities in the transplantation experiment. PNP activity was measured by enzyme assay (Kurashige et al. 1982; Rao et al. 1990). ALT was measured by enzyme assay with an autoanalyzer (model 736-60 HITACHI). Values present mean±S.E.M. obtained from 5 to 7 dogs. n.s., not significant; ■, lactated Ringer's (LR, $n=7$); ▨, Collins' M (CM, $n=5$); ▤, Ep4 ($n=6$).

group. Hepatocellular damages, such as cytoplasmic piknosis, nucleolysis, and/or intracellular vacuoles, were observed at 24 hours of preservation in the LR and CM groups, and at 36 hours in the Ep4 group (Figs. 4 and 5).

Transplantation experiment

All implanted livers produced bile juice. At the time of reperfusion, PNP and ALT levels in the Ep4 group were lower than those in the two other groups, but there was no significant difference among the three groups (Fig. 6). Microscopically, hepatic cord derangement was obvious in each group, and hepatocellular damage, cytoplasmic pyknosis and intracellular vacuoles were also seen. The differences among three groups were not apparent. (Fig. 7).

Discussion

The gold standard of liver preservation for transplantation to date is cold

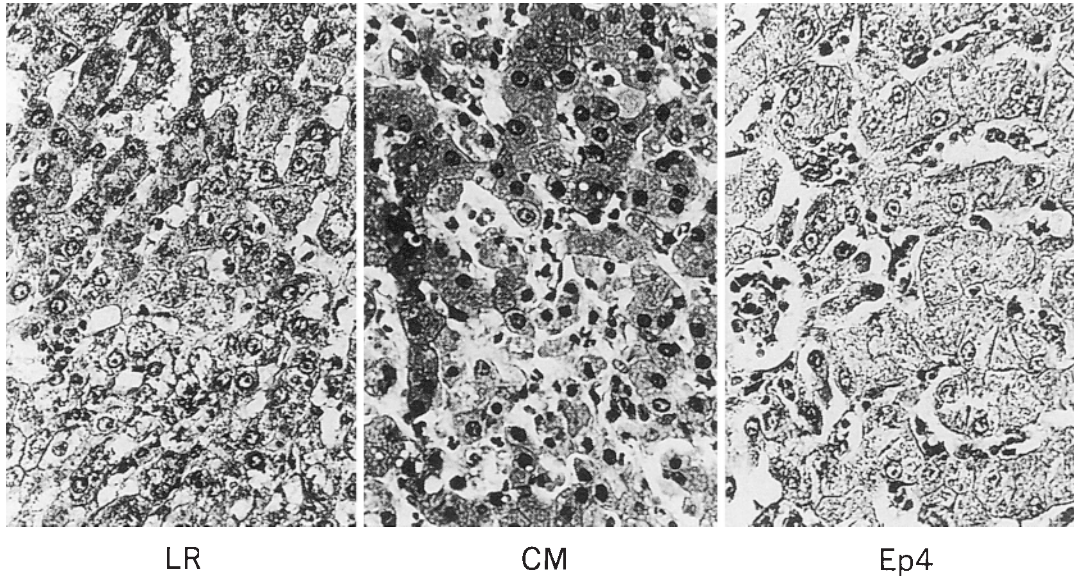

Fig. 7. Histological findings 6 hours after reperfusion (Hematoxyline-eosin, 200×). LR, lactated Ringer's; CM, Collins' M.

(4°C) flushing and immersion with the UW solution (Belzer 1988). This solution contains two impermeant anions, one colloid, two anti-oxidants, insulin, dexamethazone, adenosine, phosphate buffer, antibiotic agent and intracellular-type electrolytes. This solution is so complicated that it is difficult to determine which component is effective for liver preservation and also difficult to compare it with the simple Ep4 solution on the same theoretical ground. Therefore, we used three simple solutions for both the initial flushing and subsequent storage in this study. The results were as follows. The CM solution revealed high levels of PNP and ALT activities in the hepatic venous effluents compared to the Ep4 solution up to 48 hours after preservation. There was a significant difference between the CM and Ep4 groups in PNP levels. Histological damages of the endothelium were also lower in the Ep4 group compared to the CM group. In transplantation study, levels of PNP and ALT, and histological damages after reperfusion did not have differences among three groups.

