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K. Oikawa
N. Ohkohchi
M. Sato
S. Satomi

The effects of the elimination of Kupffer cells in the isolated perfused liver from non-heart-beating rat

K. Oikawa (✉) · N. Ohkohchi · M. Sato · S. Satomi
Second Department of Surgery, Tohoku University, School of Medicine, 1–1 Seiryo-machi, Aoba-ku, Sendai, 980–8574, Japan
e-mail: k-oikawa@mail.cc.tohoku.ac.jp
Tel.: + 81-22-7177214
Fax: + 81-22-7177217

Abstract We examined the effect of elimination of Kupffer cells on the sinusoidal microcirculation in graft harvested from non-heart-beating donors (NHBD), focusing on the arachidonic acid cascade and cytokines. Cardiac arrest was induced by thoracotomy. Livers were harvested 30 min after thoracotomy and perfused by Krebs-Henseleit bicarbonate buffer for 60 min after 6 h cold preservation. For the elimination of Kupffer cells, rats were pretreated liposome-encapsulated dichloromethylene diphosphonate (KE group). Eicosanoids (TXB_2, 6-keto-$PGF_{1\alpha}$, LTB_4) and cytokines (TNFα, IL-1β) in the perfusate were measured. Histological examination was also carried out. In the KE group, the value of TXB_2 was suppressed completely and cytokines were reduced, and sinusoidal structures and hepatocytes were well protected. These results indicated that the elimination of Kupffer cells improved sinusoidal microcirculation in NHBD and liver transplantation using grafts from NHBD could be made to succeed by modulation of Kupffer cells.

Key words Kupffer cell · Liver transplantation · Non-heart-beating donor · Thromboxane · Cytokine

Introduction

The shortage of donors has become a serious problem in liver transplantation (LTx). In recent years, to resolve this problem, LTx of the graft from non-heart-beating donors (NHBD) has become the object of attention all over the world. But liver grafts from NHBD have been considered unsuitable for LTx because the graft viability is deteriorated by warm ischemia and severe reperfusion injury [1, 2]. Many studies have been carried out to clarify the mechanisms of reperfusion injury. Recent studies showed that Kupffer cells are activated at reperfusion [3] and that inactivation of Kupffer cells contributes to the prevention of reperfusion injury [1, 2, 4–6]. We reported the success of LTx using grafts from agonal NHBD by elimination of Kupffer cells [1]. In this study, we investigated the mechanism by which the elimination of Kupffer cells could prevent ischemia/reperfusion injury of grafts from NHBD, focusing on the production of eicosanoids and cytokines.

Materials and methods

Animals and operative procedure

Experiments were conducted according to the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institutes of Health. Male Wistar rats weighing 240–300 g were allocated to four groups. In the control group ($n = 6$), livers were harvested while the heart was still beating and were perfused without cold preservation. In the HB group ($n = 6$), livers were harvested while the heart was still beating and were perfused after 6 h cold preservation in University of Wisconsin (UW) solution. In the NHB group ($n = 6$), cardiac arrest was induced by thoracotomy through the diaphragm and 30 min after thoracotomy the livers were harvested and perfused after 6 h cold preservation in UW solution. In the KE group ($n = 6$), rats were administered 2 ml/body (i.v.) liposome-encapsulated dichloromethylene diphosphonate (DMDP) to eliminated Kupffer cells 42 h before the graftectomy.

Under anesthesia with pentobarbital sodium (50 mg/kg, intraperitoneally), the liver was flushed with 10 ml 4 °C saline and 20 ml 4 °C UW solution (DuPont Pharmaceuticals, Wilmington,

