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# Kupffer cell activation in the survival discrepancy between liver grafts from enterally and parenterally fed donors

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**Abstract** This study was designed to investigate the effects of differences in the route of nutritional support of the donor on cold ischemia/reperfusion injury. Participation of Kupffer cells in these effects, based on the analysis of hepatic energy metabolism in early phases of reperfusion was also investigated. Orthotopic liver transplantation was performed between Large-White pigs weighing 20–30 kg after a 4-h cold preservation of the graft in Euro-Collins solution at 4 °C. One group was fed orally with a standard laboratory diet (FED group, $n = 5$), a second group was fasted and given 20% glucose intravenously (12 kJ/kg per day) (PEF group, $n = 5$), and a third group was fed orally with a standard laboratory diet and given $GdCl_3$ (10 mg/kg) intravenously 24 h before operation (FEDGD group, $n = 5$). These treatments were given for 7 days prior to harvesting. The survival time was significantly longer in the PEF (34.8 ± 5.5 days) and FEDGD (28.0 ± 11.9 days) groups than in the FED (9.8 ± 2.0 days) group ($P < 0.05$). The serum hyaluronic acid elimination rate determined from 1 to 2 h after reperfusion was significantly lower in the FED group than in the other two groups ($P < 0.001$). The glycogen content of the livers 1 h after reperfusion in all three groups had been consumed rapidly, but the ATP content of the livers was significantly reduced in the FED group alone ($P < 0.01$). Hepatic FFA clearance ($C_{FFA}$) was moderately increased in all three groups in the early phase after reperfusion, but it was higher in the FED group than in the other two groups, with significant differences 1 and 2 h after reperfusion ($P < 0.05$). In conclusion, parenteral nutrition of the donors reduced cold ischemia/reperfusion injury which is related to Kupffer cell activation and, thus, was better than enteral nutrition for donor management.

**Key words** Liver transplantation · Kupffer cell · Energy metabolism · Glycogen · Free fatty acid

## Introduction

Liver transplantation (LTx) has been established as the definitive treatment for end-stage liver disease, but nutritional management of the donor is still a matter of debate [1–6]. We have previously reported that nutritional repletion of the donor with a high level of glycogen storage in the liver improves the outcome of LTx, and that parenteral feeding of the donor is superior to oral feeding in a pig LTx model [7, 8]. We sought to determine whether activated macrophages play a role in the survival discrepancy between LTx from orally and parenterally fed donors, and analyzed the energy dynamics in the reperfused liver grafts using a pig model.

## Materials and methods

### Animals

Fifteen pairs of Large-White pigs weighing 20 to 30 kg were used as the donors and recipients. They were divided into three groups each of five animals according to the nutritional pretreatment for the donors administered for 7 days prior to harvesting: one group was fed orally with a standard laboratory diet (360 kJ/day) and allowed to feed until 3 h before harvesting (FED group); a second group was fasted and given 20% glucose intravenously (12 kJ/kg per day) (PEF group); and a third group was fed orally with a standard laboratory diet (360 kJ/day) and given gadolinium chloride (10 mg/kg) intravenously 24 h preoperatively (FEDGD group) as outlined by Clavien et al. [9]. Transient respiratory distress was observed in pigs treated with 10 mg/kg $GdCl_3$, but all of these pigs recovered uneventfully by 6 h after $GdCl_3$ administration without any supportive therapy. Water was available *ad libitum* to all groups. The standard laboratory diet comprised (per 100 g) crude protein 14.6 g, crude fat 5.2 g, crude fiber 10.5 g, water 4.3 g, calcium 2.30 g and phosphorus 1.85 g, together with vitamins, amino acids and fatty acids. The standard diet contained 74.5 kJ/100 g and the total amount given per day was about 500 g.

