Highlight

- Despite existing various steatosis models in rodent, method to produce moderate (30 to 60%) macro-steatosis for basic research on liver preservation and reperfusion has not yet been precisely evaluated.
- Steatosis model was examined in male SHRSP5-Dmcr rat with HFC feedings for 5 days to 2 weeks, and evaluated by NAFLD activity score (NAS) and MRI-based PDFF.
- Since MRI-based PDFF correlates with NAS steatosis grade and HFC-fed time, preoperative evaluation of MRI-based PDFF can minimize the variance of HFC-induced steatosis.
- The steatotic grafts produced in this study were of good quality and can be used for cold preservation and reperfusion experiments.

Abstract

Interventions for liver grafts with moderate macrovesicular steatosis have been an important issue in enlarging donor pools. Here, we tested a high-fat and cholesterol (HFC) diet to create a steatosis model for hepatic cold preservation and reperfusion experiments. The aim of the present study was to assess the reliability of the steatosis model and to show quality of the resulting graft for cold preservation and reperfusion experiment. Male SHRSP5-Dmcr rats were raised with HFC diet for up to two weeks. The fat content was evaluated using magnetic resonance imaging (MRI) proton density fat fraction (PDFF). The NAFLD activity score (NAS) was evaluated after excision. Steatosis created by 2 weeks of HFC diet were subjected to 24-hour cold storage in UW and heavy water-containing test solution (New sol.). Grafts were applied to isolated perfused rat livers (IPRL) for simulating reperfusion. NAS were 2.2 (HFC5d), 3.3 (HFC1W), and 5.0 (HFC2W). Ballooning and fibrosis were not observed in any group. MRI-PDFF showed 0.2 (HFC0d), 12.0 (HFC1W), and 18.9 (HFC2W). NAS and MRI-PDFF values correlated. Many indices in the IPRL experiment showed a tendency to improve in the New Sol. group but were insufficient. Although new solution failed to show complete efficacy, it acted at multiple sites under difficult conditions. In conclusion, HFC diet for 2 weeks in SHRSP5-Dmcr rats together with MRI-PDFF evaluation is a reliable method for creating simple steatosis, and good quality for cold preservation and reperfusion experiments.

Title Rapid and reliable steatosis rat model SHRSP5-Dmcr for cold storage experiment

Introduction

The exacerbation of ischemia and reperfusion injury (IRI) in steatosis is an obstacle in enlarging the donor pool. Severe macrovesicular steatosis (>60%) is a contraindication for liver transplantation, whereas the outcome of moderate macrovesicular steatosis (30–60%) remains controversial [1]. Therefore, basic research should be performed using the moderate steatosis model. There are many rodent models of non-alcoholic steatosis and steatohepatitis with various methods, reproducibility, and feasibility for human fatty liver disease [2]. We previously reported the time course of warm IRI using rat steatosis produced by fasting and subsequent re-feeding with a high-carbohydrate diet [3]. Unfortunately, it was difficult to control steatosis grade in this model. If we can produce moderate macrovesicular steatosis in a short period with high reliability and reproducibility, it would contribute to the progression of basic research on cold preservation and reperfusion injury in steatosis.

Here, we tested the reliability of rat steatosis using SHRSP5-Dmcr rats fed a high-fat, high-cholesterol (HFC) diet [4]. The aims of the present study were to establish a method to produce moderate (30 to 60%) macrovesicular steatosis and to show that the resulting graft was of good enough quality to be used for the cold preservation and reperfusion experiment.

Materials and Methods

Animals

Animal experiments were conducted with the approval (No. 17-0032) of the Institutional Review Board of Hokkaido University, according to the protocol for the care and use of laboratory animals at Hokkaido University. Laboratory conditions and animal care were the same as those previously described [3]. Male SHRSP5-Dmcr rats, 4–6 weeks old, were purchased from Japan SLC Inc. (Hamamatsu, Japan) [4]. Animals were fed a standard laboratory diet (Oriental Yeast, Tokyo, Japan) for at least 3 days before starting the experiment. A high-fat and high-cholesterol (HFC) diet (Funabashi Farm, Chiba, Japan) was then fed for up to 2 weeks. The HFC-diet comprised lipids (35.3%), carbohydrates (39.6%), and proteins (14.1%) [4]. After 5 days, 1 week, and 2 weeks of HFC diet, animals were subjected to functional magnetic resonance imaging (fMRI) under modified neuroleptanalgesia. A mixture of medetomidine hydrochloride (DOMITOR®; ZENOAQ, Fukushima, Japan), midazolam (Dormicum®; Maruishi Pharmaceutical, Osaka, Japan), and butorphanol tartrate (Vetorphale®; Meiji Seika Pharma, Tokyo, Japan) was administered intraperitoneally at doses of 0.3, 4, and 4 mg/kg, respectively [5]. After fMRI examination, the animals were euthanized by deep inhalation of isoflurane (Mylan EPD, Tokyo, Japan) and massive blood sampling from the lower vena cava.

