主論文

題目

Causes of liver steatosis influence the severity of ischemia reperfusion injury and survival after liver transplantation in rats

脂肪肝の成因が肝移植における虚血再灌流障害に与える影響

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Key words

primary non-function; hemeoxygenase-1; liver sinusoidal endothelial cell; microcirculation; methionine and choline deficient diet

Footnote page

1. Abbreviations

ALT; alanine aminotransferase

AST; aspartate aminotransferase

ATP; adenosine triphosphate

eNOS; endothelial nitric oxide synthase

ET-1; endothelin-1

FHA; fasting and hyper alimentation

H & E; hematoxylin-eosin stain

HO-1; hemeoxygenase-1

IL; interleukin

iNOS; inducible nitric oxide synthase

IRI; ischemia reperfusion injury

LSEC; liver sinusoidal endothelial cell

MCDD; methionine and choline deficient diet

MDA; malondialdehyde

NAFLD; nonalcoholic fatty liver disease

Nqo1; NAD(P)H Quinone Dehydrogenase -1

OLT; orthotopic liver transplantation

ORO; oil-red-O stain

PNF; primary non-function

RP1, RP3 and RP24; 1, 3 and 24 hours after reperfusion

SEM; scanning electron microscopy

TEM; transmission electron microscopy

TNF; Tumor necrosis factor

TG; triglyceride

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Abstract

Liver steatosis is a leading cause of graft disposal in liver transplantation; however, the degree of steatosis has been so far the almost single factor determining whether the grafts is acceptable or not. We investigated how the cause of liver steatosis affects graft function in rat orthotopic liver transplantation (OLT). OLT was performed using two types of steatotic liver grafts; the fasting and hyper alimentation (FHA) and methionine and choline deficient diet (MCDD) models. The FHA and 4-week MCDD feeding (MCDD4wk) groups showed similar liver triglyceride levels without signs of steatohepatitis; therefore, the two groups were compared in the following experiment. With 6-hour cold storage, 7-days survival rate after OLT was far worse in the FHA than the MCDD4wk group (0% vs. 100%, $P = 0.002$). With 1-hour cold storage, the FHA group showed higher aspartate and alanine aminotransferase levels and histological injury score in zone 1 and 2 at 24 hours after reperfusion than the Normal liver and MCDD4wk groups. Intrahepatic microcirculation and tissue ATP level were significantly lower in the FHA group after reperfusion. Hepatocyte necrosis, sinusoidal endothelial cell injury and abnormal swelling of mitochondria were also found in the FHA group after reperfusion. Tissue malondialdehyde levels were higher in the MCDD4wk group before and after reperfusion; however, the grafts upregulated several antioxidant enzymes soon after reperfusion.

Conclusion: Even though the degree of steatosis was equivalent, the two liver steatosis models

possessed quite unique basal characteristics and showed completely different response against ischemia reperfusion injury and survival after transplantation. Our results demonstrated that the degree of fat accumulation is not a single determinant for the fate of steatotic liver graft.

1. Introduction

Because of the fear of post-transplant primary non-function (PNF), liver steatosis is a leading cause of graft disposal in liver transplantation.[1] While livers with less than 30% steatosis have no increased risk of PNF, the risk increases if graft steatosis exceeds more than 30%[1]; therefore, grafts with more than 60% steatosis are deemed unacceptable in US or UK.[2, 3] On the other hand, as the prevalence of patients with overweight, obesity or diabetes has increased,[4, 5] the rate of liver steatosis in donor candidates will almost certainly rise. However, considering the severe donor shortage, using as many liver grafts as possible, even though they may have severe steatosis, is essential to help patients who suffering from end-stage liver disease.

Some authors argued that even severe steatotic liver grafts can be used safely under certain clinical conditions. Wong et al. reported that none of 19 patients who received severe (more than 60%) steatotic liver grafts experienced PNF nor early allograft dysfunction.[6] McCormack et al. also reported that PNF did not occur in the 20 cases of liver steatosis group (median 90%, range 65-100%) and postoperative survival rate was equivalent to those in the control group.[7] Considering these results,[6, 7] steatosis itself seems to be a superficial phenomenon and there may be another key factor that determine whether the graft is acceptable for transplantation or not.

Liver steatosis is not a homogenous disease.[8] Various factors can cause fat accumulation and its pathophysiological process is also not uniform.[8] Therefore, it seems natural that two different types of steatotic liver grafts, even though they have the same degree of steatosis, show different graft function and survival after transplantation. Currently, various rat liver steatosis models have been established worldwide,[9-12] some of these are now available in our laboratory. Therefore, we can now precisely mimic the aforementioned clinical situation. From the above, the aims of this study are to i) create the same degree of liver steatosis using two different methods, ii) compare the graft functions using rat orthotopic liver transplantation (OLT) and iii) investigate the factors affecting function or survival of steatotic liver grafts.

2. Material & methods

2.1 Animals

A total of 114 male LEW/CrlCrlj rats weighting 250 to 350g were purchased from Charles River Laboratories Japan, Inc., Yokohama, Japan. The animals were housed under specific pathogen-free conditions in a temperature- and humidity-controlled environment with a 12-hour light/dark cycle. Except for the following liver steatosis models, rats were fed with a standard diet (F-2; Oriental Bio Service, Kyoto, Japan) and tap water ad libitum. The experimental protocol was approved by the institutional ethics committee of Kyoto University (Medkyo 18536), which met the ethical guidelines of the Declaration of Helsinki. And all animals received humane care according to criteria outlined in 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 (NIH Publication No 86-23, revised in 1985).