### Orthotopic liver transplantation

Following intramuscular premedication with 5 mg/kg ketamine chloride, general anesthesia was induced intravenously with 5 mg/kg thiopental sodium and 0.5 mg/kg suxamethonium chloride, and maintained with intravenous pancronium bromide and 1% halothane gas. After a broad median incision was made, the liver was mobilized by division of the falciform, left triangular, and gastrohepatic ligaments. Dissection of the hepatoduodenal ligament was performed and the splenic and superior mesenteric veins were ligated near their point of confluence. Then the left gastric and splenic arteries were identified and divided. In the last step, the terminal and supraceliac aortas were ligated for cannulation and clamping. Prior to hepatectomy, the liver was cooled in situ by perfusion with 1000 ml Euro-Collins (EC) solution at 4 °C via irrigation catheters inserted into the infrarenal aorta and the superior mesenteric vein. The liver was then retrieved by detachment of the supra- and infrahepatic vena cavae, the abdominal aorta and the portal vein.

The livers were preserved in cold EC solution for 4 h, and then transplanted orthotopically using a previously described technique [10]. Catheter cholecystostomy was then performed. The blood glucose level was maintained above 120 mg/dl by continuous infusion of 10% glucose solution during and after surgery. Two units of fresh whole blood were transfused during the surgery to maintain the systemic blood pressure above 90 mm Hg. Biopsy of the transplanted liver graft was performed before preservation, after preservation, and after reperfusion. All biopsy specimens were frozen and stored in liquid nitrogen at – 70 °C. These specimens were also subjected to histopathological staining with hematoxylin and eosin and periodic acid-Schiff (PAS).

### Evaluations

The serum aspartate amino transferase (AST) levels were measured as an indicator of hepatocyte injury. The serum hyaluronic acid levels, reflecting sinusoidal endothelial cell damage and specifically metabolized by sinusoidal endothelial cells [11, 12], were measured using blood samples obtained from the hepatic vein. The rate of decrease of the serum hyaluronic acid concentration between 1 h and 2 h after reperfusion was then calculated as an index of sinusoidal endothelial cell damage.

Since we considered free fatty acids (FFA) as a potential energy substrate in the early postreperfusional phase, the serum FFA levels in the portal vein and in the hepatic vein were also measured. Serial hepatic FFA clearances ($C_{FFA}$) were calculated to evaluate the demand of the liver graft for energy substrates. $C_{FFA}$ was originally defined as follows: $C_{FFA} = (100 - HCT)/100 \times PBF \times (P - V)$, where PBF is the portal blood flow (ml/min), P is the portal FFA level (mequiv/ml), and V is the hepatic venous FFA level (mequiv/ml). PBF was measured using ultrasound transit time flow probes and a flowmeter (Transonic Systems, Ithaca, N.Y.).

The glycogen contents of the livers were measured using an enzymatic hydrolysis method using amyloglucosidase and are expressed as milligrams glucose per gram wet weight of tissue. The concentrations of ATP in the livers were determined in perchloric acid-extracted tissue. ATP was separated from other nucleotides by high-performance liquid chromatography and the concentrations were determined by comparison of absorbance, as previously described [13].

### Statistics

The significance of differences between mean values was determined using Student's *t*-test. *P*-values less than 0.05 were considered to indicate statistical significance.

## Results

There were no significant differences in the body weight of the donors, graft weight, total ischemic time, anhepatic time or operation time among the three groups (data not shown).

### Survival

Mean survival times in the PEF group (34.8 ± 5.5 days) and FEDGD group (28.0 ± 11.9 days) were significantly greater than in FED group (9.8 ± 2.0 days, $P < 0.05$; Table 1).

### Light microscopic findings of liver biopsy specimens

Liver biopsy specimens were fixed in 10% neutral buffered formaldehyde and embedded in paraffin.