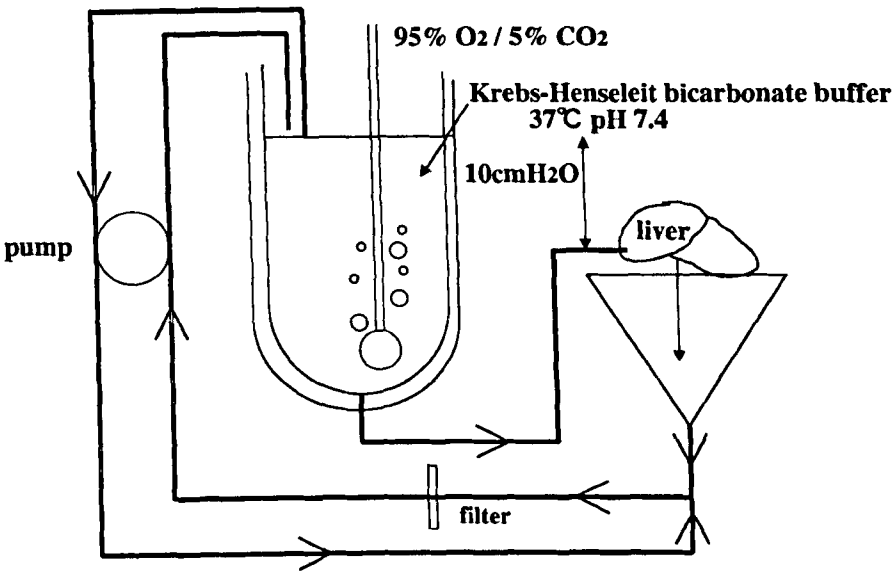

Fig. 1 Perfusion system ex vivo. Liver was perfused for 60 min at a pressure of 10 cm H_2O with 100 ml Krebs-Henseleit bicarbonate buffer (37 °C, pH 7.4) saturated with a 95 % O_2 and 5 % CO_2 mixture in a blood free recirculating system

Del., USA) via the portal vein and in the HB, NHB, and KE groups preserved in UW solution at 4 °C for 6 h.

Elimination of Kupffer cells

Liposome-encapsulated DMDP was prepared using a modification of the methods of Van Rooijen [7]. Briefly, 75 mg phosphatidylcholine and 19 mg cholesterol (Sigma, St. Louis, Mo., USA) were dissolved in 20 ml of methanol-chloroform (1:1) in a round-bottomed flask. The thin film coating the flask after low-vacuum rotary evaporation at 37 °C was dispersed in 10 ml phosphate-buffered saline (PBS; pH 7.4) containing 1.89 g DMDP (Boehringer Mannheim, Laval, Quebec, Canada) by shaking for 1 h in a water bath at 68 °C. After 2 h incubation at room temperature, free DMDP was removed by rinsing the liposomes with PBS and centrifuging them for 30 min at 100,000 g at 16 °C. Liposomes were resuspended in 4 ml PBS and 2 ml/body was injected intravenously 42 h before graftectomy as described previously. Previous studies have described a maximal effect on depletion of Kupffer cells between 24 h and 48 h after injection of DMDP liposome.

Perfusion after cold preservation ex vivo

Before perfusion, the liver was flushed with 10 ml cold saline to wash out UW solution and transferred to the perfusion system (Fig. 1) The liver was perfused for 60 min at a pressure of 10 cm H_2O with 100 ml Krebs-Henseleit bicarbonate buffer (pH 7.4, 37 °C) saturated with 95 % O_2 and 5 % CO_2 mixture in a recirculating system as described previously [8]. Flow rate of the portal was measured every 15 min, and bile juice was collected.

Examination items

Eicosanoids: thromboxane B_2 (TXB_2), 6-ketoprostaglandina (6-ketoPGF$_{1\alpha}$), and leukotriene B_4 (LTB_4)

Eicosanoids release from the perfused liver were assessed by measuring eicosanoid concentration in the perfusate using commercially available enzyme-linked immunosorbent assay kits (Cayman Chemical, Ann Arbor, Mich., USA).

*Cytokines: tumor necrosis factor*α (TNFα) and interleukin-1β (IL-1β)

Cytokines release from the perfused liver were assessed by measuring cytokine concentration in the perfusate using commercially available enzyme-linked immunosorbent assay kits (Biosource International, Camarillo, Calif., USA).

Aspartate aminotransferase (AST) and lactate dehydrogenase (LDH)

AST and LDH release, markers of liver injury, of the perfusate were assessed by measuring AST and LDH concentrations in the perfusate using commercially available kits (Wako Pure Chemical Industries, Tokyo, Japan).

Histological examination

Light microscopic study was performed. Specimens were fixed in 10 % neutral-buffered formalin and subsequently processed in paraffin wax. Sections (5 μm) were stained with hematoxylin and eosin.

Statistical analysis

All values in the text and figures are the mean ± SD. The difference between two groups was analyzed statistically using Student's *t*-test. $P < 0.05$ was considered statistically significant.

