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Successful liver transplantation from agonal non-heart-beating donors in pigs

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Abstract An effective way to overcome shortage of donors in liver transplantation (LTx) is to consider such from non-heart-beating donors (NHBDs). We investigated how a liver graft should be treated before and/or after procurement for successful LTx from an NHBD. Porcine LTx was performed with FR167653 (FR), a dual inhibitor of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and/or prostaglandin E_1 (PG). Animals were allocated to an FR group ($n=4$, donors and recipients were treated with FR), a PG group ($n=4$, donors and recipients were treated with PG), or an FRPG group ($n=4$, donors and recipients were treated with both FR and PG). No recipients in the FR group and only two of four recipients in the PG group survived, whereas all recipients in the FRPG group survived. Suppression of TNF-α and IL-1β and maintenance of microcirculation are the key to successful transplantation from NHBDs.

Keywords Liver transplantation
Non-heart-beating donor · Warm ischemic injury · Reperfusion injury

Introduction

The shortage of donors is a serious problem in liver transplantation (LTx) all over the world. It has generally been accepted that liver grafts could only be transplanted from heart-beating donors (HBDs). However, if it were possible to transplant liver grafts from non-heart-beating donors (NHBDs), the number of LTxs would increase remarkably. Clinically, LTx from controlled NHBDs is being attempted in some institutions [3, 5, 16], but it has also been reported that the grafts from NHBDs failed due to primary graft non-function more often than those from HBDs. In previous studies [6, 20] we showed that microcirculatory disturbance after reperfusion caused primary graft non-function in liver grafts from NHBDs. Microcirculatory disturbance is caused by the deterioration of sinusoidal endothelial cells (SECs) and sinusoidal narrowing. SEC deterioration can be prevented by the elimination of Kupffer cells and sinusoidal narrowing with nafamostat mesylate (NM) rinse [21]. However, in clinical practice it seems difficult to transplant liver grafts in which Kupffer cells have been eliminated. On the other hand, we also reported that tumor necrosis factor-α (TNF-α) induced the generation of superoxide by Kupffer cells, and this led to mediate neutrophil accumulation [18]. Thus, in our present study, we performed porcine orthotopic LTx of liver grafts retrieved from agonal NHBDs using FR167653 (FR), a dual inhibitor of TNF-α and interleukin-1β (IL-1β) production, to control the chain reactions after reperfusion mentioned above, instead of eliminating Kupffer cells. The aim of this study

was to establish a method leading to successful LTx from agonal NHBDs in clinical practice.

Materials and methods

All experiments were conducted according to the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health.

Experimental design

White Landrace pigs, weighing 20–30 kg, were allocated to five groups as follows:

1. Control group ($n=4$): donor and recipient pigs received no treatment. Immediately before anastomosis of the portal vein, the liver grafts were rinsed with 400 ml Ringer's lactate.
2. FR group ($n=4$): 1.0 mg/kg/h of FR167653 (FR; Fujisawa, Tokyo, Japan), a dual inhibitor of TNF-α and IL-1β production [25], was infused continuously during both donor and recipient surgery. The liver grafts were rinsed with 400 ml NM rinse (Table 1; Torii, Tokyo, Japan).
3. PG group ($n=4$): 20 ng/kg/min of prostaglandin E_1 (PG; Ono, Tokyo, Japan) was administered intravenously during donor and recipient surgery. The liver grafts were rinsed with NM rinse immediately before anastomosis of the portal vein.
4. FRPG group ($n=4$): donor and recipient pigs were treated with both FR and PG in the same manner as above. The liver grafts were rinsed with NM rinse.
5. HBD group ($n=4$): donor and recipient pigs did not receive any treatment, and the liver grafts were rinsed with NM rinse. The rinsing solution was kept at approximately 4 °C. LTx was performed after the animals had been starved for 24 h, during which time they were allowed to drink water ad libitum.