**Table 1** Survival times of individual animals and the mean ± SD *survival time for each group*

| Group | Survival (days) | Mean survival time (days) |
|---|---|---|
| FED | 6, 9, 9, 11, 13 | 9.8 ± 2.0* |
| PEF | 27, 33, 34, 39, 41 | 34.8 ± 5.5 |
| FEDGD | 8, 28, 32, 34, 38 | 28.0 ± 11.9 |

* $P < 0.05$ vs PEF and FEDGD groups

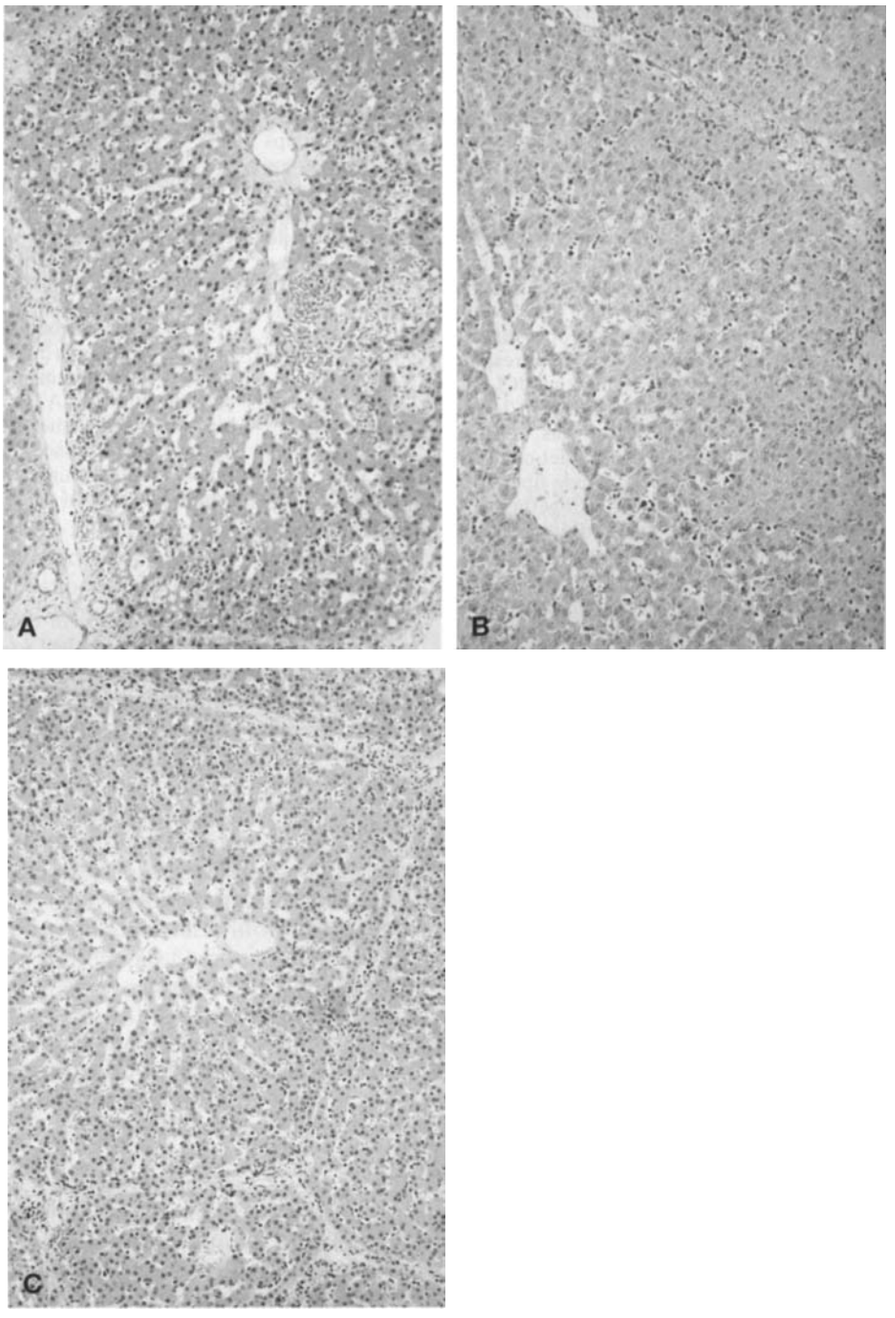


**Fig. 1 A–C** Light micrographs of liver biopsy specimens (H & E, × 125) obtained 2 h after reperfusion. Specimens obtained from the FED group (**A**) revealed marked vacuolar degeneration of hepatocytes, detachment of sinusoidal endothelial cells and congestion. In contrast, these changes were not obvious in the PEF group (**B**) or the FEDGD group (**C**)