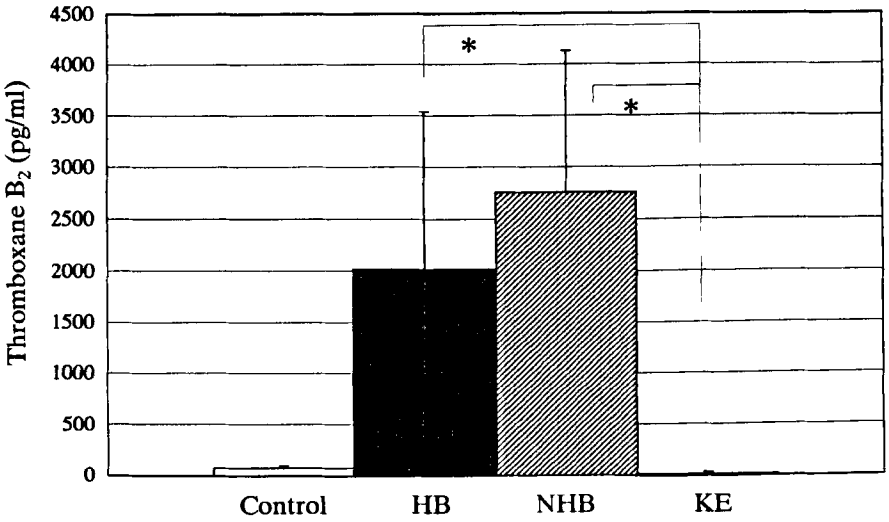

Fig. 2 The concentrations of thromboxane B_2 (TXB_2) in the perfusate 60 min after reperfusion. Each value represents the mean ± SD. In the KE group, production of the TXB_2 was completely suppressed. * $P < 0.01$, KE versus HB and NHB groups. *Control* heart-beating donor without preservation, *HB* heart-beating donor with 6 h cold preservation, *NHB* non-heart-beating donor with 6 h cold preservation, *KE* non-heart-beating donor with elimination of Kupffer cells and 6 h cold preservation

Table 1 Portal venous flow and bile production 60 min after reperfusion. Values of aspartate aminotransferase *(AST)* and lactate dehydrogenase *(LDH)* in the perfusate. Each value represents the mean ± SD. *Control* Heart-beating donor without preservation, *HB* heart-beating donor with 6 h cold preservation, *NHB* non-heart-beating donor with 6 h cold preservation, *KE* non-heart-beating donor with elimination of Kupffer cells and 6 h cold preservation

Groups	Portal venous flow (ml/kg body weight per h)	Bile production (µl/kg body weight per h)	AST (Karmen units)	LDH (Wroblewski units)
Control	4382 ± 543	1197 ± 543	1.0 ± 1.8	6.7 ± 2.7
HB	3277 ± 548	920 ± 318	0.5 ± 1.0	13.7 ± 10.8
NHB	3466 ± 1213	767 ± 148	17.0 ± 14.7	163.7 ± 154.4
KE	4830 ± 603*	1124 ± 143*	0.7 ± 1.6*	12.4 ± 7.9*

* $P < 0.05$ vs NHB group

Results

Portal venous flow

In the KE group, the portal venous flow was significantly increased compared with those in the HB and NHB groups ($P < 0.05$; Table 1).

Bile production

In the KE group, the bile production was significantly increased compared with those in the HB and NHB groups ($P < 0.01$; Table 1).

Eicosanoids

TXB_2 in the perfusate

In the NHB group, the concentration of TXB_2 was significantly higher compared with the other group. In the KE group, the concentration of TXB_2 was completely suppressed (Fig. 2).

6-Keto $PGF_{1\alpha}$ in the perfusate

In the HB group, the concentration of 6-keto $PGF_{1\alpha}$ was significantly higher compared with the other groups. In the NHB group, the concentration was reduced to the level in the control group (Fig. 3).

TXB_2/6-keto $PGF_{1\alpha}$ ratio

In the NHB group, the ratio was significantly larger compared with the other groups. In the KE group, the ratio was completely suppressed (Fig. 4).

LTB_4 in the perfusate

LTB_4 could not be detected in any group.