Donor operation

After the pigs had been pre-medicated with atropine injected intramuscularly, anesthesia was maintained with an isoflurane/oxygen mixture and pancuronium bromide. A catheter was placed in the external jugular vein to monitor central vein pressure, and another was placed in the common carotid artery to monitor blood pressure. After laparotomy had been performed, 10 mg/kg methylprednisolone and 30,000 U/kg ulinastatin (UTI; Mochida, Tokyo, Japan) were administered intravenously. The liver was retrieved as in humans, with minor modifications. First, the splenic vein and common bile duct were cannulated after heparin (300 U/kg) had been administered intravenously. We cannulated the abdominal aorta and stopped the donor's respiration by using pancuronium bromide and detaching the respirator. Central vein pressure was measured and kept below 14 cm H_2O until cardiac arrest was identified on the electrocardiogram. Ten minutes after cardiac arrest, perfusion of the liver with University of Wisconsin (UW) solution was started. UW solution (500 ml) was infused through the portal vein and the abdominal aorta, after which the liver was retrieved. The liver was promptly flushed with 50 ml UW solution through the hepatic artery and was stored in two plastic bags in an ice-slush bath for 3 h. In the HBD group, the liver was procured as mentioned above, but not in the agonal state.

Table 1 Composition of NM rinse

Parameter	Value
Na-lactobionate	110 mmol/l
NaH_2PO	25 mmol/l
Raffinose	30 mmol/l
Allopurinol	1 mmol/l
Glutathione	3 mmol/l
NM	0.8 mmol/l
pH	7.44
Osmotic pressure	290–300 mosmol/l

Recipient operation

The recipient pigs were anesthetized in the same way as the donors were. The right carotid artery and external jugular vein were cannulated for monitoring of blood pressure and for blood sampling and intravenous fluid infusion, respectively. After laparotomy, the liver was dissected free and removed. During the anhepatic phase, the blood of the portal vein and infra-hepatic inferior vena cava was shunted to the left external jugular vein with a biopump (Medtronic Bio-Medicus, Eden Prairie, Minn., USA). As anticoagulant, NM was infused continuously at 1 mg/kg/h until the biopump was stopped. The liver graft was implanted orthotopically with end-to-end anastomosis of the supra-hepatic inferior vena cava, the portal vein, the infra-hepatic inferior vena cava (all with 4-0 Prolene, running suture), and the hepatic artery (8-0 Prolene, intermittent suture) in that order. The liver graft was reperfused with portal blood after the portal vein anastomosis had been completed. The bile duct was drained via an external tube fistula. FK506 was administered postoperatively at 0.1 mg/kg/day to recipient pigs for immunosuppression.

Examination items

Tissue specimens were taken from the edges of each liver graft at laparotomy, 7 min after cardiac arrest, and 1 h after reperfusion.

Energy charge of liver tissue

To measure energy charge, we took tissue specimens, using tongs, pre-cooled the specimens in liquid nitrogen, and then stored them at –80 °C. After extraction with 0.6 N perchloric acid, adenosine triphosphate, diphosphate, and monophosphate were measured by high-performance liquid chromatography (Jasco HPLC Analyzer System; Nihon Bunko, Tokyo, Japan). The eluent was 60-mM phosphate buffer (pH 6.0), and the flow rate was 1.0 ml/min at room temperature. The energy charge was calculated according to the formula proposed by Atkinson and Chapman [1].

Proton ATPase activity of liver tissue

Proton ATPase activity was determined as described by Seya et al. [17]. Briefly, mitochondrial pellets were suspended in a potassium buffer solution, and Dis3C, a fluorescent dye, was added. After the electron transport chain had been stopped by the addition of 250 ng of antimycin A, changes in the mitochondrial membrane potential upon the addition of 2 μmol of ATP to the mixture were measured by fluorometry (Jasco FP-777; Nihon Bunko) at 23 °C, at an excitation wavelength of 625 nm and an emission wavelength of 670 nm.

Histological examination

Tissue specimens for microscopy were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin wax. Sections 5 μm thick were stained with hematoxylin and eosin. Tissue specimens for electron microscopy were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). After treatment with 1% osmium tetroxide, they

were dehydrated through a graded series of ethanol and embedded in epoxy resin. Ultra-thin sections were stained with uranyl acetate and lead citrate, and zone 1 of the liver acini was examined under an electron microscope (JEM-1010; Japan Electron Optics Laboratories, Tokyo, Japan). The reasons we selected zone 1 for examination were that injuries of SECs are more prominent in the early period of cold preservation [11] and that more severe injuries may occur due to Kupffer cells at reperfusion.

Aspartate aminotransferase and lactate dehydrogenase

Serum aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were measured with commercially available kits (AST: Iatron, Tokyo, Japan; LDH: Wako Pure Chemical Industries, Osaka, Japan).