2.2. Experimental protocol

2.2.1 Experiment I: Comparison of hepatic steatosis models.

The following two types of liver steatosis models were compared.

i) Fasting and hyperalimentation (FHA) model

The modified protocol by Delzenne et al. was used.[9, 13] In short, the rats (n = 5) were fasted for 2 days, followed by refeeding with a fat-free, carbohydrate-rich diet (saccharose 38%, starch 38%, casein 16%, and a mineral and vitamin mix 8%, CLEA Japan, Inc., Tokyo, Japan) for 3 days. During this period, access to tap water was not limited.

ii) Methionine and choline deficient diet (MCDD) model

The rats were fed a methionine and choline deficient diet (sucrose 10%, starch 39%, amino acid mix without methionine 17%, and a mineral and vitamin mix without choline 1%, Oriental Yeast co., Ltd, Tokyo, Japan) for 2-, 4-, 6-weeks (n = 3, 5, 3 respectively). As with the previous model, access to tap water was not limited during the feeding period.

2.2.2 Experiment II-A: Comparison of survival rates with 6-hour cold storage

Whole liver grafts from the FHA and MCDD groups (n = 5, each) were preserved in University of Wisconsin solution (Belzer, Astellas Pharma Inc., Tokyo, Japan) for 6-hours at 4℃. After cold storage, OLT was performed using a modified method by Kamada.[14] Briefly, the portal vein was reconstructed using the cuff method, and anastomosis of the infra-hepatic vena cava was hand-sewn using 8-0 nylon. The hepatic artery was also reconstructed using a modified sleeve method.[15]

2.2.3 Experiment II-B: Evaluation of liver graft with 1-hour cold storage

Whole liver grafts from the Normal liver, FHA and MCDD group were preserved in University of Wisconsin solution for 1 hours at 4℃. After cold storage, OLT was performed as previously described. Subsequently, 3, and 24 hours after portal reperfusion, serum and liver graft tissue samples were collected prior to euthanasia by exsanguination (RP-3 and RP-24, respectively, n = 5, at each time point for each group). The samples of 3 groups before procurement were also collected and presented as controls (n = 5, for each group).

2.3 Assay procedure

2.3.1 Measurement of serum transaminase and albumin levels

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and albumin levels were measured using a standard spectrophotometric method using an automated clinical analyzer (JCA- BM9030, JEOL Ltd., Tokyo, Japan).

2.3.2 Measurement of liver tissue triglyceride, adenosine triphosphate and malondialdehyde

The left lateral segment of liver in each sample was snap frozen and stored at -80℃ until subsequent assays. Triglyceride (TG) was extracted from liver tissues according to the method of Folch et al.[16] Briefly, snap frozen liver (more than 100mg) was homogenized and extracted using a chloroform-methanol solution. The organic phase was dried and resolubilized in 2-propanol. TG was then determined using a commercial kit (Sekisui. Medical Co., Tokyo, Japan). The liver tissue adenosine triphosphate (ATP) was measured using AMERIC-ATP(T) kit (Applied Medical Enzyme Research Institute Co., Tokushima, Japan) according to the manufacturer's directions. Liver tissue malondialdehyde (MDA) was measured using the thiobarbituric acid assay (NWLSS malondialdehyde assay, Northwest Life Science Specialties, LLC, WA, USA) according to the manufacturer's protocol.

2.3.3 Measurement of tissue microcirculation

The tissue microcirculation was evaluated at the left lateral segment and middle lobe (left and right side separately) using an advanced laser flow meter (Advance Bio Research Center Co., Ltd, Nagoya, Japan). The time points were before procurement, RP-1 (before abdominal closure) and RP-3. Hepatic microcirculations before procurement and at RP-1 of rats euthanized at RP-24 were also recorded and included as the result.

2.3.4 Histological analysis

Formalin-fixed, paraffin-embedded sections (4μm thickness) were stained with hematoxylin-eosin (H & E) and oil-red-O (ORO) stain. Two independent investigators examined all tissue section in a blinded fashion. The nonalcoholic fatty liver disease (NAFLD) activity score was calculated using the existing scoring system.[17] The severity of ischemia reperfusion injury (IRI) was quantified at RP-24 with hepatocyte swelling, vacuolization, necrosis, apoptosis, and neutrophil infiltration, and degree of each factor was graded as 1 = no change or negligible lesions, affecting 0%–10% of the field; 2 = mild, lesions affecting 10%–40% of the field; 3 = moderate, lesions affecting 40%–70% of the field; 4 = severe, lesion affecting > 70% of the field.[18] The scores were evaluated in 10 random fields (magnification ×200) per slide and averaged for each slide.[18]

2.3.5 Electron microscopy

The rat livers were perfused through the portal vein with a mixture of 2% glutaraldehyde and 4% paraformaldehyde and then extracted. The livers were cut into small pieces and stored overnight at 4℃. The sections were stained with saturated uranyl acetate and lead citrate and were observed with a Hitachi H-7650 electron microscope (Hitachi, Tokyo, Japan) for transmission electron microscopy (TEM). Additionally, the samples were ion-sputter-coated and observed with a Hitachi S-4700 scanning electron microscope for scanning electron microscopy (SEM). The area of sinusoidal space was identified in 10 randomly selected TEM images (×700) in each group and calculated using Image J version 1.46r (National Institutes of Health, USA). The time points were before procurement and at RP-24 in three groups. Two independent investigators including an expert pathologist of the liver evaluated the sample findings.