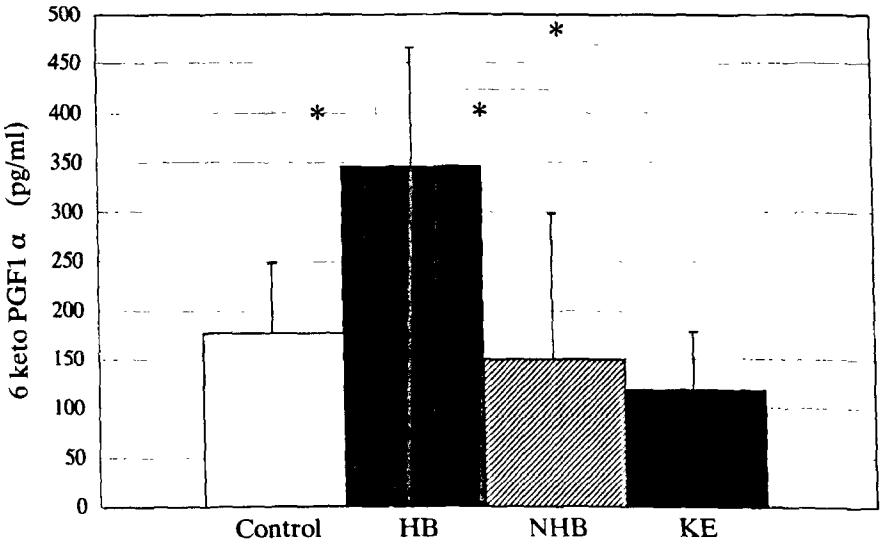

Fig. 3 The concentration of 6-ketoprostaglandinα (6-keto $PGF_{1\alpha}$) in the perfusate 60 min after reperfusion. Each value represents the mean ± SD. In the HB group, the concentration of 6-keto$PGF_{1\alpha}$ was significantly higher compared with other groups. In the NHB group, the concentration of 6-keto$PGF_{1\alpha}$ was the same as that in the control group. * $P < 0.05$, HB versus control, NHB, and KE

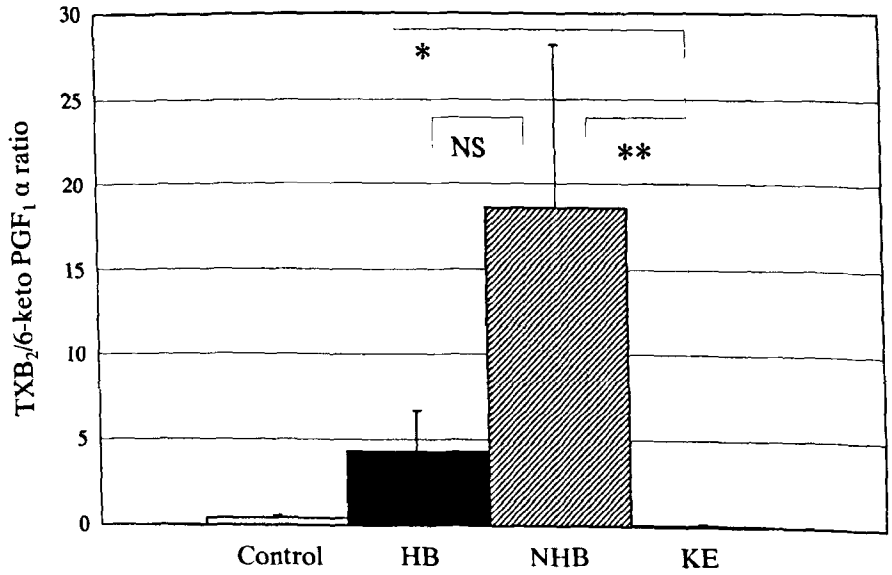

Fig. 4 Ratio of TXB_2/6-keto-$PGF_{1\alpha}$. Each value represents the mean ± SD. In the NHB group, the ratio was significantly higher compared with other groups. In the KE group, the ratio was completely suppressed. * $P < 0.05$, KE versus HB; ** $P < 0.01$, KE versus NHB. There was no significant difference between HB and NHB

Cytokines

IL-1β in the perfusate

In the NHB group, the concentration of IL-1β was significantly higher compared with other groups. In the KE group, the production of IL-1β was suppressed to the same level as the control group (Fig. 5).

TNFα in the perfusate

There was no significant difference between each group, but in the KE group, the production of TNFα was suppressed to the same level as the control group (Fig. 6).