Statistics

All values are presented as mean ± SD. We analyzed the differences among the five groups, using one-factor ANOVA, and then made pairwise comparisons using a post-hoc test. P values of below 0.05 were considered statistically significant.

Results

Recipient survival

The mean duration of warm ischemia from the time the respirator was turned off until cardiac arrest was 31.2 ± 2.9 min, and there were no statistical differences between the groups except for the HBD group. The mean time of cold ischemia was 3.0 ± 0.3 h, and there were no statistical differences between the five groups. In the control and the FR groups, no recipient survived for longer than 24 h. Massive hemorrhagic ascites were found at autopsy. In the PG group, two recipients died within 24 h, and findings similar to those observed in the control and FR groups were found at autopsy. Two of four recipients survived for more than 7 days. In the FRPG and HBD groups, all four recipients survived for more than 7 days (Table 2).

Table 2 Recipient survival after LTx

Group	Survival	Cause of death
Control group ($n=4$)	< 24 h	Primary graft failure
	< 24 h	Primary graft failure
	< 24 h	Primary graft failure
	< 24 h	Primary graft failure
FR group ($n=4$)	< 24 h	Primary graft failure
	< 24 h	Primary graft failure
	< 24 h	Primary graft failure
	< 24 h	Primary graft failure
PG group ($n=4$)	< 24 h	Primary graft failure
	< 24 h	Primary graft failure
	> 7 Days	Killed
	> 7 Days	Killed
FRPG group ($n=4$)	> 7 Days	Killed
	> 7 Days	Killed
	> 7 Days	Killed
	> 7 Days	Killed
HBD group ($n=4$)	> 7 Days	Killed
	> 7 Days	Killed
	> 7 Days	Killed
	> 7 Days	Killed

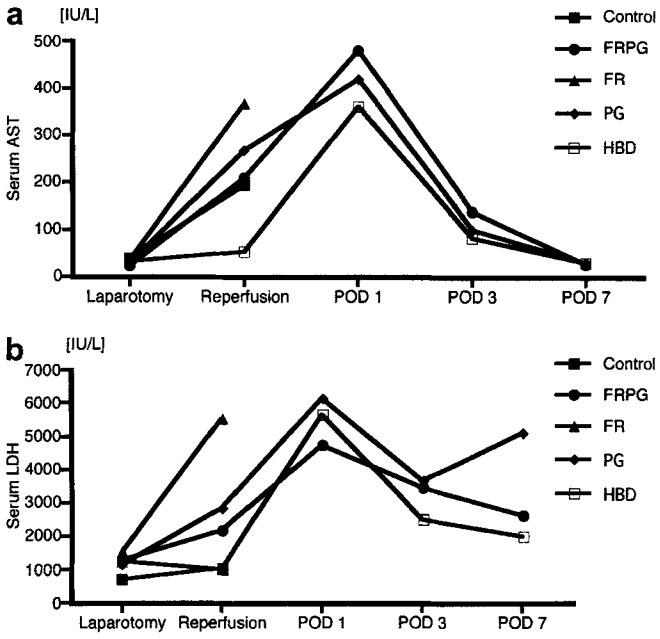


Fig. 1 Serum AST level (**a**) and serum LDH level (**b**). Both AST and LDH increased after reperfusion in all but the HBD group, and especially in the FR group. On postoperative day 1, AST and LDH remained high and then decreased. AST levels normalized by postoperative day 7. There were no statistical differences between the groups in each phase. (*Laparotomy* at laparotomy of the recipients, *Reperfusion* 5 min after reperfusion, *POD* postoperative day)

Serum AST and LDH

Serum AST and LDH increased after reperfusion in all but the HBD group and especially in the FR group. On postoperative day 1, AST and LDH remained high and then decreased. AST levels normalized by postoperative day 7. There were no statistical differences between the groups in each phase (Fig. 1).

Energy charge of liver tissue

The energy charge at laparotomy was 0.601 ± 0.134. The values decreased significantly to less than 50% of the initial value in all but the HBD group at 7 min after cardiac arrest. There were no statistical differences between the four NHBD groups. One hour after reperfusion, the value recovered to 50–85% of that at laparotomy in the FR, PG, and FRPG groups, but did

not recover in the control group. In the FR group, the recovery was less than in the PG and FRPG groups. There were no statistical differences between the five groups in the values 1 h after reperfusion (Fig. 2).