2.3.6 Real-time polymerase chain reaction

For the analysis of gene expression, real-time polymerase chain reaction, using TaqMan technology, was performed. The probe and primers for interleukin-1β (IL-1β, assay ID Rn00580432_m1), IL-6 (assay ID Rn01410330_m1), tumor necrosis factor-α (TNF-α, assay ID Rn01525859_g1), endothelin-1 (ET-1, assay ID Rn00561129_m1), endothelial nitric oxide synthase (eNOS, assay ID Rn02132634_s1), inducible nitric oxide synthase (iNOS, assay ID Rn00561646_m1), heme oxygenase-1 (HO-1, assay ID Rn01536933_m1), NAD(P)H Quinone Dehydrogenase 1 (Nqo1, assay ID Rn00566528_m1) and beta-actin (β-actin, assay ID Rn00667869_m1) were obtained for TaqMan gene expression assays from Applied Biosystems, Life Technologies Japan Ltd., Japan.

2.4 Statistics

Data are expressed as means ± standard error and are compared statistically using Student's t-test (Experiment I, Table 2) or Tukey-Kramer analysis. For the survival study, a log-rank test was performed. $P < 0.05$ was considered as a threshold; however, according to the previous recommendation of American Statistical Association,[19, 20] we avoided describing $P < 0.05$ as "statistically significant"; instead, we described the measured values and *P* values as continuous quantities in the text, tables or figures as possible. All statistical analyses were performed using JMP Pro, version 14.0.0 (SAS Institute, Inc., Cary, NC, USA).

3. Results

3.1 Experiment I: Comparison of hepatic steatosis models

According to the definition of liver steatosis,[8] liver tissue TG was quantified and used to compare the degree of steatosis between the FHA and MCDD models (Table 1). Compared with the Normal liver, both the FHA and MCDD protocols created considerable fat accumulation in the liver. Among them, the FHA and 4-week feeding of MCDD (MCDD4wk) showed the nearest TG accumulation ($P = 0.64$). The two groups also showed almost equivalent serum AST, ALT and albumin level ($P = 0.28$, 0.24 and 0.49 respectively, Table 2).

H & E and ORO staining of the FHA group showed mixed (micro- and macro-vesicular) steatosis exceeding 60%; on the other hand, the MCDD4wk group showed pure macro-vesicular steatosis exceeding 80% (Fig. 1). The NAFLD activity scores of the two liver steatosis were equivalent ($P = 0.99$) and did not reach the category of steatohepatitis (Table 2).[17] Accordingly, we compared the FHA and MCDD4wk groups in the following experiments.

3.2 Experiment II-A: Comparison of survival rates under 6-hour cold storage

Fig. 2 showed the survival rate of the FHA and MCDD4wk group under 6-hour cold storage. While all five rats in the MCDD4wk group survived 7 days after transplantation, only one survived 1 day and none survived more than 2 days after transplantation in the FHA group ($P = 0.002$). With this 6-hour cold storage model, we could not obtain the samples of 24-hour after reperfusion in the FHA group; therefore, we conducted the experiment II-B using 1-hour cold storage. The 24-hour survival rate of the FHA group with 1-hour cold storage improved to 80%.

3.3 Experiment II-B: Evaluation of liver grafts under 1-hour cold storage

3.3.1 Results of serological study, liver tissue ATP and MDA (Table 3)

The FHA group showed higher AST level at RP-3 and RP-24 than the Normal liver (both $P <$ 0.001) and MCDD4wk groups ($P = 0.001$ and < 0.001, respectively). The FHA group also showed higher ALT levels at RP-3 and RP-24 than the Normal liver ($P = 0.001$ and < 0.001, respectively) and MCDD4wk group ($P = 0.09$ and < 0.001, respectively).

Liver tissue ATP level was lower in the MCDD4wk group before procurement (vs the Normal liver and FHA groups, both $P = 0.02$, respectively); however, after reperfusion, while the MCDD4wk group showed a gradual increase in the ATP level, the FHA group showed a significant decrease in ATP (vs. the Normal liver and MCDD4wk groups, at RP-3, $P < 0.001$ and 0.17 and at RP-24, $P = 0.005$ and 0.04, respectively). MDA level was higher in the MCDD4wk group before procurement (vs. the Normal liver and FHA groups, $P = 0.15$ and 0.03), at RP-3 (both $P < 0.001$) and RP-24 ($P = 0.11$ and 0.04, respectively).

3.3.2 Intrahepatic microcirculation (Table 4)

At the left lateral segment, the FHA group showed the lowest microcirculation before procurement (vs. the Normal liver and MCDD4wk groups, $P < 0.001$ and 0.01), at RP-3 ($P =$ 0.04 and 0.02) and RP-24 ($P < 0.001$ and 0.004, respectively). Before procurement, the MCDD4wk group also showed lower micro circulation than the Normal liver group ($P = 0.02$). The same trend was also observed at the left and right sides of the middle lobe.

3.3.3 Histological analysis of liver ischemia reperfusion injury

Unlike the Normal liver and MCDD4wk groups, the FHA group showed a patchy necrotic area in zones 1 and 2 (Fig. 3A). The FHA group showed a higher IRI score in zones 1 (vs. the Normal liver and MCDD4wk groups, both $P < 0.001$, Fig. 3B) and zone 2 (both $P < 0.001$, respectively). In zone 3, the score was also higher in the FHA group than in the Normal liver group ($P = 0.04$). The MCDD4wk group showed a higher score than the Normal liver group in zone 1 ($P = 0.04$).