AST and LDH release

In the NHB group, AST and LDH release increased significantly more than those in the other groups. In the KE group, their release was suppressed to the same level as the control group (Table 1).

Histological study

In the NHB group, the sinusoidal space became narrow, and hydropic changes in hepatocytes were observed. But in the KE group, sinusoidal structures and hepatocytes were as well preserved as in the control group (Fig. 7).

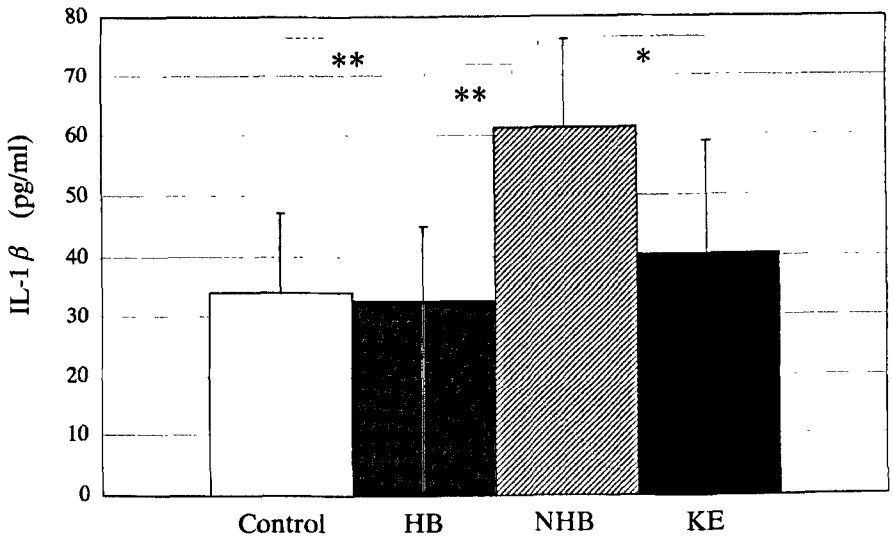

Fig. 5 The concentrations of interleukin 1β (IL-1β) in the perfusate 60 min after reperfusion. Each value represents the mean ± SD. In the NHB group, the concentration of IL-1βα was significantly higher compared with the other groups. In the KE group, it was suppressed to the control level. * $P < 0.05$, NHB versus KE; ** $P < 0.01$, NHB versus control and HB

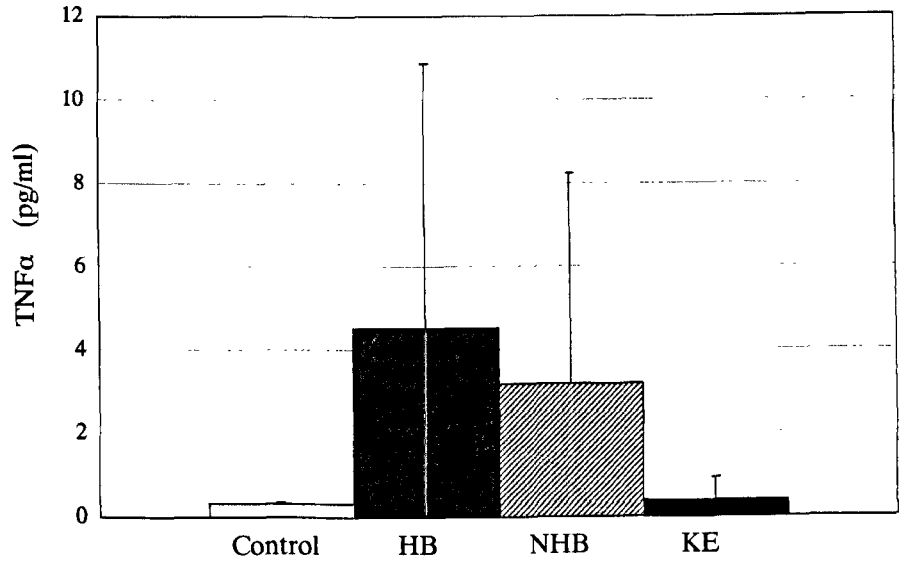

Fig. 6 The concentration of tumor necrosis factorα (TNFα) in the perfusate 60 min after reperfusion. Each value represents the mean ± SD. In the KE group, the production of TNFα was reduced to the same level as in the control group but there was no significant difference among the groups