Sections were stained with Mayer's hematoxylin and eosin. Specimens at the end of the cold ischemic time in each group maintained almost normal hepatic architecture. At 2 h after reperfusion, specimens obtained from the FED group showed marked vacuolar changes, steatosis and necrosis of hepatocytes, detachment of sinusoidal endothelial cells and hepatic congestion (Fig. 1).

Serum levels of AST

Serum AST levels in the FED group at 12 and 24 h after reperfusion were significantly higher than those in the other two groups [1019 ± 226.8 IU/l vs 726.6 ± 114.1 IU/l in the PEF group ($P < 0.05$) and 600.6 ± 187.5 IU/l in the FEDGD group ($P < 0.05$) at 12 h, and 1367.2 ± 279.1 IU/l vs 987.8 ± 144.5 IU/l in the PEF

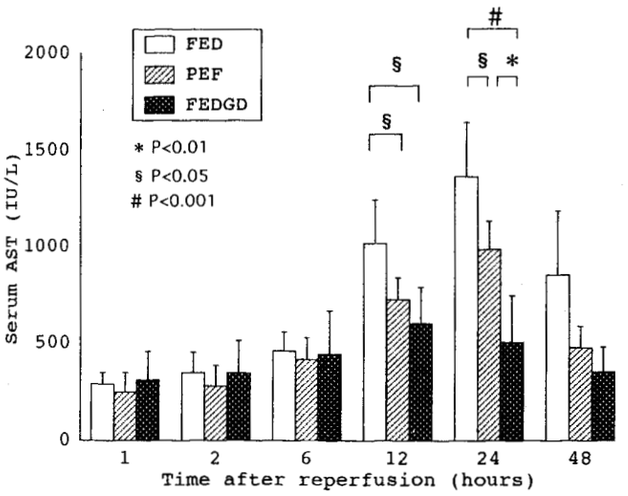


**Fig. 2** Changes in serum levels of AST up to 48 h after reperfusion in the three nutritional pretreatment groups

group ($P < 0.05$) and 505 ± 242.4 IU/l in the FEDGD group ($P < 0.001$) at 24 h; Fig. 2]. Furthermore, the elevation of serum AST levels in the FEDGD group was significantly less than in the PEF group at 24 h after reperfusion.

## Serum levels of hyaluronic acid

Serum hyaluronic acid levels were determined 1 and 2 h after reperfusion (Fig. 3). The serum hyaluronic acid levels in the FED group were significantly higher than those in the PEF and FEDGD groups up to 2 h after reperfusion. The hyaluronic acid levels in the FEDGD group were also significantly less than in the PEF group 2 h after reperfusion. The percentage decreases in serum hyaluronic acid levels between 1 and 2 h after reperfusion were 13.1 % in the FED group, 47.3 % in the PEF group and 59.7 % in the FEDGD group. The significant decrease in the 1-h percentage decrease in the FED group indicated more severe postreperfusional sinusoidal endothelial cell damage than that occurring in the PEF and FEDGD groups.

## ATP concentrations in the liver

The ATP concentrations were almost the same (900 nmol/g tissue) in the liver grafts of the three groups (Fig. 4). The initial ATP concentrations had decreased by 30 % in the three groups following 4 h preservation. However, the levels of recovery of the ATP concentrations 1 h after reperfusion were different in each group. The ATP concentrations 1 h after reperfusion in the FED, PEF and FEDGD groups were 43 %, 69 % and 71 % of the initial concentrations, respectively. The recovery of energy status in the early postreperfusional phase was significantly disturbed in the FED group.