3.3.4 Electron microscopy

Unlike the Normal liver and MCDD4wk group, the FHA group showed necrotic cell death at RP-24 (Fig. 4). Hepatocyte mitochondria of the FHA group showed marked swelling and an obscured silhouette at RP-24 (Fig. 4). Liver sinusoidal endothelial cells (LSECs) were also evaluated. Before procurement (Fig. 5), while rats in the Normal liver groups showed an intact sinusoidal structure, the FHA group showed abnormal LSEC swelling, widening of the space of Disse and sparse hepatocyte microvilli. The MCDD4wk group also showed a subendothelial basal lamina and collagen deposition in the space of Disse. Sinusoidal space was quantified using the aforementioned method. The FHA and MCDD4wk group showed a decrease in sinusoidal space compared with the Normal liver group ($P < 0.001$ and 0.008, Supplementary Figure 1), even before procurement. The FHA group also showed a trend toward a smaller sinusoidal space than the MCDD4wk group ($P = 0.30$). At RP-24 (Fig. 6), LSEC injury, that is, swelling or detachment of the LSEC, was apparent in the FHA group. SEM also showed an irregular surface and diminished fenestrae and sieve plate structure in the FHA group. The MCDD4wk groups also showed slightly disrupted fenestrae.

3.3.5 Real-Time Polymerase Chain Reaction (Table 5)

The mRNA expression of IL-1β and IL-6 showed a trend toward a higher level in the FHA group at RP-24 compared with the Normal liver ($P = 0.12$ and 0.17) and MCDD4wk group ($P =$ 0.16 and 0.20, respectively). TNF-α expression at RP-24 was also higher in the FHA group than in the Normal liver and MCDD4wk groups ($P = 0.002$ and 0.008, respectively). Before procurement, the MCDD4wk group showed higher ET-1 expression than the Normal liver and

FHA groups ($P = 0.01$ and 0.003, respectively); however, after reperfusion, the value became higher in the FHA group at RP-3 (vs. the Normal liver and MCDD4wk groups, $P = 0.01$ and 0.07) and RP-24 ($P = 0.003$ and 0.009, respectively). The mRNA expression of eNOS was almost equivalent among the three groups at all time points. The MCDD4wk group showed a higher iNOS expression at RP-3 (vs. the Normal liver and FHA groups, $P = 0.006$ and 0.06, respectively). In contrast, the FHA group showed a lower expression of HO-1 (vs. the Normal liver and MCDD4wk groups, $P = 0.03$ and 0.30) and Nqo1 ($P = 0.10$ and 0.04, respectively) at RP-3. The MCDD4wk group also showed higher Nqo1 expression before procurement (vs. the Normal liver and FHA groups, both $P < 0.001$, respectively).

4 Discussion

The results of this study provide us fundamental knowledge about transplantation using steatotic liver grafts. The FHA and MCDD4wk groups had similar liver tissue TG contents; however, the two liver steatosis models possessed quite unique basal characteristics and showed completely different response against IRI and survival after transplantation.

The FHA protocol was first introduced by Delzenne et al. and increased synthesis of free fatty acid after refeeding is thought to be the main mechanism of fatty liver formation.[9] This model mimic a liver steatosis acutely formed by short-term fasting during resuscitation and subsequent high caloric refeeding; e.g. the situation sometimes seen in traumatic brain-dead donors.[21] On the other hand, methionine and choline are essential to create very-low-density-lipoprotein in rat liver; therefore, if these elements are lacking, the rat liver cannot create the lipoprotein, which leads to the accumulation of fat droplets in the hepatocytes.[22, 23] Although some discrepancies in the pathophysiological process were suggested,[24] this liver steatosis has long been regarded as a model for NAFLD.[25, 26] As shown in Table 1, the TG level of the MCDD4wk group was almost comparable with that of the FHA group. With H & E or ORO staining, the MCDD4wk group had an equal or even more prominent fat accumulation than the FHA group (Fig. 1). The type of steatosis was also disadvantageous for the MCDD4wk group because in clinical situations, it is a macro- (not micro-) vesicular steatosis that increases the risk of PNF.[27] However, it was the FHA group that showed far worse graft function and survival after transplantation.

As described by several authors,[28-31] impaired microcirculation is an important factor affecting IRI and worse graft outcome after liver transplantation. Our results showed that rats in the FHA group had lower microcirculation than those in the Normal liver and MCDD4wk groups before and after reperfusion. We must discuss the two points separately. First, the microcirculation of rats in the FHA group was already compromised before procurement. In the steatotic liver, fat droplets in the hepatocytes increase the cell volume, which leads to the obstruction of the hepatic sinusoidal space and impaired microcirculation.[28] This is consistent with our result that the FHA and MCDD4wk groups had a smaller sinusoidal space than the Normal liver group before procurement. Although, a subendothelial basal lamina and collagen deposition in space of Disse, which are the signs of microvascular change in liver steatosis,[32] were apparent in the MCDD4wk group, the FHA group also showed a trend toward a smaller sinusoidal space and more severe LSEC swelling. Previous study demonstrated that LSEC can responsively act like sphincters by swelling or contracting and narrow the sinusoidal lumen.[33] Therefore, the changes in LSEC seen in the FHA group might also contribute to lower microcirculation than in the MCDD4wk group.