Discussion

The shortage of donors has become a serious problem in LTx. It is generally accepted that liver can be transplanted only when harvested prior to cardiac arrest because warm ischemia causes liver damage and eventually graft failure. Recently it was reported that an increment of 20–25% in organ donors could be expected if NHBD were used [9]. LTx from NHBD has three major obstacles, namely, injuries of warm ischemia, cold preservation, and reoxygenation. The biggest difference from LTx from heart-beating donors is the existence of warm ischemia. Clinical and experimental studies suggest that the liver can tolerate warm ischemia, even for 60 or 90 min [10]. However, in the LTx from agonal NHBD, there are various unstable conditions of the donor and many clinical courses leading to cardiac arrest. It is still unclear why the usage of liver grafts from agonal NHBD has been unsuccessful. There are many studies about the mechanism of warm ischemic injury, and many factors have been implicated, for example, ATP depletion, increment in cytosolic calcium, mitochondrial calcium accumulation, activation of phospholipase A2, membrane phospholipid degradation, and free radical formation [11–15]. We hypothesized that the microcirculatory disturbance after LTx from agonal NHBD was the main cause, following the deterioration of sinusoidal endothelial cells and hepatocytes caused by activated Kupffer cells. In our present study, elimination of Kupffer cells suppressed the production of TXB_2 and cytokines and prevented injury of the sinusoidal microcirculation and hepatocytes in NHBD. Therefore, these