Mitochondrial proton ATPase activity in the liver graft

Proton ATPase activity at laparotomy was 55.78± 11.32%. ATPase activity decreased 7 min after cardiac arrest in the control, FR, and PG groups, but was maintained in the FRPG group. One hour after reperfusion, the values decreased remarkably in the control, FR, and PG groups. On the other hand, the value was maintained at the same level as that observed at laparotomy in the FRPG and HBD groups, and was significantly higher than those in the other groups (Fig. 3).

Microscopy findings

At 7 min after cardiac arrest, the sinusoidal spaces in zone 1 were wide in the PG and FRPG groups, but in the control and FR groups the sinusoidal spaces were narrow (Fig. 4). One hour after reperfusion, the sinusoidal spaces were narrow, and massive bleeding was recognized in the control, FR, and PG groups. Partial necrosis of hepatocytes in zone 2 was observed in the three groups. These changes were not observed in the FRPG and HBD groups (Fig. 5).

Electron microscopy findings

At 7 min after cardiac arrest, both SECs and hepatocytes were almost normal except for hypoxic vacuoles in the PG and FRPG groups, but deterioration of SECs was recognized and spillage of the cytoplasm into the sinusoidal space observed in the control and FR groups. Hypoxic vacuoles in hepatocytes were also observed in these two groups (Fig. 6). One hour after reperfusion, both SECs and hepatocytes were observed to be well preserved in the FRPG and HBD groups. SECs and Disse's space were completely destroyed in the control, FR, and PG groups and hepatocyte microvilli were hardly recognized. Spillage of the cytoplasm into the sinusoidal space was observed, and erythrocytes were found in Disse's space (Fig. 7).

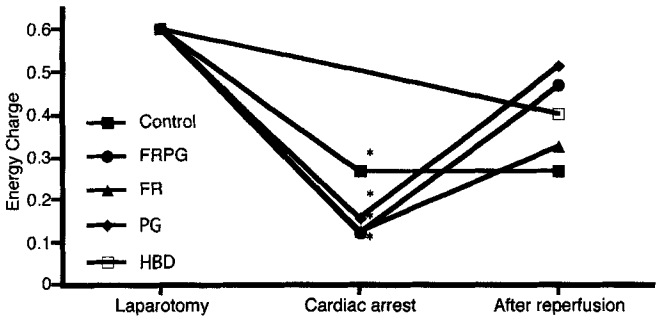


Fig. 2 Changes in energy charge of the liver grafts. After cardiac arrest, the energy charge values of non-HBD groups significantly decreased and then recovered after reperfusion, except for the control group. There were no statistical differences between the values in all groups at 1 h after reperfusion. $*P<0.05$ vs initial value (*Laparotomy* at laparotomy of the donors, *Cardiac arrest* 7 min after cardiac arrest, *After reperfusion* 1 h after reperfusion)

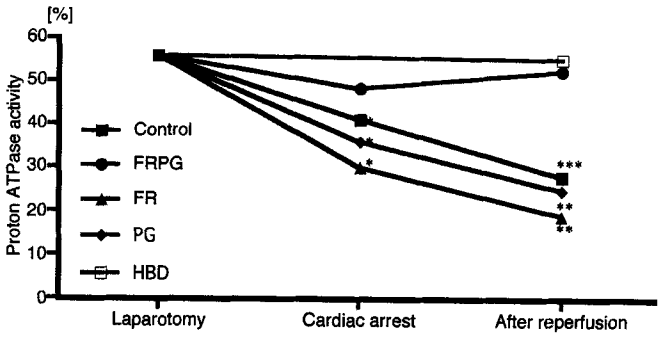


Fig. 3 Changes in proton ATPase activity of the liver grafts. The ATPase activity decreased after cardiac arrest in the control, FR, and PG groups, but was maintained in the FRPG group. After reperfusion, the values decreased remarkably in the control, FR, and PG groups. On the other hand, the value was maintained at the same level as that observed at laparotomy in the FRPG and HBD groups, and they were significantly higher than those in the other groups. $*P<0.05$ vs FRPG, $**P<0.05$ vs FRPG and HBD, $***P<0.05$ vs HBD (*Laparotomy* at laparotomy of the donors, *Cardiac arrest* 7 min after cardiac arrest, *After reperfusion* 1 h after reperfusion)