Second, the disturbance of microcirculation became more prominent in the FHA group after reperfusion. This is also consistent with the results of TEM and SEM at RP-24 demonstrating severe LSEC injury and capillarization in the FHA group. Additionally, tissue ATP was considerably lower after reperfusion in the FHA group. The lack of ATP leads to a dysfunction of the sodium/potassium ATP-dependent plasma membrane pump and results in further swelling of hepatocytes or LSEC.[34] The expression of ET-1, which is an initiator of IRI and also a strong vasoconstrictor induced by microcirculatory disturbance, was higher in the FHA group after reperfusion and this also could lead to further impaired microcirculation.[35, 36] Our results not only reinforce the previous theory that impaired microcirculation and IRI aggravate each other,[28, 29] but also add a new insight that this vicious cycle might be more strongly emphasized in some types of liver steatosis.

Liver tissue MDA levels, the marker of lipid peroxidation, were higher in the MCDD4wk group at all time points; in contrast, despite the same degree of fat accumulation, the FHA group showed MDA levels almost comparable with the Normal liver group. This seems natural because previous studies showed that the oxidative stress is strongly associated with the pathophysiology of NAFLD[37] and the MCDD model has been regarded as a mimic of the disease. Besides iNOS, both HO-1 and Nqo1 are essential antioxidant enzymes expressed against various kinds of cellular stresses.[38, 39] Therefore, higher perioperative iNOS, HO-1 and Nqo1 expressions in the MCDD4wk group should be regarded as a proper countermeasure against higher oxidative stress. In contrast, the result of tissue MDA showed that oxidative stress does not seem to weigh heavily on the FHA group; however, HO-1 and Nqo1 expression were lower in the FHA group than in the Normal liver group; this might suggest the presence of some cellular disturbances in the FHA group.

Currently, several experimental strategies have been proposed for increased utilization of extended-criteria donors; e.g. statins,[40] venous systemic oxygen persufflation[41] or ex situ machine perfusion.[42] However, the results of our study suggest that a uniform strategy might not fully address the complex pathophysiology of steatotic liver graft. Severely impaired microcirculation and acutely depleted ATP levels are thought to be the main cause of higher IRI in the FHA group and oxidative stress seems to have only a low impact on this process. Abnormally swollen hepatocyte mitochondria at RP-24 might be the sign of permeabilization and the precursor to subsequent ATP depletion and necrotic cell death in the FHA group.[43] Therefore, protecting LSEC and hepatocyte mitochondria and maintaining microcirculation and tissue ATP levels would be an effective strategy for this type of liver steatosis. On the other hand, considering the relatively well-maintained postoperative microcirculation and tissue ATP, higher oxidative stress might be the main cause of IRI in the MCDD4wk group; therefore, ameliorating oxidative stress and underpinning antioxidant capacity might be an adequate strategy for the MCDD type steatotic liver graft.

This study has several limitations. Among them, translatability to clinical practice is the most important and difficult question to address. Despite the efforts of several researchers, there remain some ambiguities over the correspondence of liver steatosis between rats and humans.[44] The study using discarded grafts in liver transplantation would be a bridge connecting the gap; however, this type of experiment is now ethically prohibited in Japan. Therefore, our study focused on demonstrating the difference between two types of liver steatosis being offered as a graft for liver transplantation. In addition, although our results showed that the survival of the FHA grafts dramatically worsened after 6-hour cold storage, it is still unclear whether the same mechanism discussed in 1-hour cold storage model could be applied in 6-hour cold storage. The FHA and MCDD groups supposedly are affected differently by cold storage. This difference would be crucial for an understanding of steatotic liver graft transplantation and will be our next research target.

In conclusion, the results of this study showed that the FHA and MCDD4wk groups, even though they have almost equivalent degree of steatosis, had quite different graft function and survival after transplantation. It should be remembered that the degree of fat accumulation is not a single determinant for the fate of steatotic liver graft.

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Figure legends

Fig. 1 Histology of the Normal liver, FHA and MCDD4wk group.

Above; H & E stain, below; ORO stain, ×400 magnification. The FHA group showed mixed (micro- and macro-vesicular) steatosis more than 60%. The MCDD4wk group showed pure macro-vesicular steatosis more than 80%. Both type of steatotic liver grafts showed no signs of steatohepatitis. FHA, fasting and hyper alimentation; H & E, hematoxylin-eosin stain; MCDD, methionine and choline deficient diet; ORO, oil-red-O stain.

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While all five rats of the MCDD4wk group survived 7-days after transplantation, only one survived 1-day and none survived more than 2 days after transplantation in the FHA group (n = 5 each, log-rank test, $P = 0.002$). FHA, fasting and hyper alimentation; MCDD, methionine and choline deficient diet.

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Normal liver and MCDD4wk group. At zone 3, in contrast, the scores were not so different between the FHA and MCDD4wk group (P = 0.36). ** $P < 0.001$, * $P < 0.05$. FHA, fasting and hyper alimentation; H & E, hematoxylin-eosin stain; MCDD, methionine and choline deficient diet.

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Supplementary Figure 1 Quantification of sinusoidal space.

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Table 1, Tissue TG contents in each liver steatosis models.

	Normal liver	FHA	MCDD2wk	MCDD4wk	MCDD6wk
	n= 3	n = 5	n = 3	n = 5	n = 3
Tissue triglyceride (mg/g tissue)	2.6 ± 0.0	116.7 ± 10.1	76.3 ± 7.1	137.2 ± 18.0*	252.8 ± 4.9* **
P value (vs. FHA)		reference	0.23	0.64	< 0.001

The data are shown as mean ± standard error.