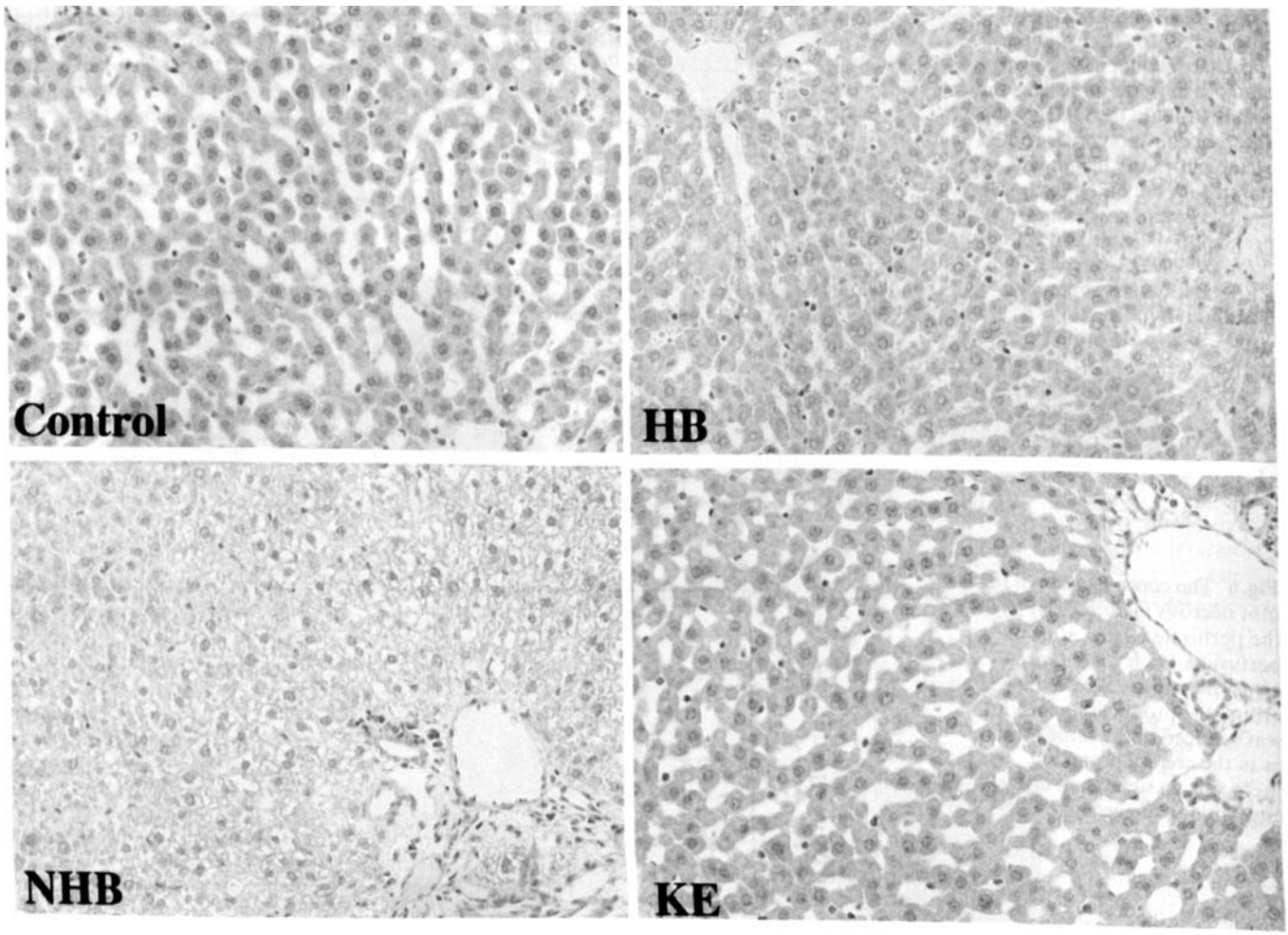

Fig. 7 Morphological findings of the livers 60 min after reperfusion (hematoxylin and eosin stain, original magnification × 100). In the NHB group, the sinusoidal space became narrow, and hydropic changes in hepatocytes were observed. In the KE group, sinusoidal structures and hepatocytes were well preserved, the same as in the control group

results indicate that Kupffer cells play an important role in the mechanism of graft injury in LTx from NHBD and that LTx using grafts from NHBD can be made to succeed by modulation of Kupffer cells.

Thromboxane A_2 (TXA_2), one of the products of the arachidonic acid cascade, is a vasoconstrictor and a stimulator of platelet aggregation [16]. Post et al. reported that concentrations of prostanoids of the hepatic vein including TXB_2 increased approximately 100- to 500-fold in the early period after reperfusion [17]. Soejima et al. reported that pretreatment of NHBD with the thromboxane synthetase inhibitor (OKY-046) in rat liver transplantation ameliorated graft viability by reducing the production of TXA_2 in the liver [18]. In general, the platelets are considered to be the main source of thromboxane production, as well as neutrophils, macrophages, Kupffer cells, and endothelial cells. In the present study, the elimination of Kupffer cells could suppressed the production of TXB_2 completely. These results suggest that Kupffer cells may be one of the main sources of thromboxane production in NHBD.

Recent investigations suggest that TNFα and IL-1 are key mediators in the early period of ischemia/reperfusion injury. Caughey et al. reported that TXA_2 was a paracrine or autocrine facilitator of TNFα and IL-1β production in zymogen-stimulated human monocytes and suggested that the capability of TNFα and IL-1β synthesis were determined by the balance between TXA_2 and PGE_2 production in human monocytes [19]. In the present study, elimination of Kupffer cells completely suppressed the production of TXB_2. The production of TNFα and IL-1β was reduced by eliminating Kupffer cells to the control level. These results support the previous study and suggest that the suppression of TXB_2 may induce the reduction of cytokine production. As mentioned above, it is interesting to made the effect of eicosanoids as a signal transmission molecule and

the relation between eicosanoids and cytokines in the mechanism of ischemia/reperfusion injury.

Kupffer cells play an important role in warm ischemia/cold preservation/reperfusion injury. A possible first step of ischemia/reperfusion injury is the production of reactive oxygen species in sinusoidal lining cells including Kupffer cells. Shibuya et al. reported that superoxide anions are generated mainly by Kupffer cells and that Kupffer cells modulate the injury in the initial phase of reperfusion after cold preservation [4]. In this study, the elimination of Kupffer cells could preserve sinusoidal endothelial cells and hepatocytes could be well preserved. These results indicated that the direct destruction of sinusoidal endothelial cells and hepatocytes by reactive oxygen species might be suppressed by the elimination of Kupffer cells.

In conclusion, elimination of Kupffer cells was strongly associated with suppression of the production of TXB_2 and cytokines and prevented disturbance of the sinusoidal microcirculation and injury of hepatocytes in NHBD. The results of our study suggest that liver transplantation using grafts from NHBD can be made to succeed by modulation of Kupffer cells.