The Ep4 solution contains extracellular-type electrolytes, phosphate buffers and low molecular weight dextran (LMD). The Collins'M solution contains intracellular-type electrolytes, phosphate buffers and mannitol. Both phosphate buffered solutions have similar osmolarity and pH. The Ep4 solution, however, has more phosphate buffer than the CM solution, and contains glucose. Handa et al. (1989) reported successful transplantation of 24-hour and 48-hour hypothermic preserved lung using the Ep4 solution. The protective effects of the Ep4 solution is thought to be due to 1) its high buffering capacity provided by $HPO_4^{2-}/H_2PO_3^-$ interaction, and 2) the oncotic pressure provided by LMD and glucose. High buffering capacity set the pH of this solution at 6.377 to 7.463 in 37°C without the influence of air composition and pressure, which is thought to suppress ischemia induced tissue acidosis. The high osmolarity provided by

LMD was significant in preventing cell swelling.

In addition, Handa et al. (1989) reported that an extracellular-type solution was effective for lung preservation because lung transplantation failed using intracellular-type solutions. An intracellular-type solution is reported to be harmful to endothelia in the initial flushing and preservation of the heart (Kinoshita and Ehara 1984; Choong and Gavin 1991; Chan et al. 1993). Kinoshita and Ehara (1984) reported that high potassium induced sudden depolarization of the cell membrane and muscle contraction due to the Ca^{2+} inflow. This muscle contraction attenuated the cardiac function after reperfusion. On the other hand, a preservation study with high sodium concentration of more than 60 mM was protected this type of muscle contraction. Choong and Gavin (1991) reported that a cardioplegic solution with high potassium increased coronary vascular resistance and induced poor coronary perfusion. Chan et al. (1993) reported an adverse effect of high potassium solution to vascular endothelial function. In addition to heart preservation, Tsubota et al. (1993) reported that a high potassium solution induced vasoconstriction of the lung and resulted in high perfusion pressure during cold perfusion preservation.

In liver preservation, Moen et al. (1989), who developed an original intracellular-type UW solution, reported good effects with a modified UW solution, electrolytes of which were completely changed from an intracellular-type to an extracellular-type. The high sodium UW solution showed significantly lower levels of asparate aminotransferase compared to the original UW solution after liver transplantation. In addition, Sumimoto et al. (1991, 1992) worked to simplify the UW solution without loosing its excellent effect and produced a histidine lactobionate (HL) solution. This solution was composed of histidine, lactobionate and extracellular-type electrolytes, and demonstrated good results compared to the original UW solution in rat liver transplantation. They emphasized that, 1) histidine had significant buffering capacity and suppressed intracellular acidosis, and 2) lactobionate did not permeate into the cell and suppressed the cell swelling during cold (4°C) simple immersed preservation. The Ep4 and HL solutions have a similar pattern of composition, such as buffering substances, oncotic agents and extracellular-type electrolytes.

In general, endothelial injuries of the hepatic sinusoid during preservation and reperfusion are one of the major causes of primary graft non-function (Clavien et al. 1992). The present study showed that a phosphate buffered extracellular-type solution demonstrated better protection of the endothelium against preservation injury than a phosphate buffered intracellular-type solution. In addition, a more simple extracellar-type LR solution, also showed lower values of PNP during first 24 hours of preservation compared with the CM solution. From these observations, extracellular-type electrolytes were significant for liver preservation, especially in preservation of the endothelium, in addition to buffers and oncotic agents. These observations are supported by the superior effect of the HL

solution in liver preservation. On the contrary, Ep4 solution needs some agents for protecting the liver tissue against reperfusion injury.

In conclusion, a phosphate buffered extracellular-type solution, Ep4 solution, is useful for liver preservation by protecting the endothelium against preservation injury. A comparative study of the Ep4 and UW solution is necessary because the latter solution is now gold standard for liver preservation.

Acknowledgments

We greatly thank Prof. Fujimura and Dr. Kondo, Tohoku University, for generously providing Ep4 solution. We also thank Prof. Kurashige, College of Medical Care and Technology, Gunma University, Prof. Kobayashi and Mr. Fukumura, Department of Central Laboratory, Gunma University Hospital, for consulting and helping in the measurement of the enzymes.