NAFLD activity Score (NAS)

Liver tissue (appx. 5 × 5 × 5 mm) was fixed in 10% formalin neutral buffer solution (FUJIFILM

Wako, Osaka, Japan) at room temperature for 1–2 days. After formalin-fixed paraffin-embedded (FFPE) blocks were made, sections were prepared and stained with hematoxylin and eosin (H&E). NAS was evaluated according to the nonalcoholic steatohepatitis (NASH) Clinical Research Network scoring system [6]. Steatosis: 0 (none), 1 (mild; 5-33%), 2 (moderate; 33-66%), and 3 (severe; >66%). Inflammation: 0 (none) to 3 (severe). Hepatocyte ballooning: 0 (none) to 2 (many cells prominent).

Functional Magnetic Resonance Imaging (fMRI)

Animals were subjected to a 3 Tesla MRI system, MAGNETOM Prisma® (Siemens AG, Erlangen, Germany) with a dedicated 8 ch array-coil for small animals, receiver coil S3H-AL8RX-1507 (Takashima Seisakusho Co. Ltd., Tokyo, Japan). Magnetic resonance (MR) acquisition parameters were as follows: DIXON sequence; slice thickness (1 mm), TR (5.86 ms), TE (2.46 ms), and flip angle (9°). The formula for the MRI-based proton density fraction (PDFF) is he ratio of the density of mobile protons from fat (triglycerides) (F) to the total density of protons from mobile triglycerides and mobile water (W) [7, 8].

PDFF = F / (W+F) x100 (%)

Cold preservation and reperfusion on an Isolated perfused rat liver (IPRL)

The feasibility of using the steatosis model for cold preservation and reperfusion experiments was assessed (n=3). Liver samples of SHRSP5-Dmcr rats fed HFC for 2 weeks were subjected

to cold preservation for 24 h in University of Wisconsin solution (UW group) or a new solution (New Sol. Group). The preserved graft was then reperfused on an IPRL for 90 min, as previously described, where Dsol was a modified UW solution containing 30% heavy water and some ingredients [9]. In this study, we used Dsol with modification of the buffer component (New Sol.). Portal venous resistance (PVR), oxygen consumption rate (OCR), bile production (μL), and LDH activity in the perfusate (IU/L) were evaluated as previously described [9].

PVR = Pressure (cmH_2O) / Flow (mL/min/g)

OCR = ($pO2_{inflow} – pO2_{outflow}$) × flow (mmHg × mL/min/g),

Statistical analysis.

Statistical analysis was performed using software JMP® version 14 (SAS Institute Japan, Tokyo, Japan). Data were presented as average ± standard deviation. Steel-Dwass tests, a Nonparametric multiple comparison, were applied. A p value less than 0.05 is considered as significant.

Results

General status of the animals

All animals were active, lively, and had good appetite throughout the 2 weeks of the experiment. The liver appeared to be larger as the HFC-fed time increased, with a whitish color change (Figure 1).

NAS (Steatosis grade and total score)

One rat was lost during the MRI because of an overdose of supporting inhalation anesthesia. Therefore, a total of 23 rats were sampled. H&E staining showed microvesicular steatosis in rats fed HFC for five days with minute inflammatory changes. In the 1-week HFC group, micro- and macrovesicular steatosis was observed with inflammatory cell infiltration. In the 2-week HFC group, diffused macrovesicular steatosis was observed with inflammatory cell infiltration and slight congestion (Figure 2). No ballooning was observed in this study. The steatosis score (NAS_S) and total score of NAS were significantly higher in the longer HFC-fed group (n=5-6, Steel-Dwass test) (Figure 2).