Discussion

The number of patients on waiting lists for LTx has been increasing, and the waiting time has become longer [2]. Surgeons are forced to consider organs from suboptimal donors, such as NHBDs, and some trials are underway at some institutions. However, LTx from NHBDs is seldom performed, since their liver grafts are very susceptible to primary graft non-function. We have previously reported that microcirculatory disturbance was a main obstacle to successful LTx from NHBDs and that this disturbance was due to two major factors: SEC deterioration and sinusoidal narrowing. Furthermore, we also reported that elimination of Kupffer cells prevented SEC deterioration and that sinusoidal narrowing was prevented by NM rinse [22]. In that study, Kupffer cells were eliminated by liposome-encapsulated dichloromethylene diphosphonate (L-DMDP). However, to eliminate Kupffer cells, L-DMDP had to be administered 42 h before the procurement of liver grafts [22]. It seems difficult to apply this procedure to clinical practice. Therefore, a new strategy to control Kupffer-

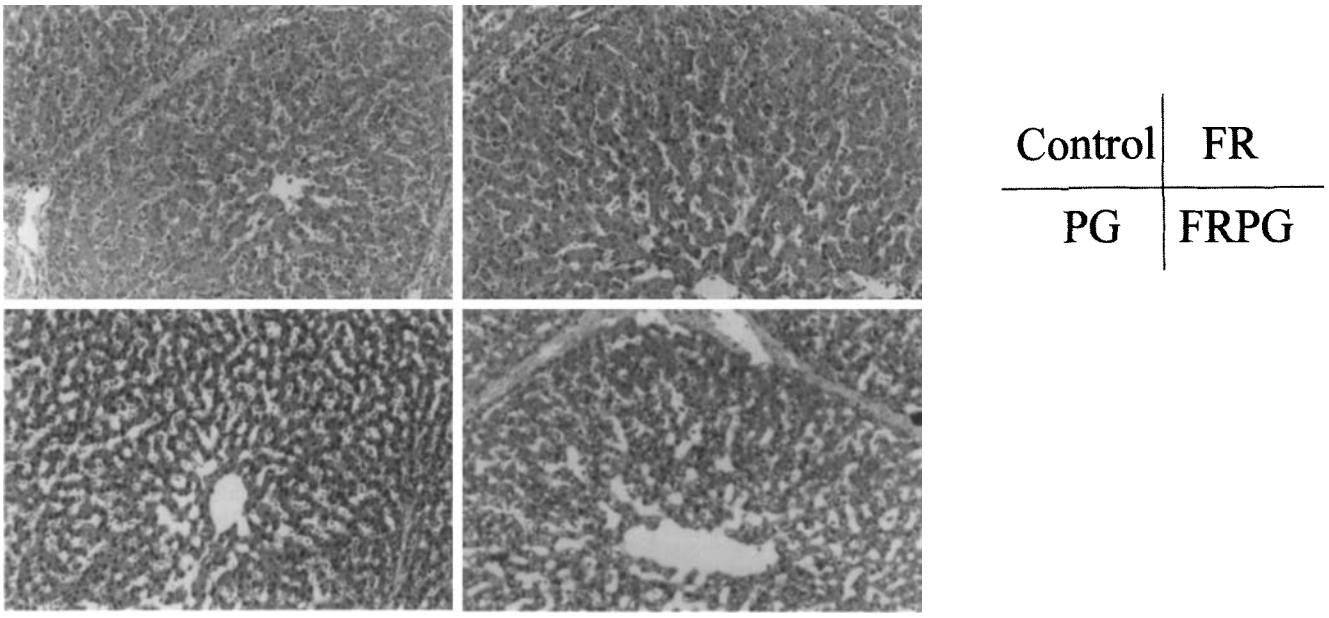
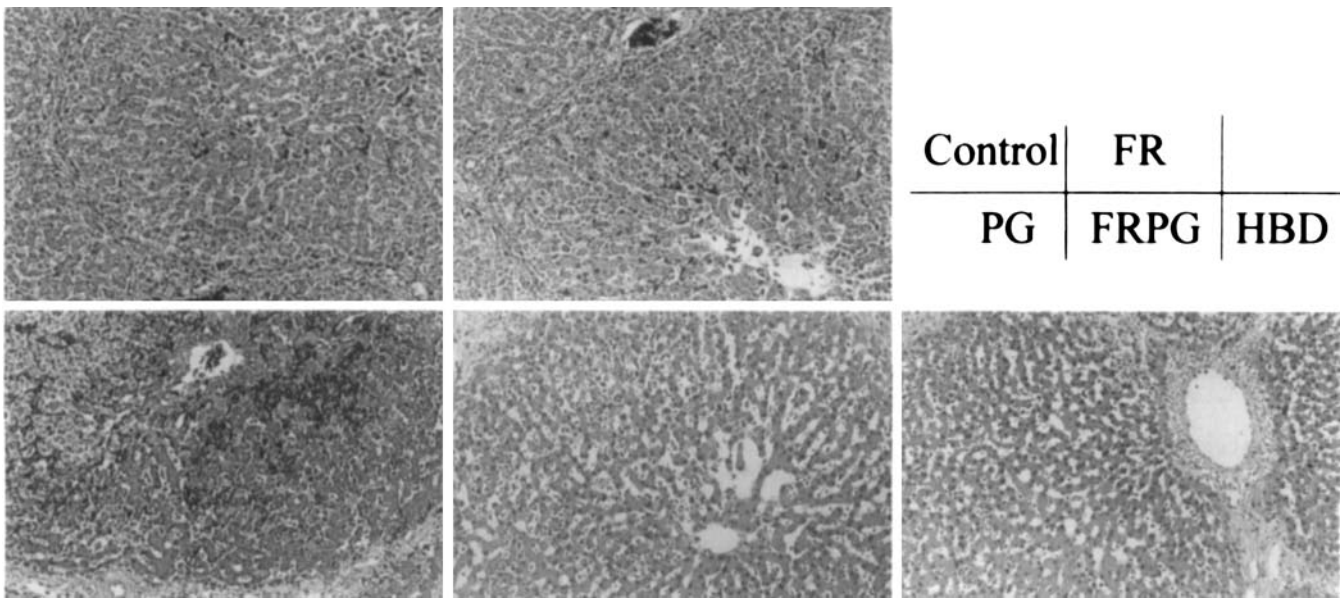