## Glycogen contents in the liver

In each group, liver glycogen was stored at rates of 23–27 mg/g tissue before cold preservation, and the rate was decreased 0.7-fold after 4 h of cold preservation. Marked decreases in glycogen deposits in the grafts were observed 1 h after and the rate of storage was only 5 % of prepreservation values (Fig. 5). Light microscopic examination confirmed that intrahepatocellular PAS-positive granules stored in hepatocytes disappeared as a result of diastase digestion and rapidly decreased in number in the postreperfusional sections of each group (Fig. 6).

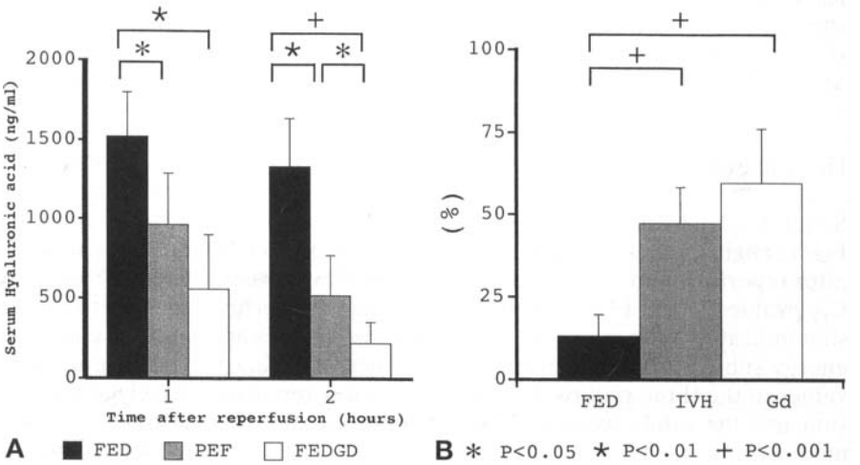


**Fig. 3 A, B** Serum levels of hyaluronic acid 1 h and 2 h after reperfusion (**A**) and its elimination rate 1–2 h after reperfusion (**B**)

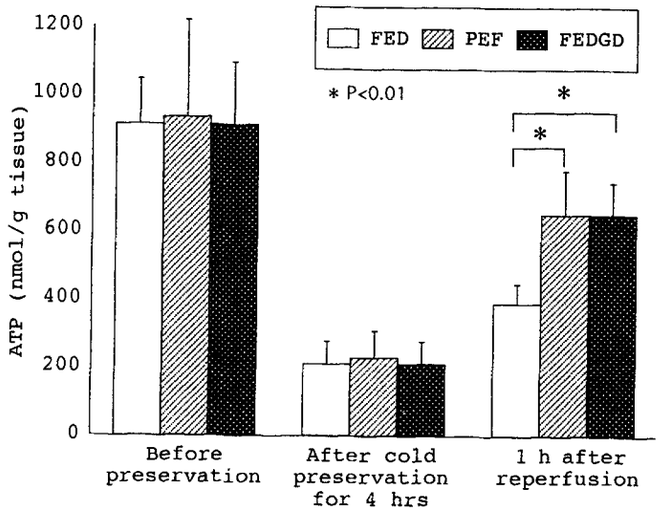


**Fig. 4** ATP concentrations in the livers before preservation, after cold preservation for 4 h and 1 h after reperfusion