Proton Density Fat Faction (PDFF)

Functional MRI revealed an increase in PDFF value dependent on HFC duration (Figure 3A). Furthermore, PDFF was correlated with steatosis grade (NAS_S) (Figure 3B): NAS_S = -0.0637 + 0.145 × PDFF. $R^2 = 0.872$, $p<0.0001$.

Cold preservation and reperfusion of steatotic liver

In the UW group, PVR was higher than that in the New Sol. group throughout the 90 min of reperfusion. Statistical significance was observed only at 5 and 10 min after reperfusion, but not at other points (Figure 4A). OCR was significantly higher in the New Sol. group at 5 min after reperfusion, but was not significant at 90 min after reperfusion (Figure 4B). Bile production

tended to increase in the New Sol. group (Figure 4C), and LDH activity in the perfusate 90 min after reperfusion tended to decrease (Figure 4D). Altogether, the graft preserved in the new solution was superior to that preserved in the UW solution at reperfusion, but the superiority was abolished during 90 min of reperfusion.

Discussion

In this study, we established a moderate macrovesicular steatosis (30-60%) model within two weeks in SHRSP5-Dmcr rats fed a high-fat and high-cholesterol diet. SHRSP5-Dmcr rats have been reported to have NASH and liver cirrhosis [5]. However, a method to produce moderate macro-steatosis for basic research on liver preservation, machine perfusion, and reperfusion has not yet been precisely evaluated.

First, we examined the steatosis grade (NAS_S) to determine the optimal length of HFC feeding to produce moderate steatosis. Although we found that 2 weeks of HFC seemed to be sufficient, the grade of steatosis and inflammation showed individual differences in rats. This variance affects the extent of ischemia and reperfusion injury as well as treatment efficacy. Therefore, a reproducible steatosis model was required.

Based on this background, we evaluated the MRI-based PDFF. As previously reported, steatosis grade is correlated with PDFF [8,9]. Accordingly, we confirmed the reproducibility of NAS grade 2 steatosis in SHRSP5-Dmcr rats after 1-2 weeks of HFC feeding. Furthermore, the

individual differences in rat steatosis models could be minimized by evaluating MRI-based PDFF without invasive evaluations such as laparotomy and biopsy. These results suggest that a reliable rat steatosis model may contribute to cold preservation and/or machine perfusion experiments.

Finally, we screened the quality of the resulting steatosis graft for the use of cold preservation and reperfusion experiment (n=3) by comparison of conventional UW solution and the new solution, which appeared to show strong cytoprotection in the cold preservation of rat hearts and livers in our preliminary study (unpublished data). Unfortunately, we failed to show efficacy of the new solution in the present study, presumably due to excess stress loaded by 24-hour cold preservation, it is not the focus of this study. Notably, OCR and microcirculation immediately after reperfusion were significantly improved in the New Sol. group. In addition, the variance in the other indices appeared to be relatively small. These results imply that the steatosis model is suitable for use in cold preservation and reperfusion experiments. Furthermore, it would contribute to the progress of the study if we could optimize the level of stress caused by cold preservation of the liver graft. Since the mechanism of lipid accumulation, lipid droplet growth, composition of phospholipids bound fatty acids, and free fatty acids is different, the extent of lipotoxicity may change, thus leading to the different stress responses during cold preservation and/or during organ perfusion [3, 10].

Conclusions

In conclusion, we established a method to produce moderate (30–60%) macrovesicular steatosis, NAS steatosis grade 2, using male SHRSP5-Dmcr rats with HFC diet for 1–2 weeks. Since MRI-based PDFF correlates with NAS steatosis grade and HFC-fed time, preoperative evaluation of MRI-based PDFF can minimize the variance of HFC-induced steatosis. The steatotic grafts produced in this study were of good quality and can be used for cold preservation and reperfusion experiments.