Fig. 4 Microphotograph of the livers 7 min after cardiac arrest (hematoxylin and eosin stain, original magnification ×40 on 35-mm film). The sinusoidal spaces in zone 1 were wide in the PG and FRPG groups, but in the control and the FR groups, the sinusoidal spaces were narrow

cell function should be considered. In the present study, we clearly demonstrated that FR, which is a dual inhibitor of TNF-α and IL-1β production, prevents SEC deterioration at reperfusion in LTx from NHBDs.

Previously, we demonstrated that suppression of Kupffer-cell function was an important key to successful LTx from agonal NHBDs. In agonal NHBDs, cytokines liberated by the gut prime the liver graft, and especially Kupffer cells. The primed Kupffer cells are stimulated and excited during cold ischemia [13]. We previously reported that TNF-α induced the generation of superoxide by Kupffer cells, and this led to mediate neutrophil accumulation [18]. Neutrophil accumulation is probably associated with microvascular damage [7], and adherent neutrophils can secrete numerous mediators including reactive oxygen species, cytokines, and proteases, greatly amplifying the inflammatory response. Our previous

Fig. 5 Microphotograph of the livers 1 h after reperfusion (hematoxylin and eosin stain, original magnification ×40 on 35-mm film). The sinusoidal spaces were narrow and massive bleeding was recognized in the control, FR, and PG groups. Partial necrosis of hepatocytes in zone 2 was observed in the three groups. These changes were not observed in the FRPG and the HBD groups