* *P* < 0.05 vs. MCDD2wk. ** *P* < 0.05 vs. MCDD4wk

FHA, fasting and hyperalimentation; TG, triglyceride; MCDD methionine and choline deficient diet.

Table 2, Comparison of the FHA and MCDD4wk group.

	Normal liver	FHA	MCDD4wk	*P*
Factors	n = 3	n = 5	n = 5	(FHA vs. MCDD4wk)
Serum study				
AST (IU/L)	82 ± 3	90 ± 5	102 ± 8	0.28
ALT (IU/L)	40 ± 1	60 ± 8	89 ± 22	0.24
Albumin (g/dL)	3.7 ± 0.0	3.7 ± 0.0	3.7 ±0.1	0.49

NAFLD activity score	0 ± 0	2.8 ± 0.2	2.8 ± 0.2	0.99

The data are shown as mean ± standard error. Student's t-test was used to directly compare the FHA and MCDD4wk group.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; FHA, fasting and hyperalimentation; MCDD4wk, 4-week feeding of methionine and choline deficient diet. NAFLD, non-alcoholic fatty liver disease.

Table 3; Summary of serum studies, ATP and MDA analysis in Experiment IIB.

		Before procurement n = 5	RP-3 n = 5	RP-24 n = 5
AST	Normal liver	75 ± 4	488 ± 88*	505 ± 59*
(IU/L)	FHA	90 ± 5	2221 ± 352[ab]*	2610 ± 256[ab]*
	MCDD4wk	102 ± 8[a]	686 ± 135*	601 ± 68*
ALT	Normal liver	40 ± 1	281 ± 61*	258 ± 53*
(IU/L)	FHA	60 ± 8	1753 ± 263[a]*	1709 ± 140[ab]*
	MCDD4wk	89 ± 22	1035 ± 272*	567 ± 58
ATP	Normal liver	1.8 ± 0.3	2.0 ± 0.2	1.9 ± 0.3
(μmol/g	FHA	1.9 ± 0.1[b]	0.6 ± 0.1[ab]*	0.7 ± 0.2[ab]*

tissue)	MCDD4wk	0.8 ± 0.2[a]	1.1 ± 0.2	1.6 ± 0.2
MDA	Normal liver	2.5 ± 0.7	1.2 ± 0.2	2.6 ± 0.6
(μmol/g	FHA	1.9 ± 0.3[b]	1.9 ± 0.3[b]	2.0 ± 0.5[b]
tissue)	MCDD4wk	3.9 ± 0.3	7.5 ± 1.5[a]	5.3 ± 1.2

The data are shown as mean ± standard error.

[a] $P < 0.05$, vs. the Normal liver group. [b] $P < 0.05$ vs. the MCDD4wk group. * $P < 0.05$ vs. before procurement, ** $P < 0.05$ vs RP3. ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATP, adenosine triphosphate; FHA, fasting and hyperalimentation; MCDD4wk, 4-week feeding of methionine and choline deficient diet. MDA, malondialdehyde; RP-3 and RP-24, 3- and 24-hour after reperfusion.

Table 4, Summary of intrahepatic microcirculation in Experiment IIB.

		Before procurement n = 10	RP-1 n = 10	RP-3 n = 5
Left lateral segment	Normal liver	15.6 ± 0.5	7.1 ± 0.9*	11.6 ± 0.3* **
(ml/min/100g tissue)	FHA	8.5 ± 0.6[ab]	4.4 ± 0.4[ab]*	4.2 ± 0.8[ab]*
	MCDD4wk	12.2 ± 1.2[a]	7.3 ± 0.8*	8.9 ± 1.1

Left side of middle lobe	Normal liver	16.4 ± 1.1	9.8 ± 1.0*	11.0 ± 1.4*
(ml/min/100g tissue)	FHA	12.1 ± 0.7^{a}	6.0 ± 0.5^{b*}	5.4 ± 0.7^{ab*}
	MCDD4wk	15.4 ± 1.3	11.1 ± 1.5	11.9 ± 0.6
Right side of middle lobe	Normal liver	15.9 ± 1.3	8.3 ± 1.0*	13.5 ± 1.8**
(ml/min/100g tissue)	FHA	11.3 ± 0.7	5.9 ± 0.5^{b*}	5.1 ± 0.7^{ab*}
	MCDD4wk	15.9 ± 2.1	9.6 ± 1.0*	11.6 ± 2.2

The data are shown as mean ± standard error.

^{a} $P < 0.05$, vs. the Normal liver group. ^{b} $P < 0.05$ vs. the MCDD4wk group. * $P < 0.05$ vs. before procurement, ** $P < 0.05$ vs RP3. FHA, fasting and hyperalimentation; MCDD4wk, 4-week feeding of methionine and choline deficient diet. RP-1 and RP-3, 1- and 3-hour after reperfusion.

Table 5, Summary of mRNA expression in Experiment IIB.