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Figure Legends

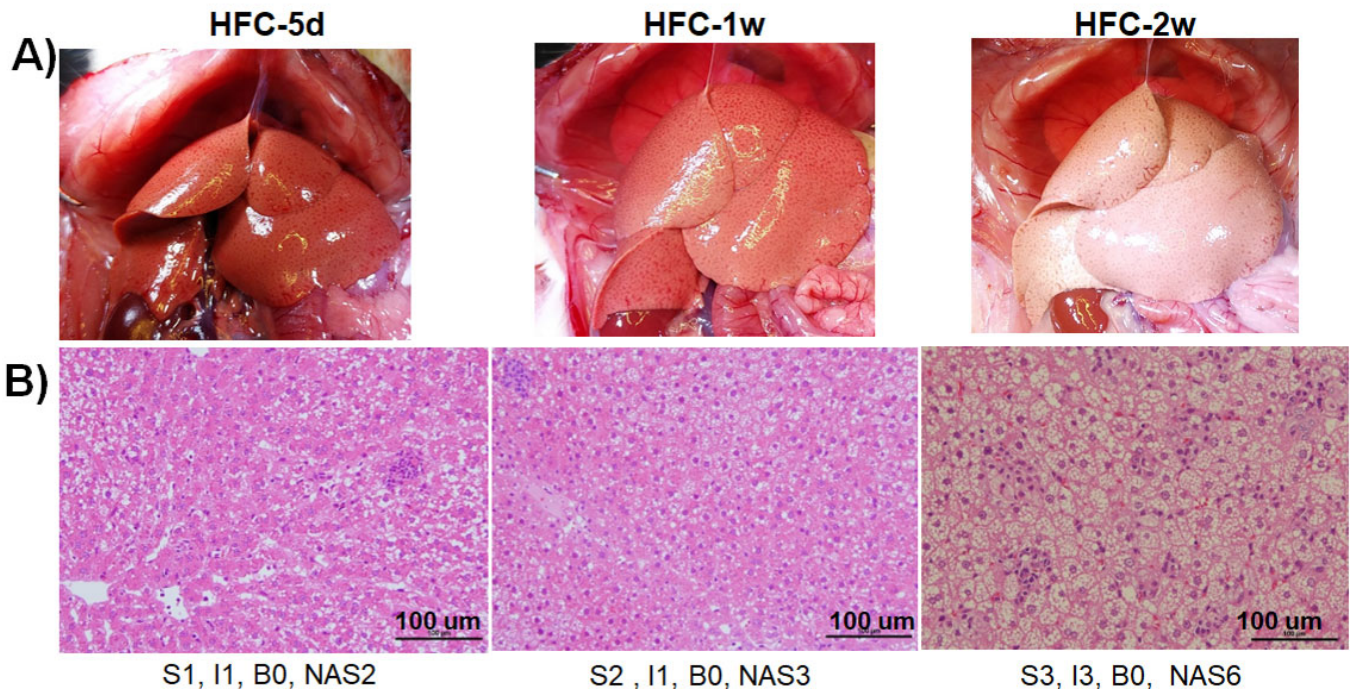
Figure 1: Livers after 5d, 1w, and 2w of HFC diet. A) Liver volume rapidly increased as HFC

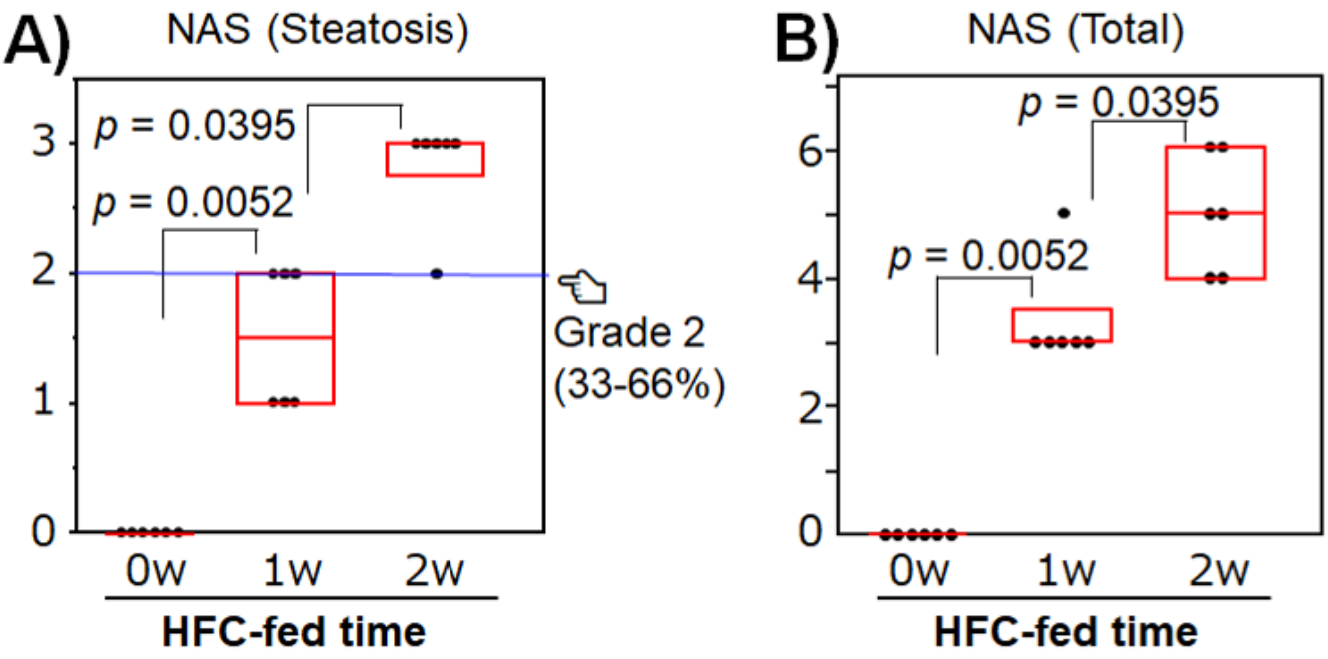
duration increased. B) H&E staining. Micro-vesicular steatosis was shown in the 1-week HFC group, whereas macro-vesicular steatosis was shown in the 2-week HFC group. The area of the lipid droplet increased as HFC duration increased.