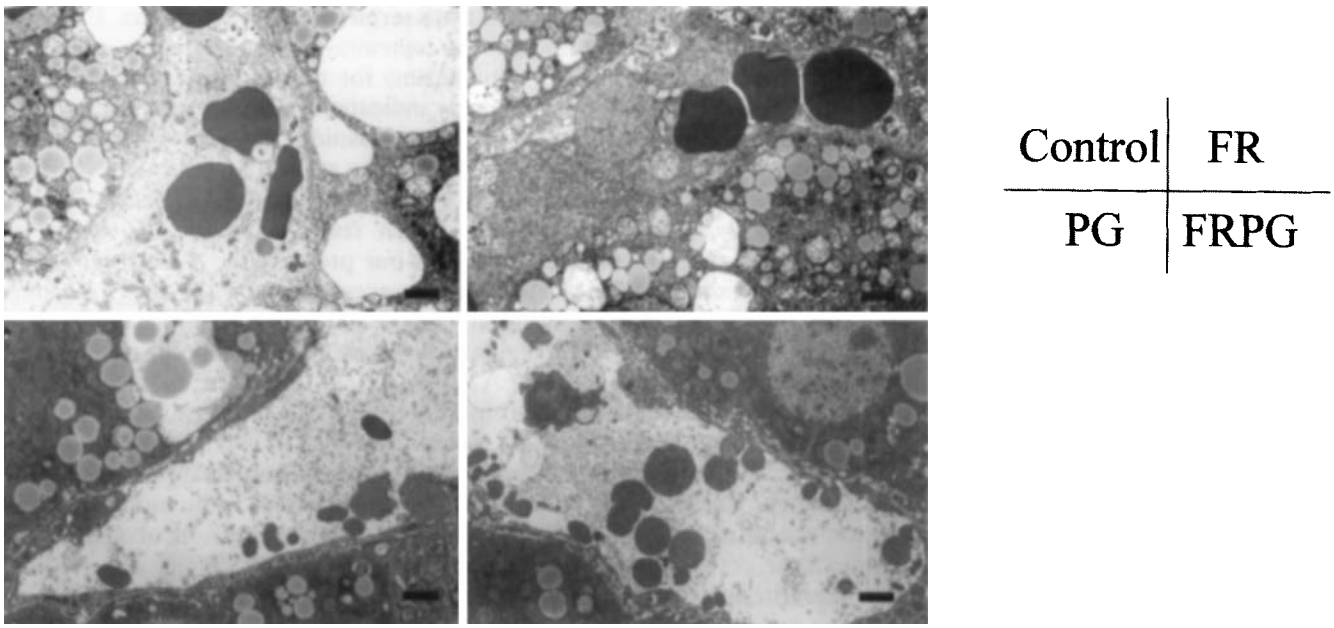
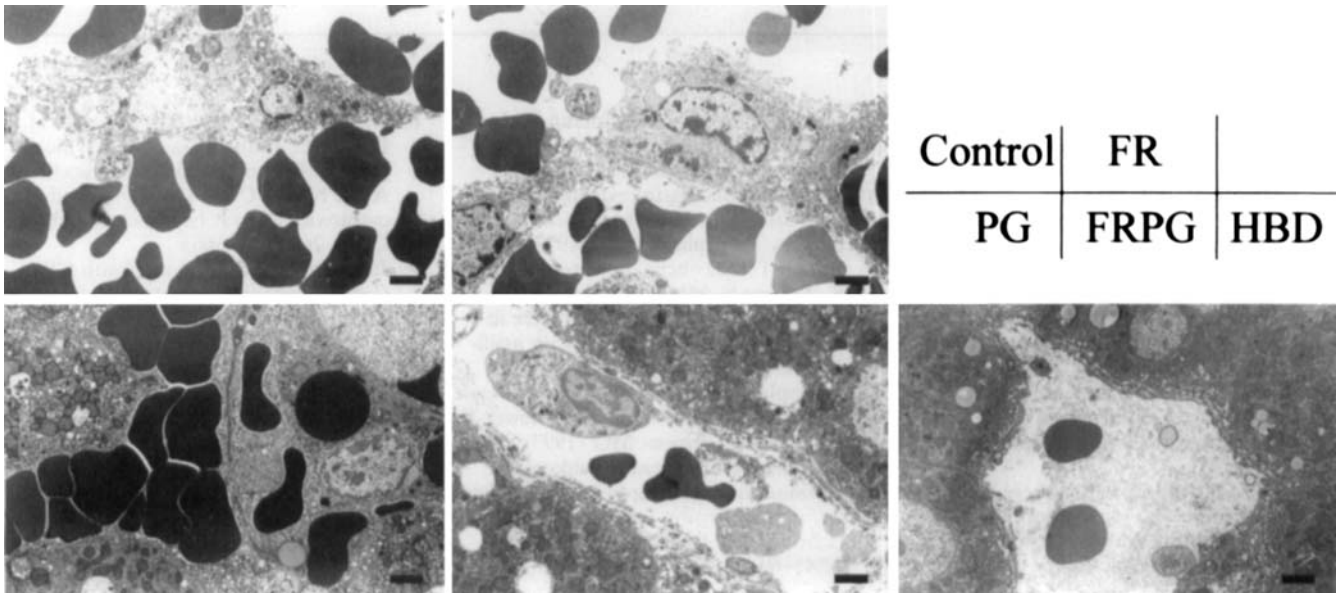