References

1) Belzer, F.O., May, R., Berry, M.N., Phil, D. & Lee, J.C. (1970) Short term preservation of porcine livers. *J. Surg. Res.*, **10**, 55-61.
2) Belzer, F.O. (1988) Principles of organ preservation. *Transplant. Proc.*, **20**, 925-927.
3) Caldwell-Kenkel, J.C., Thurman, R.G. & Lemasters, J.J. (1988) Selective loss of nonparenchymal cell viability after cold ischemic storage of rat livers. *Transplantation*, **45**, 834-836.
4) Chan, B.K., Kron, I.L., Flanagan, T.L., Kern, J.A., Hobson, C.E. & Tribble, C.G. (1993) Impairment of vascular endothel function by high-potassium storage solutions. *Ann. Thorac. Surg.*, **55**, 940-945.
5) Choong, Y.S. & Gavin, J.B. (1991) Functional recovery of hearts after cardioplegic and a storage in university of Wisconsin and in St. Thomas' hospital solutions. *J. Heart Lung.Transplant.*, **10**, 537-546.
6) Clavien, P.A., Harvey, P.R. & Strasberg, S.M. (1992) Preservation and reperfusion injuries in liver allograft. *Transplàntation*, **53**, 957-967.
7) Handa, H., Fujimura, S., Kondo, T., Ichinose, T., Shiraishi, Y. & Nakada, T. (1989) A study of preservation solution for 48- and 96-hr simple hypothermic storage of canine lung transplants. *Tohoku. J. Exp. Med.*, **159**, 205-214.
8) Kam, I., Lynch, S., Todo, S., Dewolf, A., McSteen, F., Jakob, F., Ericon, B.G., Takaya, S. & Starzl, T.E. (1986) Low flow venovenous bypass in small dogs and pediatric patients undergoing replacement of the liver. *Surg. Gynecol. Obstet.*, **163**, 33-36.
9) Kinoshita, K. & Ehara, T. (1984) Importance of sodium ions in the protective effect of high-potassium, high-glucose solution on electromechanical activities in the guinea-pig myocardium. *J. Mol. Cell Cardiol.*, **16**, 405-419.
10) Koizumi, N., Ohkouchi, N., Katoh, H., Koyamada, N., Fujimura, F., Sakurada, M., Andoh, T., Satomi, S., Sasaki, T., Taguchi, Y., Mori, S., Kataoka, S. & Yamamoto, T. (1989) Preservation and reflow damage in liver transplantation in the pig. *Transpant. Proc.*, **21**, 1323-1326.
11) Kurashige, S., Akuzawa, Y., Yoshida, T., Teshima, C. & Mitsuhashi, S. (1982) Purine metabolic enzymes in lymphocytes. I. Adenosine deaminase and purine nucleoside phosphorylase activities in mouse lymphocyte subpopulations. *Microbiol. Immunol.*, **26**, 77-85.
12) Moen, J., Claesson, K., Pienaar, H., Lindell, S., Pleog, R.J., McAnulty, J.F., Vreugdenhil, P., Southard, J.H. & Belzer, F.O. (1989) Preservation of dog liver, kidney, and pancreas using the Belzer-UW solution with a high-sodium and low-

potassium content. *Transplantation*, **47**, 940–945.
13) Nishida, Y., Hoshihara, Y. & Miyamoto, T. (1989) Activity and effect of purine metabolizing enzymes in the digestive tract. *Adv. Exp. Med. Biol.*, **253A**, 247–250.
14) Rao, P.N., Walsh, T.R., Makowka, L., Rubin, R.S., Weber, T., Synder, S.T. & Starzl, T.E. (1990) Purine nucleoside phosphorylase, a new marker for free oxygen radical injury to the endothelial cell. *Hepatology*, **11**, 193–198.
15) Rubio, R. & Berne, R.M. (1980) Localization of purine and pyrimidine nucleoside phosphorylase in heart, kidney, and liver. *Am. J. Physiol.*, **239**, H721–H730.
16) Sumimoto, R., Kamada, N., Jamieson, N.V., Fukuda, Y. & Dohi, K. (1991) A comparison of a new solution combining histidine and lactobionate with UW solution and euro-collins for rat liver preservation. *Transplantation*, **51**, 589–593.
17) Sumimoto, R., Lindell, S.L., Southard, J.H. & Belzer, F.O. (1992) A comparison histidine-lactobionate and UW solution in 48-hour dog liver preservation. *Transplantation*, **54**, 610–614.
18) Tsubota, N., Kameyama, K., Shugita, A., Hayashi, E., Kawaguchi, H., Taniguchi, H., Okada, T., Katsura, H., Nakamoto, K. & Maeda, M. (1993) Electrolyte concentration in flushing solution for lung transplantation. *Nihon Kokyuki Geka Gakkai Zasshi*, **7**, 112–117. (in Japanese with English abstract)