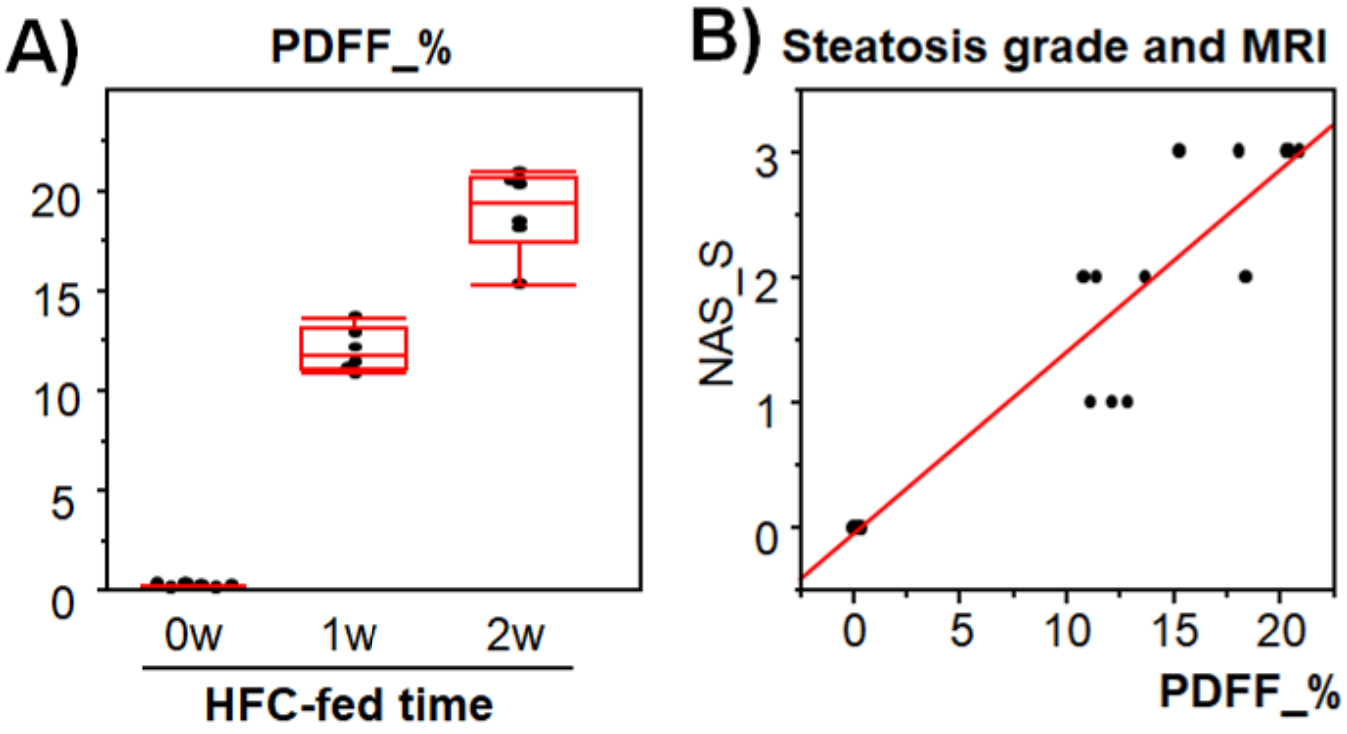
Figure 2: NAS scoring. A) Only steatosis (0-3) was evaluated. B) Total score was evaluated by the sum of steatosis (0-3), inflammation (0-3), and ballooning (0-2).