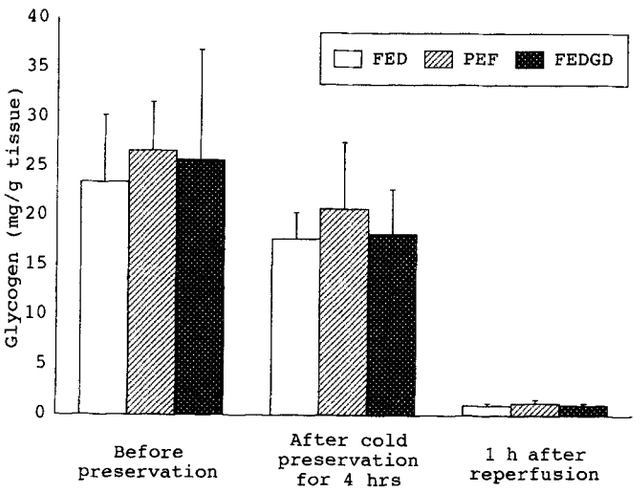


**Fig. 5** Glycogen concentrations in the livers before preservation, after cold preservation for 4 h and 1 h after reperfusion

### Hepatic FFA clearances

Serial $C_{FFA}$ changes after reperfusion are shown in Fig. 7. High levels of $C_{FFA}$ were sustained for up to 6 h after reperfusion in each group. Significant elevation of $C_{FFA}$ values in the FED group 1 and 2 h after reperfusion indicated an increase in the demand for FFA as an energy substrate in the damaged graft. Increased $C_{FFA}$ values in the three groups decreased 12 h after reperfusion, and the values were maintained at almost $20 \times 10^3$ mequiv/ml in each group.

## Discussion

Several investigators have reported the effect of nutritional management for the donors on the outcome of LTx [1–8], but nourishment protocols for the donors of liver grafts have not been standardized [1, 2]. Some investigators have emphasized the importance of glycogen deposits in the liver graft as a result of a good nutritional supply to the donor [4, 7, 8], and others have suggested the advantages of fasting which mitigates cold ischemia/reperfusion injury [5, 6]. We investigated whether differences in the donor feeding protocols affected the outcome of LTx, and how activated macrophages contribute to the outcome. We also assessed the dynamics of the energy status and supply in the liver graft in the reperfusional phase.

It is well known that Kupffer cells are activated during cold preservation [14] and play a major role in the pathogenesis of reperfusion injury [15–17]. Since Squiers et al. have reported that Kupffer cells are activated by exposure to foreign proteins and other immunogenic substances [18], the difference in the mean survival time between the FED and PEF groups might be a result of differences in Kupffer cell activity. It is not possible to detect Kupffer cell activity directly using serum levels of cytokines (TNF-α, IL-1 and IL-6) originating from Kupffer cells because of low assay sensitivity and species specificity. Therefore, $GdCl_3$, which nonspecifically suppresses macrophage activity, was used to block Kupffer cell activity in the FED group. Because $GdCl_3$ takes time to express its macrophage-blocking activity as reported by Lazar [19], an ex vivo graft perfusion model which could reduce the incidence of preoperative side-effects, was abandoned. However, the fact that the FEDGD group exhibited almost the same survival rate as the PEF group, suggested that activated Kupffer cells exacerbated the cold ischemia/reperfusion injury and resulted in shortening of recipient survival time in the FED group. AST levels and decreasing hyaluronic acid concentrations in the FEDGD group also provide indirect evidence supporting the above possibility.

Our results suggest that hepatic glycogen deposits, an emergency energy source preventing an energy crisis in other organs [4], were consumed by the liver itself during cold preservation and reperfusion. PAS staining indicated rapid exhaustion of glycogen following glycogenolytic energy supply in each group. However, the hepatic energy status as represented by the ATP concentration was different in the three groups 1 h after reperfusion. This may be explained by the fact that the degree of cold ischemia/reperfusion injury generated by Kupffer cell activation in each group affected the rate of ATP recycling and the energy status of the reperfused graft.