		Before procurement n = 5	RP-3 n = 5	RP-24 n = 5
IL-1β	Normal liver	1.5 ±0.2	4.0 ± 0.9*	1.8 ± 0.4**
(/β-actin)	FHA	1.5 ± 0.2	3.5 ± 0.5	7.8 ± 3.7
	MCDD4wk	1.8 ± 0.3	4.4 ± 0.4*	1.2 ± 0.1**

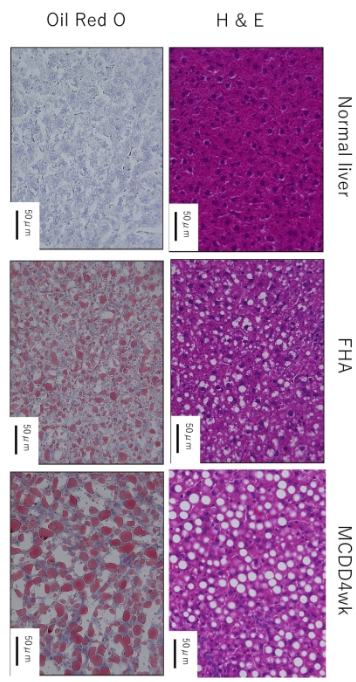
IL-6	Normal liver	2.2 ± 0.5	102.5 ± 23.1*	18.0 ± 6.7**
(/β-actin)	FHA	15.9 ± 13.3	203.9 ± 54.5	278.8 ± 165.6
	MCDD4wk	11.0 ± 6.7	673.7 ± 323.2	29.6 ± 12.6
TNF-α	Normal liver	0.8 ± 0.1	2.1 ± 0.4*	0.7 ± 0.1**
(/β-actin)	FHA	0.6 ± 0.1	2.6 ± 0.5*	2.8 ± 0.5[ab]*
	MCDD4wk	1.1 ± 0.2	3.5 ± 0.7*	1.1 ± 0.1**
ET-1	Normal liver	1.0 ± 0.1	3.6 ± 0.8*	1.7 ± 0.4
(/β-actin)	FHA	0.8 ± 0.1[b]	10.4 ± 2.0[a]*	5.8 ± 1.1[ab]*
	MCDD4wk	1.9 ± 0.3[a]	5.6 ± 0.9*	2.2 ± 0.4**
eNOS	Normal liver	0.9 ± 0.0	0.6 ± 0.1*	0.6 ± 0.1*
(/β-actin)	FHA	0.7 ± 0.1	0.7 ± 0.1	0.5 ± 0.1
	MCDD4wk	0.8 ± 0.1	0.5 ± 0.1	0.4 ± 0.1*
iNOS	Normal liver	0.7 ± 0.2	24.4 ± 6.0*	6.3 ± 3.1**
(/β-actin)	FHA	0.5 ± 0.2	42.2 ± 7.8	430.0 ± 386.9
	MCDD4wk	1.4 ± 0.3	78.7 ± 14.2[a]*	7.7 ± 1.8**
HO-1	Normal liver	1.5 ± 0.4	36.3 ± 3.5*	4.4 ± 0.6**
(/β-actin)	FHA	2.5 ± 0.3	16.1 ± 5.6[a]*	3.8 ± 0.8
	MCDD4wk	2.0 ± 0.4	26.5 ± 4.9*	1.7 ± 0.1[a]**

Nqo1	Normal liver	1.2 ± 0.2	1.4 ± 0.3	3.2 ± 0.4* **
(/β-actin)	FHA	0.8 ± 0.1[b]	0.7 ± 0.1[b]	0.7 ± 0.2[a]
	MCDD4wk	2.5 ± 0.2[a]	1.6 ± 0.2	1.1 ± 0.2[a]* **

The data are shown as mean ± standard error.

[a] $P < 0.05$, vs. the Normal liver group. [b] $P < 0.05$ vs. the MCDD4wk group. * $P < 0.05$ vs. before procurement, ** $P < 0.05$ vs RP3. eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; FHA, fasting and hyper alimentation; HO-1, heme oxygenase-1; IL interleukin; iNOS, inducible nitric oxide synthase; MCDD4wk, 4-week feeding of methionine and choline deficient diet; Nqo1, NAD(P)H Quinone Dehydrogenase 1; RP-3 and RP-24, 3- and 24-hour after reperfusion; TNF, Tumor necrosis factor.

Fig. 1



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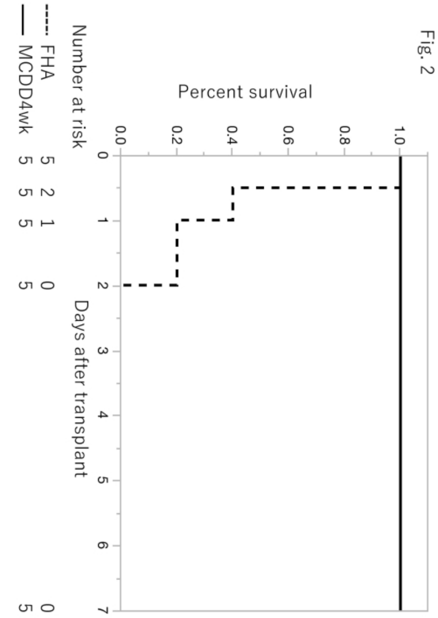