Fig. 6 Electron microphotograph of the livers 7 min after cardiac arrest. SECs were almost normal in the PG and FRPG groups, but deterioration of SECs was recognized and spillage of the cytoplasm into the sinusoidal space was observed in the control and the FR groups. *Scale bar* = 2 μm

studies suggested that the inhibition of TNF-α production would suppress Kupffer cell activation and prevent the chain reaction following reperfusion injury, as mentioned above [18]. We have also reported that the production of IL-1β was significantly high in liver grafts from NHBDs [14]. Thus, in our present study we administered FR to control the chain reactions mentioned above instead of eliminating Kupffer cells. In this study, electron microscopy after reperfusion revealed distinct disruption and detachment of SECs in liver grafts in the PG group, whereas in those in the FRPG group the injury was much milder. These results indicate that inhibition of TNF-α production to suppress Kupffer-cell function as mentioned above plays an important role in SEC deterioration after LTx from agonal NHBDs.

Fig. 7 Electron microphotograph of the livers 1 h after reperfusion. Both SECs and hepatocytes were observed to be well preserved in the FRPG and HBD groups. SECs and Disse's space were completely destroyed in the control, FR, and PG groups, and hepatocyte microvilli were hardly recognizable. Spillage of the cytoplasm into the sinusoidal space was observed and erythrocytes were found in Disse's space. *Scale bar* = 2 μm



The question of difference between liver grafts from HBDs and those from NHBDs is whether the liver grafts suffer warm ischemia or not. The viability of liver grafts from NHBDs is impaired by warm ischemia before procurement. Takada et al. reported that after 60 min of warm ischemia with no pre-treatment, liver grafts were still suitable for transplantation [19]. In this study, the donors were subjected to approximately 30 min of warm ischemia; nevertheless, the survival in the control group was worse than that reported by Takada et al. This may have been due to our NHBD model. In contrast to our model, the NHBD models used in the experimental studies conducted so far rarely pertain to the agonal state. In an as yet unpublished study we have reported that the cytokines liberated from the gut immediately before death primed the liver graft for an exaggerated response to reperfusion through cold ischemia (Miyagi et al., Role of arachidonic acid cascade in warm ischemia-reperfusion injury of liver grafts from non-heart-beating rats, manuscript submitted). It was also reported that a large increase in cytosolic free calcium concentration was an important initial step in the sequence of events leading to cell damage during warm ischemia [4, 8]. PG was reported to delay the rise in cytosolic free calcium concentration and to prevent plasma membrane bleb formation [10]. In this study, electron microscopy at retrieval revealed distinct disruption of SECs in liver grafts in which PG was not administered, whereas in those in which PG was administered the injury was much milder. Our results suggest that SECs deteriorated immediately before death and that it is important for successful LTx from NHBDs that precautionary measures against this be taken.

NM and UTI are serine protease inhibitors. Protease inhibitors prevent ischemia-reperfusion injury, but the underlying mechanisms for this action remain obscure. Our previous study indicated that NM rinse induced the widening of hepatic sinusoids and protected SECs to some degree; however, the protective effect of NM was insufficient to prevent the chain reaction elicited by reperfusion injury which starts with the activation of Kupffer cells [18]. In our present study, microscopy revealed that all grafts except those in the control group were reperfused well. Furthermore, we have reported that NM regulates arachidonate metabolites, especially thromboxaneA2 (TXA2) (Miyagi et al., manuscript submitted). Inhibition of TXA2 synthesis suppresses the liberation of other vasoconstrictive substances and neutrophil infiltration, thereby improving microcirculation [12, 26]. UTI, which is present in human urine, has two cardinal actions: (1) the suppression of neutrophil elastase (NE) and cathepsin G and (2) the stabilization of lysosomal and cellular membranes [9, 15, 22, 23]. It was also reported that UTI reduced the production of cytokine-induced neutrophil chemoattractant by Kupffer cells stimulated with NE, attenuating ischemia-reperfusion injury of the liver [24]. In this study, UTI prevented the development of acidosis after reperfusion in the FRPG group (data not shown). Our results suggest that NM rinse and UTI were effective in the protection against ischemia-reperfusion injury, although further examination is needed.

In summary, our results indicate that PG saved SECs from warm ischemic damage and that FR prevented SEC deterioration at reperfusion. The combination therapy, consisting of PG, FR, and protease inhibitors, may have a role in clinical LTx from NHBDs.

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