References

1. Tsukamoto S, Ohkohchi N, Fukumori T, Orii T, Asakura T, Takayama J, Shibuya H, Kato H, Satomi S (1999) Elimination of Kupffer cells and nafamostat mesilate rinse prevent reperfusion injury in liver grafts from agonal non-heart-beating donors. Transplantation 67 (in press)
2. Ohkohchi N, Tsukamoto S, Endoh T, Fukumori T, Satomi S (1999) Can we transplant a liver graft from an agonal non-heart beating donor? (Evaluation of effectiveness of graft conditioning by an artificial heart and lung machine and drugs on graft injury) Organs and Tissues 1: 13–30
3. Caldwell-Kenkel JC, Currin RT, Tanaka Y, Thurman RG, Lemasters JJ (1991) Kupffer cell activation and endothelial cell damage after storage of rat livers: effect of reperfusion. Hepatology 13: 83–95
4. Shibuya H, Ohkohchi N, Seya K, Satomi S (1997) Kupffer cells generate superoxide anions and modulate reperfusion injury in rat livers after cold preservation. Hepatology 25: 356–360
5. Turman RG, Lindert KA, Cowper KB, Koppele JM te, Currin RT, Caldwell, Kenkel JC, Tanaka Y (1991) Activation of Kupffer cells following liver transplantation. In: Wisse E, Knook DL, McCuskey RS (eds) Cells of the hepatic sinusoid. Kupffer Cell Foundation, Linden, 3: 358–363
6. Bremer C, Bradford BU, Hunt KJ, Kencht KT, Connor HD, Mason RP, Thrman RG (1994) Role of Kupffer cells in the pathogenesis of hepatic reperfusion injury. Am J Physiol 267:G630–636
7. Van Rooijen N (1989) The liposome-mediated macrophage "suicide" technique. J Immunol Methods 124: 1–6
8. Gores GJ, Kost LJ, LaRusso NF (1985) The isolated perfused rat liver: conceptual and practical considerations. Hepatology 6: 511–517
9. Nathan HM, Jarrell BE, Broznik R, Kochik R (1991) Estimation and characterization of the potential renal organ donor pool in Pennsylvania. Transplantation 51: 142–149
10. Deiva E, Barberousse JP, Nordlinger B, Ollivier JM, Guilmet C, Huguet C (1984) Hemodynamic and biochemical monitoring during major liver resection with use of hepatic vascular exclusion. Surgery 95: 309–318
11. Clavien PA, Harvey PRC, Strasberg SM (1992) Preservation and reperfusion injuries in liver allografts: an overview and synthesis of current studies Transplantation 53: 957–978
12. Gasbarrini A, Borle AB, Farghali H (1992) Effect of anoxia on intracellular ATP, Na^+,Ca^{2+},Mg^{2+} and cytotoxicity in rat hepatocytes. J Biol Chem 267: 6654–6663
13. Fukuzawa K, Emre S, Senyuz O, Acarli K, Schwartz ME, Miller CM (1995) *N*-acetylcysteine ameliorates reperfusion injury after warm hepatic ischemia. Transplantation 59: 6–9
14. Inoue T, Yoshida Y, Nishimura M (1993) Ca(2 +)-induced, phospholipase-independent injury during reoxygenation of anoxic mitochondria. Biochim Biophys Acta 1140: 313–20
15. Marubayashi S, Takenaka M, Dohi K (1980) Adenine nucleotide metabolism during hepatic ischemia and subsequent blood reflow periods and its relation to organ viability. Transplantation 30: 294–296
16. Samuelsson B, Goidyne M, Granstrom E, Hammarstorm S, Malmsten G (1987) Prostaglandins and thromboxane. Annu Rev Biochem 47: 997–1029
17. Post S, Goerig M, Otto G, Manner M, Foltis C, Hofmann W (1991) Rapid increase in the activity of enzymes of eicosanoid synthesis in hepatic and extrahepatic tissues after experimental liver transplantation. Transplantation 51: 1058–1064
18. Soejima Y, Yanaga K, Nishizaki T, Yoshizumi T, Uchiyama H, Sugimachi K (1998) Effect of thromboxane synthetase inhibitor on non-heart-beating donors in rat orthotopic liver transplantation. Surgery 123: 67–72
19. Caughey GE, Pouliot M, Cleland LG, James MJ (1997) Regulation of tumor necrosis factor-alpha and IL-1 beta synthesis by thromboxane A_2 in nonadherent human monocytes. J Immunol 158: 351–358