The graft might require another energy substrate after glycogen exhaustion until its energy metabolism is normalized. Therefore, FFA were investigated as a candidate second energy substrate. Significant elevation of

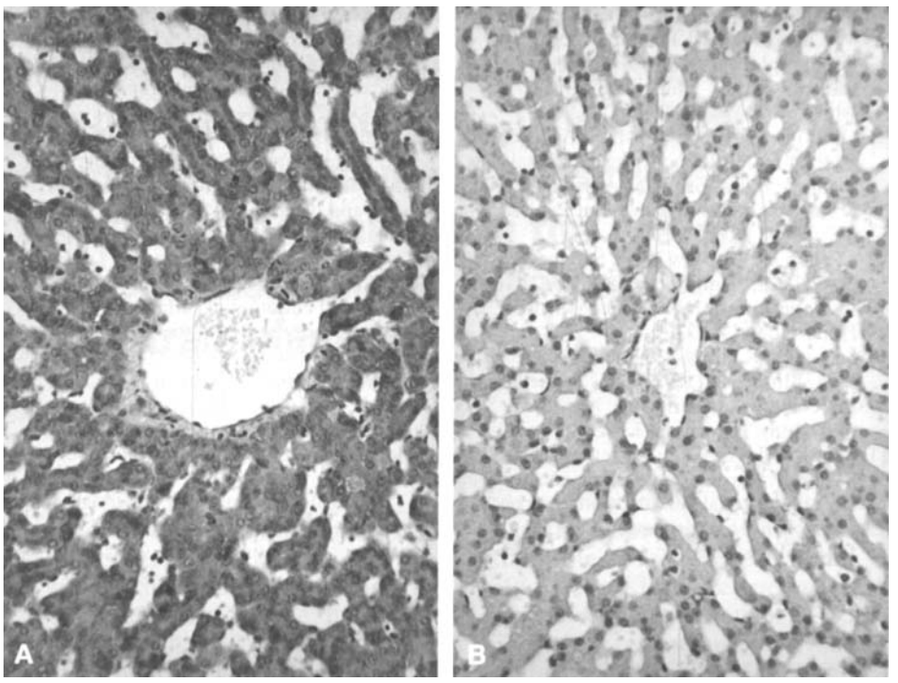


**Fig. 6 A, B** Light micrographs of liver biopsy specimens stained with PAS from the PEF group. **A** Before preservation a panlobular glycogen deposit is apparent; **B** 1 h after reperfusion, the glycogen deposits have disappeared from the entire lobular field (PAS, × 200)

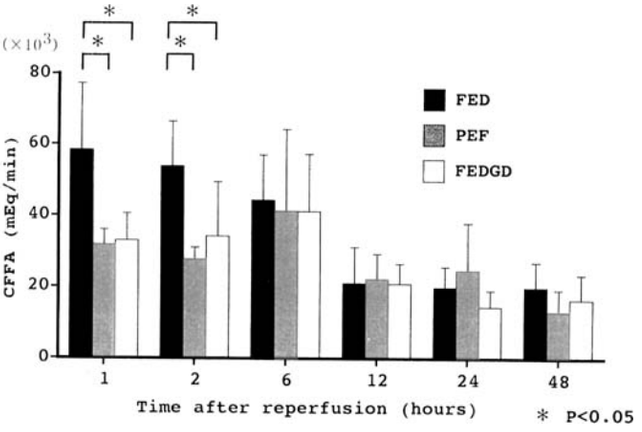


**Fig. 7** Changes in $C_{FFA}$ up to 48 h after reperfusion. The $C_{FFA}$ values were calculated by the procedures described in the Materials and methods section

$C_{FFA}$ values for the FED group reflected increased FFA demand in the graft damaged by reperfusion injury. As Seglen has reported that damaged livers do not effectively use glucose as an energy substrate [20], demand for a second energy substrate, FFA, might increase to compensate for the energy gap between glycogenolysis lasting only 1 h and normal hepatic energy metabolism.

Four striking findings emerged from the study: (1) Kupffer cell activation plays a central role in the survival discrepancy between grafts from enterally and parenterally fed donors; (2) parenteral energy repletion of the donor liver improves the outcome of LTx in a porcine model; (3) liver glycogen is the first of a series of energy substrates that support the energy demand of reperfusion; and (4) FFA are potent second energy substrate candidates in the damaged graft. Parenteral feeding for the donor may be of use in human LTx in the case of long-term brain-dead donors.

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