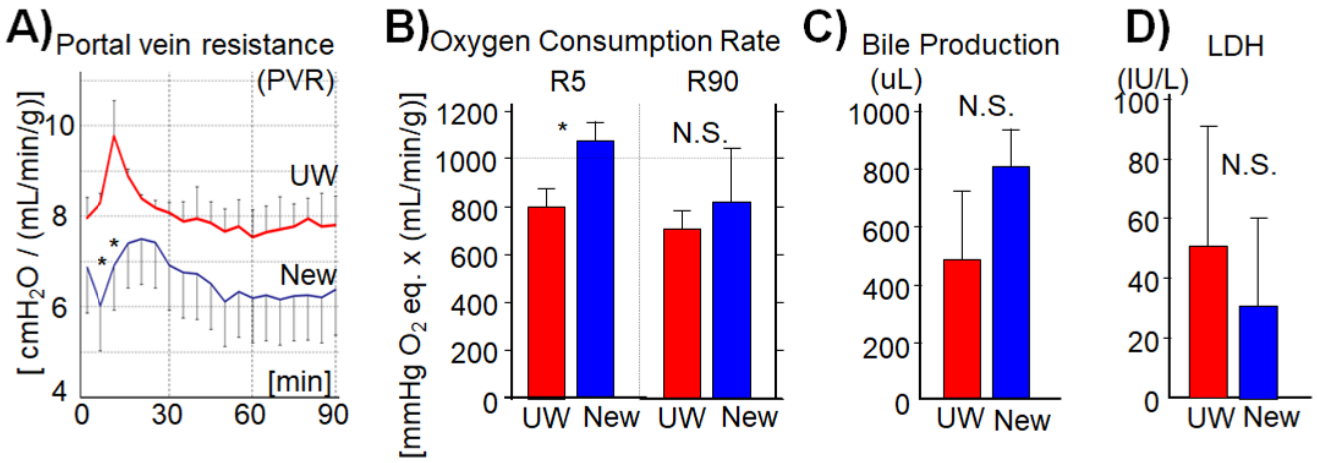
Figure 3: MRI-based Proton Density Fat Faction (PDFF). A) PDFF correlates to the HFC duration. B) NAS steatosis grade correlates to the PDFF. NAS_S = -0.0637 + 0.145 x PDFF. $R2 = 0.872$, $p<0.0001$

Figure 4: Cold preservation and reperfusion of the steatotic liver. A) Portal venous resistance (PVR) during 90 min of reperfusion. B) Oxygen consumption rate (OCR) at 5 min and 90 min after reperfusion. C) Bile production during 90 min of reperfusion. D) LDH activity in the perfusate at 90 min after reperfusion.


A)
HFC-5d
HFC-1w
HFC-2w
B)
100 um
100 um
100 um
S1, I1, B0, NAS2
S2 , I1, B0, NAS3
S3, I3, B0, NAS6


A)
NAS (Steatosis)
p = 0.0395
p = 0.0052
3
2
1
0
Grade 2
(33-66%)
0w
1w
2w
HFC-fed time
B)
NAS (Total)
p = 0.0395
p = 0.0052
6
4
2
0
0w
1w
2w
HFC-fed time


A)
PDFF_%
20
15
10
5
0
0w
1w
2w
HFC-fed time
B) Steatosis grade and MRI
NAS_S
3
2
1
0
0
5
10
15
20
PDFF_%


A)
Portal vein resistance
(PVR)
[cmH2O / (mL/min/g)]
10
8
6
4
UW
New
*
*
[min]
0
30
60
90
B)
Oxygen Consumption Rate
R5
R90
[mmHg O2 eq. x (mL/min/g)]
1200
1000
800
600
400
200
0
*
N.S.
UW
New
UW
New
C)
Bile Production
(uL)
1000
800
600
400
200
0
N.S.
UW
New
D)
LDH
(IU/L)
100
80
60
40
20
0
N.S.
UW
New