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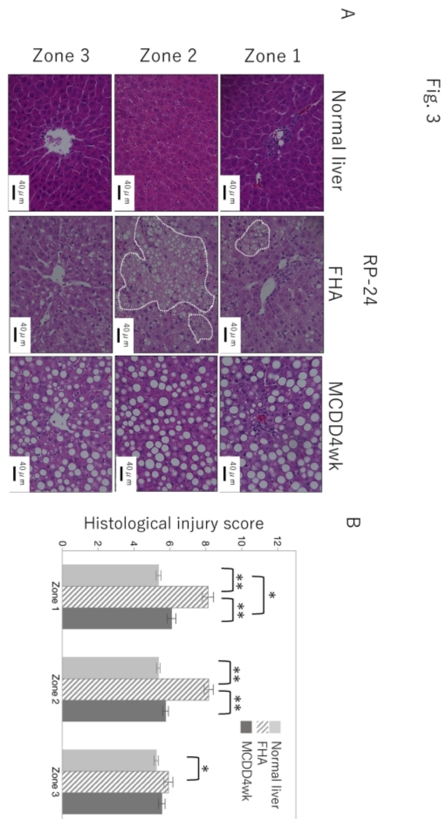

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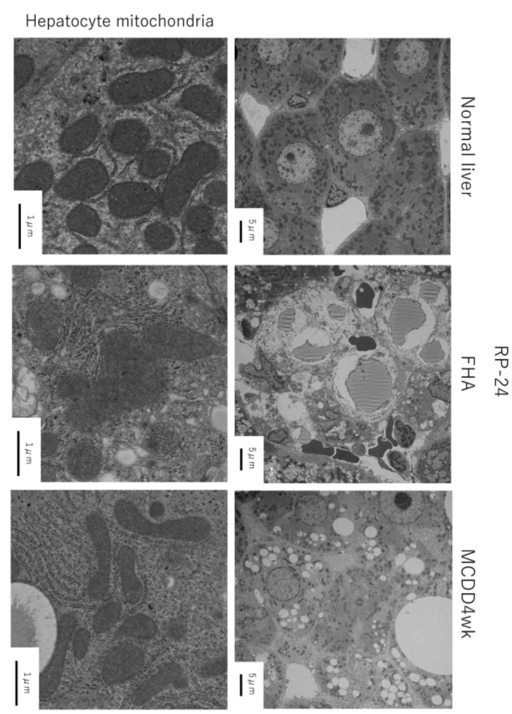

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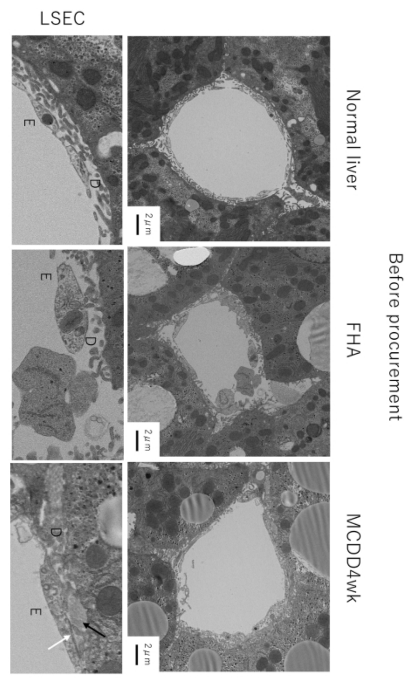

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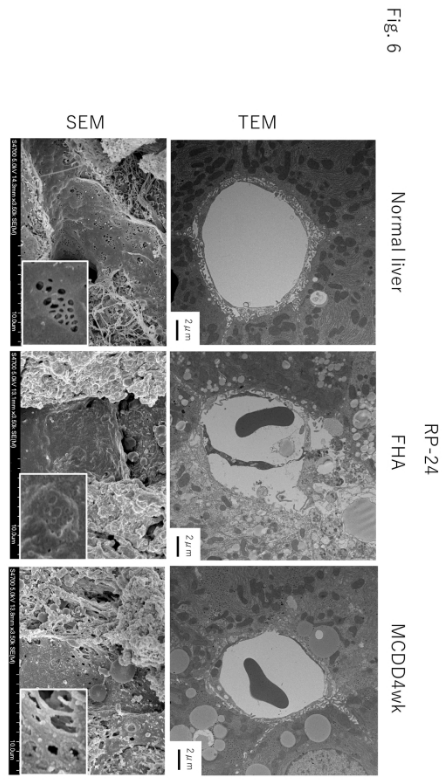

Fig. 6 Evaluation of LSEC using TEM and SEM; at RP-24.
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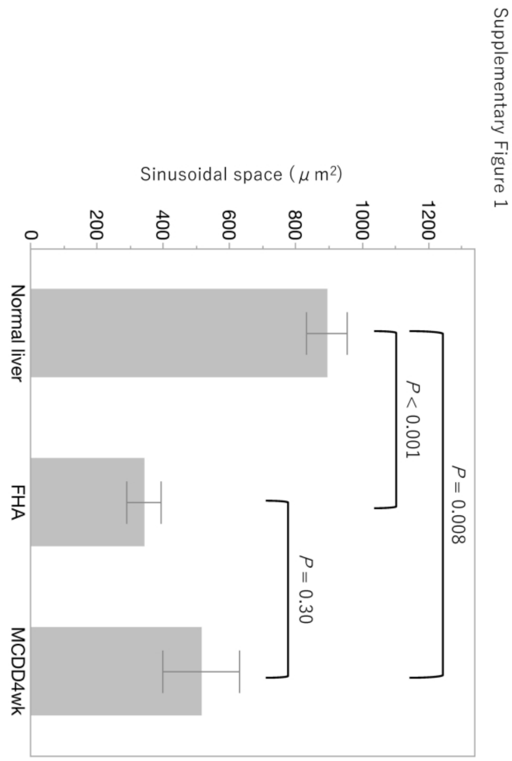

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